UNIVERSIDAD DE CÓRDOBA

DEPARTAMENTO DE GENÉTICA

TESIS DOCTORAL

Aproximaciones multidisciplinares incluyendo herramientas histológicas, bioquímicas y moleculares para estudiar los mecanismos de resistencia a marchitez vascular causada por *Fusarium oxysporum* en guisante (*Pisum sativum*)

Doctorando

Moustafa Bani

Directores

Diego Rubiales

Nicolas Rispail

Enero 2015

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DEPARTMENT OF GENETICS

PhD THESIS

Multidisciplinary approaches including histological, biochemical and molecular tools to study the resistance mechanisms to fusarium wilt caused by *Fusarium oxysporum* in pea (*Pisum sativum*)

PhD student

Moustafa Bani

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Diego Rubiales

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TÍTULO DE LA TESIS: Aproximaciones multidisciplinares incluyendo herramientas histológicas, bioquímicas y moleculares para estudiar los mecanismos de resistencia a marchitez vascular causada por *Fusarium oxysporum* en guisante (*Pisum sativum*)

DOCTORANDO/A: Moustafa Bani

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Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 19 de diciembre de 2014

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TÍTULO DE LA TESIS:

Aproximaciones multidisciplinares incluyendo herramientas histológicas, bioquímicas y moleculares para estudiar los mecanismos de resistencia a marchitez vascular causada por *Fusarium oxysporum* en guisante (*Pisum sativum*).

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This work is dedicated to my mother and father, my brothers and sisters, may wife and my gorgeous daughter Nussaiba "my daughter forgives me for not having enough time for you"

« كُنْ فِي الدُّنْيَا كَالنَّحْلَة، إنْ أَكَلَت أَكَلَت طَيِّبًا وإنْ أَطْعَمَت
أَطْعَمَت طَيِّبًا وَإن سَقَطَت عَلَى شَيْءٍ لَمْ تَكْسِرْه ولَمْ تَخْدَشْه »

ابن القيم الجوزية

« be in this world as the bee: it eats only good,
produces only good, and when it rests upon
anything it neither ruins it nor deflowers it »

Ibn Qayyim Al-Jawziyyah

Abstract

Pea is a legume crop with worldwide economic importance that has been largely grown as a source of protein for animal and human nutrition for many centuries. However, yields of pea are usually reduced by biotic and abiotic stresses. Fusarium wilt caused by *Fusarium oxysporum* f.sp. *pisi* (*Fop*) is one of the most destructive disease of this crop. The use of resistant cultivars is widely recognized as the safest, most economical and most effective method for controlling this disease. However, the constant evolution of the pathogen drives the necessity to broaden the genetic basis of resistance to *Fop*. Successful screening for disease resistance is based on (i) the availability of large and diverse germplasm collections, including wild relatives; (ii) the availability of precise and accurate screening techniques and genetic structure; (iii) the knowledge of plant and pathogen biology, variability and host–pathogen interaction. In the present work, we aimed to identify and characterize new sources of resistance to *Fop* within a *Pisum* spp. collection using different approaches and further understand mechanisms involved in resistance to this pathogen. To establish a standard, easy and useful methodology to evaluate the resistance of legumes to *Fusarium oxysporum*, a preliminary study evaluating the physiological and susceptibility response to the disease of two contrasting genotypes of model plant *Medicago truncatula* has been performed. For this we evaluated the effect of several cultural conditions known to affect the disease incidence such as plant age at inoculation, growth substrate and the method of inoculation. The results obtained indicated that the method of inoculation strongly affected fusarium wilt disease development while it was not significantly altered by the plant age or the inorganic growth substrate tested. This also allowed us to establish an optimum method of inoculation to study legume resistance to *F. oxysporum* that we then used to screen a *Pisum* spp. germplasm collection against one isolate of *Fop* race 2. This showed a large variation in the disease response among pea accessions ranging from highly resistant to susceptible, indicating the quantitative expression of the resistance. The repetition of the inoculation experiments on a subset of 19 accessions, indicated that the established method of inoculation and evaluation was robust and reproducible and confirmed the highly resistant phenotypes of 11 accessions. We then aimed the characterization of the mechanism of resistance responsible for the resistance in these pea accessions. First, we collected the root exudates of 12 pea accessions presenting differential responses to *Fop* race 2 to determine their effect on *Fop* growth. While the root exudates of most pea accessions stimulated *Fop* germination, some accessions did not and even inhibited *Fop* germination. This indicated that some pea accessions express some constitutive pre-penetration mechanisms that might contribute to decrease or delay the building of

pathogenic population. Further studies showed that the pisatin exuded by these accessions was at least partly responsible for the inhibition suggesting that pisatin play an important role in this constitutive resistance mechanism. Beside this pre-penetration mechanism, pea might have developed others mechanisms acting during or after root penetration. In preliminary macroscopic observation, we found that, in all resistant accessions, the resistance mechanisms efficiently stopped pathogen progression at the basal part of plant. Thus we initiated a histological study to identify the resistance mechanism acting in the upper root section of the plant at cellular level. The detailed comparison of 8 accessions with differential response to *Fop* race 2 showed that these resistant accessions established several barriers at the epidermis, exodermis, cortex, endodermis and vascular stele efficiently impeding the fungus progression. We found that these barriers were mainly based on three defence mechanisms including cell wall strengthening by lignification, formation of papilla-like structure at penetration site and accumulation of different substances within and between cells, and that they were differentially expressed in each resistant accession. The main important components of these different barriers are phenolic compounds, lignin and carbohydrates. Our result also supports the important role of CWDEs in *F. oxysporum* pathogenicity. The molecular characterisation of the resistance through a proteomic approach confirmed partly these observations. Indeed, the proteomic analysis of three pea genotypes showing different resistance response to *Fop* race 2, confirmed the implication of the phenolic compounds and revealed an important role of the proteins involved in the reinforcement of cell membrane and cell wall in the resistance. This analysis also revealed that ROS burst generation was not sufficient to prevent fungal colonization or reproduction within the xylem tissues of susceptible plant. On the other hand, to gain further knowledge on *Fusarium oxysporum* pathogenicity we studied the secondary metabolites produced by some isolates of *Fop* race 2 and their relationship with pathogenicity. The results obtained showed that these isolates produced mainly two toxins identified as fusaric acid and 9,10-dehydrofusaric acid. Both toxins were found highly phytotoxic on excised leaves. In addition the amount of toxin produced by each strain was correlated its virulence showing that these toxins are important determinants of *Fop* race 2 pathogenicity.

Resumen

El guisante es una leguminosa-grano muy importante al nivel mundial que ha sido cultivada como fuente de proteínas para la nutrición animal y humana durante muchos siglos. Sin embargo, el rendimiento del guisante se ve reducido frecuentemente debido a estreses bióticos y abióticos. La marchitez vascular causada por *Fusarium oxysporum* f.sp. *pisi* (*Fop*) es una de las enfermedades más destructivas de este cultivo. El uso de variedades resistentes es el método más seguro, más económico y más eficaz para controlar esta enfermedad. La constante evolución del patógeno implica la necesidad de ampliar la base genética de la resistencia a *Fop*. El éxito en la mejora genética por resistencia se basa en (i) la disponibilidad variabilidad genética, disponible en colecciones de germoplasma, incluyendo entradas silvestres; (ii) la disponibilidad de técnicas de evaluación precisa y exacta; (iii) el conocimiento de la biología y la variabilidad de la planta y del patógeno así como de la interacción entre ambos. En el presente trabajo nos propusimos buscar y caracterizar nuevas fuentes de resistencia a *Fop* en una colección de germoplasma de *Pisum* spp. y profundizar en el conocimiento de los mecanismos implicados en la resistencia a este patógeno mediante distintas aproximaciones. Con el fin de establecer una metodología estándar, fácil y útil para evaluar la resistencia de las leguminosas frente a *Fusarium oxysporum*, se realizó un estudio preliminar evaluando la respuesta fisiológica y la susceptibilidad a esta enfermedad de dos genotipos contrastantes de la planta modelo *Medicago truncatula*. Para ello se evaluó el efecto de varias condiciones culturales conocidos por afectar la incidencia de la enfermedad tales como la edad de la planta en el momento de infección, el sustrato de crecimiento y el método de inoculación. Los resultados indicaron que el método de inoculación afecta fuertemente el desarrollo de la enfermedad mientras que la edad de la planta y los sustratos de crecimiento inorgánico probados no la afectaba significativamente. Este estudio permitió también poner a punto un método de inoculación de *F. oxysporum* que se utilizó posteriormente para evaluar la respuesta de una colección de germoplasma *Pisum* spp. frente un aislado de raza 2 de *Fop.* El resultado de esta evaluación mostró una gran variabilidad en la respuesta de las entradas de guisante a la enfermedad lo que indica la expresión cuantitativa de la resistencia a *Fop* en nuestra colección. La repetición de los experimentos con 19 entradas, indicó que el método de inoculación y de evaluación era robusto y reproducible y confirmó la alta resistencia de 11 entradas. A continuación, se persiguió la caracterización de los mecanismos responsables de la resistencia en estas entradas de guisante. En primer lugar, se recogieron los exudados radiculares de 12 entradas de guisante que presentan respuestas diferenciales a la raza 2 para determinar sus efectos sobre el crecimiento del

patógeno. Mientras que los exudados de la mayoría de las entradas de guisante estimulaban la germinación de *Fop*, algunas entradas no lo hacían e incluso inhibían su germinación. Esto indicó que algunas entradas expresan un mecanismo constitutivo que actúan antes de la penetración y que podría contribuir a disminuir la población del patógeno. Este estudio permitió demostrar también que la pisatina contenida en los exudados de estas entradas juega un papel importante en este mecanismo de resistencia constitutiva. Además de este mecanismo, el guisante ha desarrollado otros mecanismos que actúan durante y después de la penetración de las raíces. Las observaciones macroscópicas preliminares, muestran que, en todas las entradas resistentes, la progresión del patógeno está eficientemente bloqueado en la parte basal de la planta. Por ello se inició un estudio histológico para identificar los mecanismos de resistencia que actúan en la parte superior de la raíz de la planta a nivel celular. La comparación detallada de 8 entradas con respuesta diferencial a la raza 2 de *Fop* demostró que las entradas resistentes desarrollan varias barreras en la epidermis, exodermis, córtex, endodermis y la estela vascular que impiden eficientemente la progresión del patógeno. Se determinó que estas barreras se basan principalmente en tres mecanismos de defensa, el fortalecimiento de la pared celular por lignificación, la formación papila en el sitio de penetración y la acumulación de diferentes sustancias entre y dentro de las células, y que estas barreras estaban expresadas diferencialmente en las entradas resistentes. Los principales componentes de estas diferentes barreras son los compuestos fenólicos, la lignina y los carbohidratos. Nuestros resultados apoyan también la importancia de las enzimas de degradación de la pared celular en la patogenicidad de *F. oxysporum*. La caracterización molecular de la resistencia a través una aproximación proteómica confirma parcialmente estas observaciones. El análisis proteómico de tres genotipos con diferentes niveles de resistencia a la raza 2 de *Fop,* demostró la implicación de los compuestos fenólicos así como de las proteínas responsables del reforzamiento de la membrana y de la pared celular en la resistencia del guisante a *Fop*. Este análisis ha revelado también que la producción de especies reactivas de oxígeno no era suficiente para prevenir la colonización de la planta susceptible por el patógeno. Por otro lado, y para profundizar en el conocimiento sobre la patogenicidad de *Fusarium oxysporum* se han estudiado los metabolitos secundarios producidos por algunos aislados de la raza 2 de *Fop* y su relación con la patogenicidad. Este estudio ha demostrado que estos aislados producen principalmente dos toxinas, el ácido fusárico y el ácido 9,10 dihydrofusárico. Ambos toxinas son fitotóxicos y la cuantidad de toxinas producidas por cada cepa está correlacionado con sus niveles de virulencia. Así, estos resultados indican claramente que estas toxinas son unos factores determinantes en la patogenicidad de la raza 2 de *Fop*.

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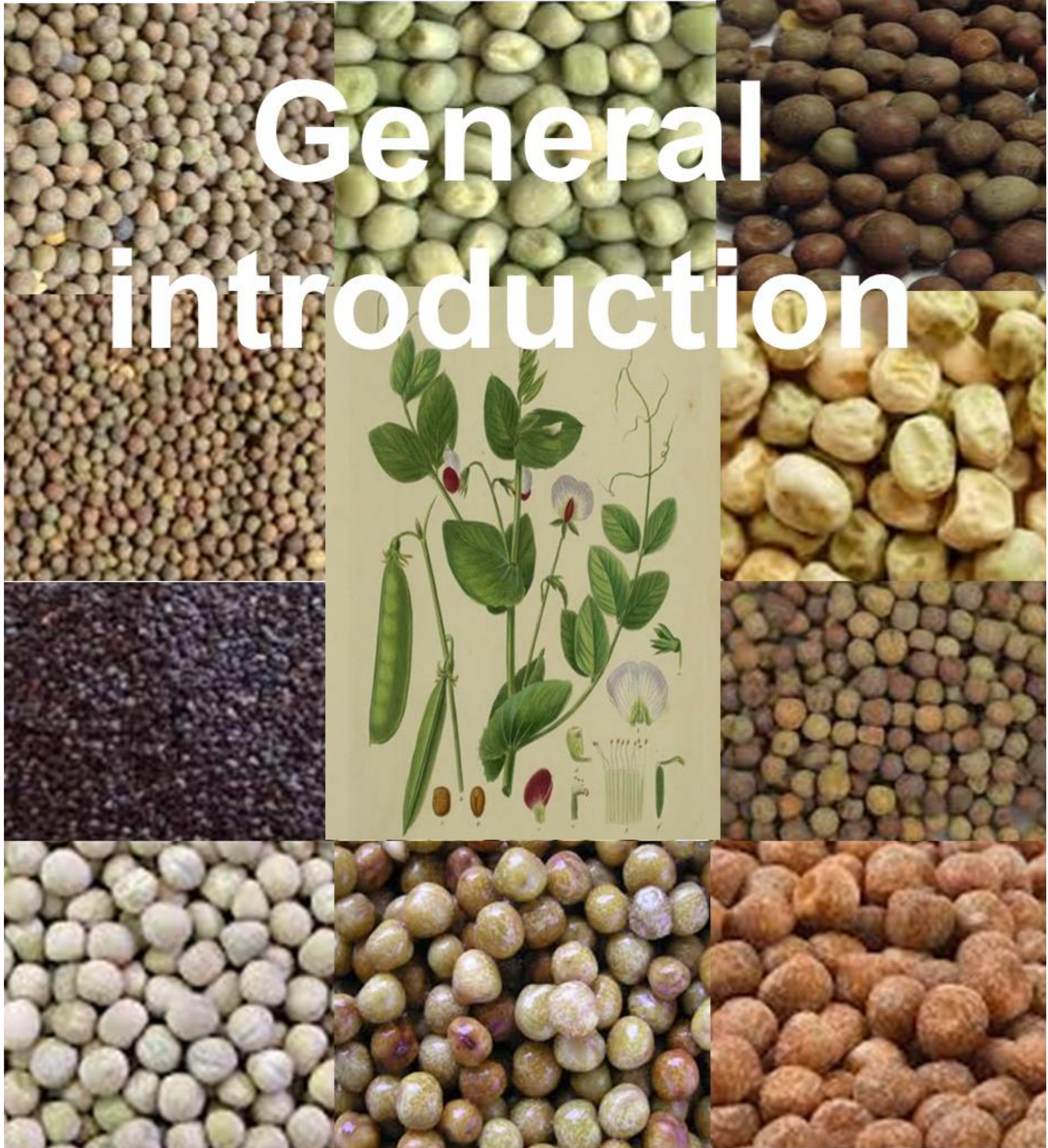
General introduction



General introduction

1. Grain legumes

The legume family (Fabaceae) is composed of more than 700 genera containing approximately 20,000 species (Doyle and Luckow, 2003). It represents the third largest plant family next to Orchidaceae and Asteraceae (Sugiyama and Yazaki, 2012). The characteristic feature of legumes is their symbiotic interaction within root nodule with soil bacteria of the *Rhizobium* and related genera that allow them to fix atmospheric nitrogen (Rispail *et al.*, 2010). Major agricultural legumes are divided into two main groups the cool-season and warm-season legumes. The cool-season legumes include *Vicia, Pisum, Trifolium, Medicago, Lens, Cicer* and *Lotus* genera. The warm-season legumes contain the *Vigna, Phaseolus, Cajanus* and *Glycine* genera (Singh, 2005).

Grain legumes, also known as pulses refer to the legume species of which the edible part is seed (Singh, 2005). Their high protein content ranging from 20 to 50% of the seeds made them second only to cereals in their dietary importance to humans and animals (Graham and Vance, 2003). Furthermore, legume use in arable crop rotations reduces the need for fertilizer application and acts as a break-crop, reducing pest and disease pressure for the subsequent crops. Grain legumes regroup the legume species common bean, pea, chickpea, faba bean, soybean, cowpea, lentil, pigeonpea, peanut, Asian *Vigna* species, grass pea and horsegram (Singh *et al.*, 2013).

Despite their benefits, the inclusion of grain legumes in European cropping system has been rather low mainly due to their yield inconsistency. In these last years, however, the European Union (EU) has developed new strategies to improve grain legumes use for food and feed. These strategies sought to mobilise and integrate European scientific research into grain legumes in order to revert this deficiency by securing production and improving legume feed and food quality and safety, while using legumes to develop sustainable agriculture. The objectives of these strategies were to devise a series of innovating genetic and genomic tools to facilitate studies and breeding of legumes. (http://cordis.europa.eu/result/rcn/51624_en.html). The every growing array of technologies and genomic tools that are being developed for model species are already starting to impact on breeding of a number of crop species. For legumes the model species are *Lotus japonicus* and *Medicago truncatula*. The developments include platform technologies and resources to link their discovery in models to application in crops (Waugh *et al.*, 2006).

The major European legume crops are soya beans, pea, faba beans, broad beans, chickpea, lentil, lucerne and clover, the majority of them are evolutionarily and genetically closely related to model legume plant (*M. truncatula*). The driving idea of European strategies is to exploit the genome conservation between model and crop species to develop a series of diverse and complementary novel tools and to understand the relationship between biological processes in crops and model by exploiting technical developments in the model system *M. truncatula*. One of the major legume crops, for which these strategies are based, is the pea (http://cordis.europa.eu/result/rcn/51624_en.html). The high degree of co-linearity between the *Pisum* and *M. truncatula*, genetic maps and the large international sequencing effort in Medicago brings an unprecedented range of tools, resources and data into the compass of those researching and breeding pea (Ambrose, 2008).

1.1. Barrel medic (*Medicago truncatula*)

Barrel medic, *M. truncatula* (2n = 16) is an important pasture legume (Crawford *et al.,* 1989) cultivated on over 4.5 million hectares in Australia alone (Hill and Donald, 1998). It is of particular importance in southern Australian cereal-livestock zone where it provides feed for livestock and plays an important role in crop rotation. *M. truncatula* originates from the Mediterranean basin and it has also been naturalized in other regions of the world following European migrations (Bataillon and Ronfort, 2006; Franssen and Geurts, 2007).

M. truncatula is an annual autogamous (selfing) species. It is a member of Fabaceae family, subfamily Faboideae, and tribe Trifoliees. On the basis of pod characteristics; three main subspecies (ssp. *truncatula*, ssp. *tricycla* and ssp. *longeaculata*) have been distinguished (Bataillon and Ronfort, 2006). Genetic resources of *Medicago* include both traditional cultivars and wild relatives are maintained in four main Stock centers, namely The South Australian Research and Development Institute (SARDI), the USDA National Plant Germplasm System (NPGS), the French National Institute for Agricultural Research (INRA) and The Samuel Roberts Noble Foundation (Nair *et al*., 2006).

M. truncatula plant is made up of a main axis that can organize either in a rosette or as an elongated axis; branches of different orders with trifoliate leaves. Each leaflet is rounded, 1-2 cm long, often with a dark spot in the center. The flowers are yellow, produced singly or in a small inflorescence of 2-5 together. The seed have morphological features typical of dicotyledons, and are born in a spine-covered spiral pod. The cotyledons are rich in protein (35-45%), soluble sugars (6-10%), the major

storage molecule, accumulate ca. 8-10% lipids and only traces of starch. Thus, seed composition is more similar to oilseed legumes such as soybean or lupin than it is to pea or field bean, despite being phylogenetically closer to these latter crop species (Moreau, 2006; Gallardo *et al.*, 2006)

The study of economically important legume crops such as soybean, pea, alfalfa, bean or chickpea is complicated because of their large genome sizes or polyploidy. Fortunately, *M. truncatula,* was originally proposed as a model plant for legume biology because it possesses a number of interesting characteristics for both molecular and classical genetics (Cook, 1999; Pérez-de-Luque *et al.*, 2006). This species has all characteristics of a plant model species: a small diploid genome (500 Mbp; equivalent to five times the size of the *Arabidopsis thaliana* genome and similar to that of rice), autogamous fertilization, a short generation time (3 to 4 months from seed to seed), and good genetic transformability, a number of available cultivars, and a well characterized nitrogen-fixing symbiont (Thoquet *et al.,* 2002; Franssen and Geurts, 2007).

The molecular genetic map of the *Medicago* genome, together with the knowledge of agronomically important genes, should pave the way for comparative genomic studies in legumes (Thoquet *et al.,* 2002). Phylogenetically, *M. truncatula* is closely related to alfalfa (the major world forage legume), lentil, pea, faba bean, and clover (Franssen and Geurts, 2007). The cross-species amplification of *M. truncatula* microsatellites across three major legumes crops revealed significant transferability of *M. truncatula* microsatellites to these legumes (40% in faba bean, 36.3% in chickpea, and 37.6% in pea) (Gutierrez *et al.*, 2005). In addition, comparative genetic maps have been created between *M. truncatula* and alfalfa (*Medicago sativa*) and pea. This comparison indicates that the two *Medicago* genomes (*M. truncatula* and *M. sativa*) are highly similar and that a high degree of synteny exists between pea and the *Medicago* species since only two major chromosomal translocations have been identified (Franssen and Geurts, 2007).

The ability to study resistance to agriculturally relevant pathogens in *M. truncatula* is likely to lead to more successful strategies to introduce resistance to these pathogens, particularly to legume crops. *M. truncatula* provides an excellent system to study plant interactions with fungal pathogens because of the range of agriculturally relevant pathogens for which *M. truncatula* is a host (Lichtenzveig *et al.*, 2006).The majority of infecting pathogens studied using *M. truncatula* are isolated from alfalfa, such as *F. oxysporum* f.sp. *medicaginis* (*Fome*) that cause fusarium wilt in alfalfa, however barrel medic can be also susceptible to *Fome.* Barrel medic has been also

described as a host for other pathogens such as *Verticillium albo-atrum* (Ben *et al.*, 2013), *Phoma medicaginis* var. *pinodella* (Ellwood *et al.*, 2006), *Didymella pinodes* (Madrid *et al.*, 2014), *Colletotrichum trifolii* (Torregrosa *et al.*, 2004), *Erysiphe pisi* (Prats *et al.*, 2007), *Uromyces striatus* (Rubiales and Moral, 2004) and *Orobanche crenata* (Castillejo *et al.*, 2009).

Based on these studies, it has been concluded that the *Medicago* genome can be effectively used as a reference to increase our understanding of the symbiotic interaction and legume disease resistance (Rispail *et al.,* 2010). The comparative mapping based on molecular markers tightly linked with resistance in *M. truncatula* is a good approach to identify candidate genes involved in the resistance of the species with less saturated maps (Rubiales *et al.*, 2015). This approach has been used for example in resistance to ascochyta blight in chickpea (Madrid *et al.*, 2012), for identifying the gene *SYM2* in pea involved in symbiosis (Gualtieri *et al.*, 2002) and in resistance to broomrape (Cobos *et al.*, 2013).

The use of model plants such as *M. truncatula* may contribute to improve our understanding of the *F. oxysporum*-legume interaction and legume resistance. The large post-genomic resources established for *M. truncatula* may serve to better understand the *F. oxysporum*-legume interaction. Application of proteomic-based techniques is particularly useful for the identification of proteins involved in stress responses in plants (Gygi and Aebersold, 2000). However, protein identification requires the existence of complete genome databses that thus far are available only for a limited number of legumes such as *M. truncatula* for which 90 to 100% of the differentially accumulated proteins can be identified (Schenkluhn *et al.*, 2010; Zhang *et al.*, 2011), while in pea only 51% of protein can be identified (Castillejo *et al.*, 2012).

1.2. Pea (*Pisum sativum*)

Pea (*P. sativum*) is an important grain legume crop that has been largely grown as a source of protein for animal and human nutrition for many centuries and has worldwide economic importance (Rubiales *et al.*, 2012). Energetically and economically, it is an advantage to utilize nitrogen inputs from this legume source for securing carbon sequestration into food, feed and biofuel crops (Nemecek *et al.*, 2008). In common with other grain legumes, pea plays a critical role in crop rotation and it can be used for fodder or green manure (Redden *et al.*, 2005; Nemecek *et al.*, 2008). Pea has also been widely used for genetic and physiological studies due to its large genetic and morphological variations.

1.2.1. Origin, taxonomy and botanical description

Pea is an old cool season annual legume crop whose origins trace back to the primary centre of origin in the near and middle east. Carbonised remains of pea have been found at Neolithic farming villages in northern Iraq, southern and south eastern Turkey and Syria indicating its cultivation and use as food as early as 7000-6000 BC (Ambrose, 2008). Their presence was also found in remain sites in Southern Europe (Zohary and Hopf, 1973). Two additional secondary centre of pea diversity have also been identified, the highland Asiatic region of the Hindukusch that runs the whole length of the southern slopes of the Himalayan mountains (Turkey, Syria, Iraq, and Lebanon) and the central highland region of Ethiopia and uplands of Southern Yemen, which covers the currently known distributional range of *Pisum* a*byssinicum* (Ambrose, 2008; Redden *et al.*, 2005). Peas were disseminated towards Europe, together with wheat and barley ca 5500 BC. They also spread towards east Africa through Egypt and Ethiopia ca 5000 BC, and towards India ca. 2000 BC (Rubiales *et al.*, 2012).

Pisum is a member of Fabaceae family, subfamily Papillionaceae, and tribe Vicieae and comprises three main species, *P. abyssinicum, P. fulvum* and *P. sativum*. *P. sativum* can be further divided into subspecies such as *P.s.* ssp. *sativum*, *P.s.* ssp. *elatius*, *P.s.* ssp. *humile*, *P.s.* ssp. *arvense* , *P.s.* ssp. *transcaucasicum* and *P.s.* ssp. *hortense* (Redden *et al.*, 2005; Ambrose, 2008; Rubiales *et al.*, 2012). *P.s.* ssp. *elatius* and *P.s.* ssp. *humile* are considered the progenitors of the modern cultivated pea *P.s.* ssp. *sativum*.

On the other hand *P.s.* ssp. *arvense* encompasses the field pea and is characterized by coloured flowers and pigmented seeds (Redden *et al.*, 2005). The species *abyssinicum,* cultivated in Ethiopia and Yemen, and also wild in Ethiopia, may have evolved independently with contributions of *P. fulvum* and *P.s.* ssp. *elatius* germplasms (Redden *et al.*, 2005). All taxa within *Pisum* are diploid (2n = 14) and the majority are fully inter-crossable and produce viable hybrids (Redden *et al.*, 2005).

Pea is an annual species with no known perennial habit and is traditionally produced as a spring annual crop. The fall-sown or winter pea is sown in late September to mid-October in northern latitudes and ideally reaches a moderately branched, rosette stage ca. 5 to 7 cm in height prior to entering a winter dormant period. As temperatures rise in the spring the rosette breaks dormancy assuming a vegetative growth pattern followed by a reproductive period and seed fill. Crop maturity is typically 2 to 3 weeks prior to spring sown types (McPhee, 2007). The first nodes, some of which give rise to branches, are vegetative, while subsequent nodes are

reproductive. Generally two flowers, from which the pods develop, are present at each reproductive node (Cousin, 1997). The pea flower is typical of the Papilionaceae family. The corolla contains five petals. The pea is cleistogamous and must be considered as a strictly self-fertilizing species (Cousin, 1997). The wild species often have tall (more than 2 meters), slender and branched stems, purple or pink flowers and small pods producing a small quantity of seeds with coloured coat.

P.s. ssp. *elatius* and *P. abyssinicum* have distinct toothed leaflets and stipules. *P.s.* ssp. *elatius* has coloured flowers, lilac-blue standards, dark purple wings and maroon veiny brown seeds. *P. abyssinicum* has pink flowers and dark purple seeds. *P. fulvum* may have two fructification types, a normal one located in the upper part of the plant, the other very peculiar with very short basal branches which push the pods slightly into the ground. *P.s.* ssp. *humile* is characterized as a medium sized climbing species with dentate leaf margins and light blue flowers (Cousin, 1997). Plant type of modern cultivars has changed significantly compared to cultivars of 15 to 20 years ago.

Overall plant height has been reduced through selection of short internodes conferred by the major recessive gene *le* responsible for tall climbing (*Le*/-) vs. dwarf bush (*le*/*le*) habit, and leaf morphology has been converted from conventional leaflets to a semileaflets type where leaflets are converted to tendrils by the recessive *af* along with other modifying genes (Goldenberg, 1965). Several genes are available that reduce leaf area including *af* that converts leaflets to tendrils and gives “semi-leafless” peas, *sf* that reduce stipule and combined to *af* results in leafless peas and *rogue* that reduce both leaflet and stipule size making them erect like hare’s ear (Cousin, 1997; Fig. 1).

1.2.2. World production and utilization

Pea is an important cool season legume crop produced worldwide, mainly in temperate regions (Rubiales *et al.*, 2012). Dry pea ranks third in production among the grain legumes after beans and chickpeas, for total production of 10.97 million tonnes produced over 6.38 million hectares worldwide in 2013 (Fig. 2).

The world average yield per hectare was 1.72 tonnes/ha (FAOSTAT, 2013). Dry peas are grown commercially in almost 100 countries, but production is concentrated in Canada, Russian Federation, and China. Jointly, these three countries produce over one half of the world’s dry peas (Fig. 3). Spain is the tenth world producer of dries pea producing 177,700 tonnes in 122,000 hectares with a yield of 1.46 tonnes/ha (FAOSTAT, 2013).

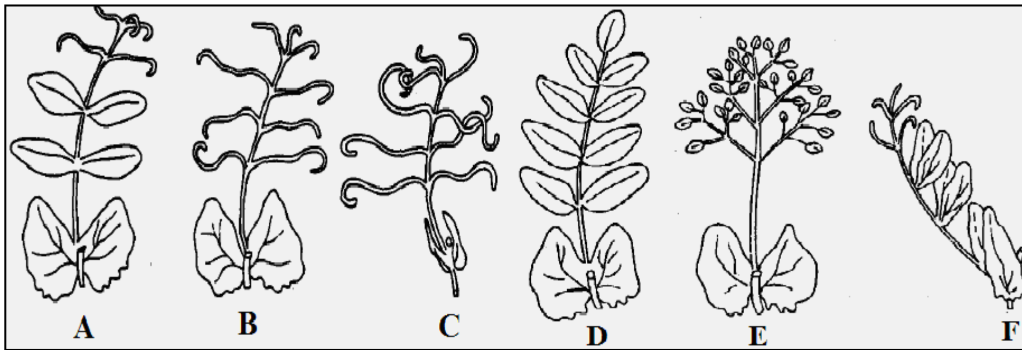


Figure 1. Different morphological types of pea leaves. (A) Normal leaf. (B) Semi-leafless or "afila", "*af*" gene transforms the leaflets into tendrils. (C) Leafless, combination of "*af*" and *"st"* genes. The "*st*" gene causes reduced stipules. (D) Acacia-type, the "*tl*" gene transforms the tendrils into leaflets. (E) Combination of "*af*" and "*tl*" genes. (F) Hate's ear, the "*rogue*" gene reduces the width of the leaflets and stipules and makes them erect. (Cousin, 1997).

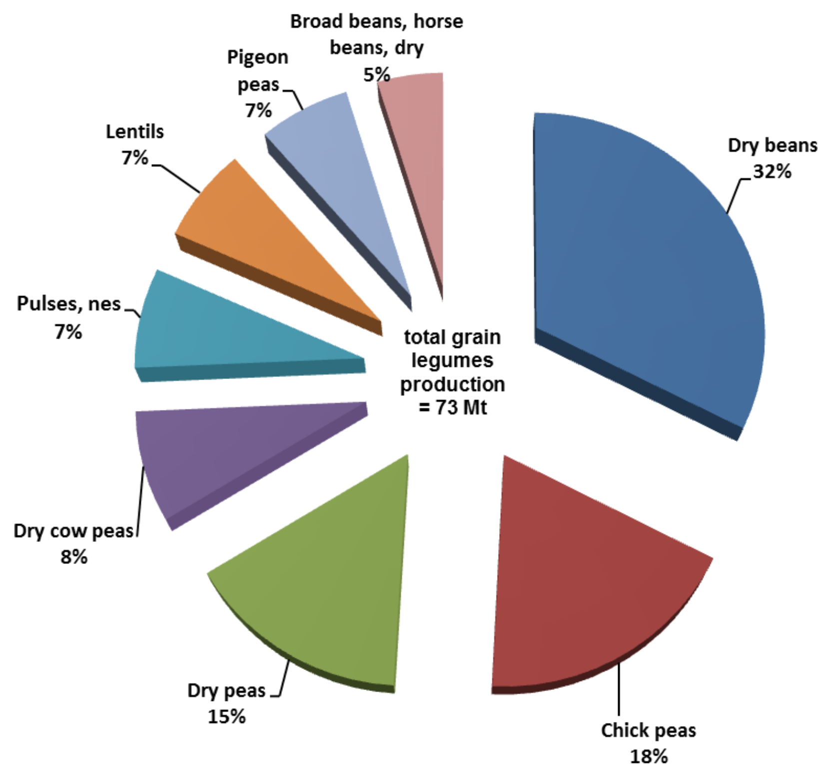


Figure 2. Composition of global grain legumes production in 2013 (FAOSTAT, 2013).

European agriculture relies extensively on nitrogen fertilizer, chemical pesticides and lacks diversity. It is thus highly important to re-introduce leguminous crops into farming systems and raise their profile through the revision of agricultural policies (Rubiales *et al.*, 2012). The EU has used subsidies to arrest declines in domestic pea production as a way to reduce reliance on imported protein crops for animal feed and to promote agroecological benefits. Subsidies were partly responsible for Spain increasing dry pea production to over 70% between 2009 and 2010, from 147,870 tons in 2009 to 251,575 tons in 2010 (FAOSTAT, 2013).

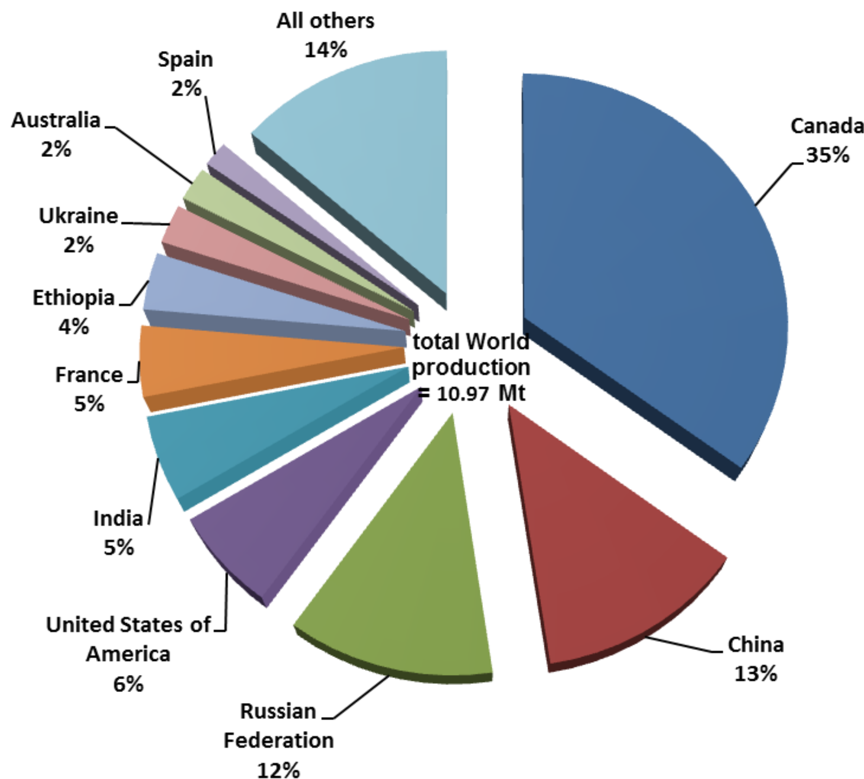


Figure 3. Dry pea production shares for the top ten producing countries worldwide in 2013 (FAOSTAT, 2013).

The pea crop is harvested for dried seed or in the immature state as fresh peas (green pea) for canning and freezing. Mature dry seeds are used in a wide range of forms. Approximately half of the dry pea world production is fed to livestock while the remaining half is used for human food, primarily in developing countries (Rubiales *et al.*, 2012). Dry mature seeds are used in diverse food specialties but can also be industrially processed to produce starch and protein concentrates. Fresh pea production for the canning and freezing industry includes the harvest of whole pods for use in oriental preparations and snap peas, which can be eaten fresh or frozen (Rubiales *et al.*, 2012). Pea is also grown for fodder or for green manure in Eastern Europe and Canada (Redden *et al.*, 2005).

Dry pea is nutritionally rich, containing 18 to 33% protein, 4 to 7% fibres, and 37 to 50% starch, and is consumed as a staple protein source in many developing countries. (McPhee, 2007; Rubiales *et al.*, 2012). Pea seed proteins are deficient in sulphur amino acids, but contain relatively high levels of lysine, making pea a good dietary complement to cereals. No major anti-nutritional factor is present in pea. Tannins, trypsin inhibitors, and lectins are relatively minor or can be easily counter-selected, and thus do not adversely affect crop use. Colour, appearance, flavour and dietary proprieties are significant criteria for pea used for human consumption (Rubiales *et al.*, 2012).

1.2.3. Genetic resources and molecular variations

Genetic resources of pea include both traditional cultivars and wild relatives. They are maintained both as *in situ* collections in centres of diversity and as *ex situ* seed collection in many centres for genetic resources distributed worldwide (Redden *et al.*, 2005). Historically, these centres were established to provide access to the large range of morphological variation and to the different gene pools found in pea accessions for taxonomic reference, research and to underpin breeding programs (Ambrose, 2008). These *ex situ* collections have a long history of active collaboration between each other and in supporting wider initiatives (Ambrose and Green, 1991). A working group for grain legumes exists as part of the European Cooperative Programme for Crop Genetic Resources which brings together the formal and informal sectors to collaborate on activities and initiatives of common interest such as the European central crop databases (ECP/GR; http://www.ecpgr.cgiar.org/Networks/Grain_legumes/grain_legumes.htm).

In the absence of a CGIAR institution with a global mandate for pea, an international consortium for pea genetic resources (PeaGRIC) has recently been formed that links together key collections within Europe, USA, ICARDA and Australia (Ambrose, 2008). A large number of *ex situ* germplasm collections for pea have been developed to provide long term conservation and ready access to the broad range of diversity from crop wild relatives, landraces, elite cultivars, mutants, mapping populations and host differentials for use by research and to underpin the requirements of breeders (Ambrose, 2008). Frankel (1984) proposed the formation of core collections, which are representative samples of the whole collection. A core comprising 10% of the whole collection should retain more than 70% of the alleles in the original collection, and can be composed to represent the major genetic and ecological categories in the collection (Redden *et al.*, 2005). Pea collections exist in the USDA (4384 *Pisum* spp. accessions), John Innes (3030 accessions), and ICARDA (5935 accessions) centers. Core collections for pea will be developed at both ATFCC Australia (2283 accessions) and at the Institute for Crop Germplasm Research (ICGR, China; 2292 accessions), as part of a collaborative project (Ambrose, 2008). These core collections can usefully include the wild relatives (Redden *et al.*, 2005). The pea collections have been found to be rich in genetic diversity for tolerance to a wide range of soil, mineral, and climatic stresses and for resistances to significant biotic stresses such as mildew, wilt, and viral diseases (Ambrose, 2008).

The pea genome size is ca. 4800 Mbp and spread across 2n = 2x = 14 chromosomes. Karyotypes were presented as early as 1931 (McPhee, 2007), showing

that chromosomes 1 and 2 have metacentric centromeres, while the remaining five chromosomes are subcentromeric. Chromosomes 4 and 7 contain secondary constrictions and possess satellites. *P. fulvum* contains a third secondary constriction on chromosome 5 (McPhee, 2007). Over the year, many pea genetic maps have been generated. These genetic maps are becoming increasingly well aligned as more markers are mapped and exchanged between mapping groups (Loridon *et al.,* 2005; Fondevilla *et al.*, 2011).

In recent years, the deployment of a range of molecular marker diversity studies in pea have had significant impact on the level of information that is available (Lu *et al.*, 1996; Ellis *et al.*, 1998; Pearse *et al.*, 2000; Burstin *et al.*, 2001; Vershinin, 2003; Baranger *et al.*, 2004; Tar'an *et al.*, 2005). The improved reliability of marker systems and the ability to develop them as high throughput systems (Flavell *et al.*, 2003) open the possibility to screen whole germplasm collections. The first such example in pea was the application of retrotranspon element markers to the entire John Innes *Pisum* collection that was initiated in 2000 (Tegerm, 2000; Jing *et al.*, 2010).

1.2.4. Breeding approach to improve peas

Pea was the original model organism used in Mendel's discovery of the laws of inheritance (1866), making it the foundation of modern plant genetics (Smýkal *et al.*, 2012). The easy cultivation of pea, its self-pollinating reproductive habit and genetic variation wealth made of pea an easy subject for genetic investigation and breeding (Mcphee, 2007). Pea genetic improvement began with domestication and has continued throughout history. Since that time, breeders discovered and introduced novel traits to adapt the indeterminate, tall, slender, bushy or climbing plants with small and coloured seeds to a short, determinate field combining crop with large seeds without tannins (Rubiales *et al.*, 2012).

Breeding objectives for pea vary depending on production region and end use. Primary objectives common to all regions and commodity types include increased yield, multiple disease resistance, and superior quality for the desired end use and agronomic adaptation to local production conditions (Redden *et al.*, 2005; McPhee, 2007). Classical breeding has made significant improvement in crop production and seed quality. The most widely practiced breeding method is the pedigree breeding system through transgressive segregation from crosses, bulk selection and single seed descent system. In addition, recurrent backcross selection is commonly used to introduce a single desired trait (Ambrose, 2008).

Many common diseases of pea can be controlled by single genes such as *er1*, *er2* and *Er3* conferring powdery mildew resistance, and plant breeding has been extremely successful in combining many of these genes in improved cultivars or germplasms (McPhee, 2007; Rubiales *et al.*, 2012). In addition, partial genetic resistance associated with quantitative resistance mechanisms have been described to several foliar fungal pathogens and parasitic weeds such as *Mycosphaerella pinodes*, *Uromyces pisi, Phoma medicaginis* var. *pinodella*, *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *Pseudomonas syringae* pv *pisi and Orobanche crenata* (Bevan *et al.*, 1995; Valderrama *et al.*, 2004; Fondevilla *et al.*, 2008; Barilli *et al.*, 2009a; Rubiales *et al.*, 2009a; Barilli *et al.*, 2010; Fondevilla *et al.*, 2010).

Several breeding programs aim the characterisation of the QTL responsible for the resistance to fungal pathogens and parasitic weeds in wild and cultivated pea genotypes, e.g., *Aphanomyces* partial resistance QTLs (*Aph1*, *Aph2*, and *Aph3*) (Pilet-Nayel *et al.*, 2005), ascochyta blight partial resistance QTLs (Timmerman-Vaughan *et al.* 2002; 2004; Prioul *et al.,* 2004; Fondevilla *et al.,* 2008) and *Uromyces pisi* partial resistance QTLs (Barilli *et al.*, 2010). However, Intraspecific and interspecific hybridization allow the exploitation of useful genes from wild, unimproved species for the benefit of the cultivated species. All known wild *Pisum* accessions are readily crossable to the cultigen (Redden *et al.*, 2005). *P. fulvum* is the only separate wild species, and it has been a useful source of disease and insect resistance (Redden *et al.,* 2005; Fondevilla *et al.,* 2007). The marker assisted selection (MAS) has been widely used in breeding programs. This approach was initiated with the mapping of isozyme in the early 1980s and the rapid improvement in marker systems in the recent years that increases its application ease and lowers its costs, has resulted in breeders becoming more interested in deploying MAS as they become available (Ambrose, 2008; Rubiales *et al.*, 2015). On the other hand, there is a long history of mutational breeding in pea where chemical or radioactive mutagenic agents are deployed on commercial cultivars to induce random mutations across the genome to induce novel traits that could be useful in crop improvement such as stem thickness or simultaneous flowering (Blixt *et al.*, 1991).

1.2.5. Main yield limitations

1.2.5.1. Abiotic stresses

Potential yields of major crops are usually reduced due to environmental constraints in addition to the biotic constraints (Rubiales *et al.*, 2012). Cold, drought and nutritional stresses are the most important environmental stresses affecting pea production. Most

pea cultivars are very susceptible to cold, especially pea lines with long internodes, large leaf area, early seed set or wrinkled seeds (Cousin, 1997). Autumn-sown peas may be exposed to prolonged freezing temperatures and snow in some regions, whereas in regions with less severe winters, frosts are very damaging, especially at flowering time (Redden *et al.*, 2005). Only a few lines from wild *Pisum* species or forage winter pea, probably originating from *P. s.* ssp. *arvense* had been identified as highly cold tolerant (Cousin, 1997). Winter hardiness is reported to be a quantitatively inherited trait (Markarian and Andersen, 1966; Auld *et al.*, 1983; Cousin, 1997; Lejeune-Henaut *et al.*, 2008).

Drought is the second most important abiotic stress of pea. Drought and high temperatures occur together in many environments important for legume production, and both stresses can interact to reduce yields (Rubiales *et al.*, 2012). Drought was shown to stop nitrogen fixation and to decrease biomass production (Cousin, 1997). On the other hand, the most important nutritional stress is salinity, critical levels of salt have been established and screening procedures developed to identify genetic tolerance to salt in pea (Saxena, 1993). Symbiotic nitrogen fixation is particularly sensitive to high salinity (Saxena, 1993).

1.2.5.2 Biotic stresses

1.2.5.2.1 Pests

Insects including wireworms, aphids, thrips, pea leaf weevil, pea weevil, loopers or cutworms can cause severe damage to crop plants and grain (Redden *et al.*, 2005). The most damaging pests of field pea is the pea weevil (*Bruchus pisorum*). The larvae of this bruchid reduce seed yield and quality by feeding on the developing seeds. Only one larva develops to maturity in an individual seed consuming large portion of that seed (Ali *et al.*, 1994). The incorporation of pea weevil resistance from the related wild species *P. fulvum* into pea appeared promising (Clement *et al.*, 2002).

O. crenata has been known to threaten pulse crops since antiquity and it is a severe constraint to the cultivation of grain legumes (Rubiales *et al.*, 2006; Rispail *et al.*, 2007). *O. crenata* is a major constraint for winter pea production in Mediterranean and East Asian countries. The problem is particularly severe for field pea, which is very sensitive to standard glyphosate treatments and in which little resistance has been identified (Rubiales, 2003; Rubiales *et al.*, 2009a). The low level of resistance and the absence of suitable control method have relegated pea cultivation to uninfested areas

which instead of controlling the broomrape problem is increasing both its intensity and infected acreage (Rubiales *et al*., 2008; Rubiales *et al*., 2009c).

Pea is also affected by a number of nematodes including root-lesion nematode (*Pratylenchus* spp.), root-knot nematode (*Meloidogyne* spp.) and pea cyst nematode (*Heterodera goettingiana*) to which little resistance is available (Rubiales *et al*., 2012).

1.2.5.2.2 Bacterial and virus diseases

Two very closely related plant pathogenic bacteria are associated with peas. *Pseudomonas syringae* pv. *pisi* is a seedborne pathogen that causes pea bacterial leaf blight, responsible to significant economic damage especially during spring and summer while the other bacterium, *P. syringae* pv. *syringae* (brown spot disease), although a less virulent pathogen, can cause severe disease following hail damage or excessively wet conditions (Mazarei and Kerr, 1991). At present seven physiological races of *P. syringae* pv. *pisi* are known (Cousin, 1997).

Viral diseases are important in most areas of the world where peas are grown (Redden *et al*., 2005). There are more than 50 viruses known to infect pea worldwide (Kraft and Pfleger, 2001), the most important among them is the Pea seedborne mosaic virus (PSbMV) that has been disseminated worldwide by infected seeds (Khetarpal *et al*., 1990; Cousin, 1997). The disease also spread by aphids which transmit the virus from plant to plant (Kraft and Pfleger, 2001). Pea is also affected by other viruses of minor importance including the Pea leafroll virus (PLRV) causing the Pea top yellow disease in Europe and the USA, Pea enation mosaic virus (PEMV) also named virus 1, Pea mosaic caused by Bean virus 2 (BV2), Pea common mosaic virus (PCMV), and Pea early browning virus (Cousin, 1997).

1.2.5.2.3 Diseases incited by oomycete

Aphanomyces root rot, caused by *Aphanomyces euteiches,* is a soil-borne pathogen responsible for soft honey-brown rotting of the roots and epicotyls resulting in yellowing and stunting of aerial plant parts, and then death of the plant (Rubiales *et al*., 2012). It is one of the most widespread and destructive pea diseases, affecting more particularly North America, New Zealand, Europe, and Japan. Despite good cultural practices, the disease remains very destructive regionally and no effective fungicides are yet available to control this disease. In addition, commercial varieties with high level of resistance are still not available (Redden *et al*., 2005). Downy mildew caused by *Peronospora viciae* f.sp. *pisi,* is widely distributed all over the world, causing systemic

infection of seedlings, local infections on leaves and pod infections (Rubiales *et al.*, 2012). This disease occurs in periods of cool moist weather and is widespread. Regionally, symptoms can vary from local to systemic (Redden *et al.*, 2005).

1.2.5.2.4 Fungal diseases

Variation in pea yield is often associated with fungal disease. Major diseases of this crop include foliar and root fungal diseases. Pea growth is subject to various necrotrophic and biotrophic fungal foliar diseases. The major foliar necrotrophic fungal disease ascochyta blight is caused by a complex of three fungal species, *Ascochyta pisi, Didymella pinodes* and *Phoma medicaginis* var. *pinodella. D. pinodes* is the most prevalent and damaging among this complex (Rubiales *et al.*, 2015). *Phoma koolunga* and *P. glomerata* have also been reported to be part of this complex in Australia (Davidson *et al.*, 2009; Tran *et al.*, 2014). These diseases are widespread wherever peas are grown and are particularly a problem where peas are grown over mild and wet growing conditions, such as Australia (Redden *et al.*, 2005) and it constitutes the second major constraint for the crop after broomrape in the Mediterranean basin (Rubiales *et al.*, 2003).

The most important pea foliar diseases caused by biotrophic pathogens are pea powdery mildew and pea rust. Pea powdery mildew caused by *Erysiphe pisi,* is an air-borne disease with a worldwide distribution (Rubiales *et al.*, 2009b). Recently *E. baeumleri* and *E. trifolii* has also been reported to cause powdery mildew on pea (Ondrej *et al.*, 2005; Attanayake *et al.*, 2009; 2010). The disease can cause severe damage if it occurs early in the season, prior to flowering. It is most prevalent in subtropical regions and in temperate areas (Kraft and Pfleger, 2001). Pea rust has become an important pathogen of dry pea from the mid-1980s particularly in regions with warm, humid weather. Pea rust has been reported to be caused either by the fungus *Uromyces viciae-fabae* (syn. *U. fabae*) or *U. pisi*. *U. viciae-fabae* is the principal causal agent of pea rust in tropical and subtropical regions such as India and China (Kushwaha *et al.*, 2006). Whereas in temperate regions *U. pisi* is predominant (Barilli *et al.*, 2009b). Other more regionally important foliar fungal diseases includes sclerotinia white mold, botrytis grey mold, anthracnose, septoria blotch and alternaria blight (Laflamme, 1998; Redden *et al.*, 2005).

Soil-borne diseases are considered the main limiting factor in increasing and stabilizing pea yields (Kraft, 1994). Among them, fusarium root rot caused by *F. solani* f.sp. *pisi* and fusarium wilt caused by *F. oxysporum* f.sp. *pisi* are the most important treat to pea cultivation. Fusarium root rot, caused by *Fusarium solani* f.sp. *pisi*

(anamorph of *Nectria haematococca*), causes brownish-black lesions which coalesce and ultimately destroy the roots, mainly in North America and Europe, and it can be highly destructive in both irrigated and dryland farming systems. Currently, control is through the use of long crop rotations from infested fields (Kraft and Pleger, 2001). No commercial cultivars are resistant to fusarium root rot. Germplasms with partial resistance are being identified, and breeding effort is leading to the development of varieties with higher level of resistance (Kraft and Pleger, 2001).

Fusarium vascular wilt disease caused by the soil-borne pathogen *F. oxysporum* f.sp. p*isi* (*Fop*) has been reported in all countries where peas are grown and can be particularly severe where short rotations are practiced (Kraft and Pleger, 2001). Four races of *Fop* have been described. Races 1 and 2 occur worldwide, while races 5 and 6 are only important in western Washington State (Infantino *et al.*, 2006). The only economic way to control wilt is through resistant varieties (Kraft and Pleger, 2001). Fungal seed and seedling diseases such as pythium (caused by *Pythium* spp.) and rhizoctonia (caused by *Rhizoctonia solani*) (seed and seedling rot) are of relatively minor importance and are mostly controlled with seed fungicide treatments and cultural practices. Thielaviopsis root rot caused by *Thieimriopsis hicola*, is considered a serious root rot pathogen of peas in some pea growing areas of the United States (Tu, 1987).

2. Fusarium wilt

Fusarium wilt caused by *Fusarium oxyporum* is a major limiting factor in the production of many agricultural and horticultural crops, including pea, banana, date-palm, cabbage, cotton, flax, onion, tomato, watermelon, china ester, carnation, chrysanthemum, gladioli and tulip (Armstrong and Armstrong, 1981; MacHardy and Beckman, 1983).

2.1. Causal agent (*Fusarium oxysporum*).

F. oxysporum is without a doubt the most economically important species of the *Fusarium* genus given its numerous hosts and the level of losses that can result when it infects a plant (Leslie and Summerll, 2006). The genus *Fusarium* belonging to the Nectriaceae family of Hypocreales order and the Sordariomycete class within the Ascomycete phylum combines many cosmopolitan species colonizing the soil, air and organic materials. Phytopathogenic *Fusarium* species can infect both the shoot and root of plant host. Most plant pathogenic *Fusarium* species are soil-borne hemibiotroph that incorporate both biotrophic and necrotrophic infection strategies (Ma *et al.*, 2013). The word fusarium derives from the Greek word "Fusiform" meaning engorged in the

middle and pointed toward the ends corresponding to the typical form of the spores (Nelson *et al.*, 1983). This genus has been deeply studied by Snyder and Hansen, who suggested nine species (Snyder and Hansen, 1945) of which *F. oxysporum* is the most common species worldwide recovered from most soils including arctic, tropical or desert (Di Pietro *et al.*, 2003) and found in almost all agricultural soils (Kistler, 2001). Soil population of *F. oxysporum* species includes both pathogenic and non-pathogenic strains (Nelson *et al.*, 1983; Gordon and Okamoto, 1992).

F. oxysporum is a haploid soil-borne fungus with no known sexual cycle although vegetative reproduction by the mean of heterokaryon formation after hyphal fusion of different isolates has been described (Di Pietro *et al.*, 2003). Members of the *F. oxysporum* species complex are capable of causing wilt diseases in over one hundred agronomically important plant species (Beckman, 1987). However, individual *F. oxysporum* isolates often exhibit a high degree of host specificity leading to the description of more than 120 *formae speciales* of *F. oxysporum* to classify isolates according to their host (Armstrong and Armstrong, 1981; Kistler, 1997). In several cases, *F. oxysporum formae speciales* consist of multiple, independent lineages that evolved polyphyletically through convergent evolution (Ma *et al.*, 2013). *Formae speciales* are further subdivided in races to classify each isolates according to their virulence pattern on different cultivar host (Armstrong and Armstrong, 1975; Kistler, 1997). Races are defined by their differential interaction with host genotypes (Armstrong and Armstrong, 1975), which, in some cases, are cultivars known to carry one or more major genes for resistance (Gordon and Martyn, 1997).

2.1.1. Morphological and genetic characterisation

Morphological characters are by far the most commonly used criteria to identify *Fusarium* species (Leslie and Summerll, 2006). The main morphological features of *F. oxysporum* include the production of microconidia in false heads on short phialides formed on the hyphae, the production of chlamydospores and the shape of the macroconidia and the microconidia (Fuskey, 1996; Watanabe, 2002; Leslie and Summerll, 2006). Microconidia are small (6-15.8 µm × 1.9-3.7 µm), oval or club shaped, hyaline and generally without septa although one septa was occasionally described. They are produced by monophialides and occasional polyphialides. Macroconidia are medium size (29.1-45 µm x 2.9-4.7 µm), slender, thin walled, hyaline and straight to slightly curved. They are initially composed of 3 septa although older macroconidia can contain up to 5 septa. In addition macroconidia apical cells are short and tapered while basal cells are notched or foot shaped. Chlamydospores are intercalary or terminal, spherical, occurring singly or in groups of two to three and

present a smooth or rough-walled surface. Chlamydospores are hyaline to pale yellow in colour and are comprised between 10.2-15 µm in diameter (Fig. 4). Sclerotia are rare in culture. When found sclerotia are 1-2 mm diameter structures distributed over the mycelium or in groups and present a variety of colour from pale brown to violet (Fuskey, 1996; Watanabe, 2002; Leslie and Summerll, 2006).

Asexual reproduction in *F. oxysporum* is accomplished by macroconidia, microconidia, chlamydospores and hyphal fragments (Leslie and Summerell*,* 2006). The absence of sexual recombination and the similarity of morphological characters between differents f*ormae speciales* and between pathogenic races and non-pathogenic strains of *F. oxysporum* imply the use of other genetic means to characterize *F. oxysporum*. Using a technique previously applied to *Aspergillus* based on the ability of nitrate reductase mutants to form a heterokaryon and to grow as wild-type on a nitrate-containing medium, Puhalla classified *F. oxysporum* isolates into vegetative compatibility groups (VCGs) (Puhalla 1985). In general individual members of the same VCG are genetically similar, having the same incompatibility alleles at all loci and therefore able to exchange nuclear material (Glass *et al.*, 2000). Studies on the vegetative compatibility of 18 *formae speciales* of *F. oxysporum* identified 167 VCGs, in which some *formae speciales* including the *F.o.* f.sp. *lycopersici* and *F.o.* f.sp. *asparagi* were represented by several VCGs, while seven *formae speciales* were only represented by one VCG. Interestingly, VCGs were always composed of isolates from a single *forma specialis* (Yoo *et al.*, 1993; Mes *et al.*, 1994; Takehara and Kuniyasu, 1994; Marlatt *et al.*, 1996; Tantauoi *et al.*, 1996; Henni *et al.*, 1998; Katan and Di Primo, 1999; Rosewich *et al.*, 1999). Studies on *F.o.* f.sp. *pisi* (*Fop*) have showed a high degree of coincidence between vegetative compatibility grouping and race structure revealing 4 VCGs. The first VCG contains all isolates from races 1 and 6, the second VCG regroups all isolates from race 5 while isolates from race 2 are found in two separate VCGs , termed 2A and 2B (Correll *et al.*, 1987; Whitehead *et al.*, 1992; Grajal-Martin *et al.*, 1993; Kraft, 1994).

Molecular tools were also used to study the relationship among individuals and classify *F. oxysporum* isolates on genetic bases. Restriction fragment length polymorphism (RFLP) was used to distinguish 3 races of *Fop* (Coddington *et al.*, 1987) and to distinguish *formae speciales* on cucurbitaceae using mitochondrial DNA (Kim *et al.*, 1992). Amplified fragment length polymorphism (AFLP) (Vos *et al.*, 1995) was used to evaluate phylogenetic relationships among different *formae speciales* and proved to be an interesting tool to distinguish between them (Baayen *et al.*, 2000). Random amplification of polymorphic DNA (RAPD) analysis has also been widely used to distinguish within non-pathogenic and pathogenic isolates of many *formae speciales*

including *F.o.* f.sp. *dianthi* (Manulis *et al.*, 1994; Migheli *et al.*, 1998), *F.o.* f.sp. *vasinfectum* (Assigbetse *et al.*, 1994), *F.o.* f.sp. *ciceris* (Kelly *et al.*, 1998), *F.o.* f.sp. *albedinis* (Fernandez and Tantaoui, 1994; Tantaoui *et al.*, 1996), and *F.o.* f.sp. *basilici* (Chiocchetti *et al.*, 1999).

The genome of *F. oxysporum* was initially sequenced by the Broad Institute in 2007 (Cuomo *et al.*, 2007). Ma *et al.* (2013), show that the size of the completely sequenced *F. oxysporum* genomes is 61 Mb. Comparative genomics analysis revealed lineage-specific (LS) genomic regions in *F. oxysporum* that include four entire chromosomes and account for more than one-quarter of the genome. LS regions are rich in transposons and genes with distinct evolutionary profiles but related to pathogenicity, indicative of horizontal acquisition (Ma *et al.*, 2010).

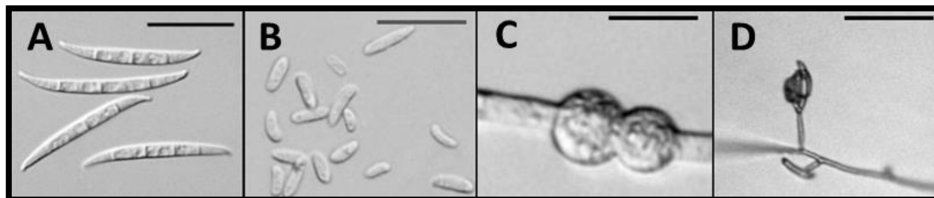


Figure 4. Microscopic characters of *F. oxysporum* (**A**: macroconidia, **B**: microconidia, **C**: chlamydospores, **D**: microconidia *in situ* produced on leaf-piece agar (CLA). A-C: scale bar = 25 µm; D: scale bar = 50 µm. (Leslie and Summerell, 2006).

2.2. Plant-pathogen interaction

The interactions between plants and pathogens involve the exchange of complex molecular dialogue. First, the pathogen is able to sense and recognize the plant host and to change its metabolism to provide favourable conditions for pathogenicity (Alfano and Collmer, 2004). Meanwhile, plants have evolved to identify structures associated with pathogens, leading to strengthening of existing defences and development of other powerful defence mechanisms.

2.2.1. Pathogenesis in *Fusarium oxysporum*.

Pathogenesis describes the complete process of disease development in the host, from initial infection to production of symptoms. Plant-pathogenic fungi have evolved a wide range of mechanisms to invade their hosts, overcome their defences, and colonize the host tissue, thereby causing disease. Successful plant infection by *F. oxysporum* is a complex phenomenon that requires a series of highly regulated processes including 1) recognition of host roots through unknown host signals, 2) root surface attachment and differentiation of penetration hyphae, 3) penetration of the root

cortex and degradation of physical host barriers such as the endodermis in order to reach the vascular tissue, 4) adaptation to the hostile plant environment, including tolerance to plant antifungal compounds, 5) hyphal proliferation and production of microconidia within the xylem vessels, and 6) secretion of virulence determinants such as small peptides or phytotoxins (Di Pietro *et al.*, 2003). During the initial stages of the interaction, fungal pathogens must sense stimuli from the plant and respond with appropriate morphogenetic and biochemical changes. This signalling process represents the first and most critical step in defining the outcome of fungal infection, i.e. success or failure in establishing disease (Roncero *et al.*, 2003).

As a soil inhabitant, *F. oxysporum* can survive extended periods in the absence of the host, mainly in the form of thick-walled chlamydospores. The proximity of roots induces the dormant propagules to germinate and initiate infection (Di Pietro *et al.*, 2003). Root exudates are reported to be responsible for chlamydospore germination of several root rot and wilt pathogens (Huisman, 1982). After germination, infection hyphae adhere to the host roots (Bishop and Cooper, 1983a; Di Pietro *et al.*, 2003) and penetrate them directly (Rodriguez-Gálvez and Mendgen, 1995) although the formation of appressorium-like structure have been sometime described prior to root penetration (Czymmek *et al.*, 2007). The mycelium then advances inter- or intracellularly through the root cortex, until it reaches the xylem vessels and colonize it (Bishop and Cooper, 1983b; Beckman, 1987; Baayen *et al.*, 1989; Benhamou *et al.*, 1996; Olivain and Alabouvette., 1999; Benhamou and Garand, 2001; Olivain *et al.*, 2006; Czymmek *et al.*, 2007; Zvirin *et al.*, 2010). After xylem vessels colonization, the fungus progress vertically through them to colonize stem host (Bishop and Cooper, 1983b; Beckman, 1987). The characteristic wilt symptoms appear as a result of severe water stress, mainly due to vessel clogging (Di Pietro *et al.*, 2003). Wilting is most likely caused by a combination of pathogen activities, such as the accumulation of fungal mycelium and/or toxin production and host defence responses, including production of gels, gums and tyloses, and vessel crushing by proliferation of adjacent parenchyma cells (Beckman, 1987; Di Pietro *et al.*, 2003) or by degradation of the host tissues (Kroes *et al.*, 1998; Olivain and Alabouvette, 1999; Ouellette *et al.*, 2002). After wilting and plant death, the fungus reaches the plant surface where it produces chlamydospores that are dispersed onto the soil for a second cycle of infection (Di Pietro *et al.*, 2003).

Several virulence factors have been reported to play a role at different stages of the infection process to induced disease and counteract the plant defence reaction (Roncero *et al.*, 2003). The soil-borne plant pathogen *F. oxysporum* have adapted a network of highly conserved signalling pathway to sense and respond to environmental cues by appropriate changes in gene expression, including those leading to host

recognition, root penetration, breakdown of host defences, proliferation within the host tissue and establishment of disease (Di Pietro *et al.,* 2001; Michielse and Rep, 2009; Rispail *et al.*, 2009; Rispail and Di Pietro, 2010). Virulence function of pathogenic fungi was shown to be mainly mediated by two highly conserved signalling pathways the cyclic adenosine monophosphate (cAMP) and a mitogen-activated protein kinase (MAPK) orthologous to the yeast mating MAPK Fus3 (Lengeler *et al.*, 2000). *F. oxysporum* virulence function was shown to be mainly controlled by the Fus3 orthologue Fmk1 and its main target Ste12 (Di Pietro *et al.*, 2001; Rispail and Di Pietro, 2009), although cAMP-PKA pathways was also shown to play a role in its virulence (Jain *et al.,* 2003; Jain *et al.*, 2005; Delgado-Jarana *et al.*, 2005; Michielse and Rep, 2009).

Once in contact with the root, the fungus needs to penetrate the cell walls (Agrios, 2005). Some of the best studied virulence factors are the cell wall-degrading enzymes (CWDEs). *F. oxysporum* secretes an array of CWDEs, such as polygalacturonases (PGs), pectate lyases (PLs), xylanases and proteases, during root penetration and plant colonization (Beckman, 1987; Agrios, 2005). Accordingly, CWDEs were greatly produced by *Fop* inside the plant implying the importance of CWDEs in the pathogenicity of *Fop* in pea (Benhamou and Garand, 2001). EndoPG is the first enzyme activity detected in *F. oxysporum* cultures on tomato cell walls (Jones *et al.*, 1972). Pectate lyases catalyse the trans-elimination of pectate and have been suggested to play an important role in the development of vascular wilt (Beckman, 1987). Indeed, the presence of a specific PL isozyme, PL1, was detected in tomato root and stem tissues infected by *F.o.* f.sp. *lycopersici* (Di Pietro and Roncero, 1996).

On the other hand, *F. oxysporum* has been known for a long time to produce phytotoxic secondary metabolites (phytotoxins) that have been implicated in pathogenesis of *F. oxysporum* (Gáumann, 1958; Roncero *et al.*, 2003). Despite the production of numerous toxins by other *Fusarium* spp., *F. oxysporum* is known to produce only a limited number of toxins (Nelson *et al.*, 1981). The most well-known toxin produced by *F. oxysporum* is fusaric acid. At high concentration fusaric acid induces many physiological responses in plant cells including alteration of cell growth, mitochondrial activity and membrane permeability while at lower concentration, it could trigger plant defence reactions and programmed cell death (Bouizgarne *et al.*, 2006; Jiao *et al.*, 2013). It was also shown to induce wilt symptoms on pepper and cucumber (Sarhan and Hegazi, 1988; Wang *et al.*, 2012). Apart from fusaric acid, some *F. oxysporum* isolates have been shown to produce additional toxins such as beauvericin, enniantin B, bikaverin, moniliformin, fumonisin and trichothecene (Mirocha *et al.*, 1989;

Moretti *et al.*, 2002; Son *et al.*, 2008; Irzykowska *et al.*, 2012) that can also contribute to their pathogenicity.

In addition, *F. oxysporum* has developed different strategies to tolerate antifungal compounds produced by host plant. These include modification of the membrane sterol content (Défago *et al.*, 1983), regulation of specific chitin synthase genes which have been recently shown to be important *F. oxysporum* pathogenicity factors (Madrid *et al.*, 2003; Martín-Urdiroz *et al.*, 2008; Pareja-Jaime *et al.*, 2010) and secretion of the enzymes that degrade and detoxify these antifungal compounds as has been shown with the pea phytoalexin pisatin. Depending on their pisatin-demethylating ability, naturally occurring *F. oxysporum* isolates are either incapable of degrading pisatin (Pda2) or degrade it slowly (PdaL) or fastly (PdaH), and their degrading ability paralleled their ability to cause disease walls (Agrios, 2005).

2.2.2. Mechanisms of plant defences

Plants evolved several pathways and different defence mechanisms to interact with pathogens, defend their invasion and protect themselves from pathogen damage. Such mechanisms are represented by a number of physical and biochemical changes (Beckman, 1987; VanEtten *et al.*, 1994; Grayer and Kokubun, 2001; Agrios, 2005).

The first plant defence against many potential pathogens is the production of antifungal compounds which inhibit spore germination and germ tube elongation (Grayer and Kokubun, 2001). If this first barrier is surpassed, the cell attempts to limit the spread of the infection by several ways. One common response is the formation of cell wall appositions that has been shown to be induced quickly after the onset of fungal penetration, sometime in a matter of only a few hours (Vidhyasekaran, 2008). These appositions are called papillae (Bishop and Cooper, 1984) and they typically comprise a callose matrix (Brammall and Higgins, 1988; Beckman *et al.*, 1989; Tessier *et al.*, 1990) variously incorporating cellulose, suberin, calcium, lignin, and silicon (Newcombe and Robb, 1988; Ouellette *et al.*, 2002; Vidhyasekaran, 2008). In the first stage of the cell wall hydrolysis, mechanical mechanisms are involved, using the suberin, lignin and other phenolic compounds of the cell wall to restrain the pathogenic CWDEs action on the host cell wall. However, lignin can be deposited over the entire wall of the infected cell or group of cells, or only at the infection site (Walters *et al.*, 2007). Lignin is a heterogeneous polymer formed by the polymerization of up to three components: coumaryl, coniferyl and sinapyl alcohols (Strange, 2003). Given its chemical and mechanical properties, the lignin represents not only a tremendous barrier against pathogens but also restricts the diffusion of enzymes and toxins from

the fungus to the plant and the nutrient and water from the plant to the fungus (Ride, 1978; Walters *et al.*, 2007). In addition, lignin precursors such as coniferyl alcohol and free radicals may be toxic to the pathogen *per se* and in some instances the hyphae of invading fungi may be lignified (Ride, 1978). Like lignin, suberin is a constituent of healthy plant tissue and its synthesis may be enhanced through pathogen-induced defences. These layers are impermeable and help strengthen the cell wall and limit water and nutrients loss (Strange, 2003). A second chemical mechanism occurs at later stages to prevent the production of CWDEs by the pathogen. This inhibition is linked to the participation of esterified phenols in the cell wall (El Modafar and El Boustani, 2002; Vidhyasekaran, 2008). Alternatively, cells of the vascular tissue may produce vascular occlusions such as gels, gums and tyloses or lead to vessel crushing by proliferation of adjacent parenchyma cells (Beckman and Halmos, 1962; Beckman, 1964; Elgersma *et al.*, 1972; Vandermolen *et al.*, 1977; Bishop and Cooper, 1983a; MacHardy and Beckman, 1983; Bishop and Cooper 1984; Baayen and Elgersma, 1985; Beckman, 1987; Charchar and Kraft, 1989; Ouellette *et al.*, 1999). The production of the vascular occlusion materials by adjacent parenchyma cells and their accumulation within xylem vessels can block the advance of fungal hyphae into plant (Beckman, 1987; Agrios, 2005).

The production of antifungal compounds is also a basic plant defence strategy (Lamb and Dixon, 1997; Dixon, 2001). It includes the production and accumulation of these antifungal compounds such as phenolic compounds and pathogenesis-related (PR) proteins in host plant tissues, including xylem fluids (Shi *et al.*, 1992). However, concentration of elemental sulphur and specific classes of pathogenesis-related (PR) proteins was shown to dramatically increase in the xylem following inoculation with *F. oxysporum* (Stintzi *et al.*, 1993; Rep *et al.*, 2002; Williams *et al.*, 2002; Rep *et al.*, 2004). Due to its instrumental role in fungal growth, the chitin is a natural target of plant antifungal proteins such as chitinases (Iseli *et al.*, 1993; Palomares-Rius *et al.*, 2011), beta-1,3-glucanase (Palomares-Rius *et al.*, 2011) or lectins (Ciopraga *et al.*, 1999). The application of these proteins to fungal mycelium results in lysis of hyphal tips, swelling and vacuolation (Iseli *et al.*, 1993; Ciopraga *et al.*, 1999). Phenolic compounds play an important role in the resistance process against vascular diseases (Beckman, 2000), they are precursors of several secondary metabolites involved in disease resistance, such as phytoalexins and structural barriers such as lignin (Matern *et al.*, 1995). Flavonoids are a large class of phenolic compounds that occur in most plant tissues especially in specific plant/pathogen combinations. Many of them are inhibitory or toxic to pathogens (Agrios, 2005). Phytoalexins are a large group of low molecular weight that are toxic and inhibit the plant pathogens (Sweigard *et al.*, 1985; Mert-Türk, 2002;

Agrios, 2005). Most of the legume phytoalexins have an isoflavonoid skeleton derived from mixed acetate-malonate and shikimate pathways. The main legume phytoalexins kievitone and phaseollidin produced by *Phaseolus vulgaris*, medicarpin and maackianin by *Medicago spp.* and *Cicer arietinum* and pisatin by *P. sativum* (Pedras *et al.*, 2005). On the other hand, phytopathogenic fungi have been shown to detoxify phytoalexins produced by plants of the Leguminosae family (Agrios, 2005; Pedras *et al.*, 2005). For instance, the pea pathogens *Ascochyta pisi* and *F. oxysporum* could degrade pisatin to a non-toxic metabolite (Uehara, 1964; Vanetten *et al.*, 2001).

Small antimicrobial peptides (García-Olmedo *et al.*, 1998) and reactive oxygen species (ROS) (Lamb and Dixon, 1997) can also be produced in response to pathogen infections. The oxidative burst is one of the earliest typical events in a plant-pathogen interaction (Averyanov, 2009). Production of ROS lead to the cross-linking of cell wall proteins, rendering plant cell walls more resistant to attack by fungal enzymes (Keen, 1999). ROS have also been considered as signalling agents leading to the hypersensitive reaction and additional active defence responses (Alvarez *et al.*, 1998). However, resistance to these compounds is considered a prerequisite for pathogenesis (Morrissey and Osbourn, 1999).

2.3. Fusarium wilt of pea

2.3.1. Disease symptoms

Disease caused by *Fop* often result in a vascular wilting and death of pea plant. Symptoms due to *Fop* races 1, 5 and 6 include downward curling of leaves and stipules, basal internode thickening, the leaves and stems becoming more brittle and rigid, progressive yellowing and necrosis of leaves from the base to the apex of the plant. Fusarium wilt develops rapidly resulting in plant death. While the external root system appears normal, the vascular tissue of root and stem may turn yellow to orange. This vascular discoloration often extends to the upper stem (Hagedorn, 1984; Kraft, 1994; Kraft *et al.*, 1998; Kraft and Pfleger, 2001; Biddle and Cattlin, 2007). In fields where races 1, 5, and 6 are prevalent, symptoms usually occur at any stage of growth in small to large patches of dead or dying plants, which gradually enlarge over time (Hagedorn, 1984; Kraft, 1994; Kraft *et al.*, 1998; Kraft and Pfleger, 2001).

Race 2 of fusarium wilt is also known as near wilt (Biddle and Cattlin, 2007). Symptoms of this race on individual plants are similar to symptoms of the other *Fop* races, with unilateral wilting sometimes moving asymmetrically up one side of the plant before the other (Kraft and Pfleger, 2001). Infection with race 2 can also cause

secondary cortical decay in roots and stems (Kraft and Pfleger, 2001). Vascular discoloration caused by race 2 is generally more intense than the other races ranging from orange to dark red (Kraft, 1994; Kraft *et al.*, 1998; Kraft and Pfleger, 2001). However, symptoms of race 2 usually occur later in the growing season, often at time of flowering and pod setting so that field symptoms only consist of occasional plants exhibiting symptoms unless the inoculum level is extremely high. For this reason these symptoms have been described as near wilt (Kraft and Pfleger, 2001; Biddle and Cattlin, 2007).

2.4. Disease history

Pea fusarium wilt disease was first described and distinguished from fusarium root rot by Jones and Linford (1925), who named it "an undescribed wilt disease". At that time, the disease was found in 50 fields in Wisconsin, and caused greater losses in some areas than those reported for root rot. The causal organism was originally named *F. othoceras* var. *pisi* (Linford, 1928). The pathogen was later named race 1 of *F. oxysporum* f.sp. *pisi* in 1935 (Goth and Webb, 1981). Resistance to this race was quickly found and introduced in pea cultivars leading to the creation of wilt resistant varieties that gave complete control. Wade (1929) determined that resistance to race 1 was inherited as a single dominant gene. With the emergence of resistant pea cultivars, the problem caused by this *Fop* race 1 were solved in the United States until 1972 (Kraft *et al.*, 1974). Since then, problems caused by *Fop* race 1 have not been eliminated but the disease is under control through the growing of resistant cultivars.

A second wilt of pea was observed and named near-wilt (race 2) because symptoms appeared later in the growing season (Hagedorn, 1984). Isolates of this new race was capable of wilting plants resistant to race 1 and plants infected are most often scattered throughout the field rather than being concentrated in specific areas as with race 1. Hare *et al.* (1949) determined that resistance to race 2 was also controlled by a single, separate, dominant gene not linked with the race 1 resistance gene. Delwiche Commando was the first cultivar developed possessing resistance genes for both races 1 and 2 (Goth and Webb, 1981). Races 3 and 4 were described in the Netherlands and Canada, respectively (Schreuder, 1951; Bolton *et al.*, 1966). However, they were later reclassified as more virulent isolates of race 2 (Huebbeling, 1974; Kraft and Haglund, 1978; William *et al.*, 1979; Kraft and Pfleger, 2001).

In 1970, race 5 was described in north-western Washington (Haglund and Kraft, 1970), where all commercial cultivars resistant to races 1 and 2 were susceptible. Because of the short crop rotations and favourable climate in that area for wilt

development, race 5 spread rapidly and affects 4,000 to 12,000 ha of planted peas each year. Resistance to race 5 was also attributed to a single dominant gene in the host. In 1979, a new race of wilt was again described from western Washington, which was pathogenic on cultivars and breeding lines resistant to races 1, 2 and 5, and was named race 6 (Haglund and Kraft, 1979). In genetic studies of inheritance, resistance was again attributed to a single dominant gene (Kraft, 1994). The pathogenicity of races 1, 2, 5 and 6 of *Fop* can be distinguished by their reaction on the differential varieties which contain dominant genes for resistance towards each race (Kraft, 1994; Kraft and Pfleger, 2001). Race 1 and 2 can be economically important in most pea growing areas of the world, whereas races 5 and 6 are reported to cause economic losses to pea crops primary in north-western Washington state and British Colombia (Haglund and Kraft, 1979).

2.5. Epidemiology and disease control

Fop fungi can survive in soil for more than 10 years in the absence of growing peas. Survival is related to the association of the fungus with the roots of non-host crops. In addition, *F. oxysporum* can be carried from one field to another on farm equipment, on contaminated soil or plant refuge by running water, wind and people. Although movement of strains as a result of human activities is clearly the dominant factor on the establishment of new infestations of fusarium wilt (Gordon and Martyn, 1997). *Fop* dissemination can also occurs through transport of contaminated and infected seed, especially in the case of race 2 (Kraft and Pfleger, 2001). Once introduced into a new field, the fungus need first to increase its number leading to a latency period of a few years before an appreciable amount of disease become evident (Hagedorn, 1984; Kraft, 1994; Kraft *et al.*, 1998; Kraft and Pfleger, 2001).

As indicated above, field symptoms caused by races 1, 5 and 6 are similar and consisted in small infected areas in the field. With the increase in inoculum potential brought about by repeated planting of susceptible cultivars, these areas enlarge and coalesce, forming irregular areas of dead plants (Hagedorn, 1984; Kraft and Pfleger, 2001). The degree of loss and spread in the individual fields depend on inoculum potential, the temperature (20-21 °C optimal) and cultivars (Kraft and Pfleger, 2001). Race 2 is almost universally distributed in pea-growing areas and often causes death of 1-3% of plant in infected fields. However, under conditions ideal for the pathogen (coarse-textured soils, 25 °C), losses may be severe, and entire fields may be destroyed (Hagedorn, 1984; Kraft and Pfleger, 2001).

The control of the pathogen is mainly based on three strategies including farming practices, application of agrochemicals and use of resistant varieties (Guimarães *et al.*, 2007). Crop rotation is the best farming practice that can contribute to reduce the damage caused by fungal pathogen. However, crop rotation has only minor effect in solving pea fusarium wilt problem not only because *Fop* chlamydospores survive for a very long time in the soil but also because inoculum can multiply on the roots of symptomless carrier plants. Nevertheless, a rotation which includes peas no more frequently than once in 5 years will help to prevent the disease building up to damaging levels (Biddle and Cattlin, 2007).

Soil fumigation with a broad-spectrum fungicide is another potential control method that provides a good initial control (Keiko and Mashita, 2005), although soil re-colonization by *Fop* occurs very quickly. In addition, soil fumigation is too expensive for field application and owing to the negative impact of chemicals on non-target organisms and the potential of environmental and health risk, the range and rate of fungicides used has been gradually limited and some of them were phased out. E.g., methyl bromide identified as Class I stratospheric ozone depleting substance, used as a pre-plant soil fumigant for over 40 years, is being phased out worldwide until 2015 (Duniway, 2002).

In the last several years, biological control of fusarium wilt has given encouraging results. Control may use the antagonistic fungi such as non-pathogenic strains of *F. oxysporum*, *Trichoderma* and *Gliocladium*, *Pseudomonas fluorescens* and *Burkholderia cepacia* bacteria, and others (Pal and McSpadden-Gardener, 2006). Although promising, none of these organisms have been used to control fusarium wilt in practice so far. While these control methods had only moderate effect, the use of resistant pea varieties was found the only practical and economical measure to control the disease in the field (Kraft and Pfleger, 2001).

As previously stated, Genetic resistance to race 1, 2, 5 and 6 of *Fop* is reported to be conferred by independent single dominant genes (Coyne *et al.*, 2000; Kraft and Pfleger, 2001). Resistance to race 1 is controlled by a single dominant gene, *Fw*, which is bred into most cultivars currently grown. The gene was localized to linkage group III of pea genetic map (Grajal-Martin and Muehlbauer, 2002). Resistance to race 2 is also conferred by a single dominant gene, *Fwn* (Kraft and Pfleger, 2001) although it has recently been suggested that more genes might be involved (Infantino *et al.*, 2006). Resistance to race 5 is controlled by a single dominant gene *Fwf* located on linkage group II close to the RAPD marker “U693a” (Coyne *et al.*, 2000; Okubara *et al.*, 2002).

The gene for resistance to race 2 has not yet been assigned to a chromosomal location and the gene for resistance to race 6 has not yet been determined.

Horizontal resistance has been observed but it is not complete, so when disease potential is high, the cultivars are killed or mature earlier than disease-free plants, causing a loss in yield as well as quality of freezing or canning pea (Hagedorn, 1984; Kraft and Pfleger, 2001). To date, only the single dominant gene for resistance to *Fop* has been used in the development of commercial cultivars. However, breeding programmes based on only a few dominant genes are in serious risk of resistance breakdown. This breakdown of formerly effective resistance, although not as rapid as experienced with airborne pathogens such as rusts or powdery mildews, is the nightmare of breeders, and forces them to search continually for new sources of resistance within pea germplasm collection also including wild pea relatives.

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Objectives

To achieve the general objective of this work; the following specific objectives were raised:

(1) Establishment of a detailed methodology to study the resistance of legumes to *F. oxysporum* using a model plant *M. truncula*ta (treated in Chapter 1) and *Pisum* spp. (treated in Chapter 2).

(2) Identification of novel sources of resistance to *F. oxysporum* f.sp. *pisi* race 2 in a collection of wild *Pisum* spp. (treated in Chapter 2).

(3) Evaluation of the role of root exudates in resistance of pea to *F. oxysporum* f.sp. *pisi* (treated in Chapter 3).

(4) Characterization of the resistance mechanisms occurring in different pea accessions resistant to *F. oxysporum* f.sp. *pisi* race 2 at cellular level (treated in Chapter 4).

(5) Molecular characterisation of the defence reaction of pea plant after infection by *F. oxysporum* f.sp. *pisi* race 2 through a proteomic approach (treated in Chapter 5).

(6) Identification of the main toxins produced by *Fusarium oxysporum* f.sp. *pisi* race 2 and their relation with its pathogenicity (treated in Chapter 6).

Chapter 1

Chapter 1

Resistance reaction of *Medicago truncatula* genotypes to *Fusarium oxysporum:* effect of plant age, substrate and inoculation method.

Nicolas Rispail, Moustafa Bani and Diego Rubiales

Abstract

Fusarium wilt caused by several *formae speciales* of *Fusarium oxysporum* is an important disease of most crop and pasture legumes including pea, chickpea, alfalfa and barrel medics. *Medicago truncatula* is an important pasture legume and a model legume species. Thus it can be used to increase our knowledge on the resistance mechanisms efficient to block *F. oxysporum* infection provided that its response to the disease is characterized. Here we evaluated the physiological and susceptibility response to the disease of two contrasting *M. truncatula* genotypes, and the effect of several cultural conditions known to affect the disease incidence such as plant age at infection time, growth substrate and the method of inoculation. Our results indicated that the A17 accession harbours a moderate level of resistance to the disease. We also showed that the method of inoculation strongly affected fusarium wilt disease development in this model species while it was not significantly altered by the plant age or the inorganic growth substrate tested. In addition we describe a rapid change in leaf temperature after infection that can be used as indirect parameter to confirm fungal infection at a very early stage of the interaction.

Keywords
Barrel Medic, Fungal pathogen, Disease resistance, Infra-red imaging system, Phenotyping.

1. Introduction

Barrel medic, *Medicago truncatula* is an important pasture legume (Crawford *et al.,* 1989) cultivated on over 4.5 million hectares in Australia alone (Hill and Donald, 1998). It is of particular importance in southern Australian cereal-livestock zone where it provides feed for livestock and plays an important role in crop rotation. It contributes to improve soil fertility due to its capacity to fix atmospheric nitrogen through symbiosis and provides a disease break for various cereal root pathogens (Nair *et al.,* 2006). Beside its agronomic importance, barrel medic is also an important model plant species for structural and functional genomic due to its small diploid genome and short life cycle (Cook, 1999; Frugoli and Harris, 2001). As such, it has been widely used to increase our understanding of the symbiotic interaction and legume disease resistance (Rispail *et al.,* 2010).

Fusarium wilt caused by the soil-borne ascomycete *Fusarium oxysporum* is a recurrent disease that causes important losses in many crops including alfalfa, pea, chickpea and tomato (Gordon and Martyn, 1997; Rubiales *et al.,* 2015). Barrel medic can be also susceptible to fusarium wilt caused by *F. oxysporum* f.sp. *medicaginis* (*Fome*). While both monogenic and quantitative resistance mechanisms have been described in different legumes (Hijano *et al.,* 1983; Infantino *et al.,* 2006; Bani *et al.,* 2012), only monogenic resistance has been used in breeding programmes which are at risk of breakdown by the emergence of new races and/or pathotypes (Sharma *et al.,* 2010). This is in part due to the difficulty to include the quantitative resistance mechanisms within current breeding programmes for which a better understanding of disease resistance mechanisms at molecular level are needed. The large post-genomic resources established for *M. truncatula* may serve to better understand the *F. oxysporum*-legume interaction. However, before large-scale “-omics” analyses could be applied, the disease response of *M. truncatula* accessions, and in particular of the A17 accession for which most post-genomic resources have been developed, should be well-characterised.

Previous works have described the A17 genotype as either susceptible or moderately resistant to *Fome* (Ramirez-Suero *et al.,* 2011; Rispail and Rubiales, 2014). The controversy of these data could have arisen by differences in the plant age, plant substrate or in the inoculation method used since the severity of fusarium wilt disease is influenced by host, pathogen and environmental factors (Ben-Yephet and Shtienberg, 1997). Among them, the pathogenic strain, the concentration of inoculum and the temperature are the most limiting factors (Ben-Yephet and Shtienberg, 1997).

However, under optimal conducive conditions, plant age at infection, plant growth substrate and the inoculation methods can also greatly influence the establishment and intensity of fusarium wilt disease (Nyvall and Haglund, 1976; Swanson and van Gundy, 1985; Latin and Shell, 1986; Sedra and Besri, 1994; Cohen *et al.*, 2008).

Currently the best way to check successful fusarium wilt infection involves the isolation of the fungus from root or stem tissues which requires the destruction of the sample (Zhou and Everts, 2004). Thus, an efficient non-destructive and fast phenotyping method suitable for large scale analysis is required to confirm plant infection. The recent development of imaging-based phenotyping such as the evaluation of superficial temperature by infra-red imaging system or the measurement of chlorophyll fluorescence may offer an alternative to detect the pathogenic infection. This method has been applied to detect the presence of a few plant diseases both in fields and laboratory including virus, downy mildew, cercospora leaf spot and verticilium wilt (Chaerle *et al.*, 2004; Chaerle *et al.*, 2006; Stoll *et al.*, 2008; Calderón *et al.*, 2013). *F. oxysporum* inoculation has been recently shown to increase superficial leaf temperature in banana and cucumber (Dong *et al.*, 2012; Wang *et al.*, 2012). Thus, monitoring the changes in superficial leaf temperature by infra-red imaging may be an adequate method to confirm fusarium wilt infection.

The impact of plant age at inoculation, inorganic growth substrate and inoculation method on the disease response of *M. truncatula* accessions to *Fome* is still unknown. Thus, we aimed, here, to determine their effects on the fusarium wilt disease severity in contrasting *M. truncatula* accessions including the reference genotype A17. We also explore the possibility to use infra-red imaging system as a fast phenotyping method to confirm the successful infection at an early stage of the interaction. Since the commonly used root dipping method may induce uneven and uncontrolled stress to the plants, it may be inappropriate for highly sensitive purposes such as advanced imaging-based phenotyping or molecular studies of resistance, that are strongly affected by slight changes in plant physiology, the possibility to use alternative inoculation methods for such applications is discussed.

2. Material and Methods

2.1. Fungal isolate and culture conditions

Fome isolate *605* (MIAE00930), was obtained from the Microorganismes d'Intérêt Agro-Environmental (MIAE) collection of UMR1347 Agroecology, INRA Dijon, France (http://www2.dijon.inra.fr/umrmse/spip.php?rubrique47) and was used for all

experiments. The fungal strain was routinely stored as microconidial suspensions at -80 ºC with 30% glycerol. For microconidia production, cultures were grown in potato dextrose broth (PDB; Difco, Detroit, MI) at 28 ºC with shaking at 170 rpm.

2.2. Plant material and growth conditions

M. truncatula genotypes, PI577607 and A17, were used in all experiments to determine the impact of inorganic substrate, plant age at inoculation and the inoculation methods on disease development. The accessions PI516927 and PI577600 were also used in additional experiments to confirm the influence of the inoculation method on fusarium wilt disease development. PI577607 and PI516927 were highly susceptibles, PI577600 was resistant while the A17 showed moderate resistance to this *Fome* isolate (Rispail and Rubiales, 2014). All PI accessions were kindly provided by the Agricultural Research Service of the United States Department of Agriculture (USDA-ARS, USA) germplasm collection while the A17 genotype was provided by the Institut National de Recherche Agronomique (INRA, France).

In all cases, *M. truncatula* seeds were scarified with sulphuric acid solution for 5 minutes and surface-sterilized for 2 minutes with a 3% chlorine solution and left to imbibe in sterile water before being plated onto water-agar plates. Then, they were stratified for 2 days at 4 °C in the dark and incubated at 20 ± 2 °C until germination still in the dark. Once germinated, seedlings were transferred to pots (36 cm^2 x 8 cm) filled with sterile inorganic substrate and grown in a controlled environment chamber under a 16/8 h light-dark photoperiod at constant 26 ± 2 °C temperature regime with 200 µmol m^{-2} s^{-1} of illumination before inoculation. Plants were watered every three days with tap water and weekly with Hoagland nutrient solution (Hoagland and Arnon, 1950).

To test the impact of plant substrate on disease development, plants were grown either in sterile fine grade vermiculite (1-3mm Ø) or in a sterile mixture of sand: perlite at 1:3 proportion (V/V) both medium commonly used for *M. truncatula* growth. To define the level of susceptibility of the contrasting *M. truncatula* accessions, plants were grown in vermiculite for all subsequent experiments, *M. truncatula* plants were grown in the sand : perlite mixture.

2.3. Inoculation and disease assessment

To estimate the impact of plant age on disease development, the response of *M. truncatula* to the disease was evaluated after inoculating 10 days old young seedlings (2-3 leaves) or 30 days old established plants (10 leaves). To define the level of

susceptibility of the contrasting *M. truncatula* accessions and determine the impact of plant substrate, plants were inoculated after 10 days of growth. In all additional experiments, plants were inoculated after 30 days of growth. In all experiments, *M. truncatula* plants were inoculated with the standard root dipping method as described previously (Rispail and Rubiales, 2014). Briefly, the plants were removed from the substrate, cleaned gently under tap water and submerged in a *Fome* spore solution for 30 min. Then the plants were transplanted to new pots containing sterile vermiculite and maintained in the above mentioned growth chamber.

To establish the impact of the method of inoculation on disease development, plants were also inoculated with two alternative watering methods. For these methods, the inoculum was supplied either by adding 50 ml of inoculum at the base of the plants in each pot (pot watering method) or by placing the pots in a tray containing the spore inoculum until complete absorption (pot dipping method). Two days before watering inoculation, water supply of these plants was stopped to avoid the washing through of fungal spores at inoculation and to maximise the contact of these spores with the host root. In all cases, the inoculum consisted on a solution of $5x10^6$ *Fome* spores ml^{-1} in water and control plants were maintained non-inoculated by replacing the spore inoculum by sterile water. Only one control was used for both watering methods. For all experiments, five plants were used per treatment and each experiment was repeated twice independently. *Fome* symptoms were rated every three days from 7th to 30th day post-inoculation (dpi) by estimating the percentage of leaves with symptoms (leaf yellowing, necrosis and wilting all together) per plant (Bani *et al.*, 2012). These data were used to calculate the area under the disease progression curve (AUDPC) value as described previously (Bani *et al.*, 2012).

2.4. Infection bioassays

To confirm the pathogen presence within inoculated plants, the fungus was re-isolated from root and stem tissues of two inoculated plants per genotype and treatment after 10 dpi as described previously (Bani *et al.*, 2012). In addition, infrared images of *M. truncatula* plants were obtained with a Thermovision A40M (FLIR, USA) thermal camera equipped with a 43° FOV lens and connected to computer via IEEE-1394 protocol. The image sensor was a Focal Plane Array (FPA) based on uncooled microbolometers with a resolution of 320 × 240 pixels, a spectral response in the range 7.5-13 µm, with 0.08 ºC sensitivity at 30 ºC and 0.1 mm minimal focus distance. Digital thermograms were acquired with the temperature range set between + 10 and + 55 ºC with the spectral rainbow colour scheme with FSCAP software (FLIR, USA). The leaf temperature for each plant was determined by calculating the mean

temperature of three 0.3 mm spots placed on three distinct leaves. Leaf temperature was monitored at 4 and 10 dpi. To take into account natural variation in the superficial leaf temperature, 10 plants were used per genotype and inoculation method.

2.5. Statistical Analysis

All experiments followed a completely randomized design and were repeated independently twice. Preliminary one-way ANOVA was conducted to compare the results between independent repeats. No significant differences were detected and the results of both replicates were combined before further analysis. Percentage of symptom values was subjected to an angular transformation to normalise the data and stabilize the variances before being subjected to the analysis of variance (Baird *et al.*, 2002). The significance of the differences in leaf number, disease symptoms and superficial leaf temperature according to the treatment or genotype was estimated by one and two-way ANOVA. All statistical analyses were performed using SPSS statistics v.22 software (IBM Corp., USA).

3. Results

3.1. Response to *Fusarium oxysporum* inoculation

Clear differences in disease ratings were observed between A17 and PI577607 genotypes when 10 day old plants grown in vermiculite were inoculated according to the traditional root dipping method (Fig. 1). Thus, whereas PI577607 plants were dead after 15 dpi, plants of A17 genotype remained alive until the end of the experiment although it was moderately affected by the pathogen (Fig. 1).

3.2. Effect of plant substrate on disease severity

To determine the influence of inorganic plant substrate, the overall plant growth and disease ratings were compared between plants grown in vermiculite or in a sand:perlite (1:3, v/v) mixture. A more profuse plant growth was obtained with the sand:perlite medium in which plants reached around 70 leaves after 55 days whereas the PI577607 and A17 plants only grew up to 18 and 30 leaves in vermiculite respectively (Fig. 2a). However, both media were similarly conducive to the disease when 10 day old seedlings were inoculated with the root dipping method. Inoculated plants growing in both substrates showed clear Fome symptoms after 7 and 10 dpi for the PI577607 and A17 genotypes respectively. Although the level of disease was significantly different between genotypes, the symptoms developed by inoculated plants were similar for both substrates reaching AUDPC values of 2126.7 and 1902.1 in sand:perlite and

vermiculite, respectively for the susceptible accession PI577607 and of 948.2 and 1122.3 for the A17 genotype (P > 0.05, Fig. 2b).

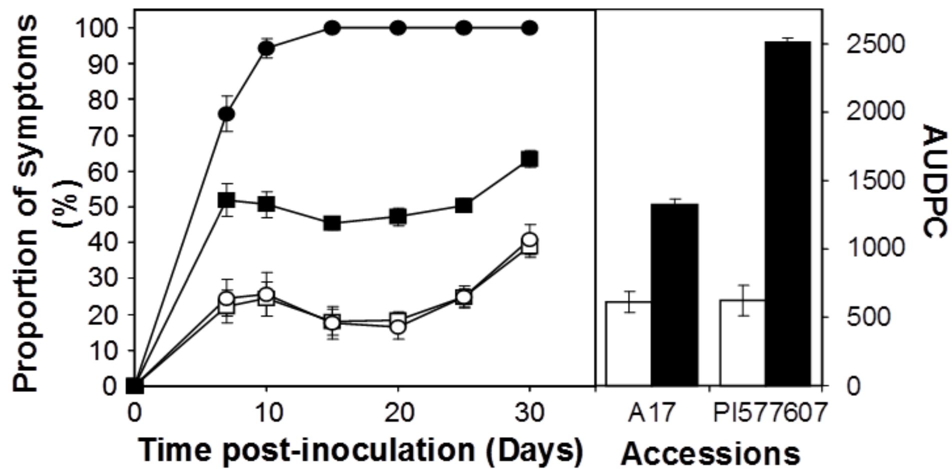

Figure 1. Clarification of the response of *M. truncatula* A17 accessions to *F. oxysporum* f.sp. *medicaginis*. Comparison of the susceptibility of the PI577607 (circle symbols) and A17 (square symbols) genotypes grown in vermiculite and inoculated by *F. oxysporum* f.sp. *medicaginis* with the root dipping method (closed symbols/bars) or maintained non-inoculated (open symbols/bars). The susceptibility was determined by plotting the progression over time of disease symptoms estimated with a visual scale ranging from 1 (healthy) to 10 (completely dead plants) and the corresponding area under the disease progression curve (AUDPC) histogram. Vertical bars are standard errors for n = 10.

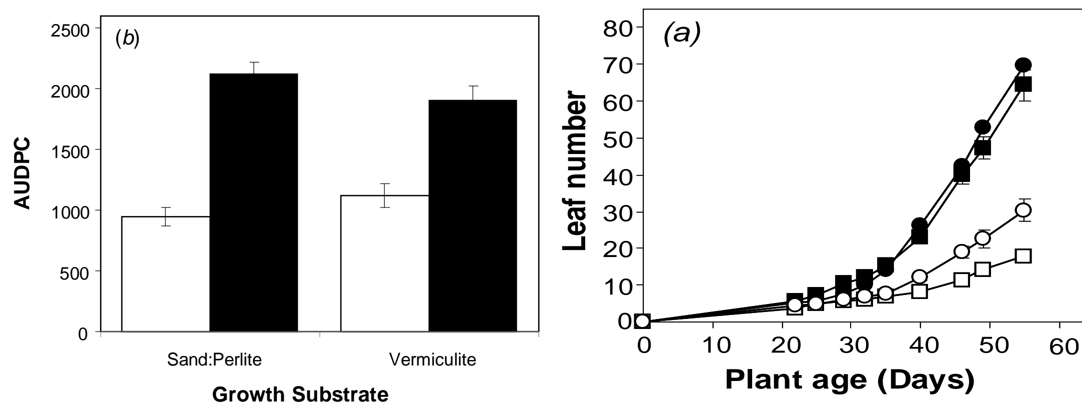

Figure 2. Effect of growth substrate on *Medicago truncatula* growth and susceptibility to *F. oxysporum* f.sp. *medicaginis*. (*a*) Comparison of the overall growth, estimated as the total number of leaves per plants over time, of the accessions PI577607 (circle symbols) and A17 (square symbols) grown on vermiculite (open symbols) or on a mixture of sand:perlite (1:3, V/V) (closed symbols) as determined by the number of leaves. (*b*) Comparison of the susceptibility of the PI577607 (closed bars) and A17 genotypes (open bars) grown in vermiculite or a sand:perlite mixture to *F. oxysporum* f.sp. *medicaginis* as determined by their AUDPC values. Inoculation was performed on 10 days old seedlings with the root dipping methods. Vertical bars are standard errors for n = 10.

3.3. Effect of plant age on disease severity

M. truncatula seedling establishment in growth substrate is a very long process that require about a month in a controlled environment. During this period, *M. truncatula* seedling grows very slowly reaching 2-3 leaves after 10 days and 10 leaves only after 30 days of growth in inorganic substrate (Fig. 2a). Once established, approximately 30 days after sowing, *M. truncatula* grows exponentially producing profuse ramifications each harbouring several leaves and flower buds. Thus we determined the response of the contrasting *M. truncatula* genotypes to *Fome* when inoculated with the root dipping method at seedling stage (10 days after sowing) or after plant establishment (30 days after sowing). Significant differences ($P < 0.05$) between genotypes were detected at both plant ages, with the disease ratings of the susceptible accessions PI577607 reaching 2127 ± 88 and 2022 ± 29 when inoculating 10 and 30 days old plants respectively while it only reached 891 ± 56 and 789 ± 45 for the A17 accession (Fig. 3). By contrast, the differences detected in the AUDPC values of each genotype when comparing plants inoculated at 10 or 30 days after sowing were not statistically different ($P > 0.05$, Fig. 3).

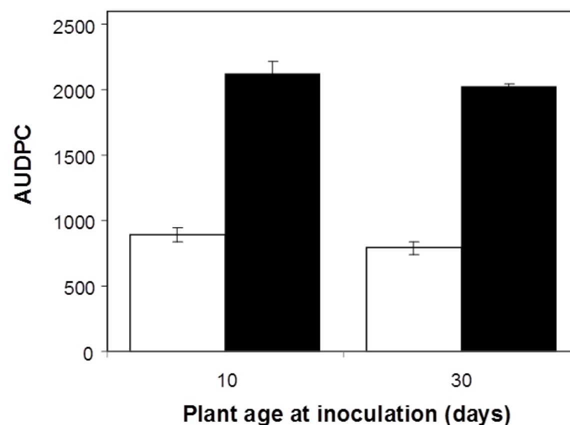

Figure 3. Effect of plant age on *M. truncatula* susceptibility to *F. oxysporum* f.sp. *medicaginis*. Comparison of the susceptibility of the PI577607 (closed bars) and A17 (open bars) genotypes inoculated after 10 days or 30 days of growth by *F. oxysporum* f.sp. *medicaginis* with the root dipping method as determined by their AUDPC values. Vertical bars are standard errors for n = 10.

3.4. Effect of the inoculation method on disease severity

To determine the impact of the inoculation method on fusarium wilt disease development on contrasting *M. truncatula* genotypes, we tested the pot watering and the pot dipping method as alternative to the standard root dipping method. Following the three inoculation methods, we detected the characteristic wilt symptoms on susceptible plants. However, the root dipping method induced faster and stronger

symptoms development (Fig. 4). Compared to the fast symptoms appearance at 7 dpi, symptoms induced by pot watering initiated at 15 dpi in both accessions reaching 28% and 65% of the leaves at the end of the experiment for the A17 and PI577607 genotypes respectively (Fig. 4c and d). Thus, although both genotypes showed a lower level of disease it was possible to distinguish between healthy and inoculated plants. It was also possible to distinguish the genotypes since the A17 genotype showed a moderately resistant phenotype as previously observed while the PI577607 genotype was susceptible (Fig. 4c). By contrast, the pot dipping method induced milder and delayed symptoms on inoculated plants (Fig. 4e and f). While it was still possible to distinguish between accessions (*P* < 0.001), it was not possible to discriminate between healthy and inoculated A17 plants following this inoculation method (*P* > 0.05) (Fig 4e).

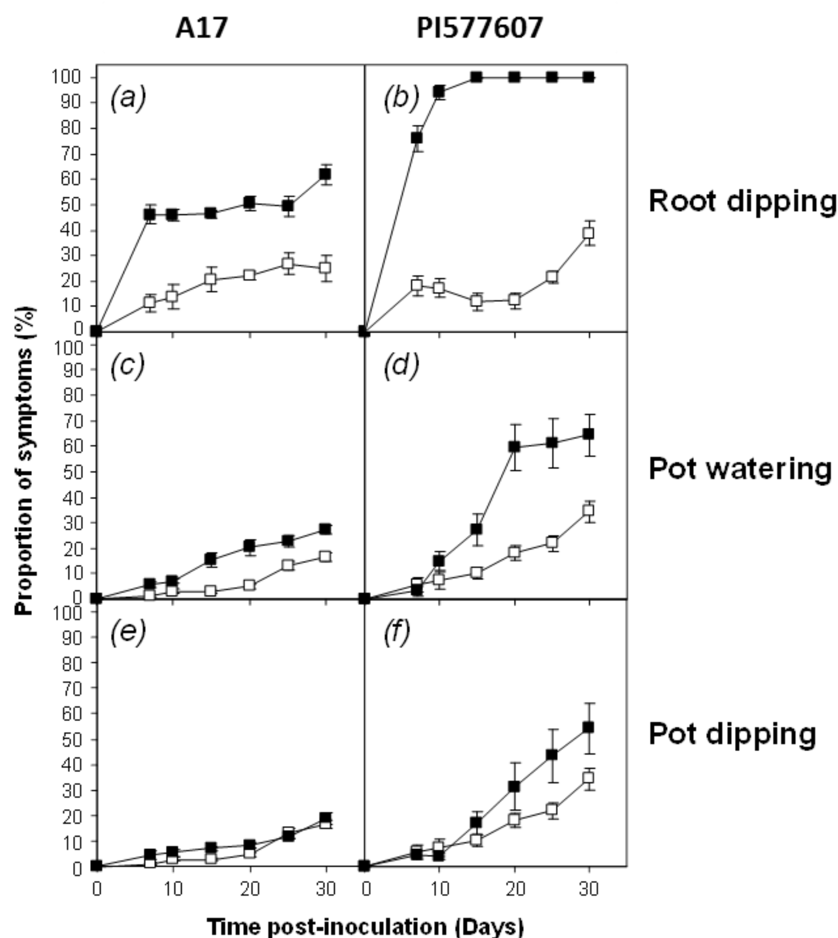

Figure 4. Effect of inoculation methods on *M. truncatula* susceptibility to *F. oxysporum* f.sp. *medicaginis.* Comparison of the susceptibility of the moderately resistant A17 *(a, c, e*) and the susceptible PI577607 (*b, d, f*) accession inoculated 30 days after sowing by *F. oxysporum* f.sp. *medicaginis* (closed symbols) or maintained non-inoculated (open symbols). Plants were inoculated with the root dipping (*a, b*), the pot watering (*c, d*) or the pot dipping (*e, f*) The susceptibility was determined by plotting the progression over time of disease symptoms estimated as the percentage of leaves with symptoms per plants. Vertical bars are standard errors for n = 10.

Inoculation of susceptible accessions PI516927 with these methods showed very similar disease ratings as the susceptible reference PI577607 while a resistant accession PI577600 remained symptomless in all conditions tested (Fig. 5).

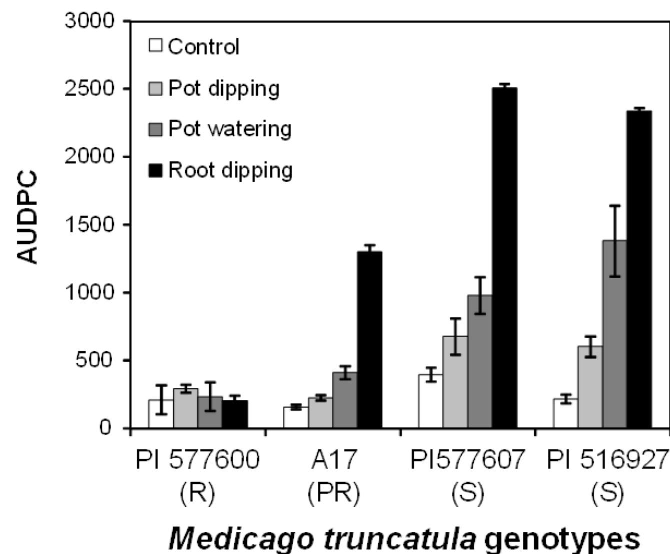

Figure 5. Comparison of the susceptibility of different *M. truncatula* genotypes according to the method of inoculation. The histogram compares the AUDPC values obtained for the resistant accession PI577600, moderately resistant accession A17 and the susceptible accessions PI577607 and PI516927 inoculated 30 days after sowing by *F. oxysporum* f.sp. *medicaginis* according to the method of inoculation. Vertical bars are standard errors for n = 10.

3.5. Validation of *Fusarium oxysporum* f.sp. *medicaginis* infection

Fome was easily re-isolated from stems of both genotypes when inoculated with the root dipping and pot watering methods, however, few colonies could be re-isolated from stems of plants inoculated with the pot dipping method, confirming the low efficiency of this method of inoculation (Fig. 6a). In all cases, lower numbers of *Fome* colonies were isolated from the stems of A17, confirming its partial resistance. *Fome* inoculation led to changes on the leaf surface temperature on both PI577607 and A17 accessions (Fig. 6b and c). However, differences could be detected according to the genotype and the method of inoculation. Overall, *Fome* inoculation led to a rapid and significant (P = 0.02) increase in leaf temperature of about 1 ºC for the A17 genotype and of 0.5 ºC for the PI577607 accession at 4 dpi with the root dipping method (Fig. 6c). Thereafter, the leaf temperature continuously rose to reach a significant difference of 3 ºC at 10 dpi for both accessions (P < 0.001; Fig. 6c). By contrast, inoculation by pot watering and pot dipping led to long-lasting differences between PI577607 and A17 accessions. For PI577607 plants both methods of inoculation led to a rapid and significant temperature increase of approximately 1 ºC at 4 dpi that was maintained at 10 dpi. For the moderately resistant A17 genotype, this rapid temperature increase was also observed at 4 dpi for both methods of inoculation (Fig. 6c), but it was only

transient and it reduced in successive days (Fig. 6c), albeit slightly faster for the pot dipping method. Thus, although the susceptible and the moderately resistant genotypes followed a different trend at longer times following inoculation, both accessions showed a significant temperature increase at the early stages of the infection process ($P < 0.001$ and $P = 0.003$ for PI577607 and A17 genotypes, respectively) that could be used to check the successful plant infection by *Fome* before appearance of the first visible symptoms.

4. Discussion

Fusarium wilt is a major constraint of most legume crop worldwide including pea, chickpea, lentil and alfalfa (Rubiales *et al.,* 2015). The use of model plants such as *M. truncatula* may contribute to improve our understanding of the *F. oxysporum* - legume interaction and legume resistance. To this aim we characterised the response to the disease of two genotypes reported to have contrasting levels of resistance. Although A17 has been adopted as the reference genotype by *M. truncatula* researchers worldwide (Choi *et al.,* 2004; Young *et al.,* 2005; Mun *et al.,* 2006; Kamphuis *et al.,* 2007) contradictory reports have been given on its actual response to the same isolate of *Fome* (Ramirez-Suero *et al.,* 2011; Rispail and Rubiales, 2014). As for other species, *F. oxysporum* disease severity on *M. truncatula* might be affected by different parameters such as the growing substrate or plant age at time of inoculation (Swanson and van Gundy, 1985; Cohen *et al.,* 2008). For instance, the use of organic substrate such as peat and compost has been shown to affect disease severity leading to highly variable results mainly depending on the microorganism they contains (Cohen *et al.,* 2008). By contrast inorganic substrates such as sand, perlite or vermiculite had often little influence on disease severity observed on susceptible accessions although they may lead to slight variations for partially resistant accessions (Ben-Yepthet and Shtienberg, 1997; Cohen *et al.,* 2008). In addition, these alternative substrates may sustain *M. truncatula* growth differently (Barker *et al.,* 2006). Thus, we tested their influences on the disease response of two contrasting *M. truncatula* genotypes. Our results indicated that the A17 accession harbours a moderate level of resistance to the disease (Figs. 1 and 2). Although the plant growth was affected by the substrate, the disease developed similarly on both substrates and the A17 genotype was in all cases moderately resistant to *Fome* compared with the response of the susceptible genotype PI577607 (Fig. 2).

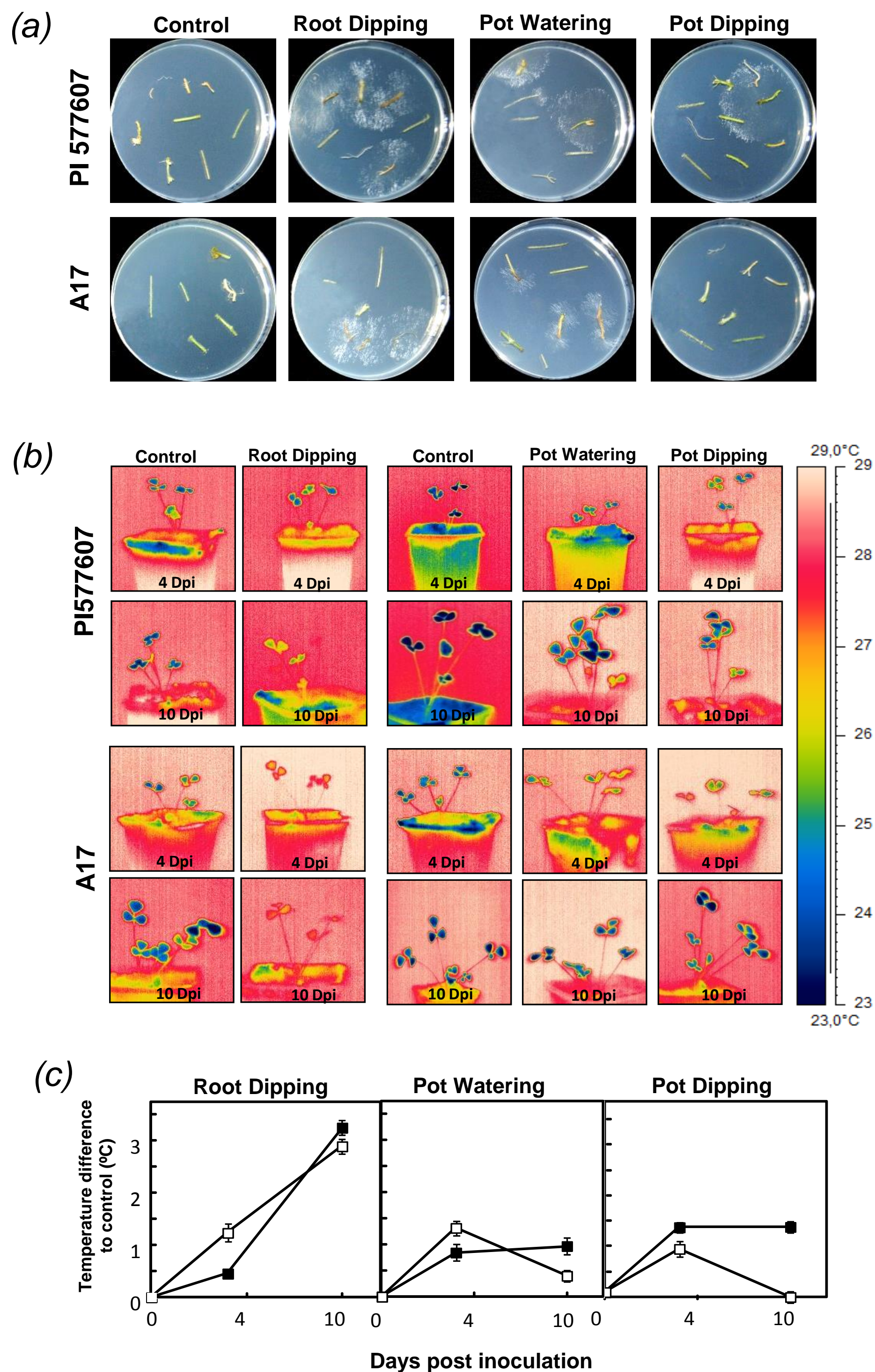

Figure 6. Verification and physiological characterisation of *M. truncatula* plant infection by *F. oxysporum* f.sp. *medicaginis* according to the method of inoculation. (*a*) Detection of *Fome* presence by re-isolation of the fungus from inoculated stems. Photographs compare the extension of *Fome* colonies growing out of plant tissue from the susceptible PI577607 and the moderately resistant A17 accessions control non-inoculated plants or inoculated by the root dipping, the pot watering or the pot dipping method. (*b*) Characterisation of the leaf temperature of *M. truncatula* control non-inoculated plants or inoculated by *Fome* with the root dipping, the pot watering or the pot dipping method as determined by infra-red imaging thermographic measurement at 4 and 10 dpi. (*c*) Evolution of the leaf temperature changes induced by the method of inoculation in the susceptible PI577607 (closed symbols) or the moderately resistant A17 (open symbols) as determined by the difference in infra-red thermographic measurement between inoculated and control non-inoculated plants. Vertical bars are standard errors for n = 10.

It has been also reported that older plants may possess higher resistance levels to fusarium wilt compared with younger ones (Nyvall and Haglund, 1976). Adult plant resistance has been frequently reported against airborne diseases (Fondevilla *et al.,* 2006; Sánchez-Martín *et al.,* 2011; Bansal *et al.,* 2014) and has been detected for some crops against fusarium wilts (Nyvall and Haglund, 1976; Cirulli and Ciccarese, 1982; Latin and Snell, 1986) but not for others such as cowpea or some tomato cultivars (Cirulli and Ciccarese, 1982; Swandon and van Gundy, 1985). At the ages tested in this study, *M. truncatula* plants showed no differences in their response to the infection and A17 and PI577607 showed a similar response to that observed in the above mentioned experiments (Fig. 3). Thus, our data on these genotypes are in accordance to the results found for other legumes in which no adult plant resistance against *F. oxysporum* was observed (Swanson and van Gundy, 1985). The differences detected in the susceptibility of the A17 in previous studies (Rámirez-Suero *et al.,* 2011; Rispail and Rubiales, 2014) might be hence more likely due to the methods of inoculation used than to the plant age. On the other hand, the fact that *M. truncatula* susceptibility to *Fome* is not dependent on plant age at inoculation time opens the possibility to use older plants when high amount of plant tissue is needed for genomic, proteomic or metabolomics studies. Simultaneous high through-put molecular studies require large amount of material and early sampling time after inoculation. Given the very slow growth of *M. truncatula* seedlings before establishment, many *M. truncatula* young seedlings would be required for each time point considering the needs of biological replicates which may not be possible for space limitation in most growth rooms. Thus, the use of established *M. truncatula* plants (30 days old) at inoculation may contribute to reduce the number of plants needed to establish the molecular basis of fusarium wilt resistance with such high through-put molecular approaches while the time of 10 days for inoculation may be optimal for other applications when no large samples are required (Barker *et al.,* 2006; Rispail and Rubiales, 2014).

In most plant species including *M. truncatula*, pea, tomato and melon, the standard method for *F. oxysporum* inoculation is the root dipping approach with or without root trimming that favours *F. oxysporum* root colonization through wounds (Latin and Snell, 1986; Di Pietro and Roncero, 1998; Lichtenzveig *et al.,* 2006; Bani *et al.,* 2012; Rispail and Rubiales, 2014). *F. oxysporum* infection in other species have also been performed by transplanting or sowing healthy plants in inoculated substrate obtained by mixing plant substrate with either infected seeds or fungal material (Kraft *et al.,* 1994; Riccioni *et al.,* 2003). Alternatively, inoculum may also be applied to the plant as a liquid solution by supplying the spores to each pot at the base of the plants or by dipping plant pots or trays in the spore solution (Latin and Snell, 1986; Baya *et al.,*

1995; Schreuder *et al.,* 2000; Riccioni *et al.,* 2003). For rapid screening, continuous dipping methods involving the transfer of seedlings to flask or vials containing diluted spore solution has also been proposed (Freemand and Rodriguez, 1993; Chikh-Rouhou *et al.,* 2010). Since the inoculation method may led to variation of the severity of fusarium wilt according to the plant species considered (Latin and Snell, 1986; Sedra and Besri, 1994; Bayaa *et al.,* 1995), we compared the response to the disease of the contrasting *M. truncatula* accessions obtained after inoculating the plants with the root dipping methods without root trimming or two alternative watering-based methods of inoculation. Both methods reduced the level of symptoms observed on the susceptible and partially resistant accessions indicating a lower efficiency of the inoculation compared to the standard root dipping method. Differences between these watering-based methods were also detected (Fig. 4). *F. oxysporum* enters host root preferentially through the root epidermis (Di Pietro *et al.,* 2003). It has been also reported to enter through wounds and by crack entry at site of lateral root emergence (Michielse and Rep, 2009). The root dipping method wounds the root system and allows direct contact with the pathogenic spores leading to massive entry of the pathogen directly in the xylem through these wounds. In addition, by entering through wounds, the pathogen might escape defence mechanisms acting at the root epidermis or cortex such as the formation of papilla-like structures that otherwise would prevent fungal penetration (Michielse and Rep, 2009). For these reason, the root dipping method might induced faster and stronger symptom development than the watering methods. Recent studies have also shown that normal penetration through root epidermis is preceded by proliferation of the fungus along the root system (Czymmek *et al.,* 2007) suggesting that the fungus should reach a certain density before root infection. This would delay further the host colonization when inoculated with the watering methods. It may also explain the differences we observed in the efficiency of both watering-based inoculation methods. Indeed, we reckon that a lower number of *Fome* spores may reach the root system by the tray watering method than with the pot watering method and thus infection with the pot dipping method would require additional delays to reach the minimum density of mycelium before root penetration.

Although it induces delayed and milder symptoms on both susceptible and partially resistant accessions, the pot watering method still allow differencing the susceptible accessions PI577607 and PI516927 from the A17 accession that maintained its moderate resistance. By contrast with the pot dipping method, the A17 accessions remained symptomless similarly to the resistant accession PI577600 while only mild symptoms were detected on the susceptible accessions (Figs. 4 and 5). Thus the use of the pot dipping method for resistance screenings may led to the erroneous

classification of the A17 accession as resistant and PI577607 and PI516927 as partially resistant accessions. From all the parameters evaluated here, the inoculation method is the only one that affected the severity of fusarium wilt in *M. truncatula.* Thus, the difference in the A17 susceptibility to *Fome* previously detected may thus be explained by the differences in inoculation method or the pathogenic strain used. The A17 was previously shown to be susceptible when inoculated with the strain *179.29* by root dipping in a hydroponic system (Rámirez-Suero *et al.,* 2011) while it presented a partial resistant when inoculated with the strain *605* in our conditions (Fig. 1; Rispail and Rubiales, 2014). Previous comparison of both strains indicated that the strain *605* was also pathogenic on the A17 accessions (Rámirez-Suero *et al.,* 2011) and more virulent than the strain *179.29* (Rispail and Rubiales, 2014). Thus, it is unlikely that the difference in the susceptibility of the A17 to *Fome* was due to differences in strain virulence. Rather, this difference in susceptibility might be due to the experimental conditions. Altogether, this would indicate that the optimal method for resistance screening of *M. truncatula* collection to *Fome* is the traditional root dipping method. This method was specifically developed for resistance screening or to assess the efficiency of pathogen control methods (Haglund, 1989; De Cal *et al.,* 1999; McPhee *et al.,* 1999; Wang *et al.,* 1999; Chikh-Rouhou *et al.,* 2010; Bani *et al.,* 2012; Rispail and Rubiales, 2014). This method that involve uprooting seedlings from their substrate and dipping the root system in a solution of *F. oxysporum* spore before transplanting to new pot (Di Pietro and Roncero, 1998) generally allowed a rapid and strong infection as shown here for *M. truncatula.* However, it does not take into account the general host welfare and induces additional stresses to the plant at inoculation even when plant roots were not trimmed at inoculation. Thus it may not be adequate for more sensitive purposes such as advanced imaging-based phenotyping or molecular studies of resistance, that are strongly affected by slight changes in plant physiology. Although the pot watering method induced delayed and reduced disease symptoms, it provided reproducible and homogeneous infection, maintained the phenotypic difference between accessions and did not involve any plant manipulation at inoculation. Thus it may be an adequate alternative to the root dipping method to elucidate the molecular basis of *F. oxysporum* resistance in legume.

The recent development of imaging-based phenotyping such as the evaluation of superficial temperature by infra-red imaging system is revolutionizing agriculture and plant science. Infra-red thermal imaging is a non-contact, non-destructive and rapid technique which provides a temperature map of the targeted material or plant. Our results showed that infra-red imaging might be used for controlling the successful infection of plants which is critical for large scale molecular analysis (Fig. 6). Thus, by

infra-red imaging we could confirm the infection by *F. oxysporum* of *M. truncatula* plants as with the traditional, destructive and time-consuming re-isolation method. In general, plant superficial temperature is dependent on transpiration rates. At high transpiration rate, the leaf temperature is cooling while at lower transpiration rate, this temperature rises. The increase of temperature following *F. oxysporum* infection suggest a closure of stomata in the *M. truncatula* plants, which is a feature also observed upon infection with *F. oxysporum* (Dong *et al.,* 2012; Wang *et al.,* 2012) and other fungal or bacterial plants infection (Melotto *et al.,* 2006; Prats *et al.,* 2006; Prats *et al.,* 2007; Mur *et al.,* 2013). Stomata are important regulators of the interaction between plants and their environment. Stomata movement controls plant transpiration, thus stomata closure under drought stress limit water losses by the plant. While the function of stomata closure after challenge by pathogen is still unclear, it have been often described as a direct consequence of plant defence or pathogen action (Mur *et al.,* 2013). In the case of *F. oxysporum,* the increase in leaf temperature and associated stomatal closure might reflects the perturbation of the water flux in infected plants due to the vessel plugging induced by the intensive fungal growth within xylem cells and the plant defence and/or the production of fusaric acid (Mepsted *et al.,* 1995; Dong *et al.,* 2012; Wang *et al.,* 2012; Wang *et al.,* 2013).

Independently of the mechanism controlling stomatal movement during plant infection, monitoring superficial temperature is a good indicator of plant stresses. On the other hand, the relation between stomatal closure and resistance is still unclear and depends on the plant species, the pathogen and the resistance mechanisms involved at least for aerial pathogens (Prats *et al*., 2006; Prats *et al*., 2007; Mur *et al*., 2013). Here, by monitoring the temperature changes induced by infection at 4 days post inoculation it was possible to discriminate between genotypes since the temperature increase was slightly higher in the partially resistant A17 than in the susceptible PI577607 (Fig. 6c). Thus monitoring changes in superficial temperature might be useful not only to check for *Fome* infection but also to screen for resistance to *F. oxysporum* although further studies with a larger number of discriminating genotypes would be needed to confirm the usefulness of infra-red imaging as an alternative screening method.

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Chapter 2

Chapter 2

A detailed evaluation method to identify sources of quantitative resistance to *Fusarium oxysporum* f.sp. *pisi* race 2 within a *Pisum* spp. germplasm collection.

Moustafa Bani, Diego Rubiales and Nicolas Rispail

Abstract

Fusarium oxysporum f.sp. *pisi* (*Fop*) is an important pathogen of field pea (*Pisum sativum*) worldwide. The constant evolution of the pathogen drives the necessity to broaden the genetic basis of resistance to *Fop*. To achieve this, it is important to have a large germplasm collection available and an accurate and efficient method for disease assessment. Here, a detailed evaluation method coupling disease incidence, disease rating over time and its related area under the disease progression curve (AUDPC) was established and used to screen a *Pisum* spp. germplasm collection against one isolate of *Fop* race 2. A large variation in the disease response of specific pea accessions ranging from highly resistant to susceptible was observed within the collection, indicating the quantitative expression of the resistance. The repetition of the inoculation experiments on a subset of 19 accessions, including two susceptible accessions, indicated that the scoring method was robust and reproducible and confirmed the highly resistant phenotypes of 11 accessions. To initiate the characterization of resistance mechanisms within these accessions, the external and internal stem symptoms were compared between these selected pea accessions, together with the extent of fungal colonization within plants. All these tests indicated that, in all resistant accessions, the resistance mechanisms efficiently stopped pathogen progression at the crown. Incorporation of these sources of resistance to breeding programmes will contribute to improved Fop resistance in pea cultivars.

Keywords
Pisum sativum; Fusarium oxysporum f.sp. *pisi;* Quantitative resistance; Resistance mechanisms; Screening method.

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1. Introduction

Fusarium wilts are among the most important diseases affecting grain legumes throughout the world (Kraft *et al.*, 1998). *Fusarium oxysporum* f.sp. *pisi* (*Fop*) is an important and destructive pathogen of field pea (*Pisum sativum*). It has been reported in every country where pea is grown (Kraft and Pfleger, 2001). This soil-borne pathogen can survive as thick-walled chlamydospores, which remain viable in the soil for more than 10 years (Kraft, 1994). The infection cycle of *F. oxysporum* is initiated by the germination of spores in the soil in response to an undetermined signal within the host root exudates (Di Pietro *et al.*, 2003). Upon germination infective hyphae adhere to the root surface and penetrate root epidermis directly without the formation of any distinctive structure (Bishop and Cooper, 1983a; Rodriguez Galvez and Mendgen, 1995). The mycelium then advances inter- or intracellularly through the root cortex, until it reaches the xylem vessels and enters them through the pits (Bishop and Cooper, 1983b; Beckman, 1987). At this point, the fungus switches to an endophytic mode of host colonisation, during which it remains exclusively within the xylem vessels, using them as avenues to rapidly colonise the host (Bishop and Cooper, 1983b). At this stage, the characteristic wilt symptoms appear as a result of severe water stress, which ultimately lead to complete plant death. Upon plant death, pathogenic hyphae grow outward from the vascular tissue and begin to intensely sporulate on the plant surface (Di Pietro *et al.*, 2003).

Characterisation of *Fop* isolates according to their capacity to induce disease in a set of differential lines, their assignment to specific vegetative compatibility groups and the establishment of their molecular fingerprint profiles allowed the identification of four different races of *Fop* (races 1, 2, 5 and 6) (Haglund and Kraft, 1979; Correll *et al.*, 1987; Grajal-Martin *et al.*, 1993). Races 1 and 2 occur worldwide, while races 5 and 6 are, to date, only important in western Washington State (Infantino *et al.*, 2006). In addition, *Fop* is continually evolving with new variants of the pathogen that continue to emerge (Bodker *et al.*, 1993; Kraft and Pfleger, 2001). As for many soil-borne pathogenic fungi, the use of fungicides is not necessarily effective in controlling fusarium wilt (Sharma *et al.*, 2010). As a consequence, control of this disease is achieved mainly by integration of different disease management procedures including agronomic and farming practices (Navas-Cortes *et al.*, 1998), soil disinfestation (Momma *et al.*, 2010), biocontrol (Alabouvette *et al.*, 2009) and breeding for resistance (Sharma *et al.*, 2010). Among these methods, the use of resistant cultivars is widely recognized as the safest, most economical and effective method for protecting crops from this disease. Fortunately, resistance to *Fop* in pea is conferred by single race-

specific genes that have been successfully transferred to pea cultivars (Infantino *et al.*, 2006). Although the use of these resistant pea cultivars has proven effective in controlling this disease, there is a constant risk of resistance breakdown, since monogenic resistance can be easily overcome by the emergence of new pathogen variants. A continuous search for novel resistance sources to complement and strengthen the resistance of elite cultivars is thus essential with an emphasis on resistance sources based on quantitative and polygenic mechanisms. However, sources of fusarium wilt resistance in pea are limited (Ali *et al.*, 1994). To broaden the genetic basis of resistance it is important to evaluate large and diverse germplasm collections, including wild species and to use precise and accurate screening techniques (Infantino *et al.*, 2006).

Different screening methods for *Fop* resistance have been described although most of them only consider the disease incidence (DI) or the proportion of asymptomatic plants to classify accessions as resistant or susceptible (Haglund, 1989; McPhee *et al.*, 1999; Sharma *et al.*, 2010). This disease scoring method may not be adequate for quantitative resistance giving the continuous gradient of symptom severity (Russell, 1978). Here, a more detailed disease scoring method that considered not only DI, but also the progression of disease symptoms was established and tested on a set of differential lines and a *Pisum* spp. germplasm collection for resistance to *Fop* race 2. This screening method detected the existence of quantitative resistance to the pathogen within this collection and identified several *Pisum* accessions with high resistance that have a good potential for improving pea resistance to race 2 of *Fop*.

2. Material and Methods

2.1. Fungal isolates and cultural conditions

Fusarium oxysporum f.sp. *pisi* (*Fop*) race 2 strain R2F42 was kindly provided by Dr. W. Chen (USDA-ARS, Pullman, USA) for use in all the experiments. The fungal strain was stored as microconidial suspensions at -80 ºC in 30% glycerol. For microconidia production, cultures were grown in potato dextrose broth (PDB; Difco, Detroit, MI) at 28 ºC in a shake culture set at 170 rpm (Di Pietro and Roncero, 1998). To determine the extent of *Fop* colonization, the fungus was re-isolated from the root and the basal, middle and apical stem regions of three inoculated plants of four susceptible and 13 resistant accessions after 7 dpi. pea tissue following the method described by Lichtenzveig *et al.* (2006) except that the 1 cm long fragment were plated on PDA containing 0.1 mg/ml kanamycin and incubated at 28 ºC for 3 days. With this method,

typical colonies of *Fop* can be easily detected as white filaments emerging from the plant tissue and colonizing the PDA medium.

2.2. Plant material and growing conditions

A collection of eighty accessions of *Pisum* spp. of diverse origins was used in this study. The collection was composed of 7 *P. sativum* cultivars from the USDA core collection of the differential set for the four races of *Fop* (Table 1) and 73 accessions from the John Innes pea collection that had been obtained from different countries.

Pea seeds were surface-sterilized for 20 min in a 20% solution of sodium hypochlorite and then rinsed three times with sterile water. Then the seeds were wrapped in wet filter paper in a petri dish, stratified for two days at 4°C in the dark and incubated at 26 ± 2°C until germination. Once germinated, the seedlings were transferred to pots (36 cm^2 x 8 cm) containing sterile vermiculite (1-3 mm diameter) and grown in a controlled environmental chamber under a 16/8 h light-dark photoperiod at 26 ± 2°C temperature regime with 200 µmol m^{-2} s^{-1} of illumination. Plants were watered every three days with tap water.

Table 1. Disease Ratings of differential lines of *Pisum sativum* to *Fop 2*.

Accession	Fop 2	DI (%)	Disease Ratings[1]			
			Plant Mean Disease Index		Symptomatic Leaves (%)	
			MVI	AUDPC	PSL	AUDPC
Little Marvel	S	100	5.0 ± 0.00^b	84.6 ± 5.40^b	100.0 ± 0.00^b	2250 ± 0.0^b
Dark Skin Perfection	S	100	5.0 ± 0.00^b	57.9 ± 8.92^b	100.0 ± 0.00^b	1519 ± 206.6^b
New Season	R	0	2.3 ± 0.24^a	13.2 ± 2.56^a	43.4 ± 6.18^a	514 ± 74.7^a
Mini	R	20	3.1 ± 0.52^a	12.1 ± 4.79^a	61.3 ± 10.57^a	438 ± 137.1^a
Mini 93	R	40	3.4 ± 0.73^a	23.3 ± 8.42^a	65.0 ± 15.00^a	741 ± 184.9^a
Wsu31	R	20	3.0 ± 0.52^a	24.7 ± 4.31^a	59.9 ± 10.78^a	846 ± 215.7^a
74SN5	R	20	3.0 ± 0.62^a	18.6 ± 2.73^a	66.9 ± 12.52^a	622 ± 97.1^a

[1] Data are means of 5 replicates, different letters indicates significant difference between value according to Duncan Multiple Range Test at α = 0.05. DI, disease incidence; MVI, mean value of visual index for all leaves on an individual plant; PSL, percentage of leaves showing symptoms for each individual plant.

2.3. Inoculation and disease assessment

Seven-day-old *Pisum* spp. seedlings (2-3 node stage) were inoculated following a modified version of the dip technique described by Haglund (1989). For this procedure, vermiculite was removed from the roots which were trimmed by a third and immersed for 5 min in a suspension containing 5 x 10^6 microconidia per ml of water. Control plants were treated in the same way and were immersed in sterile water. Seedlings were planted in individual pots containing sterile vermiculite and maintained in the same growth chamber. Screening of the whole collection including the differential lines

was first performed on five seedlings per *Pisum* accessions. Then the 15 most resistant accessions were tested twice in independent experiments along with two partially resistant and two susceptible accessions with five seedlings per accession and per experiment.

Disease symptoms were assessed every three days from 10th to 30th days post-inoculation (dpi). Two different leaf symptom-based approaches were used to estimate the disease symptom rate (DR) at the leaf and the whole plant level. At the whole plant level DR was evaluated as the percentage of symptomatic leaves for each individual plant (PSL) (Fig. 1a). At the leaf level, evaluation of DR was established by assigning a visual index ranging from 1 (healthy leaf) to 5 (dead leaf) to each leaf within a plant and reporting these values for each individual plant by calculating the mean value of the visual index (MVI) of all its leaves (Fig. 1b). These data were used to calculate the area under the disease progression curve (AUDPC) using the formula: $AUDPC = \Sigma[(x_i + x_{i+1}) / 2] * (t_{i+1} - t)$, where x_i = estimated proportion of disease severity at date i, x_{i+1} = estimated proportion of disease severity at date i+1, and $t_{i+1} - t_i$ = number of days between scoring dates i and i + 1.

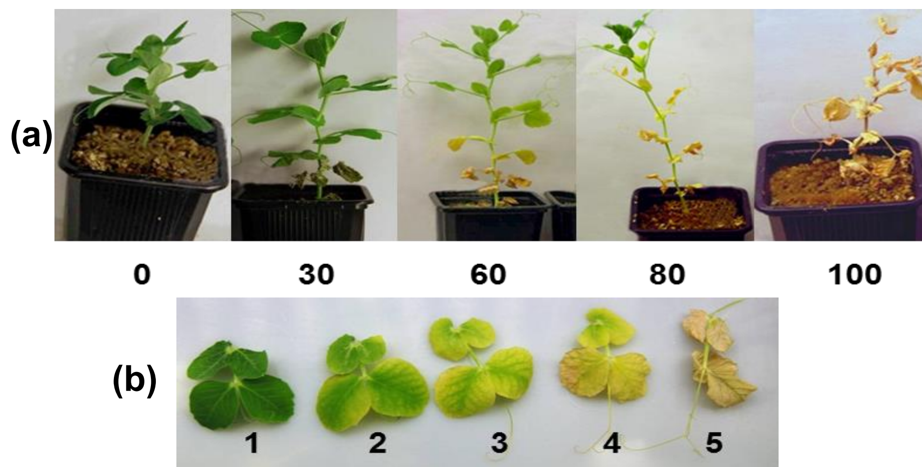

Figure 1. Evolution of disease symptoms induced by *Fusarium oxysporum* f.sp. *pisi* (*Fop*) race 2 on susceptible pea accessions. (a) The photographs represent the evolution of fusarium wilt symptoms at whole plant level of susceptible pea accessions inoculated with one isolate of *Fop* race 2. Numbers below each photograph represent their respective disease ratings, estimated as the percentage of leaves showing symptoms. (b) Typical progression of fusarium wilt disease of susceptible pea accessions inoculated with *Fop* race 2 at leaf level. Numbers under each leaf indicate its respective disease ratings value based on a disease index scale ranging from 1 (healthy leaf) to 5 (dead leaf).

Disease incidence (DI) determined as the proportion of dead plants was also scored at 30 dpi. To classify accessions as resistant or susceptible, their disease symptoms were compared to those of the accessions P627 and P21 used as resistant and susceptible controls respectively. In addition, the differential lines New Season

(resistant) and Little Marvel (susceptible) were also included in order to confirm their response to the infection.

2.4. Whole plant staining

To detect the extent of fungal colonization, three plants of the 12 most resistant accessions and the susceptible accession P21 were harvested 30 dpi, washed with sterile water to remove any un-adhered *Fop* microconidia, cleared with 2.5% KOH at 90 ^{o}C for 1 h, rinsed twice with deionized water and incubated overnight at room temperature in a solution of 1% HCl. The root and stem regions were then stained in a 1% Parker blue Quink ink aqueous solution for 30 min at 60 ^{o}C and destained 16 h at room temperature in lactoglycerol. The resulting stained tissues were then stored at room temperature in 100% glycerol until observation under a binocular microscope. Following this treatment stained fungal structures were clearly visible.

2.5. Detection of internal symptoms

A red-brown discoloration within plant tissue has been previously shown to be associated with *F. oxysporum* infection in field peas (Kraft and Pfleger, 2001). In order to observe this red-brown discoloration within pea plant tissue, the basal and middle part of the stem and the upper part of the root system of three plants from the 12 most resistant accessions were harvested at 30 dpi, longitudinally cut with a razor blade and observed under a Nikon SMZ1000 binocular microscope (Nikon Europe B.V., Badhoevedorp, The Netherlands). For comparison, three plants of the susceptible accession P21 were harvested at 10 dpi and treated in the same way.

2.6. Statistical analysis

To analyse the significance of the differences in DR and DI between the different pea accessions to *Fop*, all data obtained from the DI, MVI, PSL and AUDPC values were subjected to an analysis of variance (One-way ANOVA). Percentage PSL and DI data were subjected to an angular transformation to normalize the data and stabilize the variances before being subjected to the analysis of variance (Baird *et al.*, 2002). Whenever the ANOVA was statistically significant ($p \leq 0.05$) for a specific variable, a Duncan's multiple range test was conducted to assess the differences of the means between each accession. The coefficient of correlation existing between the different disease parameters were calculated using the non-parametric Spearman's rank correlation coefficient analysis. All statistical analyses were performed with the Genstat release 11.1 software (VSN International Ltd., Hemel Hempstead, UK).

3. Results

3.1. Disease development

The assessment of susceptible cultivars including Little Marvel and Dark Skin Perfection differential lines showed that the initial symptoms appeared on the primary leaves around 10-15 dpi and sequentially reached the later-formed leaves until the whole plant withers and dies (Fig. 1a). At the leaf level, the disease symptoms initiated at the leaf margins, which yellowed and/or curled downward. Leaf yellowing was associated with necrosis until the whole leaf wilted and became dry and brittle (Fig. 1b). These observations allowed the development of the two scales to estimate Disease Rate (DR).

3.2. Validation of the scoring method on differential lines of *P. sativum*

Before applying these screening methods to the *Pisum* spp. germplasm collection they were tested on seven well-described differential lines. As expected, Little Marvel and Dark Skin Perfection lines were highly susceptible to our isolate of *Fop* race 2 showing a DI of 100% and DR values at 30 dpi of 5 and 100% according to the MVI and PSL evaluation method respectively (Table 1). Similarly, the resistant differential cultivars (Haglund and Krafts, 1979) were all resistant with DI value ranging from 0 for New Season to 40% for Mini93. DR scores at 30 dpi ranged from 2.3 for New Season to 3.4 for Mini93 for the MVI scale and from 43.4% for New Season to 66.9% for 74SN5 according to the PSL scale. The AUDPC values calculated from the DR data sets ranged from 12.1/438 (AUDPC MVI/AUDPC PSL) for Mini to 24.7/846.4 for Wsu31 over the whole experiments according to the MVI/PSL scales respectively (Table 1). The statistical analysis performed on both DR scales and their associated AUDPC values indicated significant differences between race differential cultivars ($P < 0.001$) and a clear discrimination between the susceptible and resistant genotypes (Table 1).

3.3. Screening of wild *Pisum* spp. collection against race 2 of *F. oxysporum* f.sp. *pisi*

The same scoring parameters were used to screen a collection of 73 *Pisum* spp. accessions to identify new sources of resistance to *Fop* race 2 (Tables 2 and 3). Large variation in the disease response was detected among the *Pisum* spp. accessions for all the parameters monitored (Fig. 2 and Table 2). Thus, DI ranged from 0 to 100%, DR ranged from 1.3/20% to 5/100% according to MVI / PSL parameter and from 4.0/125 to 90.0/2220 for their respective AUDPC values (Table 2). The continuous distribution of

the pea accessions for the different parameters monitored, as shown for their AUDPC values, indicated that resistance in this germplam collection is quantitative (Fig. 2). The one-way ANOVA performed for all parameters detected statistically significant differences between accessions. Mean comparison analysis performed for the DR and AUPDC parameters failed to separate them in discrete groups except for the most resistant and susceptible accessions. To simplify classification, we separated the accessions in three main groups according to the result of the mean comparison test of their AUDPC values. All accessions not significantly different according to the Duncan's multiple range test for both AUDPC parameters, to the resistant check P627 were considered resistant. Accessions not significantly different from the susceptible check P21 were considered susceptible and the rest of the accessions were considered partially resistant. As expected the differential line New season and Little Marvel were classified within the resistant and susceptible group respectively (Table 2). According to this classification, 18 accessions were categorized as resistant (24.7% of the collection), 25 accessions as partially resistant (34.3% of the collection) and 30 as susceptible (41% of the collection) (Fig. 2).

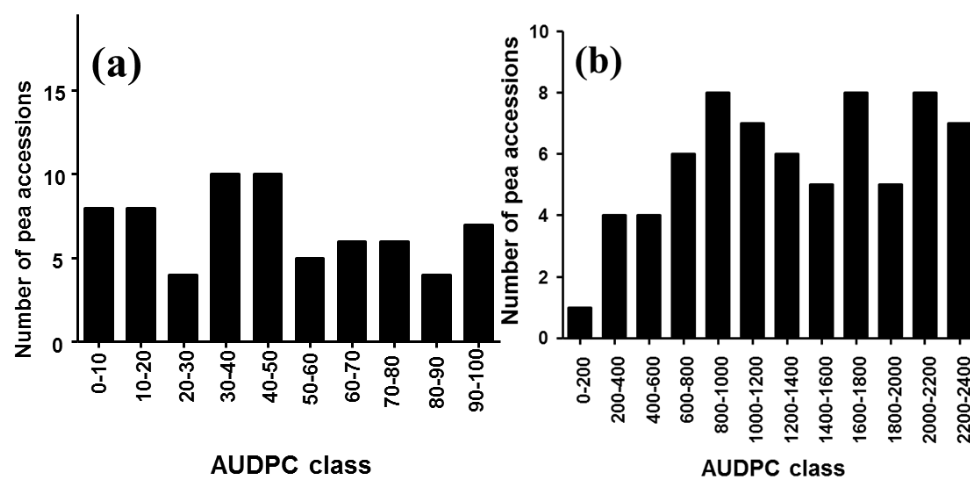

Figure 2. Distribution of the pea accessions upon inoculation with one isolate of *Fusarium oxysporum* f.sp. *pisi* race 2 according to AUDPC values calculated from the evaluation of disease ratings for mean value of visual index for all leaves on an individual plant, MVI (a) and percentage of leaves showing symptoms for each individual plant, PSL (b).

The independent repetitions of the assessment of 17 resistant and partially resistant accessions confirmed their low incidence and severity compared to the highly susceptible accessions P21 and P662 (Table 3). As expected, P21 and P662, the susceptible accessions showed a DI value of 100% and DR values of 5 and 100% for MVI and PSL measurements, respectively. Conversely, the DI of the resistant accessions ranged from 0 to 53% while MVI and PSL values varied from 1.8 and 30.0% for P633 to 3.5 and 68.8% for P18 (Table 3). Similarly, the AUDPC values of resistant accessions remained relatively low ranging from 9.7/343.2 for the highly

resistant P23 to 37.1/1264.6 for the partially resistant P316 according on the MVI and PSL scales while these values ranged from 73.9/2028 and 83.5/2274 for the susceptible accessions P662 and P21 respectively(Table 3). Statistical analysis confirmed the differences between genotypes at $P < 0.001$ for all parameters evaluated.

Table 2. Disease Ratings of the *Pisum* spp. accessions to *Fop* 2.

Accession	Species	Disease Incidence (%)	Disease Ratings[1]			
			Plant Mean Disease Index		Symptomatic Leaves (%)	
			MVI	AUDPC	PSL	AUDPC
New Season (R	*P. sativum*	0	2.3 ± 0.24^{a-h}	13.2 ± 2.56^{a-e}	43.4 ± 6.18^{a-f}	514 ± 74.7^{a-c}
P627	*P. sativum* ssp. *arvense*	0	1.8 ± 0.79^{a-d}	4.0 ± 3.51^{a}	24.0 ± 19.39^{ab}	118 ± 84.5^{a}
P656	*P. fulvum*	40	2.9 ± 0.79^{b-j}	5.2 ± 1.96^{a-c}	53.3 ± 18.50^{b-h}	244 ± 82.9^{ab}
P23	*P. sativum* ssp. *elatius*	0	2.12 ± 0.72^{a-f}	5.4 ± 3.16^{a-c}	48.8 ± 13.25^{a-g}	279 ± 75.8^{ab}
JI1760	*P. sativum* (cv. Consort-*af*)	20	1.9 ± 0.40^{a-d}	5.0 ± 2.54^{ab}	37.0 ± 12.93^{a-e}	309 ± 141.0^{ab}
P633	*P. sativum* ssp. *arvense*	0	1.4 ± 0.09^{ab}	6.5 ± 1.85^{a-c}	22.3 ± 1.72^{a-c}	311 ± 63.9^{ab}
JI1559	*P. sativum* (cv. Mexique 4)	0	1.8 ± 0.14^{ac}	6.9 ± 1.91^{a-c}	30.4 ± 3.88^{a-d}	475 ± 124.4^{a-c}
JI1412	*P. sativum* (cv. Marlin)	0	2.1 ± 0.19^{a-e}	12.5 ± 0.67^{a-g}	35.3 ± 4.38^{a-e}	485 ± 57.2^{a-c}
P669	*P. fulvum*	20	2.5 ± 0.45^{a-g}	8.6 ± 1.68^{a-d}	67.3 ± 13.01^{d-l}	529 ± 84.0^{a-d}
P42	*P. sativum* ssp. *arvense*	0	1.3 ± 0.18^{a}	8.1 ± 1.95^{a-d}	20.0 ± 9.35^{a}	536 ± 106.7^{a-e}
P638	*P. sativum* ssp. *arvense*	0	2.8 ± 0.09^{a-h}	10.7 ± 1.30^{a-f}	53.9 ± 4.82^{a-g}	577 ± 67.8^{a-f}
P632	*P. sativum* ssp. *arvense*	0	2.5 ± 0.26^{a-g}	17.0 ± 3.45^{a-j}	43.4 ± 9.00^{a-e}	649 ± 103.8^{a-g}
P613	*P. sativum* ssp *tibetanicum*	20	2.2 ± 0.64^{a-f}	21.0 ± 17.26^{a-k}	48.2 ± 11.90^{a-g}	687 ± 393.2^{a-h}
P650	*P. sativum* ssp. *jormadi*	0	1.9 ± 0.32^{a-d}	12.6 ± 4.16^{a-g}	34.1 ± 10.18^{a-f}	712 ± 149.3^{a-i}
P615	*P. sativum* ssp. *elatius*	40	3.3 ± 0.75^{c-l}	14.1 ± 4.96^{a-i}	84.0 ± 16.00^{h-n}	716 ± 215.0^{a-i}
P614	*P. sativum* ssp. *elatius*	0	2.0 ± 0.18^{a-d}	13.1 ± 2.10^{a-h}	38.0 ± 4.18^{a-e}	731 ± 87.3^{a-j}
JI1766	*P. sativum* (cv. Barton-*af,st)*	100	5.0 ± 0.00^{m}	30 ± 0.00^{a-o}	100.0 ± 0.00^{n}	750 ± 0.0^{a-j}
P639	*P. sativum* ssp. *arvense*	0	1.9 ± 0.78^{a-d}	19.4 ± 14.02^{a-k}	28.0 ± 19.60^{a-c}	752 ± 395.7^{a-j}
JI1566	*P. sativum* (cv. Almota)	0	3.1 ± 0.26^{c-k}	25.1 ± 2.23^{a-n}	61.4 ± 8.09^{b-j}	840 ± 56.1^{b-k}
P634	*P. sativum* ssp. *arvense*	20	2.4 ± 0.70^{a-g}	19.6 ± 10.18^{a-k}	37.3 ± 16.81^{a-e}	841 ± 275.7^{b-k}
JI1747	*P. sativum* (cv. Almires)	40	3.6 ± 0.61^{f-m}	26.0 ± 4.24^{a-n}	70.7 ± 14.39^{e-n}	888 ± 98.8^{b-l}
P641	*P. sativum* ssp. *arvense*	0	3.0 ± 0.14^{c-j}	21.5 ± 1.44^{a-l}	53.9 ± 3.05^{a-g}	890 ± 41.9^{b-l}
P640	*P. sativum* ssp. *arvense*	0	2.9 ± 0.26^{b-j}	23.5 ± 3.61^{a-m}	62.8 ± 6.97^{c-k}	996 ± 126.8^{c-m}
P18	*P. sativum* ssp. *elatius*	40	3.3 ± 0.83^{c-l}	36.4 ± 20.12^{d-p}	57.6 ± 20.95^{c-j}	998 ± 514.5^{c-m}
P628	*P. sativum* ssp. *arvense*	40	3.6 ± 0.51^{f-m}	30.4 ± 6.69^{a-o}	72.2 ± 10.37^{e-n}	1090 ± 136.5^{c-n}
JI502	*P. sativum* (cv. Rondo)	80	4.7 ± 0.31^{m-o}	37.1 ± 4.33^{e-p}	92.5 ± 6.71^{l-n}	1165 ± 114.9^{d-o}
P621	*P. sativum* ssp. *jormadi*	20	2.7 ± 0.55^{a-h}	33.8 ± 7.66^{c-o}	55.4 ± 10.48^{b-j}	1181 ± 181.6^{e-o}
P637	*P. sativum* ssp. *arvense*	40	3.6 ± 0.59^{e-m}	31.7 ± 6.62^{a-o}	71.1 ± 10.59^{e-n}	1186 ± 124.6^{f-o}
P645	*P. sativum* ssp. *arvense*	20	2.9 ± 0.50^{b-i}	33.3 ± 8.77^{b-o}	55.6 ± 11.86^{b-i}	1193 ± 229.5^{f-o}
P636	*P. sativum* ssp. *arvense*	40	3.3 ± 0.71^{d-l}	32.5 ± 12.97^{a-o}	73.4 ± 9.81^{e-n}	1200 ± 316.5^{f-o}
JI2480	*P. sativum* (CGN 3352)	40	3.8 ± 0.44^{g-m}	28.3 ± 5.22^{a-o}	83.3 ± 9.43^{f-n}	1223 ± 165.1^{f-o}
JI2302	*P. sativum* (B76-197)	60	4.1 ± 0.56^{h-m}	36.2 ± 8.48^{d-p}	84.3 ± 10.20^{g-n}	1225 ± 189.1^{f-o}
P619	*P. fulvum*	80	4.4 ± 0.40^{i-m}	41.3 ± 14.80^{h-r}	93.3 ± 6.67^{l-n}	1232 ± 334.5^{g-o}
P316	*P. fulvum*	60	4.4 ± 0.60^{i-m}	39.0 ± 10.26^{f-q}	88.0 ± 12.00^{j-n}	1296 ± 201.1^{g-p}
P731	*P. sativum* ssp. *arvense*	80	3.8 ± 0.62^{g-m}	41.8 ± 15.66^{i-r}	96.7 ± 3.33^{l-n}	1331 ± 334.7^{h-p}
JI210	*P. sativum* (cv. Lucknow Boniya)	100	5.0 ± 0.00^{m}	45.7 ± 13.99^{k-s}	100.0 ± 0.00^{n}	1352 ± 315.2^{i-q}
P649	*P. sativum* ssp. *jormadi*	40	3.0 ± 0.74^{c-j}	38.0 ± 16.01^{e-p}	63.0 ± 13.97^{d-l}	1378 ± 361.6^{j-r}
P635	*P. sativum* ssp. *arvense*	60	4.6 ± 0.34^{k-m}	39.4 ± 9.32^{g-q}	93.3 ± 6.67^{l-m}	1418 ± 279.4^{k-s}
P11	*P. sativum*	40	3.7 ± 0.62^{g-m}	43.5 ± 11.02^{j-s}	72.0 ± 11.38^{e-n}	1429 ± 281.6^{k-s}
P68	*P. sativum* ssp. *elatius*	40	2.8 ± 0.92^{a-h}	40.7 ± 15.44^{g-r}	44.0 ± 23.15^{a-e}	1443 ± 329.8^{k-s}
P630	*P. sativum* ssp. *arvense*	80	4.5 ± 0.47^{k-m}	47.2 ± 9.44^{k-u}	93.3 ± 6.67^{l-n}	1471 ± 235.2^{k-s}
P626	*P. sativum* ssp. *arvense*	100	5.0 ± 0.00^{m}	50.2 ± 3.19^{l-w}	100.0 ± 0.00^{n}	1532 ± 106.3^{l-t}
P54	*P. sativum* ssp. *arvense*	80	4.6 ± 0.40^{lm}	44.4 ± 4.19^{j-s}	93.3 ± 6.67^{l-m}	1563 ± 177.0^{m-u}
P631	*P. sativum* ssp. *arvense*	80	4.7 ± 0.33^{lm}	50.2 ± 6.84^{m-w}	93.3 ± 6.67^{l-n}	1601 ± 103.6^{m-v}
P617	*P. sativum* ssp. *thebaicum*	100	5.0 ± 0.00^{m}	55.71 ± 11.47^{o-}[illegible]	100.0 ± 0.00^{n}	1656 ± 189.8^{n-v}
P643	*P. sativum* ssp. *arvense*	80	3.6 ± 0.34^{e-m}	53.0 ± 5.63^{n-x}	71.1 ± 7.00^{d-m}	1659 ± 123.8^{n-v}

Table 2. (Continued)

Accession	Species	Disease Incidence (%)	Disease Ratings[1]			
			Plant Mean Disease Index		Symptomatic Leaves (%)	
			MVI	AUDPC	PSL	AUDPC
P19	*P. sativum* ssp. *elatius*	100	5.0 ± 0.00^{m}	47.8 ± 5.04^{k-v}	100.0 ± 0.00^{n}	1684 ± 136.8^{n-v}
P670	*P. fulvum*	40	3.3 ± 0.69^{c-l}	46.2 ± 12.26^{k-t}	65.4 ± 14.49^{d-l}	1686 ± 177.9^{n-v}
P642	*P. sativum* ssp. *arvense*	80	4.6 ± 0.40^{lm}	63.4 ± 16.45^{p-z}	91.1 ± 8.89^{l-n}	1748 ± 310.9^{n-v}
P647	*P. sativum* ssp. *arvense*	100	5.0 ± 0.00^{m}	66.1 ± 9.42^{q-z}	100.0 ± 0.00^{n}	1778 ± 194.1^{o-v}
P648	*P. sativum* ssp. *arvense*	100	5.0 ± 0.00^{m}	63.9 ± 7.07^{p-z}	100.0 ± 0.00^{n}	1920 ± 105.1^{p-v}
P651	*P. sativum* ssp. *elatius*	80	4.4 ± 0.57^{i-m}	73.3 ± 16.66^{t-z}	86.7 ± 11.93^{i-n}	1942 ± 308.0^{p-v}
JI82	*P. sativum*	100	5.0 ± 0.00^{m}	67.9 ± 11.42^{r-z}	100.0 ± 0.00^{n}	1991 ± 216.7^{q-v}
P24	*P. sativum* ssp. *elatius*	80	4.4 ± 0.51^{j-m}	74.2 ± 12.37^{u-z}	90.0 ± 8.94^{k-n}	1999 ± 251.0^{q-v}
JI1951	*P. sativum*	100	5.0 ± 0.00^{m}	74.5 ± 3.00^{u-z}	100.0 ± 0.00^{n}	2021 ± 71.2^{r-v}
P691	*P. sativum* ssp. *elatius*	100	5.0 ± 0.00^{m}	69.8 ± 6.44^{s-z}	100.0 ± 0.00^{n}	2022 ± 140.0^{r-v}
P14	*P. sativum* ssp. *abysinicum*	100	5.0 ± 0.00^{m}	75.2 ± 6.37^{v-z}	100.0 ± 0.00^{n}	2063 ± 145.2^{s-v}
P629	*P. sativum* ssp. *arvense*	100	5.0 ± 0.00^{m}	75.2 ± 3.94^{v-z}	100.0 ± 0.00^{n}	2152 ± 70.1^{t-v}
JI1213	*P. sativum* (cv. erylis)	100	5.0 ± 0.00^{m}	79.0 ± 5.15^{x-z}	100.0 ± 0.00^{n}	2153 ± 97.5^{t-v}
JI1210	*P. sativum* (cv. Erygel)	100	5.0 ± 0.00^{m}	77.7 ± 3.11^{w-z}	100.0 ± 0.00^{n}	2184 ± 40.7^{uv}
P667	*P. fulvum*	100	5.0 ± 0.00^{m}	86.5 ± 3.50^{z}	100.0 ± 0.00^{n}	2190 ± 60.0^{uv}
P666	*P. fulvum*	100	5.0 ± 0.00^{m}	86.0 ± 4.00^{z}	100.0 ± 0.00^{n}	2200 ± 50.0^{uv}
JI2840	*P. sativum* (RIL 15x399_68)	100	5.0 ± 0.00^{m}	82.5 ± 4.69^{yz}	100.0 ± 0.00^{n}	2250 ± 0.0^{v}
P21	*P. sativum* ssp. *elatius*	100	5.0 ± 0.00^{m}	90.0 ± 0.00^{z}	100.0 ± 0.00^{n}	2250 ± 0.0^{v}
P312	*P. sativum* ssp. *elatius*	100	5.0 ± 0.00^{m}	90.0 ± 0.00^{z}	100.0 ± 0.00^{n}	2250 ± 0.0^{v}
P623	*P.sativum* ssp. *transcaucasicum*	100	5.0 ± 0.00^{m}	90.0 ± 0.00^{z}	100.0 ± 0.00^{q}	2250 ± 0.0^{v}
P657	*P. fulvum*	100	5.0 ± 0.00^{m}	90.0 ± 0.00^{z}	100.0 ± 0.00^{n}	2250 ± 0.0^{v}
P659	*P. fulvum*	100	5.0 ± 0.00^{m}	90.0 ± 0.00^{z}	100.0 ± 0.00^{n}	2250 ± 0.0^{v}
P661	*P. fulvum*	100	5.0 ± 0.00^{m}	90.0 ± 0.00^{z}	100.0 ± 0.00^{n}	2250 ± 0.0^{v}
P662	*P. fulvum*	100	5.0 ± 0.00^{m}	90.0 ± 0.00^{z}	100.0 ± 0.00^{n}	2250 ± 0.0^{v}
P671	*P. sativum* ssp. *elatius*	100	5.0 ± 0.00^{m}	90.0 ± 0.00^{z}	100.0 ± 0.00^{n}	2250 ± 0.0^{v}
P675	*P. sativum* ssp. *elatius*	100	5.0 ± 0.00^{m}	90.0 ± 0.00^{z}	100.0 ± 0.00^{n}	2250 ± 0.0^{v}
Little Marvel (S)	*P. sativum*	100	5.0 ± 0.00^{m}	84.6 ± 5.40^{z}	100.0 ± 0.00^{n}	2250 ± 0.0^{v}

[1] Data are means of 15 replicates, different letters indicates significant difference between value according to Duncan Multiple Range Test at α = 0.05. DI, disease incidence; MVI, mean value of visual index for all leaves on an individual plant; PSL, percentage of leaves showing symptoms for each individual plant.

In addition, the mean comparison tests allowed separation of the different accessions in three groups for all parameters confirming our classification. The results obtained largely supported the data of the initial screening with very few exceptions. For instance, 11 out of the originally 15 resistant accessions were confirmed as resistant while the remaining four were classified as partially resistant along with P18 and P316 (Table 3)

To determine the most adapted and easiest method of disease scoring for future screening of pea resistance to *Fop*, we examined the correlation existing between the different parameters evaluated using a non-parametric Spearman's correlation rank analysis. The highest and significant correlations were obtained between both AUDPC values ($r = 0.942$; $P < 0.001$) and both DR measurements ($r = 0.925$; $P < 0.001$). Similarly, a good positive correlation were observed between the AUDPC values and their respective DR evaluations at 30 dpi with Spearman's rank correlation of $r = 0.803$ and 0.716 ($P < 0.001$) for MVI and PSL, respectively (Table 4). By contrast, only a low

correlation with r ≤ 0.524 was observed between DI and any of the other parameters (Table 3).

Table 3. Disease Ratings of selected accessions to *Fop* 2

			Disease Ratings[1]			
			Plant Mean Disease Index		Symptomatic Leaves (%)	
Accession	Species	DI (%)	MVI	AUDPC	PSL	AUDPC
P23	*P. sativum* ss|	20 ± 0.0^{bc}	2.3 ± 0.42^{ab}	9.7 ± 4.65^{a}	45.8 ± 8.45^{a}	343 ± 106.4^{a}
JI1412	*P. sativum* ss|	0 ± 0.0 ^{a}	2.1 ± 0.08^{ab}	12.2 ± 1.03^{a}	34.6 ± 2.83^{a}	440 ± 20.6^{ab}
JI1760	*P. sativum* ss|	7 ± 6.7^{ab}	2.4 ± 0.30^{ab}	10.4 ± 1.86^{a}	43.9 ± 7.48^{a}	477 ± 62.4^{ab}
P627	*P. sativum* ss|	7 ± 6.7^{ab}	2.1 ± 0.36^{ab}	15.2 ± 3.69^{a}	31.3 ± 8.93^{a}	479 ± 113.9^{ab}
P656	*P. fulvum*	33 ± 6.7^{cd}	3.6 ± 0.50^{cd}	12.8 ± 3.72^{a}	68.3 ± 11.76$^{b\text{-}d}$	505 ± 109.1^{ab}
P633	*P. sativum* ss|	0 ± 0.0 ^{a}	1.8 ± 0.12^{a}	11.7 ± 1.85^{a}	30.0 ± 2.78^{a}	507 ± 60.4^{ab}
P42	*P. sativum* ss|	0 ± 0.0 ^{a}	1.8 ± 0.14^{a}	12.2 ± 1.61^{a}	32.3 ± 3.33^{a}	571 ± 66.4^{ab}
JI1559	*P. sativum* ss|	0 ± 0.0 ^{a}	2.1 ± 0.12^{ab}	15.1 ± 2.69^{a}	32.8 ± 2.30^{a}	634 ± 79.9$^{a\text{-}c}$
P614	*P. sativum* ss|	0 ± 0.0 ^{a}	2.1 ± 0.12^{ab}	14.1 ± 1.30^{a}	35.6 ± 2.84^{a}	660 ± 49.0$^{a\text{-}c}$
P639	*P. sativum* ss|	7 ± 6.7^{ab}	2.1 ± 0.35^{ab}	18.4 ± 6.07^{ab}	34.3 ± 8.82^{a}	684 ± 172.1$^{a\text{-}d}$
P650	*P. sativum* ss|	0 ± 0.0 ^{a}	1.9 ± 0.22^{a}	12.9 ± 2.29^{a}	34.0 ± 6.86^{a}	694 ± 97.4$^{a\text{-}d}$
P632	*P. sativum* ss|	0 ± 0.0^{a}	2.5 ± 0.11^{ab}	20.8 ± 1.89^{ab}	42.2 ± 4.06^{a}	715 ± 50.5$^{b\text{-}d}$
P638	*P. sativum* ss|	13 ± 6.7$^{a\text{-}c}$	2.9 ± 0.22^{bc}	19.3 ± 3.38^{ab}	54.5 ± 5.44^{ab}	755 ± 67.7$^{b\text{-}d}$
P669	*P. sativum* ss|	40 ± 20.0^{cd}	3.4 ± 0.40^{cd}	20.3 ± 4.06^{ab}	76.5 ± 7.63^{cd}	756 ± 84.6$^{b\text{-}d}$
P18	*P. sativum* ss|	53 ± 6.7^{de}	3.5 ± 0.50^{cd}	34.3 ± 9.78^{c}	68.9 ± 11.24^{bc}	965 ± 245.3$^{c\text{-}e}$
P615	*P. sativum* ss|	53 ± 6.7^{de}	3.8 ± 0.45^{cd}	29.9 ± 6.41^{bc}	83.8 ± 9.03$^{c\text{-}e}$	1010 ± 163.2^{de}
P316	*P. sativum* ss|	73 ± 0.7^{e}	4.1 ± 0.41^{d}	37.1 ± 6.25^{c}	88.6 ± 6.68^{de}	1265 ± 129.0^{e}
P662	*P. fulvum*	100 ± 0.0^{f}	5.0 ± 0.00^{e}	73.9 ± 0.43^{d}	100.0 ± 0.00^{e}	2028 ± 64.2^{f}
P21	*P. sativum* ss|	100 ± 0.0^{f}	5.0 ± 0.00^{e}	83.5 ± 2.91^{d}	100.0 ± 0.00^{e}	2274 ± 23.0^{f}

[1] Data are means of 15 replicates, different letters indicates significant difference between value according to Duncan Multiple Range Test at α = 0.05. DI, disease incidence; MVI, mean value of visual index for all leaves on an individual plant; PSL, percentage of leaves showing symptoms for each individual plant.

3.4. Detection of *F.* oxysporum f.sp. *pisi* within plant tissue

To detect the extension of *Fop* colonisation within plants, we re-isolated the fungi from the different parts (root and basal, middle and apical stem regions) of inoculated plants of 15 of the selected accessions. In the susceptible genotype P21 and the partially resistant accession P316, *Fop* colonies were detected at both extremities of all plated plant segments indicating that as early as 7 dpi, *Fop* had already colonised the whole plant in these accessions (Fig. 3). By contrast, *Fop* colonies were recovered only from root and basal stem segments of the other resistant and partially resistant *Pisum* accessions monitored (Fig. 3). While *Fop* colonies were detected on each extremity of the basal segment in nine resistant and partially resistant genotypes including P23, P42, P614, P615, P627, P632, P633, P650 and JI1760, they were detected only at the lowest extremity in the other four resistant accessions including P638, P639, P656 and P669 (Fig. 3).

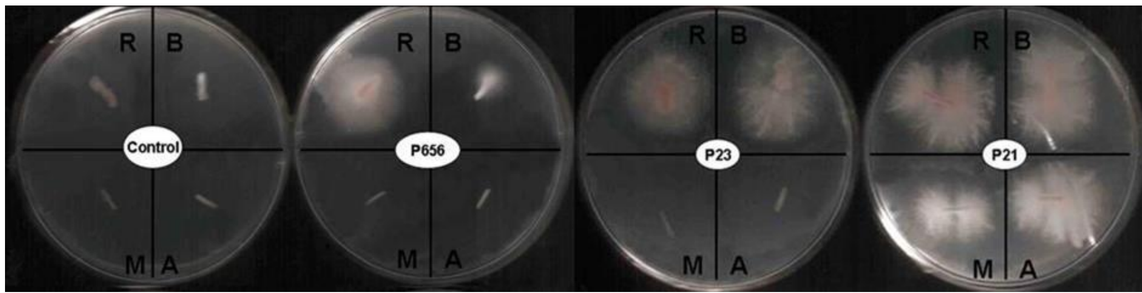

Figure 3. Isolation of *Fusarium oxysporum* f.sp. *pisi* (*Fop*) race 2 colonies from inoculated plants. Photographs compare the extension of *Fop* race 2 colonies out of plant tissues from control non inoculated plants, and plants 7 dpi of the susceptible accession P21, the partially resistant accession P656 and the resistant accession P23. R, B, M and A stand for root, basal stem, middle stem and apex sections, respectively.

3.5. Detection of external and internal symptoms

At the end of the experiment, plants from the selected accessions were removed from vermiculite and visually compared. Interestingly, the external area of the upper root system, the crown and the basal shoot region of resistant and partially resistant genotypes were black- brown in colour. The extent of this discoloration varied according to the genotype, the largest colored area being observed in the highly resistant accession P42 (Fig. 4). This black-brown external discoloration was not detected on the susceptible accessions suggesting that it might play a role in the resistance to *Fop* (Fig. 4).

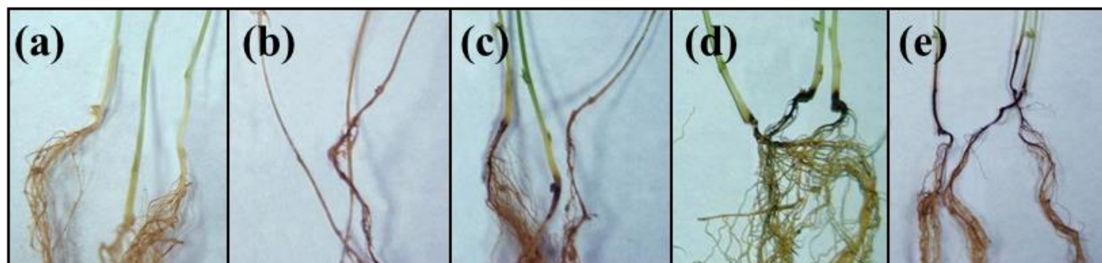

Figure 4. Comparison of *Fusarium oxysporum* f.sp. *pisi* -induced superficial crown darkening on different susceptible and resistant accessions. (a) Control plants of the susceptible accession P21, non-inoculated. (b) Susceptible accession P21 at 30 dpi. (c) Partially resistant accession P656 at 30 dpi. (d) Resistant accession P23 at 30 dpi. (e) Resistant accession P42 at 30 dpi.

Observation under a binocular microscope of longitudinal sections of resistant accessions showed that the discolored root and shoot tissues was still healthy albeit brown suggesting that this discoloration is due to an accumulation of pigmented substances within cells rather than due to cell death (Fig. 5). These sections also revealed the extent of differences in the typical vascular discoloration between accessions. As it is characteristic for *Fop* race 2 infection, the vascular tissue of the whole plant of susceptible accessions such as P21 turned dark red (Fig. 5). By

contrast, this vascular discoloration did not extend further than the plant crown in the resistant and partially resistant accessions as shown for P633 in Fig. 5.

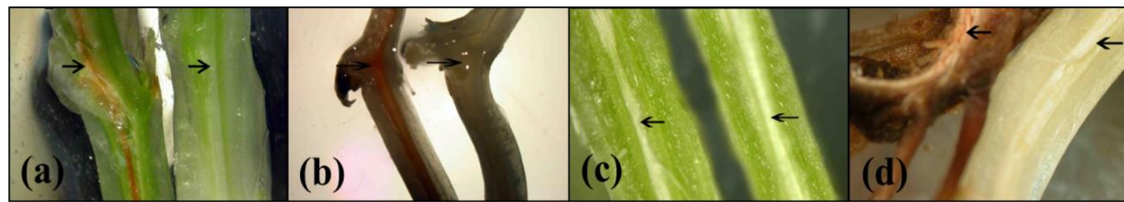

Figure 5. Comparison of the extension of the typical *Fusarium oxysporum* f.sp. *pisi* induced vascular discoloration between susceptible and resistant pea accessions. Each picture shows hand-made longitudinal sections of stem (a, c) and crown (b, d) for inoculated (left) and non-inoculated control (right) plants. (a, b) Susceptible accession P21 showing the typical dark red vascular discoloration in crown and stem 30 dpi with Fop race 2. (c, d) Resistant accession P633 showing the typical dark red vascular discoloration only within crown section. Black arrows indicate vascular tissue.

Staining with the commercial Parker blue Quink ink at 30 dpi supported this observation. With this staining method, the whole shoot of the susceptible accessions, such as P21, appeared blue (Fig. 6a and b), indicating the presence of fungi in the entire shoot of susceptible plants. In these genotypes, we could detect some patches of more intense staining within the surface of the shoot that corresponded to fungal colonies growing out of the susceptible plant tissues (Fig. 6a). By contrast, the blue staining was only observed in the crown and basal shoot section of most resistant entries while the rest of the shoot remained clear (Fig. 6c and d). Altogether, these findings indicated that in most cases the fungal progression was efficiently stopped at crown level and suggested that the most discriminating defence mechanism may be acting in the crown (Figs. 3, 4, 5 and 6).

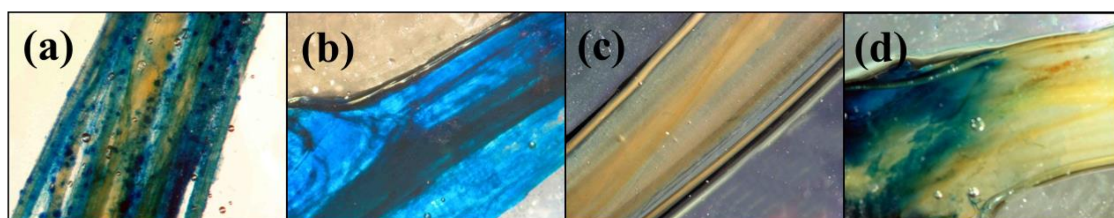

Figure 6. Comparison of the extension of *Fusarium oxysporum* f.sp. *pisi* race 2 colonization within plant tissues between susceptible and resistant pea accessions. Each picture shows a section of stem (a, c) and crown (b, d) of inoculated plants superficially stained with the commercial ink Parker Blue Quink 30 dpi, indicating fungal presence as a blue coloration. (a, b) Susceptible accession P21 showing fungal presence over the whole crown and stem surface. (c, d) Resistant accession P23 showing fungal presence only within the crown section.

4. Discussion

F. oxysporum f.sp. *pisi* is a recurring problem causing important yield losses wherever pea is grown. In this study, different methods to accurately evaluate fusarium wilt disease in a controlled environment were assessed and used to screen a *Pisum* spp. collection to identify new sources of resistance to *Fop* race 2. The different methods of disease evaluation (DI, DR and AUDPC) revealed large variability in the response of the different accessions to *Fop* race 2 ranging from resistant to susceptible and including many partially resistant accessions. Such range of responses suggests that resistance to this *Fop* isolate in this *Pisum* spp. collection is mainly of quantitative nature. As a result of this study, we identified and confirmed 11 new sources of resistance (JI1412, JI1559, JI1760, P23, P42, P614, P627, P633, P639, P650 and P656) showing a very high level of resistance to *Fop* race 2 that may be useful for a breeding program.

Fusarium wilt disease causes a series of external symptoms including vein clearing, leaf epinasty, wilting, chlorosis, necrosis, and abscission leading to the complete plant wilting and death (MacHardy and Beckman, 1983). Under the conditions of this study, the *Fop* race 2 isolate used was highly pathogenic causing fusarium wilt symptoms on susceptible and partially resistant accessions (Fig. 1 and Table 2) which allowed the development of a methodology based on leaf symptoms to evaluate the *Pisum* spp. collection.

Previous studies described pea lines as resistant (no symptoms) or susceptible (dead plants) to specific *Fop* isolates and ignored any variation in their symptom severity (Haglund and Kraft, 1979; Bodker *et al.*, 1993). As a consequence, most previous studies on the identification of resistance sources to this pathogen were based on the sole evaluation of DI (Haglund and Kraft, 1979; Haglund, 1989; McPhee *et al.*, 1999; Sharma *et al.*, 2010). In case of *Fop* race 2, variation in the DI value of pea accessions have often been detected hampering their classification within the resistant or susceptible group and the establishment of clear segregation ratios within populations (Hare *et al.*, 1949; McPhee *et al.*, 1999). In the present study, similar variation for DI was detected between experiments (Table 3). In addition, DI values did not always agree with the resistance reactions in our collection, since we observed accessions such as P11 and P670 with severe fusarium wilt symptoms, but DI values of only 40% (Table 2). This highlights the need to evaluate additional parameters to accurately estimate disease reaction to *Fop* race 2 in pea. The evaluation of disease severity is often a good method to assess quantitative resistance mechanisms (Russell, 1978), but only a limited number of studies have used a disease severity

index to evaluate pea resistance to fusarium wilt (Charchar and Kraft, 1989; Lebeda and Svabova, 1997; Neumann and Xue, 2003; Lebeda *et al.*, 2010). In these studies, the disease scoring was based on a 0-5 (Charchar and Kraft, 1989; Neumann and Xue, 2003) or 0-3 (Lebeda and Svabova, 1997; Lebeda *et al.*, 2010) rating system of the whole plant which appeared inadequate in our hand to accurately assign plant symptoms to a specific disease index. Instead two different methods to assess DR considering leaf symptoms of the whole plant was tested on a series of differential lines and used to evaluate disease reactions of a *Pisum* spp. germplasm collection. Results obtained from both methods were highly similar clearly discriminating between the resistant and susceptible genotypes and detecting intermediate reactions (Tables 1 and 3). The high correlation between both methods indicates that only one of them is require to determine the disease reaction of pea accessions (Table 4). The proportion of symptomatic leaves is the fastest method and would therefore be the method of choice to evaluate DR in future screening of pea germplasm to *Fop* race 2. Fusarium wilt disease development requires several weeks from plant infection to plant death, thus, AUDPC (Teng and James, 2002) that consider severity over time was also calculated. Although more time consuming, this method appears more reliable and reproducible to estimate with accuracy disease response of pea accessions which is supported by the high correlation existing between both AUDPC measurements and between AUDPC and the DR values (Tables 3 and 4). By contrast, the low correlation coefficient obtained when comparing DI measurements with any other disease parameters confirmed that this parameter alone is not adequate to describe disease response to *Fop* race 2 in pea (Table 4).

Despite the limited number of accessions used in this study, this *Pisum* spp. collection contained sufficient genetic variation to detect a wide range of responses to *Fop* race 2 from highly resistant to susceptible genotypes (Tables 2 and 3). Among the 73 accessions screened, 59% of the collection showed some resistance with 24.7% genotypes presenting high level of resistance to this isolate, even more than the resistant differential cultivars (Tables 1, 2 and 3). Nevertheless it should be noted that only phenotypes of the selected accessions have repeatedly shown consistent reaction to the pathogen and that other accessions may need repeated testing to confirm their relative resistance. Of the 11 highly resistant accessions, only three belong to *P. sativum* ssp. *sativum* whereas eight belong to other subspecies of *P. sativum* including two *P. sativum* ssp. *elatius*, four *P. sativum* ssp. *arvense* and one *P. sativum* ssp. *jormadi* and one belong to *P. fulvum* (Table 3). While screening for resistant to all four races of *Fop* have been extensively performed, few studies described very high level of resistance in collection of *Pisum* spp. (Lebeda and Svabova, 1997). Indeed, only one

previous study specifically dealt with wild species and subspecies and although authors reported a wide variety of responses to *Fop*, they failed to identify complete resistance (Lebeda and Svabova, 1997). On the other hand, the screening of 452 pea accessions from the USDA core collection revealed that 14% of the whole collection (62 accessions) was resistant to races 1 or 2 of *Fop* (McPhee *et al.*, 1999). Interestingly, one *P. sativum* ssp. *abyssinicum* and one *P. sativum* ssp. *elatius* accessions were also identified as resistant in this study (McPhee *et al.*, 1999). The *P. sativum* ssp. *elatius* accession PI344012 was also included in this study (P24), however, under these experimental conditions, this accession was highly susceptible to our *Fop* race 2 isolate showing a disease incidence of 80% (Table 2).

Resistance to all of the *Fop* races in pea has been considered qualitative with a monogenic inheritance (Infantino *et al.*, 2006). For race 2 of *Fop,* most previous studies identified only one genetic factor controlling resistance to this race (Hare *et al.*, 1949; Haglund, 1989; McPhee *et al.*, 1999). However, mixed phenotypes were often observed within accessions even when considering the set of differential lines used to characterise *Fop* isolates (Haglund, 1989; McPhee *et al.*, 1999). In these studies, these authors attributed the variability to heterogeneity of the seed population excessive root trimming prior to inoculation or the presence of another fungal pathogen (McPhee *et al.*, 1999). Interestingly, subsequent studies by the same authors reported intermediate resistance reactions in response to *Fop* race 2 questioning the reaction of these lines to this race (McPhee *et al.*, 2004). In the present study we detected a continuity of responses from highly resistant to susceptible (Fig. 2). A previous screening of another collection of *Pisum* spp. for resistance to *Fop* race 2 also detected large variation in the responses of individual accessions (Lebeda and Svabova, 1997). Such continuity would support the existence of quantitative resistance mechanisms in the *Pisum* spp. collection, which also can be seen in the differential lines in which small variation was detected (Table 1). Thus in accordance with the observation of McPhee *et al.* (2004), our results suggest the existence of additional genetic factors that control *Fop* race 2 resistance, although genotypic analysis of progenies of these accessions would be needed to confirm this hypothesis.

As a result of the present study, 43 accessions with quantitative resistance were identified of which 11 developed only very mild symptoms. As a next step it will be important to characterise the resistance mechanisms responsible for their phenotype to ease selection in breeding programs. Many studies in *Solanum lycopersicum*, *Arabidopsis thaliana* and *P. sativum* indicated that resistant plants to *F. oxysporum* displayed a wide and complex array of anatomical and biochemical responses to counteract pathogen infection (Beckman, 1987; Kraft, 1994; Michielse and Rep, 2009).

However, the actual resistance mechanisms acting in a specific resistant accession are still unclear (Zvirin *et al.*, 2010). As a starting point to characterise the resistance mechanisms acting in the resistant accessions, a detailed observation of internal and external symptoms was undertaken. These initial studies suggested that the strongest resistance response acted at the crown level. Indeed, fungal colonies were only isolated from roots and the basal part of the stem in resistant and most partially resistant accessions while *Fop* was present in the whole plant in susceptible accessions as early as 7 dpi (Fig. 3). In addition, observation of external and internal symptoms indicated clear differences between susceptible and resistant genotypes. Pea infection by *Fop* is usually associated with a discoloration of vascular tissue which turns orange or dark red (Kraft and Pfleger, 2001). In agreement with these observations, vascular tissue of most accessions screened in the present study turned dark red (Fig. 5). However, this discoloration was restricted to the root and basal stem section in the resistant and partially resistant accessions while it reached shoot apex in susceptible accessions (Fig. 5). This agreed with the observation that *F. oxysporum* colonization of resistant host and non-host was limited to the root and basal part of the stem of different plant species (Beckman, 1987; Charchar and Kraft, 1989). Interestingly, the restriction of the extent of vascular discoloration observed in resistant and partially resistant accessions was accompanied by a blackening of the cortical and epidemical cells around the crown region (Figs. 4 and 5). Previous studies indicated that pea infection by *Fop* race 2 was often associated with secondary cortical decay (Hagedorn, 1984; Kraft and Pfleger, 2001). However, our observations on the blackened regions did not suggest any decay of these tissues, but rather a cortical hardening as the blackened cells appeared to be still alive (Fig. 5). Further studies are now underway to determine the mechanisms acting in these resistant accessions at the cellular and molecular levels. In the meantime, the incorporation of these resistant accessions in breeding programs of elite pea cultivars together with the application of a simplified scoring method derived from the present study is expected to improve the resistance status of pea to *Fop* race 2 in the near future.

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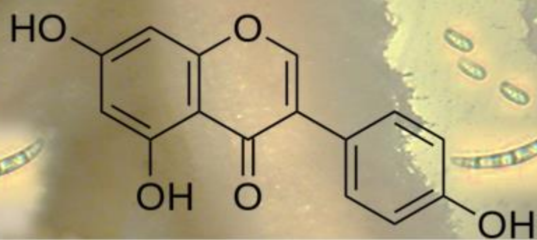
Chapter 3

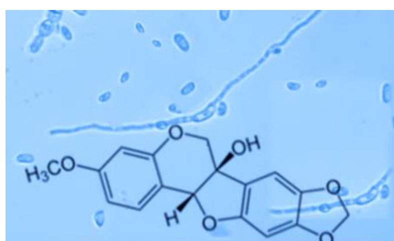


Chapter 3

Pisatin concentration in pea root exudates is an element of the pre-penetration resistance mechanism of pea accessions against *Fusarium oxysporum* f.sp. *pisi* based on inhibition of spore germination

Abstract

Fusarium oxysporum f.sp. *pisi* is one of the major constraints of pea worldwide. Control of this disease is difficult and is mainly based on the use of resistant cultivars. While monogenic resistance has been successfully used to control the disease, it is at risk of breakdown by the constant evolution of the pathogen. New sources of quantitative resistance have been recently identified from a wild relative *Pisum* spp. collection. In the present study, we aimed to identify the resistance mechanisms acting before root penetration in these novel sources of quantitative resistance. For this, we collected the root exudates of 12 pea accessions with differential responses to the disease from resistant to susceptible and determined their effect on *Fop* germination and growth. While most pea root exudates stimulated *Fop* germination, the root exudate of three pea accessions JI1412, JI2480 and P42 did not and even inhibited *Fop* germination. This indicated that some pea accessions expressed constitutive pre-penetration resistance mechanisms. Although some additional compounds might be involved, our analyses showed that the most active metabolite was the pea phytoalexin pisatin. Indeed, pisatin was identified in the active fraction of pea root exudate extracts and its amount in the root exudates was negatively correlated with the extent of *Fop* germination. This suggests an important role of pisatin in the constitutive defence of pea against one of its major constraint *F. oxysporum.*



Manuscript in preparation

1. Introduction

Fusarium wilts are among the most important diseases affecting grain legumes (Kraft *et al.*, 1998; Rubiales et *al.*, 2015). *Fusarium oxysporum* f.sp. *pisi* (*Fop*) is an important and destructive pathogen of field pea (*Pisum sativum*) worldwide (Kraft and Pfleger, 2001). This soilborne pathogen can survive as thick-walled chlamydospores, which remain viable in the soil for many years making its control difficult. Upon host recognition, the germinating fungus is able to penetrate the root and reach the vascular vessels where it grows profusely leading to a rapid plant death (Di Pietro *et al.*, 2003). Once established, this pathogen is very difficult to eradicate since it can grow saprophytically in the absence of a compatible host. Currently, the most efficient and economically viable method of control is the use of resistant cultivars (Rubiales *et al.*, 2015). *Fop* resistance, based on single race-specific genes, has been detected and successfully transferred to pea cultivars (Infantino *et al.*, 2006). However, this monogenic resistance is at risk of breakdown by the constant evolution of the pathogen leading to the emergence of four distinct races of *Fop* so far. It is thus essential to continuously search for novel sources of resistance to complement and reinforce the actual resistance of elite cultivars. Recently, a quantitative resistance has been detected in a collection of wild pea germplasm (Bani et *al.*, 2012). Characterisation of the mechanisms involved in the resistance to *Fop* in this collection is necessary for a more efficient breeding process.

Several mechanisms of resistance to fusarium wilt have been described in plants. The most efficient mechanisms are the formation of chemical and physical barriers at attacked site and within vascular tissue by means of phytoalexin accumulation, lignification and accumulation of gums, gels or tyloses within xylem cells (Beckman, 1987; Tessier *et al.*, 1990; Benhamou *et al.*, 1996). Pre-penetration mechanism inhibiting *Fop* spore germination has also been described in pea (Buxton, 1957b; Kraft, 1974). This pre-penetration mechanism may reduce the pathogenic pressure in the soil. The introduction of such mechanism into cultivars or application of the responsible bioactive compounds may thus contribute to the control of the pathogen.

Root exudates are believed to play an important role as regulator of the interaction between plants and soil organisms (Bais *et al.*, 2006). Plants exude large quantity of organic acids and amino acids that improve the nutritional quality of the soil surrounding plant roots favouring the development of many microorganisms (Rovira, 1956; Buxton, 1957a; Buxton, 1960; Bais *et al.*, 2006; Broeckling *et al.*, 2008). Plants also exude several phenolic compounds and other secondary metabolites that can modulate rhizosphere organisms (Rovira, 1956; Bais *et al.*, 2006; De-la-Pena and

Vivanco, 2010). For instance, flavonoids and strigolactones released by legume roots have been shown to play a crucial role as early signals in their symbiotic interaction with nitrogen-fixing rhizobia and arbuscular mycorrhiza fungi respectively (Akiyama *et al.,* 2005; Bais *et al.,* 2006; Rispail *et al.*, 2010). Root exudates from different crop species including tomato and pea have been shown to stimulate *F. oxysporum* growth and germination although the nature of the stimulating signal is still unknown (Whalley and Taylor, 1973; Steinkellner *et al.*, 2005). By contrast, some resistant lentil and pepper cultivars were shown to inhibit *F. oxysporum* germination. In this case, the inhibition was related to the exudation of specific amino acids and undetermined phenolic compounds (Naqvi and Chauhan, 1980; Stevenson *et al.*, 1995). In pea, an initial study showed a race-specific inhibition of *F. oxysporum* growth by some resistant accessions (Buxton, 1957b). However, further studies showed that resistant accessions induced similar level of *Fop* germination and growth as susceptible ones (Kommedahl T., 1966; Whalley and Taylor, 1973).

To determine whether some pea accessions possessed mechanisms of resistance acting before root penetration, we tested the capacity of the root exudates of resistant pea accessions to stimulate or inhibit *Fop* race 2 growth and germination. For this, we collected the root exudates of 12 pea accessions with differential responses to the pathogen (Bani *et al.*, 2012) and studied their effect on fungal growth and development. This indicated that some pea accessions expressed a constitutive pre-penetration mechanism that might contribute to control the pathogen. In addition, it revealed a key role of the pisatin in this constitutive pre-penetration resistance mechanism.

2. Material and Methods

2.1. Fungal isolates and cultural conditions

The *F. oxysporum* f.sp. *pisi* (Fop) strain R2F42 was used throughout the experiments. This strain belongs to *Fop* race 2 and was kindly provided by Dr. W. Chen (USDA-ARS, Pullman, USA). For microconidia production, cultures were grown in potato dextrose broth (PDB; Difco, Detroit, MI) at 28 ºC in a shake culture set at 170 rpm (Bani *et al.*, 2012). For the germination assays, 5 x 10^5 *Fop* conidia were incubated in 1 ml of pure root exudates or organic extracts in 0.5% methanol for 12 h at 28 ºC under constant shaking at 170 rpm. A solution of sterile water or of 0.5% methanol was used as negative control respectively while a solution of potato dextrose broth (PDB) was used as positive control. After incubation, three independent 10 µl aliquots per sample were spotted onto a microscope slides and observed under X20 magnification using a

bright field Leica DM-LB light microscope (Leica Microsystems Wetzlar GmbH, Wetzlar, Germany) to estimate the proportion of germinated conidia based on the observation of 100 conidia per aliquots. *Fop* conidia were considered germinated if their germ tube length was at least as long as the spore. Three biological replicates were used for each treatment and the whole experiment was performed at least twice with similar results.

2.2. Plant material and growth condition

Twelve accessions of *Pisum* spp. presenting differential responses to *Fop* race 2 were used in this study, five of them (JI1760, JI1213, JI1412, JI2480, 'Messire') belong to *P. sativum* ssp. *sativum*, four to *P. sativum* ssp. *arvense* (P627, P629, P633, P641), two to *P. sativum* ssp. *elatius* (P42, P615) and one to *P. abyssinicum* (P650). Among these accessions seven are resistant to race 2 of *Fop*, three are partially resistant and three are susceptible (Table 1) (Bani *et al.*, 2012). JI accessions were provided by the genetic resource center of the John Innes Center (Norwich, UK), PI accessions were from USDA (Pullman, USA) while the IFPI accessions were provided by ICARDA (Aleppo, Syria).

Table 1. List of pea accessions used in this study

Accession	Accession number	Plant Species	Reaction to *Fop R2*
JI1412		P. sativum subsp sativum	R
JI1760		P. sativum subsp sativum	R
JI2480		P. sativum subsp sativum	PR
Messire		P. sativum subsp sativum	PR
JI1213		P. sativum subsp sativum	S
P627	IFPI 2351	P. sativum subsp arvense	R
P633	IFPI 2357	P. sativum subsp arvense	R
P641	IFPI 2365	P. sativum subsp arvense	R
P629	IFPI 2353	P. sativum subsp arvense	S
P42	PI 268480	P. sativum subsp elatius	R
P615	IFPI 3370	P. sativum subsp elatius	PR
P21	PI 505059	P. sativum subsp elatius	S
P650	IFPI 2495	P. abyssinicum	R

Pea seeds were surface-sterilized with 20% sodium hypochlorite and germinated as detailed previously (Bani *et al.*, 2012). Once germinated, seedlings were transferred in 0.5 L pots containing sterile perlite at a density of 10 plants per pots and grown for 15 days in a controlled environment chamber under a 16/8 h light-dark photoperiod at a

26 ± 2 °C temperature regime with 200 µmol m^{-2} s^{-1} of illumination. Plants were watered every three days with tap water.

2.3. Collection, extraction and fractionation of root exudates

The root exudates were collected as previously described (Fernández-Aparicio *et al.*, 2009). Briefly, 15 days after emergence, three groups of 15 seedlings per accessions and plant species were transferred independently from the perlite to black containers filled with 500 ml of sterile distilled water and incubated in the same growth chamber for two days. Then, the root exudates of each replicate of 15 seedlings were collected, filtered through filter paper, frozen at -80 ºC and lyophilized at -20 ºC. The resulting dry powders were redissolved in 1/10 of the initial volume with distilled water and the solution extracted with ethyl acetate (3 x 50 ml). The resulting organic extracts were combined, dried (Na_2SO_4) and evaporated under reduced pressure yielding brown oily residues (8 ± 5 mg), for HPLC analyses.

To identify inhibitors of germination organic extract were obtained as explained previously from 30 L of root exudate from the pea cultivar 'Messire' (Fernandez-Aparicio *et al.*, 2009) and fractioned as described previously (Evidente *et al.*, 2010). Briefly, the organic extract (146.9 mg) was fractionated by column chromatography (50 x 3 cm) on silica gel [eluent chloroform-iso-propanol (95:5, v/v) yielding 5 homogeneous fraction groups according to their chromatographic profile. The residues of fractions F1-F2 were combined and further purified by thin layer chromatography (TLC) on silica gel [eluent chloroform-iso-propanol (9:1, v ⁄v)], to give the main metabolites as a homogeneous compound which was identified as pisatin by ^{1}H NMR (400 MHz, $CDCl_3$, Bruker) and ESI MS (Agilent Technologies 6120 Quadrupole LC/MS). Column chromatography (CC) was performed on silica gel (Kieselgel 60, 0.063-0.200 mm). Analytical and preparative TLC were performed on silica gel (Kieselgel 60, F254, 0_25 and 0.5 mm respectively, Merck) plates. The spots were visualized by exposure to UV radiation (280 and 354 nm), or by spraying first with 10% H_2SO_4 in methanol and then with 5% phosphomolybdic acid in ethanol, followed by heating at 110 °C for 10 min, or by exposure to iodine vapours.

2.4. HPLC quantification of pisatin in crude pea root exudates extracts

The pisatin content of the organic extracts was established by high performance liquid chromatography (HPLC) analysis according to the method of Preisig *et al.* (1990) following slight modifications, using an Agilent 1200 Series HPLC equipped with a photodiode array (PDA) detector set at 309 nm. The organic extracts dissolved in

methanol were separated on an Agilent Eclipse XDB-C18 column with a mobile phase of acetonitrile:water (25:75) v/v 46:54 which was transformed by a linear gradient to acetonitrile:water (46:54) v/v, over 30 min. The initial conditions were then restored according to a linear gradient over 5 min., and the column was re-equilibrated under these conditions for 10 min. before the next run was initiated. The flow rate was 1.0 ml min^{-1} and 20 µl aliquots of the samples were injected for analysis. Retention time for pisatin was 23.5 ± 0.7 min. Pisatin content in the organic extracts was confirmed by coinjection.

The HPLC calibration curve (the relevant data are in Table 2) for quantitative determination of pisatin was performed with absolute amount of pisatin previously isolated dissolved in methanol in the range between 1 and 40 µg with eight concentrations in triplicate for each concentration. HPLC linear regression curve (absolute amount against chromatographic peak area) for pisatin was obtained based on weighted values calculated from eight amounts of the standards in the above range. The samples were dissolved in methanol at 2 mg extract ml^{-1} and aliquots (20 µl) were injected into the HPLC instrument. Each sample was assayed in triplicate. The quantitative determination of pisatin was calculated by interpolating the area of chromatographic peaks with the data of the calibration curves.

Table 2. Analytical characteristics of calibration curve[a] for pisatin.

R_t (min)	Range (µg)	Slope	Intercept	r^2	Number of data point	LOD(µg)[b]
23.50 ± 0.7min	1-40	665.43	+253.94	0.996	7	0.5

[a]Calculated in the form y = a + bx where y = chromatographic peak area and x = µg of metabolite injected. [b]Limit of detection.

2.5. Statistical Analysis

All experiments followed a completely randomized design. Percentage of germination values was subjected to an angular transformation to normalise the data and stabilize the variances before being subjected to the analysis of variance (Baird *et al.*, 2002). The significance of the differences in *Fop* germination and pisatin content between treatments was estimated by one-way analysis of variance (ANOVA). Whenever the ANOVA test was statistically significant ($p \leq 0.05$), a Duncan's multiple range test assessing the differences of the means between each treatment was performed. All statistical analyses were performed using SPSS statistics v.22 software (IBM Corp., USA).

3. Results

3.1. Effect of pea root exudate on *Fop* germination

To determine whether some pea accessions secreted specific compounds with stimulatory or inhibiting effects on *Fop* development, we collected the root exudate from a selection of 12 wild pea relative accessions with differential responses to the pathogen. Significant differences ($p < 0.001$) were detected in the extent of *Fop* germination according to the sample tested (Fig. 1). As expected, a low level of germination of 14.2% was detected in the control sample incubated in sterile water while all spores germinated when incubated in rich growth medium (Potato dextrose broth). The root exudates of most pea accessions (9 out of 12) stimulated *Fop* germination and induced from 30% to 67% of germination for JI1213 and P615 root exudates respectively. The root exudates of the last three pea accessions, JI1412, JI2480 and P42 did not induced germination and even inhibited *Fop* germination (Fig. 1). Indeed, incubation of *Fop* spores in JI1412 and P42 root exudates led to 13.3 and 12.3% of germination which was not statistically different from the negative control. Interestingly, the root exudate of the partially resistant JI2480 accession inhibited *Fop* germination since only 6.4% of the conidia had germinated at the end of the incubation time (Fig. 1). Initially, we also tested the effect of pea root exudates on additional fungal growth parameter including colony length, hypha aggregation through anastomosis and sporulation. However, no significant differences were detected for these parameters (data not shown).

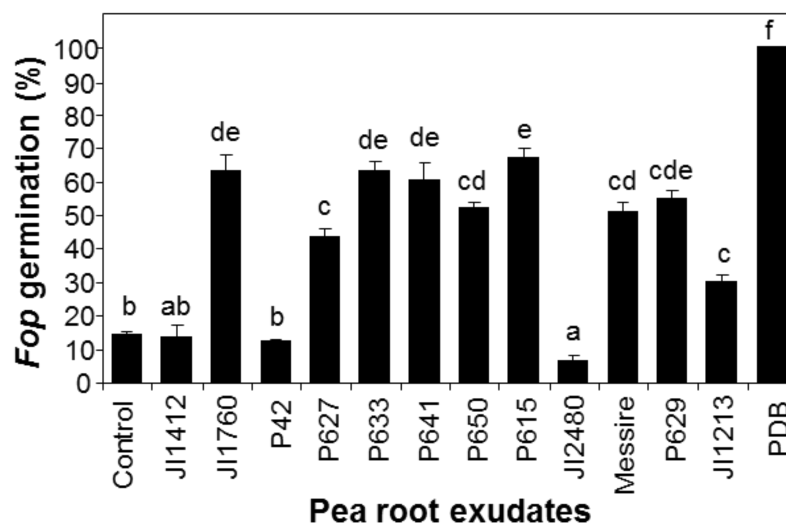


Figure 1. Effect of pea root exudates on *F. oxysporum* f.sp. *pisi* (*Fop*) germination rates. The histogram shows the extent of germination of one isolate of *Fop* race 2 after incubation for 12 h in presence of 1ml of sterile water (Control) or 1 ml of filtered root exudates from a selection of pea accessions with differential responses to *Fop*. The extent of *Fop* germination was estimated by calculating the proportion of germinated conidia from the observation of 100 conidia. Vertical bars are standard errors for n= 3.

3.2. Effect of legume and non-legume species on *Fop* germination

To determine whether the stimulation of germination detected for most pea accessions was due to the presence of species specific compounds, the effect of root exudates from legume and non-legume plant species was tested. As for the experiments with the different pea accessions, some variation in the extent of *Fop* germination was detected according to the root exudate tested (Fig. 2). All root exudates tested stimulated *Fop* germination since the level of germination was in all cases significantly higher than the negative control in water that only reached 10.4% of germination. Nevertheless, the root exudate from non-host legume species such as lentil and chickpea induced a significantly lower germination rates than that of pea. As shown in Fig. 2, the pea root exudate induced the highest level of germination reaching 47.1% while the lentil and chickpea root exudates induced the lowest level of germination reaching only 18.5% and 19.7% respectively (Fig. 2). The incubation of *Fop* in tomato root exudate led to 33.4% of *Fop* germination which was not statistically different from the incubation in pea root exudate (Fig. 2). This would indicate a non-specific stimulation of *Fop* germination by root exudates although some components of the legume root exudates may contribute in host recognition between closely related legume species.

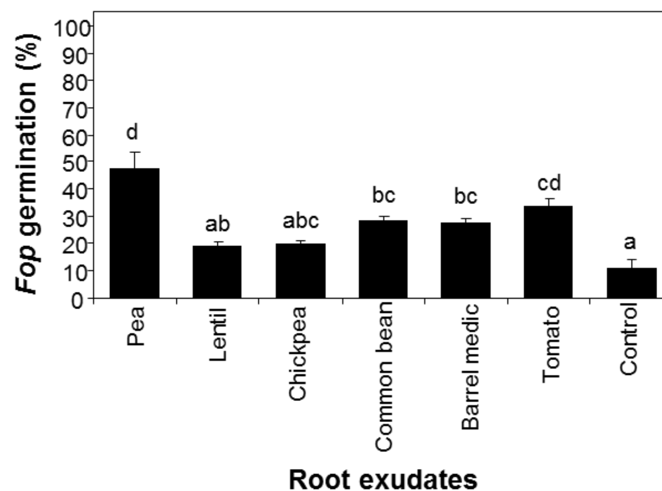


Figure 2. Effect of root exudates from legume and non-legume crop species on *F. oxysporum* f.sp. *pisi* (*Fop*) germination rates. The histogram shows the extent of germination of one isolate of *Fop* race 2 after incubation for 12 h in presence of 1 ml of sterile water (Control) or 1 ml of filtered root exudates from legume and non-legume species. The extent of *Fop* germination was estimated by calculating the proportion of germinated conidia from the observation of 100 conidia. Vertical bars are standard errors for n = 3.

3.3. Effect of root exudate organic extract on *Fop* germination

As a first step toward the identification of stimulators and/or inhibitors of *Fop* germination, the different pea root exudates were extracted with ethyl acetate. As previously, we detected significant differences in the rate of *Fop* germination according

to the root exudates extract as determined by ANOVA ($p < 0.001$) (Fig. 3). By contrast to the crude root exudates, most of these organic extracts did not stimulate *Fop* germination. As shown in Fig. 3, extracts of seven of the accessions (JI1412, JI1760, JI2480, P42, P627 and P633) induced similar level of germination than the negative control in water, while four (P641, P650, 'Messire' and JI1213) induced even lower germination rates (Fig. 3). Extracts from the root exudates of the last two accessions (P615 and P629) stimulated *Fop* germination with levels of 16.3% and 16.1% of germinated spores which was significantly higher than the control that only induced 10% of germination (Fig. 3) but still lower than their respective crude root exudates (Fig. 1). Altogether these results indicated that most stimulatory compounds are contained in the aqueous phase of the root exudates while the organic fraction may contain inhibitors of *Fop* germination.

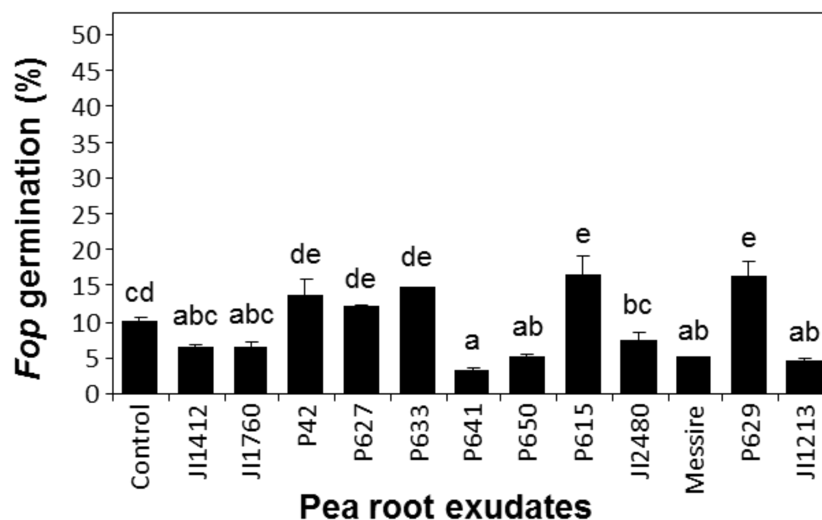


Figure 3. Effect of ethyl acetate extract of pea root exudates on *F. oxysporum* f.sp. *pisi* (*Fop*) germination rates. The histogram shows the extent of germination of one isolate of *Fop* race 2 after incubation for 12 h in presence of 1ml of sterile 0.5% methanol in water (Control) or 1 ml of root exudate extracts from a selection of pea accessions with differential responses to *Fop* obtained after extraction with ethyl acetate and resuspended in 0.5% methanol. The extent of *Fop* germination was estimated by calculating the proportion of germinated conidia from the observation of 100 conidia.Vertical bars are standard errors for n = 3.

To identify the potential inhibitors, the organic extract obtained from the root exudate of the cultivar 'Messire' was fractionated by chromatographic techniques (CC and TLC). From the five homogeneous fractions obtained, two (F1 and F2) inhibited *Fop* germination by comparison with the control. Only 3.7% and 2.5% of *Fop* spores were germinated in the F1 and F2 fractions respectively which were significantly lower than the control for which 6.7% of germination was detected (Fig. 4). Similar level of *Fop* germination (4.9%) was detected with the original organic extract of 'Messire' root exudate (Fig. 4). The other three fractions either led to similar or higher germination rates as the control. This suggested that the compounds responsible for the inhibition

of germination observed in the organic extract (Fig. 3) were contained in the fractions F1 and F2 (Fig. 4).

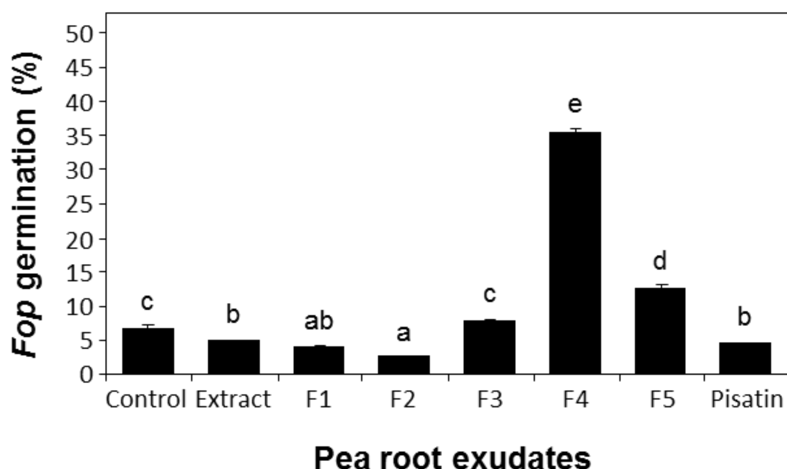


Figure 4. Effect of fractions of root exudate extract on *F. oxysporum* f.sp. *pisi* (*Fop*) germination rates. The histogram shows the extent of germination of one isolate of *Fop* race 2 after incubation for 12 h in presence of 1ml of 0.5% methanol in water (Control) or 1 ml of the homogeneous fractions (F1 to F4) of Messire root exudate extract. As positive and negative control, the effect of the four homogeneous fractions was compared to that of pure pisatin resuspended in 0.5% methanol or of the whole Messire root exudate extract. The extent of *Fop* germination was estimated by calculating the proportion of germinated conidia from the observation of 100 conidia.Vertical bars are standard errors for n = 3.

Further purification of these two fractions by TLC gave one main metabolite that was identified as pisatin by comparison of its spectroscopic properties (^{1}H NMR and ESI MS) with those reported in literature (Dewick, 1997). Pisatin a well-characterised pea phytoalexin, is a rearranged and polysubstituted isoflavonoid representing the subgroup of pterocarpans together with the closely related legumes phytoalexins maackiain and medicarpin (Fig. 5) (Dewick, 1979, Evidente *et al.*, 2010). Thus the effect of pisatin at 50 ppm in 0.5% methanol on *Fop* germination was also tested. As shown in Fig. 4, the percentage of germination of *Fop* spores in presence of pisatin (4.6%) was not different from that of whole organic extract of 'Messire' root exudate and significantly lower than the control.

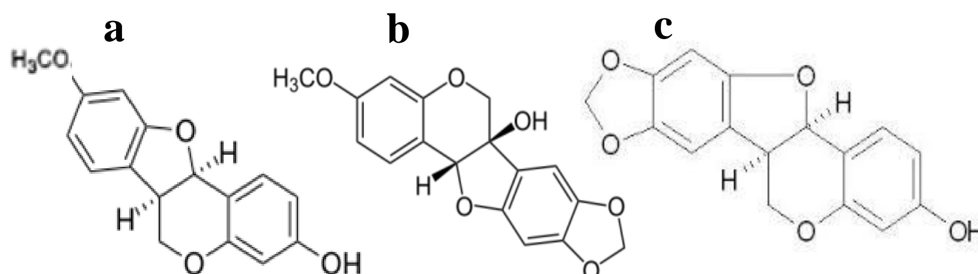


Figure 5. Chemical structure of the pterocarpan péhytoalexins isolated from various plant legumes species. (a); medicarpin produced by alfalfa and chickpea (b); pisatin, produced by garden pea (c); maackiain, produced by red clover.

3.4. Quantification of pisatin content in pea root exudates

To test whether pisatin may be also responsible for the inhibition of germination observed after incubating in the root exudates extracts of the other of pea accessions, we quantified their pisatin content. HPLC quantification (Fig. 6) of pisatin revealed large variation between extracts ($p < 0.001$). Mean comparison revealed three distinct groups according to their pisatin content (Fig. 7). Five accessions (P615, P629, P633, P641 and P650) had very low level of pisatin ranging from 30.2 to 86.8 µg pisatin ml^{-1} extract for P629 and P615 respectively. The extract of four accessions (JI1760, 'Messire', P42 and P627) contained intermediate level of pisatin ranging from 134.4 to 202.1 µg pisatin ml^{-1} extract (Fig. 7). By contrast, three accessions (JI1213, JI1412 and JI2480), all belonging to *P. sativum* spp. *sativum*, contained the highest amounts of pisatin from 813.6 to 933.6 µg pisatin ml^{-1} extract for JI1213 and JI2480 respectively (Fig. 7). Interestingly, the *P. sativum* spp. *sativum* accessions contained higher levels of pisatin than the other *P. sativum* related subspecies.

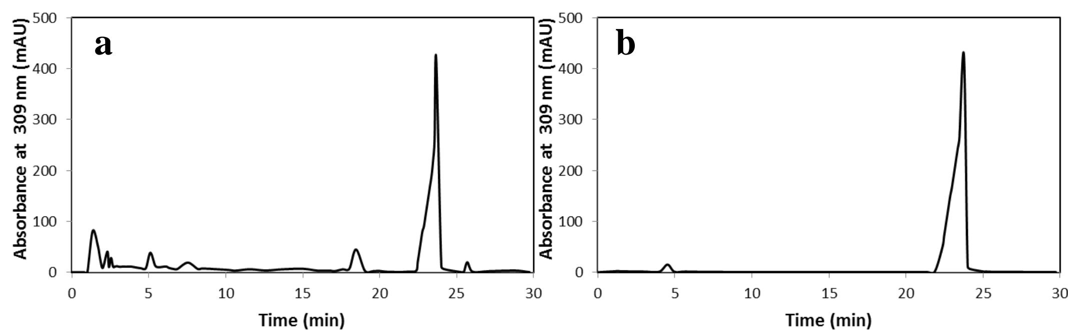


Figure 6. HPLC profile of basic organic extract of root exudate from the *P. sativum* ssp. *sativum* accession JI2480 (a) and pure pisatin (b). The peaks were identified by injection of 20 µg pure pisatin in methanol (b) or 20 µl aliquots of the root exudate extract of JI2480 at the concentration of 2 mg of organic extract ml^{-1} in methanol (a).

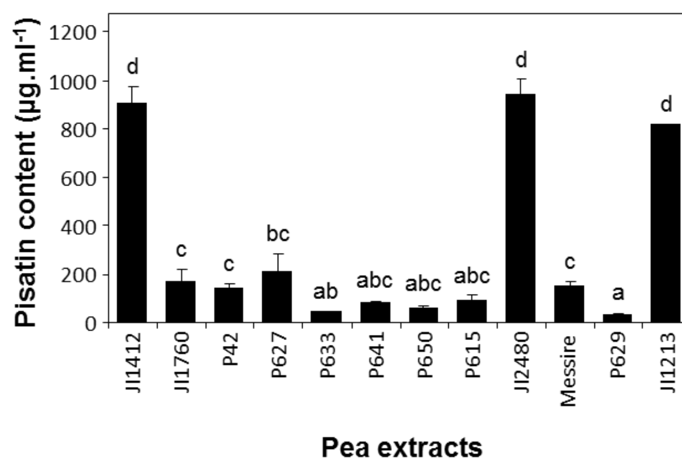


Figure 7. Quantification of pisatin in pea root exudate extract. The histogram shows the pisatin content estimated in the root exudate extracts of each pea accession. Vertical bars are standard errors for n = 3.

To confirm the involvement of pisatin in the inhibition of *Fop* germination previously observed, we established the relationship between the percentages of *Fop* germination induced by the root exudates with their pisatin content (Fig. 8). Spearman's rank correlation analysis indicated a significant negative correlation between the pisatin content of root exudate extract and the percentage of *Fop* germination (r^2 = -0.695; $p <$ 0.001). This correlation was supported by linear regression (r^2 = 0.56; Fig. 8a). Root exudates from the *P. sativum* ssp. *elatius* accession P42 contained only low amount of pisatin while they inhibited *Fop* germination. This suggests that in this accession pisatin was not responsible for the inhibition detected and explain the moderate support of the linear correlation when all accessions were considered in the analysis. Accordingly, removing this accession and restricting the analysis to the *P. sativum* ssp. *sativum* accessions increased the linear regression coefficient (r^2 = 0.89) (Fig. 8b) and the coefficient of correlation (-0.71; $p < 0.001$). This suggested that pisatin might be at least partly responsible for the inhibition of *Fop* germination detected in the *P. sativum* ssp. *sativum* accessions.

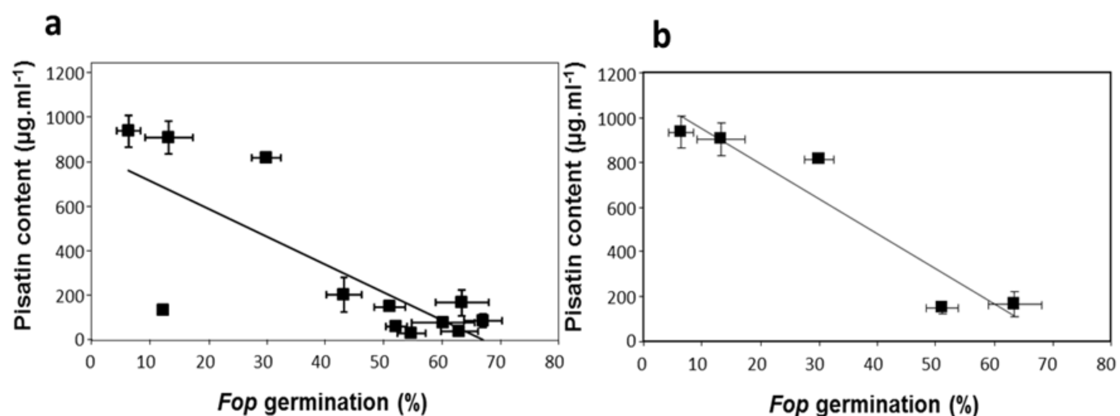


Figure 8: Relationship between the extents of germination of *F. oxysporum* f.sp. *pisi* (*Fop*) induced by the root exudates and their corresponding pisatin content. The graphic represents the linear regression calculated for the amount of pisatin in root exudate extract of all accessions (**a**) or only the *P. sativum* ssp. *sativum* accessions (**b**) with the extents of *Fop* race 2 germination. Vertical and horizontal bars are standard errors for n = 3.

4. Discussion

Root exudates are important determinants of the plant-rhizosphere interactions that may stimulate or inhibit germination and growth of microorganisms (Bais *et al.*, 2006). Several studies have shown that root exudates can either stimulate or inhibit *F. oxysporum* germination and growth (Steinkellner *et al.*, 2005; Li *et al.*, 2009; Ling *et al.*, 2013). However, the signals involved in the stimulation of *F. oxysporum* germination are still unknown (Steinkellner *et al.*, 2005; Steinkellner *et al.*, 2008). As previously reported for many plant species including tomato, pepper, watermelon, peanuts,

chickpea and pea (Whalley and Taylor, 1973; Naqvi and Chauhan, 1980; Steinkellner *et al*., 2005; Hao *et al*., 2010; Li *et al*., 2013), incubation of *Fop* race 2 microconidia in root exudates from most pea accessions largely stimulated its germination (Fig. 1). Pea root exudates have been shown to contain primarily sugars, amino acids and secondary metabolites including phenolic compounds (Rovira, 1956). Stimulation of the germination of soil-borne pathogen and other rhizosphere microorganisms have been either related to broad spectrum signals such as nutrients including both sugars and amino acids or species specific signals often of flavonoid natures (Rovira, 1956; Ruan *et al*., 1995; Cooper, 2007). In a previous study, Ruan *et al.* (1995) showed that the main inducing compounds of *F. solani* f.sp. *pisi* germination were the phenolic compounds present in pea root exudates and that they contributed to some extent to the host specificity. However, we observed here that the apolar fraction of the pea root exudates that contain the soluble phenolic compounds and related secondary metabolites did not induce or even inhibited *Fop* race 2 germination (Fig. 3). This result discards the possible role of flavonoids and other secondary metabolites as the inducing signals of *Fop* germination. The apolar fraction of chickpea root exudate also inhibited *F. oxysporum* f.sp. *ciceris* germination while the removal of phenolic compounds from tomato root exudates stimulated *F. oxysporum* f.sp. *lycopersici* germination (Stevenson *et al*., 1995; Steinkellner *et al*., 2005). Altogether these results indicate that the negative role of phenolic compounds and related secondary metabolites on *F. oxysporum* might be a general feature. Thus by contrast to the situation found for the closely related species *F. solani*, these results suggest that these metabolites are not involved in the stimulation of the germination of *F. oxysporum.* On the other hand, we observed the non-specific induction of *Fop* race 2 germination after incubation in non-host root exudates since root exudates of tomato were equally efficient in stimulating *Fop* race 2 germination than pea exudates (Fig. 2). This confirms the capacity of *F. oxysporum* to germinate in the presence of root exudates from non-host species previously shown for *F. oxysporum* f.sp. *lycopersici* (Steinberg *et al*., 1999; Steinkellner *et al*., 2005). Thus, these results would support the hypothesis that *F. oxysporum* germination in the soil is a host-independent process that might be induced by nutrients present in the soil in the form of sugar or amino acids. As a consequence, the *F. oxysporum* germination might be more likely depending on the nutritional level of the root exudates than to the level of specific inducing compounds. Nevertheless, some component of the root exudates may reduce this nutrient-driven germination, and contribute to host recognition between closely related species since the root exudates from non-host legume species closely related to pea, such as lentil and chickpea induced far less germination of *Fop* than that of pea. Although further studies would be needed to ascertain that this lower induction is species dependent.

Besides, the hypothesis of a general nutrient-dependent stimulation of *F. oxysporum* germination does not exclude the possibility that some root exudates may contain inhibiting molecules to reduce this overall stimulation. This second theory might explain the accession-dependent differences in *Fop* race 2 germination rates that have been detected here (Fig. 1) and in many other studies for other isolates of *Fop* (Buxton, 1957b) or other *formae speciales* of *F. oxysporum* (Naqvi and Chauhan, 1980; Stevenson *et al*., 1995; Li *et al*., 2009). In addition, we observed that the root exudates of a low number of pea accessions such as the partially resistant accessions JI2480 even inhibited *Fop* race 2 germination (Fig. 1). Previous studies in different plant species showed that inhibition of germination and growth of *F. oxysporum* by exuded antifungal metabolites is an important constitutive mechanism of resistant genotypes. Thus, the root exudates of several chickpea, pepper and cotton resistant genotypes were all shown to inhibit germination of their respective *forma specialis* of *F. oxyporum* (Naqvi and Chauhan, 1980; Stevenson *et al*., 1995; Li *et al*., 2009). Despite several studies on pea, the existence of such constitutive pre-penetration mechanism in resistant genotypes is still under debate (Buxton, 1957b; Whalley and Taylor, 1973). Buxton (1957b) showed that exudates of resistant genotypes were able to inhibit the germination of *F. oxysporum* spores. However, further studies using other pea accessions failed to observe differences in the germination rate of *Fop* between resistant and susceptible accessions that were all found to stimulate *Fop* germination (Kommedah, 1966; Whalley and Taylor, 1973). Here, we observed that the root exudates of three pea accessions inhibited *Fop* germination. Among these only 2 were resistant to the disease while the most inhibiting JI2480 was partially resistant. In addition, the susceptible accession JI1213 only moderately stimulated germination (Fig. 1). This indicates that a constitutive pre-penetration mechanisms depressing *F. oxysporum* germination also exist in pea. Since this mechanism was detected only on a limited number of pea accessions, this mechanism does not appear to be common within resistant accessions. However it may contribute to the quantitative resistance of some accessions by reducing the pathogenic pressure in the soil and thus allowing the plant to withstand the disease for much longer time.

The identification of the compound(s) responsible of the inhibition of *Fop* germination would be very valuable as it may contribute to control this important disease either by exogenous application in the soil or by potentiating its (their) production and release from crops through breeding. As indicated previously, the apolar fraction that mainly contains phenolic-like secondary metabolites might contain the responsible compound(s) (Stevenson *et al*., 1995; Steinkellner *et al*., 2005) since the apolar fraction of most pea accessions tested here inhibited *Fop* race 2 germination

(Fig. 3). Fractionation of the root exudate extract of the cultivar Messire that presented high inhibitory activity on *Fop* germination revealed that the inhibitor was contains in two complex fractions (Fig. 4). Although each fraction contained several compounds, pisatin was identified as their major component. Incubation of *Fop* in pure pisatin show similar inhibition than each of these fractions suggesting that pisatin may be at least partly responsible for the inhibition of *Fop* germination detected after incubation in pea root exudate extracts and or whole exudates (Figs. 1, 3 and 4). Interestingly, two of the three most active root exudates in inhibiting *Fop* germination (JI1412 and JI2480) also contained high level of pisatin. In addition, a significant negative correlation was observed between the pisatin content of most root exudates and the extent of *Fop* germination they induced. Altogether this would support an important role of pisatin in the inhibition of *Fop* germination in the soil (Fig. 8).

Pisatin is a potent antifungal molecule that was shown to inhibit the germination, growth and/or sporulation of several fungal pathogen including *F. solani* and *F. oxysporum* f.sp. *pisi* (Pueppke and Van Etten, 1974). In addition, it is largely induced in response to pathogen attack (Cannesan *et al.*, 2011). Thus pisatin is considered the major pea phytoalexin and a key element of the inducible pea defence reactions against fungal pathogens (Pueppke and Van Etten, 1974; Van Etten and Stein, 1978; Wu and Van Etten, 2004; Cannesan *et al.*, 2011). Here we found that the root exudates of several *P. sativum* ssp. s*ativum* accessions that inhibited or only moderately stimulated *Fop* germination contained constitutively high amount of pisatin. This contrast the result of Ruan *et al.* (1995) showing that pisatin possibly present in pea root exudate stimulated *F. solani* f.sp. *pisi* germination and rather confirmed the important role of pisatin in pea defence against fungal pathogen. Thus, our results suggested an important role of pisatin in the constitutive defence of pea additionally to its function in the inducible defence at least for the *P. sativum* ssp. *sativum* accessions.

Surprisingly, we found that the root exudates of the susceptible accession JI1213 contained a high level of pisatin. Accordingly, it induced lower *Fop* germination than the root exudates of other accessions such as the cultivar Messire that contained lower level of pisatin. However, it induced significantly more germination than the root exudates of the resistant accessions JI1412 and JI2480 that contain similar amount of pisatin. As stated previously, the overall stimulation of germination of *F. oxysporum* might depend on the nutritional level of the root exudates and to the presence of antifungal compounds. The accession JI1213 may thus secrete a higher amount of inducing sugars and/or amino acids than the accessions JI1412 and JI2480 counteracting the inhibition caused by the high amount of pisatin and leading to the moderate stimulation of *Fop* germination observed. On the other hand, many isolates

of *F. oxysporum* are able to degrade pisatin (Cruickshank, 1962; Delserone *et al.*, 1999; Milani *et al.*, 2012) although it depends on the carbohydrate source available in the medium since this capacity is controlled by the catabolite repression pathway (De Wit-Elshove and Fuchs, 1971). Thus the moderate stimulation of *Fop* germination might be alternatively explained by the exudation of lower amount of repressing sugars such as glucose by the JI1213 accession leading to the partial degradation of pisatin. However, a detailed analysis of sugar and amino acid content from these different pea accessions would be needed to clarify the differences observed.

While most *P. sativum* ssp. *sativum* accessions produced and secreted high level of pisatin in the rhizosphere, root exudates from the other pea subspecies only contained very small amount of pisatin (Fig. 7). However, the root exudate extracts of the accessions P641 and P650 strongly inhibited *Fop* race 2 germination suggesting the involvement of additional apolar metabolites in the inhibition of *Fop* germination (Fig. 3). This would support the hypothesis that the main phytoalexin of these wild relative of pea is not pisatin but a related phenolic compounds not detected by the HPLC analysis performed. However, further studies would be needed to identify the additional compounds involved.

On the other hand, we observed that the root exudate of the resistant *P. sativum* ssp. *elatius* accession P42 did not stimulate *Fop* race 2 germination while its corresponding ethyl acetate extract slightly stimulated it (Figs. 1 and 3). This would thus also suggest the involvement of additional compounds in the inhibition of *Fop* germination. Beside pisatin and related phenolic compounds, specific amino acids have also been identified as potent inhibitors of *F. oxysporum* germination. Crop roots have been shown to secrete several amino acids at variable concentration (Rovira, 1956; Claudius and Mehrotra, 1973; Naqvi and Chauhan, 1980). From these studies, several additional amino acids including phenylalanine, glycine, methionine, valine and citrulline were identified in root exudates of specific resistant accessions of lentil and pepper and were shown to strongly inhibit *F. oxysporum* growth (Claudius and Mehrotra, 1973; Naqvi and Chauhan, 1980). Thus it is possible that some amino acids are involved in the inhibition of *Fop* germination although further studies would be needed to ascertain this hypothesis.

Altogether, our results clearly showed that some pea accessions express a constitutive pre-penetration mechanism effective against *Fop* by allowing the inhibition of its germination and thus delaying the building of pathogenic population. This pre-penetration mechanism was at least partly dependent on the constitutive amount of pisatin, the main pea phytoalexin, present in these exudates. This points to an

important role of pisatin in this constitutive resistance mechanism although additional metabolites might also be involved.

Acknowledgement

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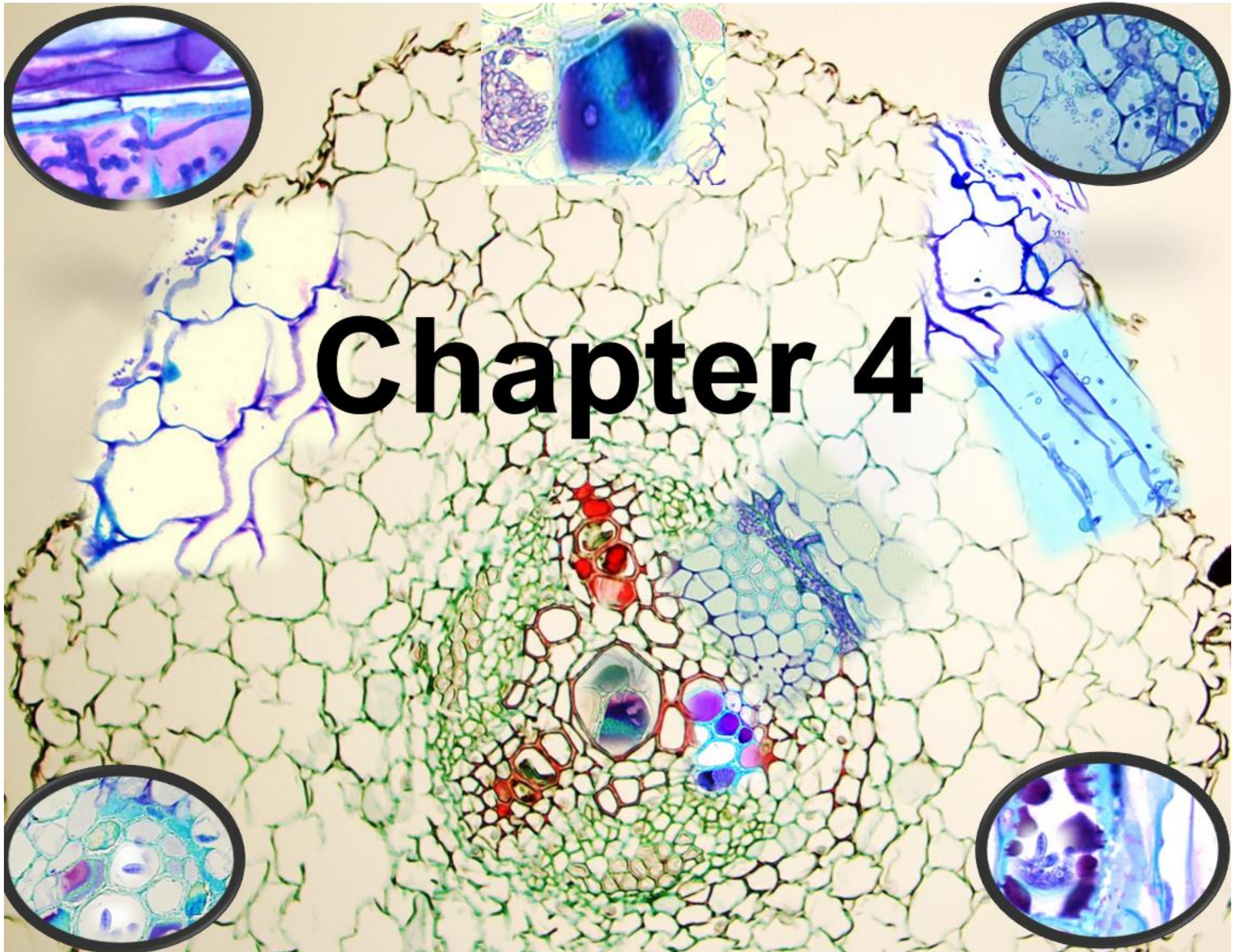
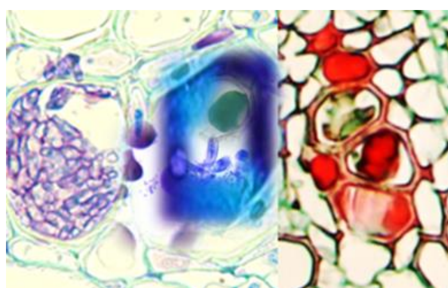

Chapter 4

Chapter 4

Establishment of physical and chemical barriers in root cells is responsible for the quantitative resistance of pea accessions to *Fusarium oxysporum* f.sp. *pisi* race 2

Abstract

Fusarium wilt caused by *Fusarium oxysporum* f.sp. *pisi* (*Fop*) is one of the most destructive diseases of pea worldwide. Control of this disease is difficult and is mainly based on the use of resistant cultivars. While monogenic resistance has been successfully used in the field to control the disease, it is at risk of breakdown by the constant evolution of the pathogen. New sources of quantitative resistance have been recently identified from a wild relative *Pisum* spp. collection. In this study, we aimed to characterize the resistance mechanisms occurring in these novel sources of quantitative resistance. For this, a detailed comparison of 8 accessions with differential response to *Fop* race 2 at cellular level showed that these resistant accessions established several barriers at the epidermis, exodermis, cortex, endodermis and vascular stele efficiently impeding the fungus progression. We found that these barriers were mainly based on three defence mechanisms including cell wall strengthening by lignification, formation of papilla-like structure at penetration site and accumulation of different substances within and between cells. In addition, these defence reactions were differentially expressed in each resistant accession. The main important components of these different barriers are phenolic compounds, lignin and carbohydrates. Our result also supports the important role of cell wall degrading enzymes in *F. oxysporum* pathogenicity.



Manuscript in preparation

1. Introduction

Fusarium oxysporum f.sp. *pisi* (*Fop*) is an important pathogen causing vascular wilt of field pea (*Pisum sativum*) worldwide (Kraft and Pfleger, 2001). Four different races of *Fop,* races 1, 2, 5 and 6 have been described (Infantino *et al.*, 2006). Races 1 and 2 have been reported in every country where pea are grown, while races 5 and 6 are, to date, only important in western Washington State (Infantino *et al.*, 2006). Control of soilborne fungal diseases, is mainly achieved by the integration of different disease management procedures. Among these methods, the use of resistant cultivars is widely recognized as the safest, most economical and most effective method for protecting crops from disease (Ciancio and Mukerji, 2008; Rubiales *et al.*, 2015). Resistance to *Fop* race 1, 5 and 6 is conferred by single dominant genes while resistance to race 2 has been recently shown to be quantitative (Bani *et al.*, 2012; Mc Phee *et al.*, 2012). Similarly to other *formae speciales* of *F. oxysporum, Fop* behaves as a classical soilborne pathogen in that its propagules can survive in the soil during extended periods of time in the absence of the host (Roncero *et al.*, 2003). The infection cycle of *F. oxysporum* is initiated by the germination of spores in the soil in response to an undetermined signal within the host root exudates (Di Pietro *et al.*, 2003). Upon germination infective hyphae adhere to the host roots and penetrate it through wounds or by direct penetration of root epidermis (Nelson, 1981; Bishop and Cooper, 1983a; Benhamou and Garand, 2001; Zvirin *et al.*, 2010). The mycelium then advances inter- or intracellularly through the root cortex, until it reaches the xylem vessels and colonize it (Bishop and Cooper, 1983b; Beckman, 1987; Baayen *et al.*, 1989; Benhamou *et al.,* 1996; Olivain and Alabouvette., 1999; Benhamou and Garand, 2001; Olivain *et al.*, 2006; Czymmek *et al.*, 2007; Zvirin *et al.*, 2010). After xylem vessels colonization, the fungus progress vertically through them to colonize the aerial region of the host (Bishop and Cooper, 1983b; Beckman, 1987). After wilting and plant death, the fungus reaches the plant surface where it produces chlamydospores that are dispersed onto the soil for a second cycle of infection (Di Pietro *et al.*, 2003). During root invasion and colonization, *Fop* is exposed to various plant defence mechanisms, such as physical barriers and chemical compounds (Beckman, 1987; Michielse and Rep, 2009). Several histological studies have been conducted to characterize infection process and resistance mechanisms in several *F. oxysporum* - plant pathosystems at cellular level, such as in carnation (Baayen, 1988; Baayen and Niemann, 1989; Baayen *et al.*,1989; 1996; Ouellette *et al.*, 1999; 2002), chrysanthemum (Barbara and Nelson, 1981; Emberger and Nelson, 1981), common bean (Pereira *et al.*, 2013), tomato (Beckman *et al.*, 1972; Bishop and Cooper, 1983a; Chamberland *et al.*, 1991; Lagopodi *et al.*, 2002; Jonkers *et al.*, 2009), Arabidopsis (Czymmek *et al.*, 2007), cotton (Shi *et*

al., 1991; 1992; Hall *et al*., 2011), lupine (Morkunas *et al*., 2005), melon (Zvirin *et al*., 2010) and pea (Bishop and Cooper, 1983a; b; 1984; Tessier *et al*., 1990). These studies revealed that the successful invasion of the host by *F. oxysporum* was dependent on a multitude of factors which may vary according to the patho-system considered. Similarly, hosts were shown to develop different physical and chemical barriers to block the pathogen progressions at different level depending on the host or cultivar. The most efficient mechanisms described are the production of antifungal compounds which inhibit pathogen growth, the formation of papillae at attacked site, suberization and lignification of the cell wall to restrain the pathogen advance and the action of its cell wall degradation enzymes (CWDEs), the accumulation of gums, gels or tyloses within xylem cells and the vessel crushing by proliferation of adjacent parenchyma cells (Beckman, 1964; Vandermolen *et al*., 1977; Bishop and Cooper, 1983a; 1984; Baayen and Elgersma, 1985; Beckman, 1987; Charchar and Kraft, 1989; Ouellette *et al*., 1999; Grayer and Kokubun, 2001).

Quantitative resistant varieties have been identified in several species including pea. However, the defence mechanisms that confer resistance in these varieties remain obscure. Although some reports described the histopathology of stem and root tissues of resistant and susceptible pea cultivars infected by *Fop* (Bishop and Cooper, 1983a; b; Tessier *et al.,* 1990), detailed studies on the infection process and resistance mechanisms of genotypes with different levels of resistance are scarce. Therefore, the objective of this study was to observe the infection process of *Fop* race 2 in eight pea accessions showing different levels of quantitative resistance and characterize the resistance mechanisms occurring in these accessions.

2. Materials and methods

2.1. Fungal isolates and cultural conditions

Fusarium oxysporum f.sp. *pisi* (*Fop*) race 2 strain R2F42, kindly provided by Dr. W. Chen (USDA-ARS, Pullman, USA), was use in all experiment. The fungal strain was stored as microconidial suspensions at -80 ºC in 30% glycerol. For microconidia production, cultures were grown in potato dextrose broth (PDB; Difco, Detroit, MI) at 28 ºC in a shake culture set at 170 rpm (Bani *et al*., 2012).

2.2. Plant material, growth conditions, and inoculation

Eight pea accessions with a wide range of response to *Fop* race 2 was used including the susceptible accessions P21 and P629, the partially resistant accessions JI2480

and Messire and the resistant accessions P42, P633, JI1412 and JI1760. Germinated pea seedlings were sown in vermiculite and grown in controlled environment under a 16/8 h light-dark photoperiod at 26 ± 2 °C constant temperatures with 200 µmol m^{-2} s^{-1} of illumination. Plants were watered every three days with tap water. Seven days old pea seedlings were inoculated with the root dipping technique with root trimming as described previously (Bani *et al.*, 2012). Control plants were similarly dipped in sterile distilled water. To study the influence of root trimming on the infection process, additional pea seedlings were also inoculated by the root dipping method without root trimming. For this the whole root system of each plant was immersed in a conidial suspension for 15 minutes. Inoculated and control plants were maintained in the same growth condition as above. Three plants were used per accessions and per method, each experiment was repeated thrice independently. Simultaneously, three plants per accession were inoculated and used to follow the disease symptoms (Bani *et al.*, 2012). The fungi were re-isolated from the different parts (root and basal, middle and apical stem regions) of inoculated plants of different accessions 7 days after inoculation as described previously (Bani *et al.*, 2012).

2.3. Collection and fixation of samples

Root and stem tissues from three plants per accessions were sampled at 4 or 7 days after inoculation. For each plant, 5-6mm long segments of root and stem were fixed. Two procedures were used to fix and section the plant samples: (i) the sampled material was fixed in FAA (50% ethanol + 5% formaldehyde + 10% glacial acetic acid, in water) for 48 h. Fixed samples were then dehydrated in ethanol series (50, 80, 95, 100, 100% for 12 h each) and transferred to xylene (Merck KGaA, Darmstadt, Germany) through a xylene-ethanol series (30, 50, 80, 100, 100% xylene for 12 h each) and finally saturated with paraffin (Paraplast plus®; Sigma, Switzerland) to prepare the blocks. 7 µm-thick sections were then cut with a rotary microtome (Nahita 534; Auxilab SA, Beriain, Spain) and attached to adhesive-treated microscope slides (polysine slides; Menzel GmbH and Co KG, Braunschweig, Germany). (ii) The sampled material was fixed in Karnovsky solution (5% glutaraldehyde + 4% formaldehyde in 0.025M cacodylate buffer + 0.5mg $CaCl_2.ml^{-1}$) for 4 h at room temperature. Fixed samples were then washed three times with 0.025M cacodylate buffer for 20 minutes each, and transferred in ethanol series in water (50, 80, 95 for 12 h each), samples were then transferred in a 1:1 ethanol-infiltration solution (basic resin Leica Historesin + basic resin activator Leica Historesin, Leica microsystems Nuβloch GmbH, Heidelberg, Germany) for 12h. After this, the solution was replaced by pure infiltration solution, and finally the blocks were prepared by polymerizing the resin with Hardener Leica

Historesin (Leica microsystems Nuβloch GmbH, Heidelberg, Germany). Semi-thin sections (2 µm) were cut with a glass knife in a semi-automated rotary microtome (Leica RM2245, Leica microsystems Nuβloch GmbH, Heidelberg, Germany) and attached to microscope slides.

2.4. Cytochemical methods for light and epifluorescence microscopy

After paraffin removal, FAA-fixed sections were stained with a mixture of alcian green: safranin (AGS) (Pérez-de-Luque *et al.*, 2006; 2007). The slides were dried and mounted with DePeX (BDH). With this staining method, carbohydrates (including cell walls and mucilage) appeared green, yellow or blue, while lignified, cutinized and suberized walls, as well as tannin and lipid material inside cells appeared red (Pérez-de-Luque *et al.*, 2006; 2007). The sections were also stained with 0.05% toluidine blue O (TBO) in phosphate buffer (pH 5.5) during 5-10 min. In this case the dye was applied before removal of paraffin (Ruzin, 1999; Pérez-de-Luque *et al.*, 2007). Semi-thin (2 µm) historesin sections of Karnovsky-fixed samples were stained with 0.1% toluidine blue O (TBO) in citrate buffer (pH 5) for 5 min. The TBO staining method allows detection of phenolics as well as tannins, lignin and suberin (Baayen *et al.*, 1996; Bordallo *et al.*, 2002; Mellersh *et al.*, 2002; Crews et *al.*, 2003; Pérez-de-Luque *et al*, 2006; 2007). The sections were observed using a light microscope (Leica DMLB, Leica Microsystems Wetzlar GmbH, Wetzlar, Germany) and photographed using a digital camera (Nikon DXM1200F; Japan). The samples were also observed by epi-fluorescence under excitation at 450-490 nm (blue-violet) with the same microscope.

3. Results

3.1. Macroscopic events

As expected from previous studies, the accessions P21 and P629 were highly susceptible and died within 21 days after inoculation (Fig. S1). Similarly, the partially resistant accessions Messire and JI2480, while remaining alive throughout the experiment, showed high disease severity estimated at 76.53% and 62.87% respectively at 30 days post inoculation (dpi). In turn the phenotype of the resistant accessions (P42, JI1412, JI1760 and P633) after inoculation remained similar to the non-inoculated control plants throughout the experiment. No significant differences in disease severity were detected between the inoculation methods. To confirm that all plants had been equally inoculated, we also re-isolated *Fop* colonies from inoculated plants at 7 dpi. As expected from previous studies (Bani *et al.*, 2012), *Fop* colonies were detected in root fragments of all accessions. However, fungal colonies were only

recovered from stem fragments of the susceptible accessions indicating that as early as 7 dpi, *Fop* had already colonized the whole plant in these accessions (Fig. S2).

3.2. Histology of susceptible plants

As expected, sections of non-inoculated control pea root presented the characteristic structure of dicotyledonous root (Fig. 1A) (James, 1998). Four dpi, the roots of susceptible accessions were extensively colonized by the fungus. At this stage, hyphae had already penetrated the epidermal cells, colonized the exodermis and the cortex inter- and intra-cellularly (Fig. 1B), and reached the vascular system. Accordingly, at 7 dpi, an abundant inter- and intracellular development of fungi was observed in endodermis, pericycle, fiber cells, parenchyma cells and xylem vessels even when the plants were inoculated without root trimming (Fig. 1C, D, E, and F). At this stage, the mycelial growth was associated with the degradation of xylem parenchyma, fiber and cortical cells surrounding the endodermis (Fig. 1E, F). As an attempt to counteract the massive infection, we observed the development of some papillae-like structures at some sites of hyphal penetration within cortical cell layer although the pathogen was able to overcome it (Fig. 1B). Limited cell wall lignification was also sometime observed in the epidermis, exodermis and cortical cells (Table 1 and Fig. 7). However, these two defensive reactions were not effective to block pathogen progression and colonization of stele in these susceptible accessions. Consequently we observed at 7 dpi, the presence of fungal structure within xylem cells and the associated degradation of xylem parenchyma in the upper stem sections (Fig. 6A and B).

3.3. Histology of resistance plants

In resistant and partially resistant accessions, fungi was observed within and between the epidermal and exodermal cells at 4 dpi (Fig. 2D, E and G). Fungal structure was also eventually observed in the intercellular spaces of cortical cells (Fig. 2F), but it was never seen inside these cells. The fungi have also been abundantly observed in the intercellular spaces of cortex, endodermis and pericycle in the partially resistant accession Messire (Fig. 3E and F). In the stele, fungal colonization was only detected in the xylem vessel of the resistant accessions after inoculation with root trimming, and in this case it was restricted to these vessels (Figs. 4A, B, D and 5A). By contrast the pathogen was able to colonize the xylem vessels of the partially resistant accessions independently of the inoculation methods, although the intensity of the colonization was lower than that observed in the susceptible accessions (Fig. 5B, C and D).

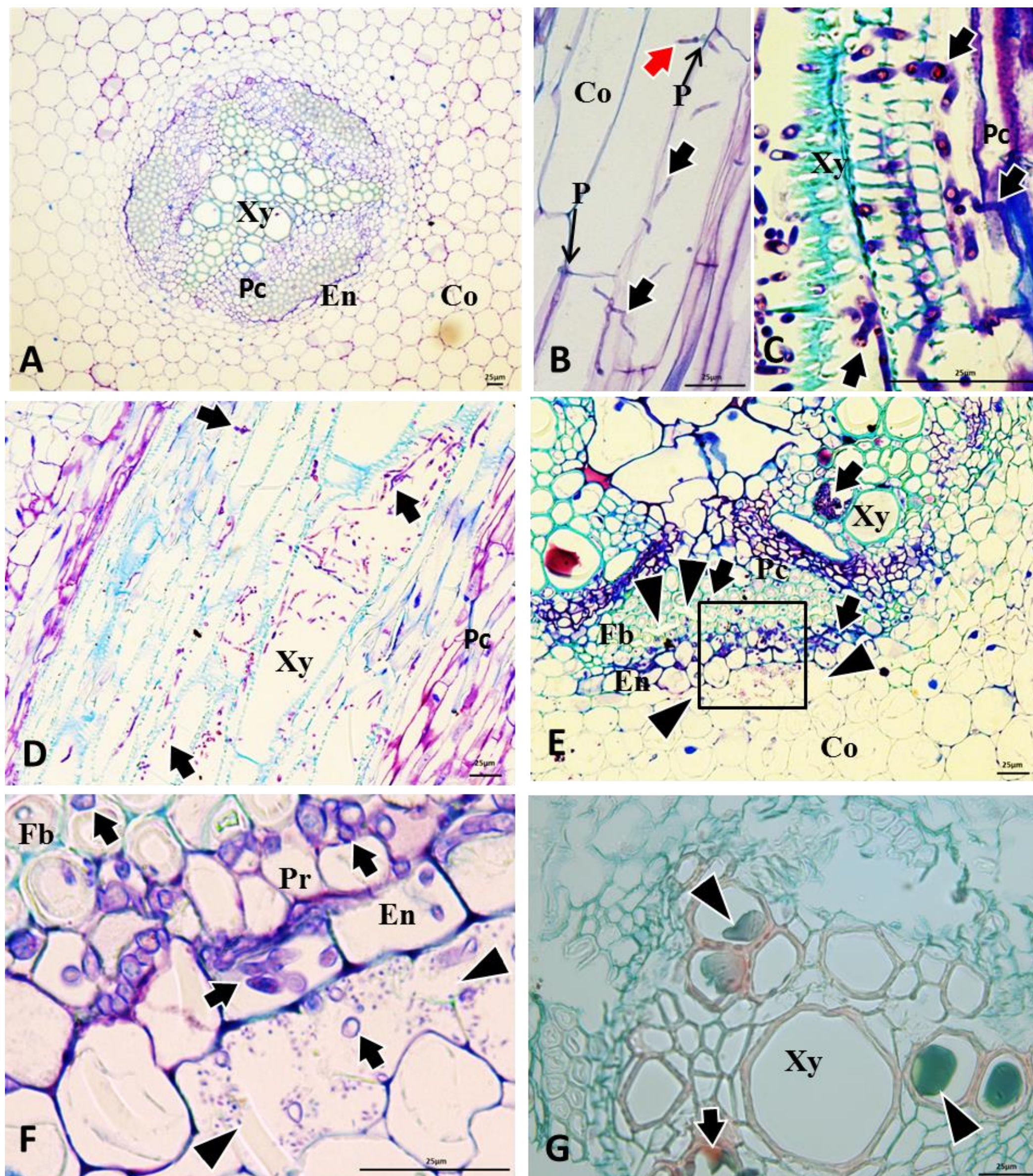


Figure 1. Longitudinal (B, C, D) and cross-sections (A, E, F, G) of roots of susceptible pea accessions, control (A) and after inoculation with *Fop* race 2, stained with TBO or AGS. (A) Cross-section of control plants of the P21 accession stained with TBO showing a general view of pea root histology: cortex (Co), endodermis (En), parenchyma cells (Pc) and xylem vessels (Xy). (B) Longitudinal section of *Fop*-inoculated root (4 days after inoculation) of the susceptible accession P21 stained with TBO showing the development of fungi (black arrow) in the epidermis (Ep) and cortex (Co), at this level the plant cells develop papillae to block the hyphae penetration (P) but the pathogen can overcome it (red arrow). (C, D) Longitudinal sections of *Fop*-inoculated root (7 days after inoculation) of the P21 accession stained with TBO showing the abundant development of fungi (arrow) in parenchyma cells (Pc) and xylem vessels (Xy). (E) Cross-section of *Fop*-inoculated root (7 days after inoculation) of the P629 accession stained with TBO sowing the abundant development of fungi (arrow) through cortex (Co), endodermis (En), and vascular tissues, this colonization is associated with the degradation (arrowhead) of xylem parenchyma cells (Pc) and cortical cells surrounding the endodermis. (F) Detail of (E) showing that the hyphae of *Fop* (arrow) develop abundantly through the inner cortex, endodermis, pericycle (Pe) and fibers (Fb). The intercellular and intracellular fungal growth is associated with the cells degradation (arrowhead). (G) Cross-section of *Fop*-inoculated root (7 days after inoculation) of the P21 accession stained with AGS showing the accumulation of carbohydrates (green color indicated with arrowhead) and other filling substances (red color indicated with arrow) in xylem vessels (Xy). Bar = 25 µm. Epidermis (Ep), exodermis (Ex: the outer cortex), cortex (Co), endodermis (En), pericycle (Pr), fibers (Fb), xylem vessels (Xy) and vascular parenchyma cells (Pc).

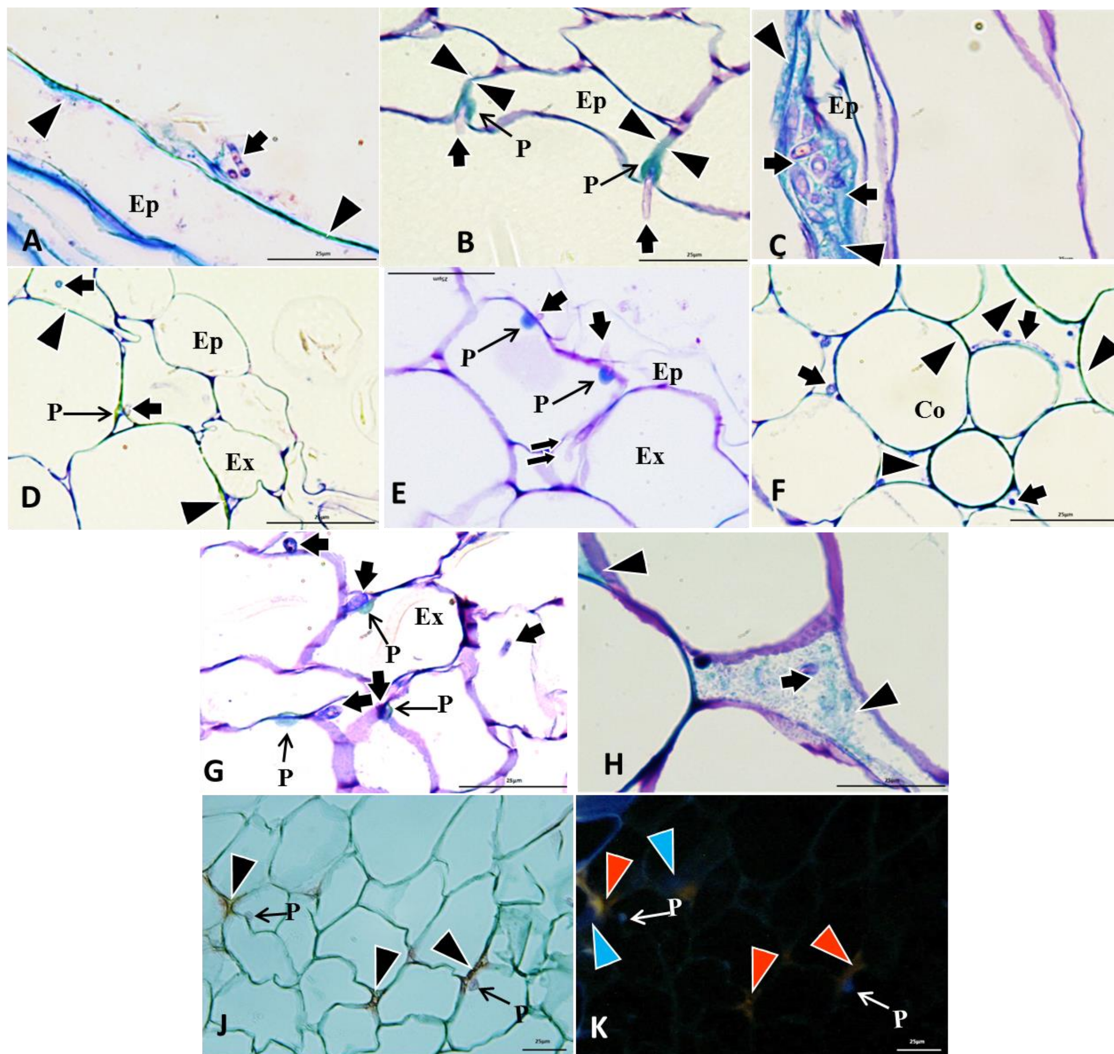


Figure 2. Defence responses observed in epidermal and cortical tissues of roots of resistant pea accessions after inoculation with *Fop* race 2, stained with TBO (A, B, C, D, E, F, G, H) or AGS (J). Arrows indicate fungus. (A) Longitudinal section of the resistant accession JI1760 (4 days post inoculation) showing the lignification of epidermal (Ep) cell walls (arrowhead) to prevent the hyphae penetration. (B) Cross-section of the resistant accession P42 (4 days post inoculation) showing the lignification of epidermal (Ep) cell walls (arrowhead) and the formation of papillae (P) at the point of hyphae penetration. (C) Longitudinal section of the resistant accession P633 (4 days post inoculation) showing the accumulation of a blue green staining indicated by arrowhead) in presence of fungi. (D) Cross-section of the P42 accession (4 days post inoculation) showing the lignification of cell walls (arrowhead) of outer cortex (Ex: exodermis) to prevent the hyphae penetration. (E) Cross-section of the resistant accession JI1412 (4 days post inoculation) showing the formation of papillae (P) at the point of hyphae penetration in the exodermis (Ex), although the fungi can penetrate the cortex intercellularly (double arrow). (F) Cross-section of the partially resistant accession Messire (4 days post inoculation) showing the lignification of cortical (Co) cell walls (arrowhead) to prevent the hyphae penetration from the intercellular spaces. (G) Cross-section of the Messire accession (4 days post inoculation) showing the formation of papillae (P) to prevent cortical cell colonization. (H) Cross-section of the P633 accession (4 days post inoculation) showing the accumulation of phenolic substances (blue green staining indicated by arrowhead) between cortical cells. (J) Cross-section of JI1760 accession (7 days post inoculation) stained with AGS showing the formation of papillae (P) and the accumulation of other substances (red staining indicated by arrowhead) between cortical cells. (K) The same cross-section as (J) observed by epi-fluorescence showing a blue-violet fluorescence of papillae (P) and two fluorescence emission types of the substances accumulated between cortical cells: blue (blue arrowhead corresponding to phenolic substances) and orange (orange arrowhead; undetermined substance). Bar = 25 μm. Epidermis (Ep), exodermis (Ex: the outer cortex), cortex (Co).

Therefore, clear differences in the extent of fungal colonization were observed between susceptible, partially resistant and resistant accessions. In the resistant accession, pathogen growth was usually restricted to the outermost root tissues, including the epidermis and the outer cortex (exodermis), and in the xylem vessel, when the plants were inoculated with root trimming. By contrast the fungus progression was blocked at the endodermis and within xylem vessel in the partially resistant accession. Consequently, the pathogen cannot colonize the plant stem of resistant and partially resistant accessions which was confirmed by the total absence of fungi in stem sections of all these accessions (Fig. 6E). However, successive cross stem sections of the partially resistant accession Messire showed the degradation of xylem parenchyma cells in the absence of fungi (Fig. 6C).

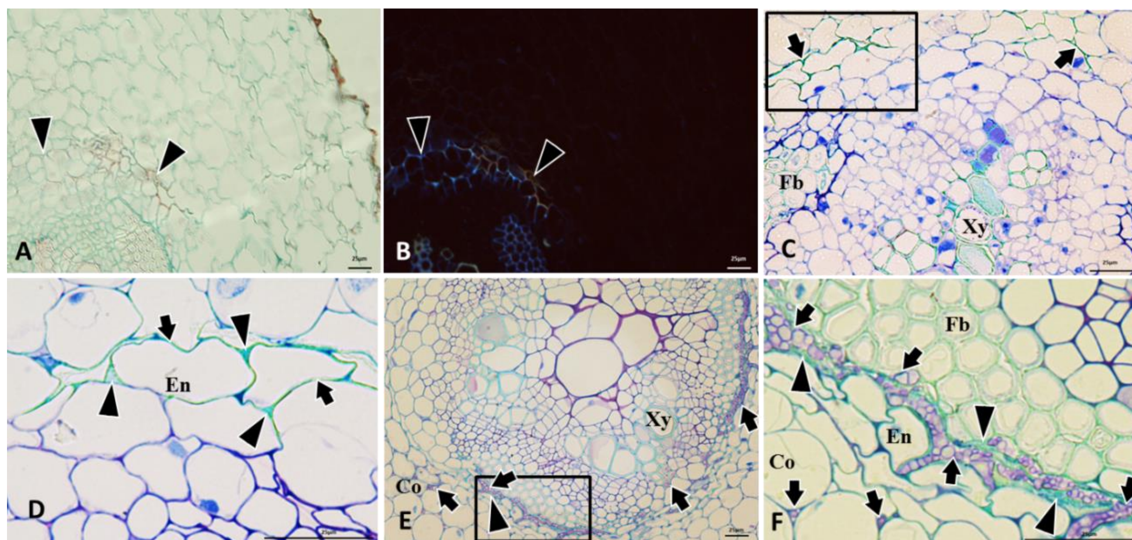


Figure 3. Defence responses observed in root endodermis of resistant pea accessions after inoculation with *Fop* race 2 and stained with AGS (A, B) or TBO (C, D, E, F). (A) Cross-section of the resistant accession JI1412 (7 days post inoculation) showing the strengthening of the endodermis cell wall (red staining indicated by arrowhead). (B) The same cross-section as (A) observed under epi-fluorescence showing a fluorescence emission of the endodermis cell wall (arrowhead). (C) Cross-section of the partially resistant accession JI2480 (4 days post inoculation) showing the strengthening of the endodermis cell wall (blue green staining indicated by arrow). (D) Detail of (C) showing the strengthening of the endodermis cell wall (arrow) and the accumulation of phenolic substances (blue green staining indicated by arrowhead) between endodermal cells. (E) Cross-section of the partially resistant accession Messire (7 days post inoculation) showing the accumulation of phenolic substances (blue green staining indicated by arrowhead) in the intercellular spaces of endodermis and pericycle, arrows indicate the colonization of the intercellular spaces of endodermis and pericycle by fungi. (F) Detail of (E) showing the accumulation of phenolic substances (blue green staining indicated by arrowhead) around the fungi (arrow) in the intercellular spaces. Bar = 25 µm. Cortex (Co), endodermis (En), pericycle (Pe), fibers (Fb), xylem vessels (Xy) and vascular parenchyma cells (Pc).

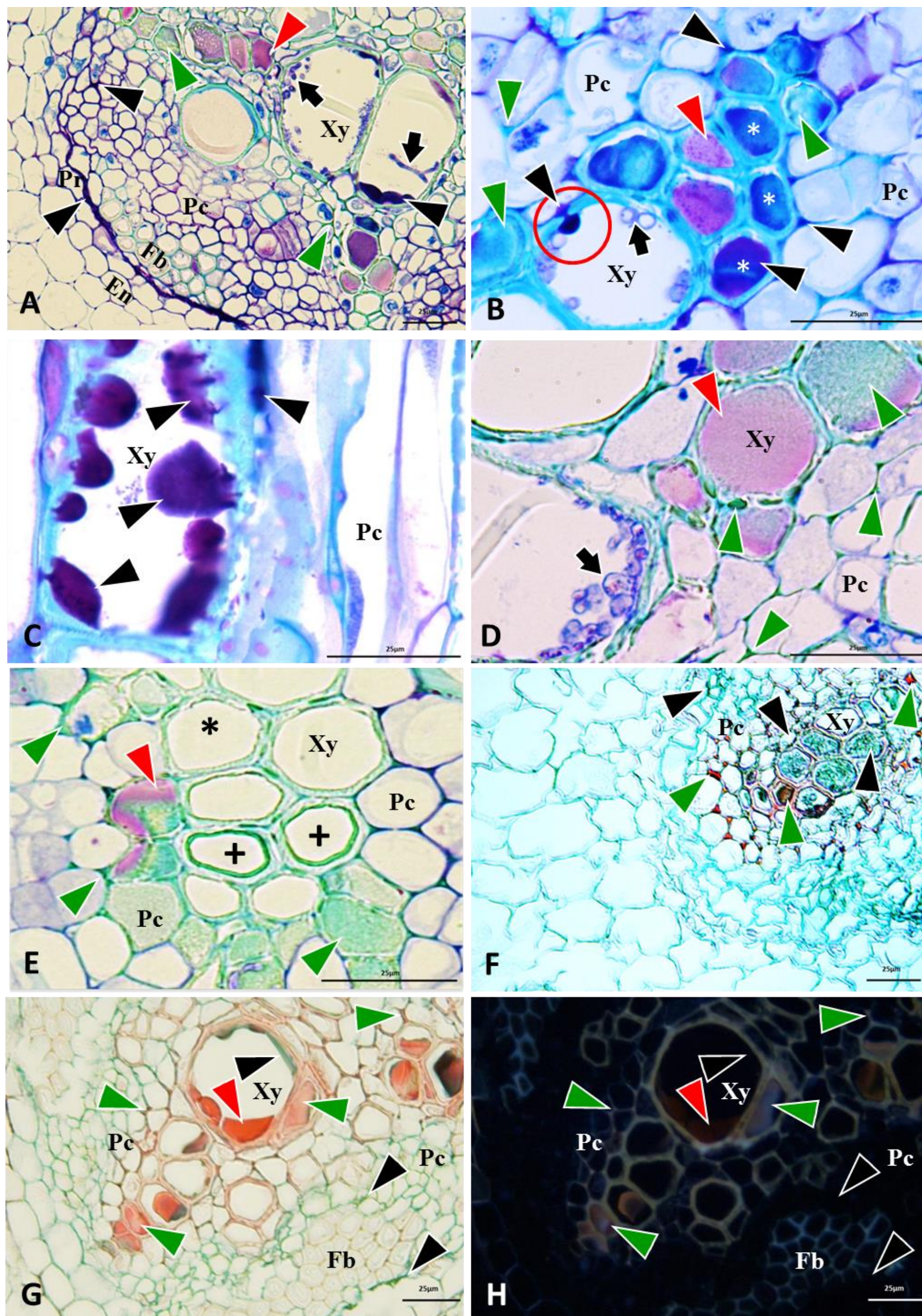


Figure 4. Defence responses observed in root vascular tissues of resistant pea accessions after inoculation with *Fop* race 2 and stained with TBO (A, B,C, D, E) or AGS (F, G). Arrows indicate fungus. (A) Cross-section of the resistant accession P42 (4 days post inoculation) showing the accumulation of carbohydrates (dark purple staining indicated by arrowheads) in the xylem cells, intercellular spaces of pericycle and vascular parenchyma cells (Pc). Blue green staining accumulated in the xylem cells and between vascular cells (green arrowheads) corresponds to phenolic substances and the clear purple staining (red arrowhead) to other filling substances. (B) Cross-section of the resistant accession JI1412 (7 days post inoculation) showing the accumulation of carbohydrates (dark purple staining indicated by arrowheads) between vascular cells and entering the xylem cells (red circle) until complete plugging of the vessels. Asterisks indicate completely plugged xylem vessels. The blue green coloration accumulated in and between completely plugged vascular cells (green arrowheads) corresponds to phenolic substances and the clear purple staining (red arrowhead) to other filling substances.

Figure 4 (legend continued) (C) Longitudinal section of the JI1412 accession (7 days post inoculation) showing the formation of xylem vessel plug by the accumulation of carbohydrates (dark purple staining indicated by arrowheads). (D) Cross-section of the resistant accession P633 (4 days post inoculation) showing the accumulation of phenolic compounds (blue green staining indicated by green arrowheads) in and between vascular cells. The clear purple staining (red arrowhead) corresponds to other filling substances. (E) Cross-section of the JI1412 accession (7 days post inoculation) showing the strengthening of vessels cell walls (+). Black asterisk indicates a normal vessel as reference. The blue green coloration (green arrowheads) corresponds to phenolic compounds . (F) Cross-section of the P633 accession (7 days post inoculation) stained with AGS showing the accumulation of phenolic substances(red staining indicated by green arrowheads) and carbohydrates (green staining indicated by black arrowheads) in and between vascular cells. (G) Cross-section of the P42 accession (7 days post inoculation) stained with AGS showing the accumulation of phenolic substances(red staining indicated by green arrowheads) and carbohydrates (green staining indicated by black arrowheads) and other filling substances (dark red staining indicated by red arrowhead) in and between vascular cells. (H) The same as (G) observed under epi-fluorescence showing the fluorescent emission of phenolic substances (green arrowheads), black arrowheads indicate carbohydrates and red arrowheads indicates other non-fluorescent filling substances. Bar = 25 µm. Xylem vessels (Xy) and vascular parenchyma cells (Pc).

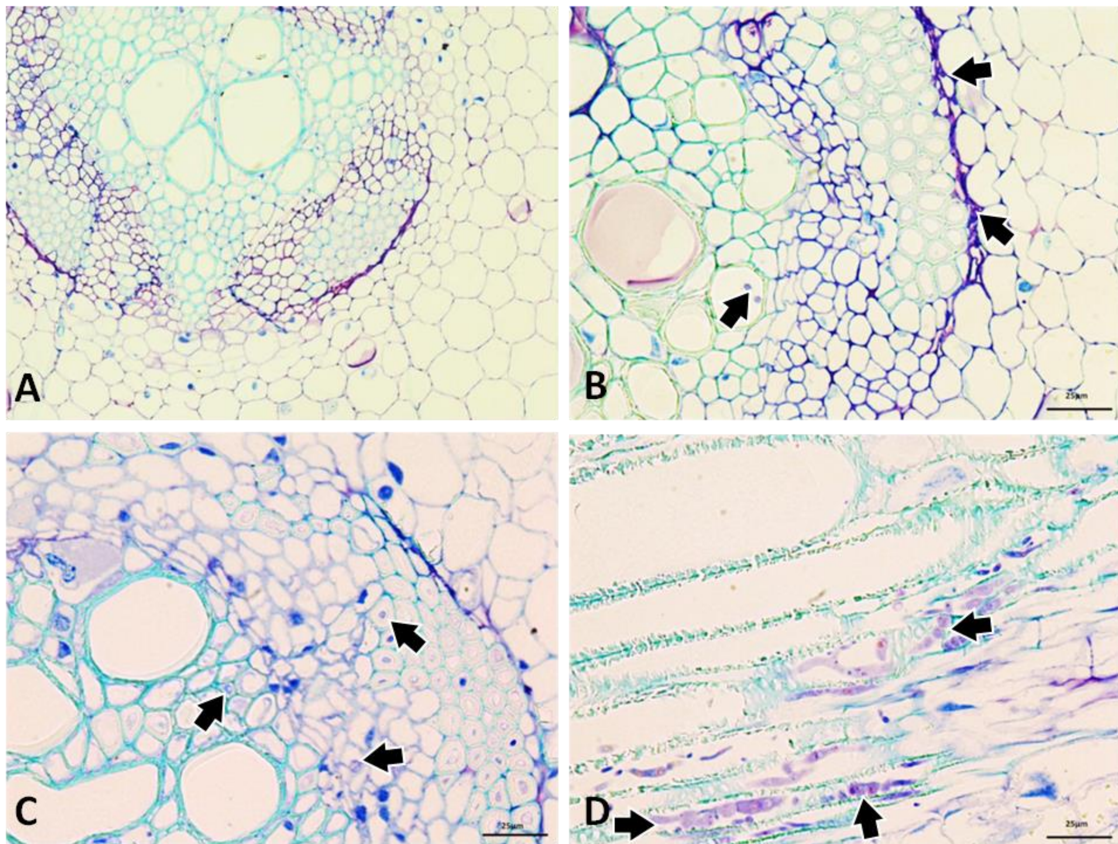


Figure 5. TBO stained cross-sections of pea roots 7 days after inoculation with *Fop* race 2 by root dipping without root trimming. Arrow indicates fungal cells. (A) Cross-section of resistant accession JI1412. (B) Cross-section of the partially resistant accession Messire. (C) Cross-section of the susceptible accession P21. (D) Longitudinal section of the susceptible accession P21. Bar = 25 µm.

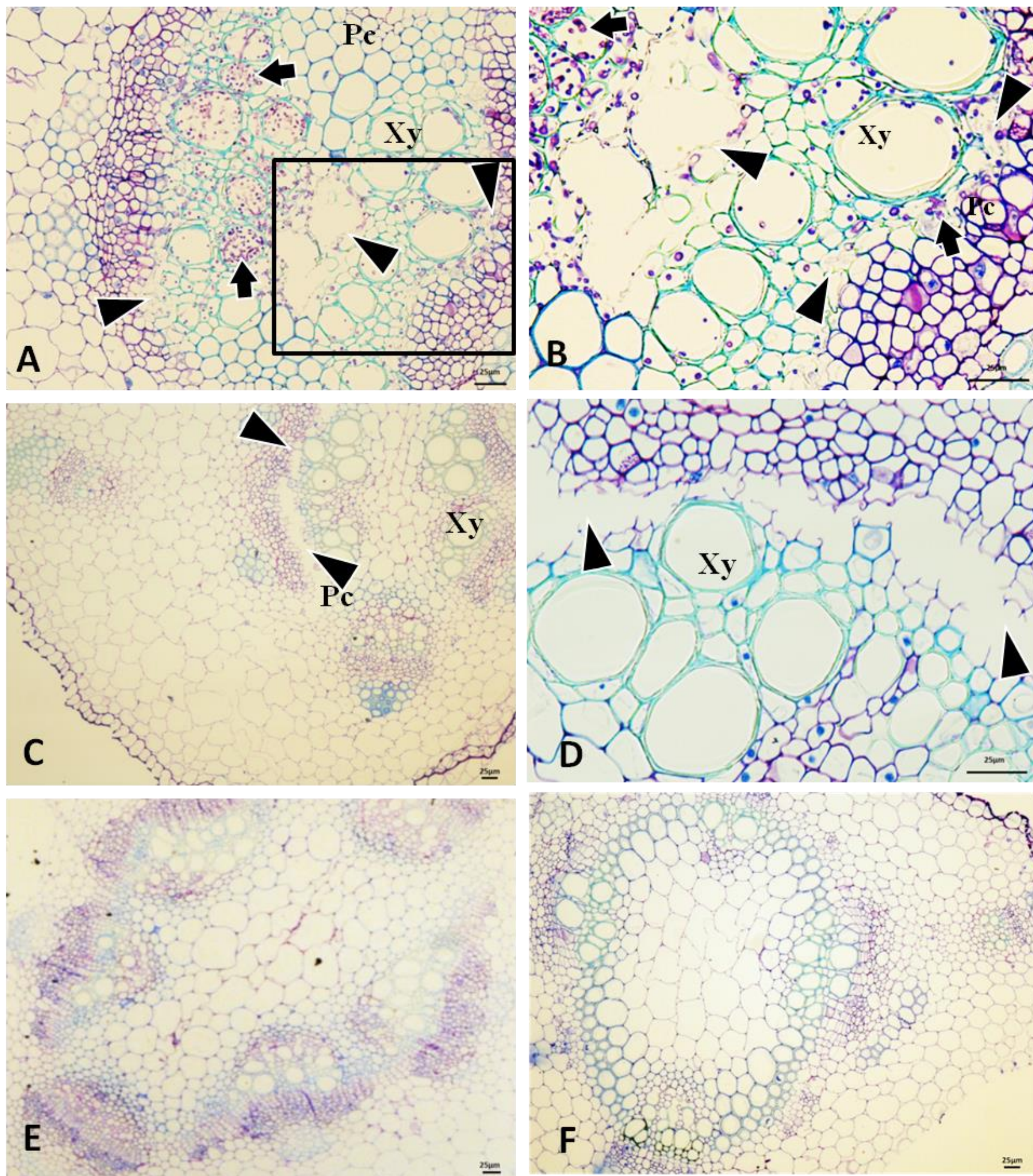


Figure 6. Cross-sections of stem of pea plants 7 days after inoculation with *Fop* race 2 and stained with TBO. (A) Cross-section of stem of the susceptible accession P21 showing abundant colonization of xylem cells (arrow), this colonization is associated with the degradation of xylem parenchyma cells (arrowhead). (B) Detail of (A) showing the degradation of xylem parenchyma cells (arrowhead) by the fungi (arrow). (C) Cross-section of stem of the partially resistant accession Messire showing the fragility and degradation of xylem parenchyma cells (arrowhead) in the absence of fungi. (D) Detail of (C) showing the degradation of xylem parenchyma cells (arrowhead) in the absence of fungi. (E) Cross-section of stem of the resistant accession JI1760. (F) Cross-section of stem of the susceptible accession P21 maintained non-inoculated as control. Bar = 25 µm. Xylem vessels (Xy), Vascular parenchyma cells (Pc).

Table 1. Host cell reaction observed at 7 days post inoculation by *Fop* of different accessions.

Tissue	Host cell reactions		Microscopic observation	Figures	Frequencies in each accession							
					P42	JI1412	JI1760	P633	JI2480	Messire	P21	P629
Epidermis	Cell wall strengthening	Lignification	Blue-green staining with TBO	Fig.2.A	2	2	4	2	2	1	1	1
		Formation of papillae	Intense blue points in the penetration sites of cell wall by the fungus	Fig.2.B	2	0	1	0	1	0	0	0
	Production of toxic substances	Substance accumulation around the fungus	Blue-green staining with TBO	Fig.2.C	0	0	1	3	0	0	0	0
Exodermis	Cell wall strengthening	Lignification	Blue-green staining with TBO	Fig.2.D	4	3	3	1	3	2	2	1
		Formation of papillae	Intense blue points in the penetration sites of cell wall by the fungus	Fig.2.D,E	4	3	2	2	1	2	1	1
Cortex	Cell wall strengthening	Lignification	Blue-green staining with TBO	Fig.2.F	2	3	1	1	2	3	1	1
		Formation of papillae	Intense blue points in the penetration sites of cell wall by the fungus	Fig.2.G	1	3	1	1	2	3	1	3
	Production of toxic substances	Accumulation of phenolic compounds between cortical cells	Blue-green staining with TBO in the intercellular spaces	Fig.2.H	1	0	1	3	0	0	0	0
		Accumulation of other substances between cortical cells	Red staining with AGS showing an orange fluorescence	Fig.2.J,K	0	0	2	1	nd	nd	0	nd
Endodermis/ pericycle	Endodermic cell wall strengthening	Lignification	Red staining with AGS showing a blue fluorescence, blue-green staining with TBO	Fig.3.A,B, C,D	3	4	1	1	3	3	1	1
	Production of toxic substances	Accumulation of phenolic compounds	Blue-green staining with TBO between endodermic and pericycle cells	Fig.3.E,F	2	0	0	0	2	3	0	0
	Carbohydrates accumulation	Carbohydrates accumulation around the pericycle	Dark purple staining with TBO surrounding the pericycle	Fig.4.A	3	2	1	2	1	2	2	2
Vascular stele	Accumulation of different substances and plugging of xylem vessels	phenolic compounds	Blue-green staining with TBO in xylem lumen	Fig.4.A,B, D,E	3	3	1	2	2	1	1	1
			Red staining with AGS showing a blue fluorescence in xylem lumen	Fig.4.G,H	4	3	2	2	nd	nd	1	nd
		carbohydrates	Dark purple staining with TBO in xylem lumen	Fig.4.A,B, C	3	3	1	3	1	2	1	2
			Green staining with AGS in xylem lumen	Fig.4.F,G, H	2	2	1	4	nd	nd	2	nd
		undetermined substances	Clear purple staining with TBO in xylem lumen	Fig.4.A,B, D,E	2	3	2	3	1	3	3	1
	Accumulation of different substances in the intercellular space	phenolic compounds	Red staining with AGS showing a blue fluorescence	Fig.4.F,G, H	3	2	1	3	nd	nd	1	nd
			Blue-green staining with TBO	Fig.4.D,E	2	3	1	3	3	3	1	1
		Carbohydrates	Dark purple staining with TBO	Fig.4.A,B	3	3	2	3	1	2	2	2
	Cell wall strengthening	Vessels wall thickening	Blue-green staining with TBO	Fig.4.E	1	3	0	0	0	0	0	0

Visual scale: 0 (absent), 1(rare), 2 (less frequent), 3(frequent), 4(Very frequent), nd: no data.

3.4. Host cell reaction to *Fop* invasion

Blocking of the pathogen progression at the different levels was mainly due to cell wall strengthening, formation of papillae and accumulation of different substances. However, quantitative differences were detected between accessions in the intensity that these mechanisms were displayed at each cell type. Thus, we established a visual

scale to estimate the intensity of reaction ranging from 0 to 4 (0 = absent, 1 = rare, 2 = low frequency, 3 = frequent, 4 = high frequency). The results of this comparison are presented in Table 1 and Fig. 7.

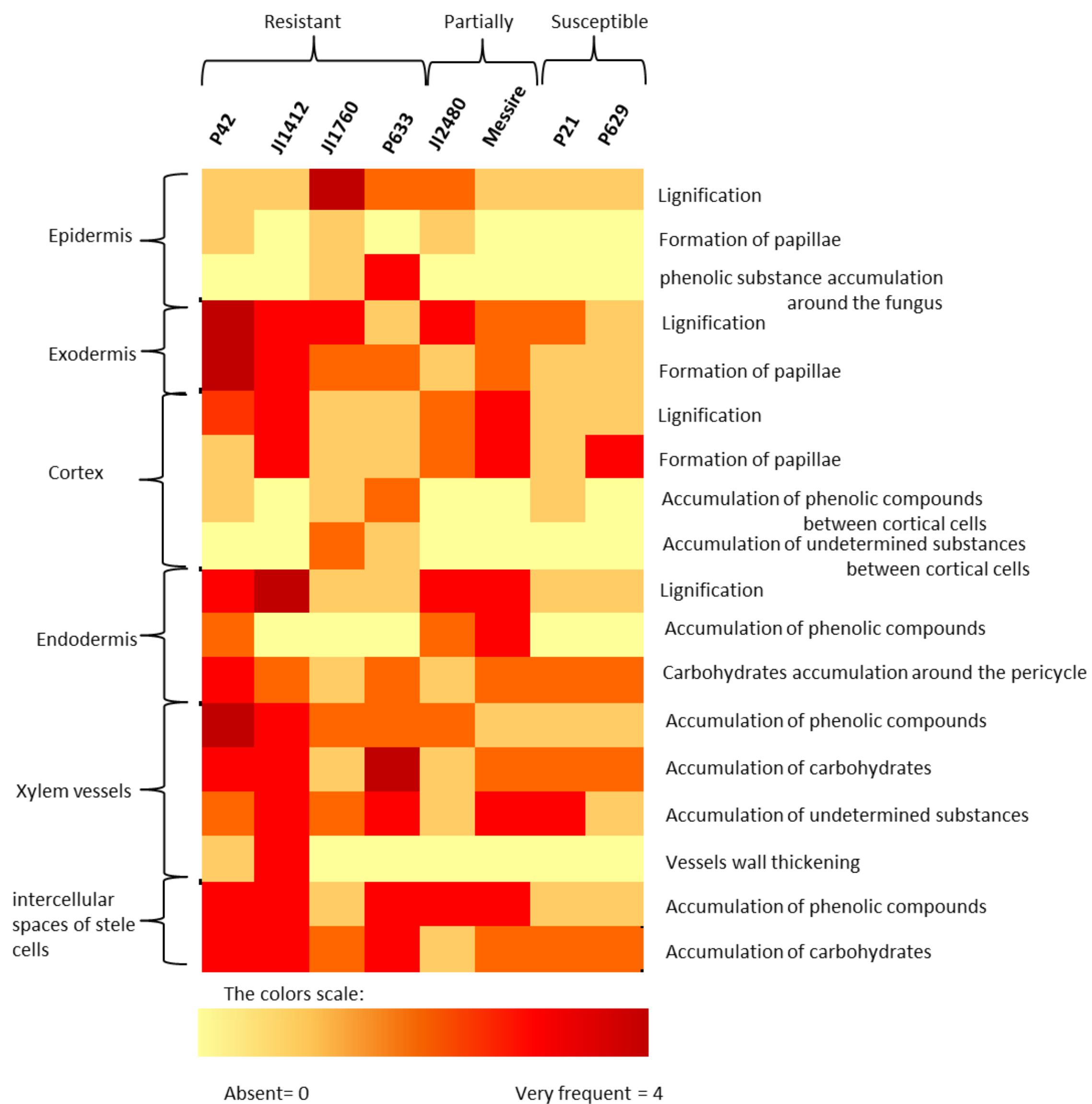


Figure 7. Heat Map represents the intensities of the host cell reactions observed at 7 days post inoculation by *Fop* in the different accessions, from yellow (0 = absent reaction) to dark red (4 = very frequent expression).

3.4.1. Outer root tissues

Staining the slides with TBO indicated that the restriction of fungal growth at the outer root area was mainly correlated with the increase of a blue-green coloration at the host cell wall and by the formation of papillae at the site of hyphae penetration in the epidermis (Fig. 2A, B), exodermis (Fig. 2D, E), and cortex (Fig. 2D, F, G, J, K). In some accessions, it was also related with the accumulation of a blue-green substance around the fungus in the epidermis (Fig. 2C) and within intercellular spaces of the

cortex (Fig. 2H). Such staining pattern suggested the lignification of host cell walls and the deposition of soluble phenolic substances in the intercellular spaces. The staining with AGS confirmed these hypothesis since they revealed a red stained filling substances between cortical cells (Fig. 2J), that showed a blue fluorescence under UV light indicative of the presence of phenolic compounds (Fig. 2K). This staining also revealed the accumulation, in the intercellular spaces of the cortex, of other undetermined substances also stained in red by AGS but presenting an orange fluorescence under UV light (Fig. 2K).

Marked differences were detected between accessions on the extent of cell walls lignification, papillae formation and toxic substances accumulation in these different plant tissues. For instance, the accumulation of the toxic substance around the fungus in the epidermis (Fig. 2C) was mainly observed in the P633 accession and to a lesser extent in the JI1760 accession, while it was not detected in the rest of genotypes (Table 1 and Fig. 7). Although cell wall lignification was detected in all accessions, the most intense lignification of epidermal cells was detected in the JI1760 accession reaching a visual score of 4. The rest of the resistant genotypes and the partially resistant JI2480 reached a visual scale of 2 for this trait while only little lignification was detected in the susceptible genotypes and the partially resistant Messire that only reached a visual scale of 1 (Table 1 and Fig. 7). At the exodermis, intense cell wall lignification (3 or 4 scale values) was observed in all resistant and partially resistant accessions except for the P633 and Messire accessions (Table 1). By contrast this partially resistant accession (Messire) together with the resistant accession JI1412 showed high lignification of cortical cells, which was less pronounced and of lesser occurrence in the rest of accessions (Table 1 and Fig. 7). The formation of papilla (Fig. 2B) to block fungal penetration was much more frequent in the exodermis than in the epidermis and cortical cell layers. At the epidermis, papillae formation was detected only in the resistant accession P42 and to a lesser extent in JI1760 and JI2480 accession (Table 1 and Fig. 7). In the exodermis (Fig. 2E), papillae were detected in all accessions although they were more frequent in the resistant accessions and the partially resistant accession Messire (Table 1 and Fig. 7). By contrast in the cortex, papillae formation (Fig. 2D, G, J, K) was only frequently observed in the resistant accessions JI1412, the partially resistant accessions JI2480 and Messire and in susceptible accession P629 while it was rare in the other resistant accessions and in the susceptible accession P21 (Table 1 and Fig. 7).

The accumulation of phenolic compounds in the intercellular space of the cortical cell layers was mainly detected in the P633 accession (Fig. 2H). This host reaction was also sometimes detected in the P42 and JI1760 resistant accessions. In this last

accession (JI1760) and to a fewer extent in P633, we also detected the accumulation of another undetermined filling substances stained red with AGS and characterized by an orange auto-fluorescent emission between cortical cells (Figs. 2J, K, 7 and Table 1).

3.4.2. Endodermis

Several defence reactions were also detected at the endodermis and pericycle layers. The resistant accessions P42 and JI1412 and the partially resistant accessions showed an intense cell wall strengthening of endodermal cell by lignification as revealed by TBO staining (Fig. 3C, D) and under UV excitation after AGS staining (Fig. 3 A, B). Such lignification was only rarely observed in the other accessions (Table 1).

TBO staining also revealed that the partially resistant accession Messire accumulated phenolic compounds in the pericycle and the endodermal intercellular spaces (Fig. 3D, E, F). This host reaction was also detected at lower intensity in the resistant accession P42 and the partially resistant accession JI2480 but not in the other accessions (Table 1 and Fig. 7). These two host reactions create a physical and chemical barrier impeding the advance of the fungus toward the vascular stele as exemplified by the intense fungal colonization of the intercellular space just beneath the endodermis cell layer in the partially resistant accession Messire (Fig. 3E, F). In addition, we observed the accumulation of a dark purple substance around the pericycle after TBO staining indicative of the presence of some kind of carbohydrates (Fig. 4A), in all accessions although it was less frequent in the JI1760 and JI2480 accessions (Table 1 and Fig. 7).

3.4.3. Vascular stele

Several defensive reactions were also differentially expressed in response to *Fop* invasion within the vascular stele depending on the accession. These reactions mainly include (i) accumulation of different substances and plugging of xylem vessels, (ii) accumulation of different substances in the intercellular spaces surrounding colonized vessels, and (iii) thickening of vessel walls. This last reaction occurred only in the resistant accession JI1412 and to a lesser extent in the resistant accession P42 (Table 1 and Fig. 7). The blue green appearance of these vessel thickenings after TBO staining (Fig. 4E) suggested that it was formed by the deposition of polyphenolic materials.

At least three different substances were detected within xylem vessels and surrounding intercellular spaces. Staining with TBO and AGS indicated that the

chemical nature of these substances were either carbohydrates (stained in dark purple by TBO and green by AGS), phenolic compounds (stained in blue-green by TBO and red by AGS and showing a blue fluorescence emission under UV light) or another undetermined filling substance (stained in clear purple by TBO and in a non-fluorescing dark red by AGS). Accumulation of these substances was observed in all resistant accessions as early as 4 dpi albeit with different intensity. By contrast, it was only observed in the susceptible accessions after 7 dpi that appeared to accumulate a lower amount of phenolic compounds and carbohydrates than the resistant accessions (Table 1 and Fig. 7).

All resistant and partially resistant accessions except the JI1760 accession showed a high accumulation of phenolic compounds (Fig. 4D, E, F, G) in the intercellular spaces of the vascular tissue (Table 1 and Fig. 7). At this site, the resistant accessions JI1412, P633 and P42 also showed a more intense accumulation of carbohydrates (Fig. 4A, B, G) classified with a visual scale of 3, than the other accessions that only reached a value of 1 or 2 (Table 1 and Fig. 7).

Within the xylem vessel, the accumulation of phenolic compounds was more intense in the resistant accessions P42 and JI1412 (Fig. 4A, B, E, G, H) that reached a visual scale value of 3 or 4 and in which some proto-xylem cells was completely plugged by phenolics (Fig. 4A, B, E). The other resistant accessions (P633 and JI1760) and the partially resistant accession JI2480 also significantly accumulated phenolics within xylem vessel reaching a visual value of 2, while it was rarely detected in the susceptible accessions and the partially resistant accession Messire (Table 1 and Fig. 7). In turns, we observed the accumulation of carbohydrates in the xylem vessels in all accessions. However, this accumulation was only intense in the resistant accessions P633, JI1412 and P42 while it was rarely observed in the resistant accession JI1760 and the partially resistant accession JI2480 (Table 1 and Fig. 7). A detailed microscopic observation showed the carbohydrates filling the xylem vessel are produced in the intercellular space surrounding the infected xylem vessels and enter them through xylem cell pits until complete plugging of the cell (Fig. 4B, C). This suggests the formation of mucilage in xylem cells as a defence response to infection by *Fop*. Other undetermined occluding material stained in clear purple by TBO was also observed in the xylem vessels of most accessions except the susceptible accession P629 and the partially resistant accession JI2480 (Fig. 4A, B, D).

4. Discussion

Fop is one of the major constraints of pea worldwide. Currently the preferred control method resides in the introduction of resistant cultivar in the field. While monogenic resistance has been identified for all *Fop* races, they are at risk of breakdown by the constant evolution of the pathogen. Thus breeding programmes would gain in incorporating quantitative resistance mechanisms within elite resistant cultivars. In a previous study, we identified a series of wild relative pea accessions with quantitative resistance against *Fop* race 2 isolates (Bani *et al.,* 2012). Defining the defence mechanisms acting in these new sources of resistance is crucial for a more targeted and efficient breeding. Thus we performed a detailed histological study on eight pea accessions with differential responses from resistant to susceptible after the challenge by one isolate of *Fop* race 2. Although several histological studies have already been conducted to characterize resistance mechanisms in several *F. oxysporum* - plant pathosystems including pea (Bishop and Cooper, 1983a; b; 1984; Baayen *et al.*, 1988; 1989; Tessier *et al.*, 1990; Shi *et al.*, 1992; Ouellette *et al.*, 1999; 2002; Zvirin *et al.*, 2010; Pereira *et al.*, 2013), our study is the first comparative histological study on several pea resistant accessions. This revealed a large variety of responses taking place throughout the fungal colonization sites from the root epidermis to the vascular stele. These defence reactions included cell wall strengthening and the accumulation of various substances both within and between the cells. Interestingly, they were differentially expressed in each of these accessions. In addition, we also confirmed the infection process in pea susceptible accessions.

Similarly to previous studies in several pathosystems (Baayen *et al.*, 1989; Benhamou *et al.*, 1996; Olivain and Alabouvette, 1999; Benhamou and Garand, 2001; Olivain *et al.*, 2006; Czymmek *et al.*, 2007; Zvirin *et al.*, 2010) we observed that at four dpi, all root tissues of the susceptible pea accessions were already extensively colonized by the pathogen. There, the pathogen was shown to penetrate the epidermal cells without the formation of any specialized structure to progress through the cortex and endodermis both inter- and intracellularly to finally colonize the vascular stele even when plants were inoculated without root trimming (Fig. 1). As a result, we observed that the fungus had already colonized the whole plant by 7 dpi. Our observation also confirmed that *F. oxysporum* mainly penetrate its host by direct penetration and through wound as suggested by Nelson (1981) although our method of inoculation which included the trimming of a portion of the root favored the fungal entry in xylem cells through wound.

In addition we also confirmed that *Fop* colonization of the susceptible plants was associated with the degradation of the cells surrounding the endodermis as previously shown for *Fop* (Tessier *et al.*, 1990; Benhamou and Garand, 2001), *F. oxysporum* f.sp. *dianthi* in carnation (Baayen and Elgersma, 1985; Baayen *et al.*, 1988; 1989; Ouellette *et al.*, 1999; 2002), *F. oxysporum* f.sp. *lycopersici* in tomato (Olivain and Alabouvette, 1999) and *F. oxysporum* f.sp. *lini* in flax (Kroes *et al.*, 1998). Our result would thus support the hypothesis that plant death is the result of cell degradation as suggested by Tessier *et al.* (1990). Since CWDEs have been shown to be produced by *F. oxysporum* within host tissues (Beckman, 1987; Baayen *et al.*, 1997; Benhamou and Garand, 2001), it is likely that the cell degradation we observed is caused by CWDEs activities within susceptible host supporting the important role of CWDEs in *F. oxysporum* pathogenicity. On the other hand, some mild and late defence reactions were detected in these susceptible accessions in the form of cell wall lignification, papillae formation, and accumulation of carbohydrates and other filling substances in the vascular stele (Figs. 1G, 7 and Table 1). However, as for other pathosystems (Baayen *et al.*, 1988; 1989; Tessier *et al.*, 1990; Olivain and Alabouvette, 1999; Ouellette *et al.*, 1999), these mild and late defence reactions were not effective to block pathogen progression.

In the partially resistant accessions, *Fop* was shown to colonize all root tissues (Figs. 2F and 3E). However, by contrast to the situation in the susceptible accessions, fewer colonies of *Fop* were detected and the fungus was shown to progress through the cortex endodermis and pericycle only intercellularly (Fig. 3E, F). Interestingly, the fungal cells massively developed in the intercellular space between endodermis and pericycle while only few colonies were detected within vascular tissue. This was more clearly visible for the Messire accessions after inoculation without root trimming (Fig. 5B). Such accumulation of fungal cells suggested the setup of an efficient barrier in this area efficiently trapping the pathogen and reducing its access to the vascular stele and support the important defensive role of endodermis to reduce *F. oxysporum* colonization (Bishop and Cooper, 1983a).

In resistant accessions, the pattern of colonization of *Fop* race 2 markedly differed from that observed in susceptible accessions. Although their outermost root tissues (epidermis and exodermis) were highly colonized by *Fop*, thereafter, the fungus was only rarely seen in the intercellular space of the cortex, and never reached the stele by centripetal growth from outer root tissues as shown after inoculating the plants without root trimming (Fig. 5A). This support the results obtained in flax and chickpea resistant accessions (Kroes *et al.*, 1998; Olivian *et al.*, 2003; Jiménez-Fernandez *et al.*, 2013) or after inoculating pea or tomato plant with a non-pathogenic strain of *F. oxysporum* (Olivain and Alabouvette, 1999; Benhamou and Garand, 2001).

In addition, the stem sections of all resistant and partially resistant accessions were completely free of fungus (Fig. 6C, D, E, F) despite the fact that the root trimming applied at inoculation favoured the direct entrance of the fungi into vascular vessels. This indicates that the vertical progression in the infected vessels of *Fop* is rapidly blocked upon the detection of the pathogen presence in these accessions. This result explains our failure to re-isolate *Fop* colonies from stem tissue of resistant accessions (Fig. S2) (Bani *et al.*, 2012). It also confirms that the restriction of pathogen growth within the xylem vessel is an important resistance mechanism that efficiently blocks pathogen progression and diseases as demonstrated previously in pea (Bishop and Cooper, 1984; Tessier *et al.*, 1990), carnation (Baayen *et al.*, 1989; Ouellette *et al.*, 2002), chrysanthemum (Barbara and Nelson, 1981), common bean (Pereira *et al.*, 2013), tomato (Beckman *et al.*, 1972; Bishop and Cooper, 1984), melon (Zvirin *et al.*, 2010) and cotton (Hall *et al.*, 2011).

Interestingly, we observed the degradation of xylem parenchyma cells in the absence of fungi in the stem sections of the partially resistant accession Messire (Fig. 6D, F). Degradation of xylem parenchyma cell walls in the absence of the fungus has been observed in susceptible carnation genotype infected by *F. oxysporum* f.sp. *dianthii* (Ouellette *et al.*, 1999). Such observation suggested that in the partially resistant Messire, the mechanisms blocking the pathogen progression are not completely efficient and while they hamper the fungal progression toward the stem, they might allow the diffusion of fungal secreted molecules such as CWDEs or toxins through the stem which might be responsible for this degradation. This might partly explain the development of symptoms in these partially resistant in absence of massive pathogen infection of the plant. No degradation has been observed in the resistant accessions indicating that the mechanisms acting in these accessions are more efficient and blocked not only the progression of the fungus but also the diffusion and/or action of its secreted pathogenicity factors. The mechanisms that block the diffusion and action of these secreted factors might involve the lignin, the esterified phenolics and other related cell wall phenolics that may inhibit and restrain the pathogenic CWDEs action on the host cell wall (El Modafar and El Boustani, 2002; Walters *et al.*, 2007; Vidhyasekaran, 2008). Given its chemical and mechanical properties, the lignin represents not only a mechanical barrier against pathogens but also restricts the diffusion of theirs enzymes and toxins (Ride, 1978; Walters *et al.*, 2007). Additional mechanisms preventing the production of CWDEs by the pathogen or inhibiting their effects such as the production of CWDEs inhibiting enzymes might also be involved, although further studies would be needed to confirm the involvement of phenolics, and CWDEs inhibiting enzymes in these processes.

The resistance to fusarium wilt has been previously associated to several defensive mechanisms in many host species differentiating three main areas, the outer root section, the endodermis and the vascular stele. Many studies indicated the importance of the outer root region (epidermis, exodermis and cortex) in the resistance where the pathogen was efficiently blocked by the localized formation of lignified cell wall papilla-like apposition at fungal attack's sites and the secretion of antifungal molecules such as phenolics in the intercellular space surrounding fungal structures (kroes *et al*., 1998; Olivain and Alabouvette, 1999; Ouellette *et al*, 1999; Benhamou and Garand, 2001; Ouellette *et al*, 2002; Olivain *et al*., 2003). Some studies in banana and tomato, however, evidenced that the resistance was due to the cell wall lignification associated or not with the accumulation of phenolics leading to strengthening of these cell layers (Mandal and Mitra, 2007; Van den berg *et al*., 2007; Mandal and Mitra, 2008). By contrast, other studies showed that the main discriminating factor between susceptibility and resistance resided at the endodermis that was suberized or lignified upon *F. oxysporum* attack preventing the movement in and out of the vascular stele rather than in the outer region of the root (Bishop and cooper, 1983; Baayen *et al*., 1989). Here, we observed a combination of all these different mechanisms coordinately established within pea root tissues to efficiently block the progression of the pathogen before the vascular stele. Thorough comparison of the six resistant and partially resistant accessions revealed that resistance was mainly based on three main mechanisms, papillae formation, cell wall strengthening and accumulation of phenolics and/or carbohydrate substances constituting physical and chemical barriers that efficiently block and supposedly killed the invading fungus (Figs. 2, 3, 4). Papillae formation was mainly concentrated in the exodermis and cortex while the last two mechanisms were detected in five different locations within root tissues including the epidermis, exodermis, cortex, endodermis and the vascular stele forming five spatially differentiated barriers within the roots (Table 1 and Fig. 7). While all accessions combined these mechanisms, each accession, appeared to have developed its own defence strategy since we detected quantitative differences both in terms of intensity and localization of these defensive reactions between accessions (Table 1 and Fig. 7). For instance the most important defense strategy of the P633 accession was mainly based on the accumulation of toxic substances in the epidermis and intercellular spaces of the cortex surrounding progressing hyphae. In turn, the JI1760 resistant accession was principally characterized by the restriction of the pathogen penetration in the epidermis and outer cortex layers through an intense lignification of epidermal and exodermal cell walls and the papillae formation in the exodermis, while the defences of P42 and JI1412 resistant accessions were mainly based on lignification and papillae formation at the exodermis and cortex. By contrast

the key defence strategy of the partially resistant accessions was the lignification of endodermal cell wall and the accumulation of phenolic substances in this area surrounding progressing hyphae. The endodermis would thus appear as the main discriminant factor for the resistance of the partially resistant accessions while it appears to play only a minor role in the resistant accessions since the fungus progression was most often blocked before this region (Fig. 3). Despite the absence of fungus in this area, the strengthening of the endodermis may, however, have a secondary role in these resistant accessions by impeding the fungus growing out of infected xylem vessels.

In parallel, several mechanisms have been shown to limit fungal growth within the xylem vessel and blocks its vertical spread in pea (Bishop and Cooper 1984; Charchar and Kraft, 1989; Tessier *et al.*, 1990), and other species (Beckman *et al.*, 1972; Barbara and Nelson, 1981; Emberger and Nelson, 1981; Bishop and Cooper, 1984; Baayen, 1988; Baayen *et al* ., 1989; Shi *et al.,* 1992; Ouellette *et al* ., 1999; 2002; Zvirin *et al.,* 2010; Hall *et al.,* 2011; Pereira *et al.,* 2013). These mechanisms consisted mainly in the plugging or occlusion of vessels with materials usually denoted as gels (gums or mucilage). Accordingly, we detected the accumulation of various substances in and around infected vessels (Fig. 4). The mucilage has also been reported previously as a putative defensive response against parasite plant *Orobanche crenata* (Pérez-de-Luque *et al.*, 2005; 2006) indicating that it might be a general mechanism established to prevent the connection to or the spreading of invading organisms through xylem. The main components of the gels seem to be carbohydrates (specifically pectins) and polyphenols, and they can contain phytoalexins and lignin-like compounds (Niemann *et al.*, 1990; Baayen *et al.*, 1996; Hall *et al.*, 2011) or lipoidal substances (Shi *et al.*, 1992; Pérez-de-Luque *et al.*, 2006). Our results showing the important accumulation of carbohydrates, phenolic compounds and additional undetermined substances in the lumen of xylem cells would support the composition of mucilage and suggested that the formation of mucilage is an important defensive mechanism of pea (Fig. 4). Interestingly, we observed the production of carbohydrates and phenolics between vascular cells and their entry within the xylem cells (red circle in Fig. 4B) suggesting that the mucilage might be produced by the vascular parenchyma cells and progressively filled the xylem vessels until complete plugging of the cells. This is in agreement with previous reports about mucilage production and secretion (Shi *et al.*, 1992; Baayen *et al.*, 1996; Rioux *et al.*, 1998; Pérez-de-Luque *et al.*, 2006). On the other hand, the occluding material can contain other substances and components including those originated from the pathogen (Ouellette *et al.*, 1999). This could explain

the heterogeneous aspect and granular structure of the indeterminate substances observed into xylem vessels (Fig. 4).

In addition to the mucilage formation, we also detected the thickening of vessel cell walls (Fig. 4E). Vessel wall thickening has been reported as a typical defence reaction against *F. oxysporum* in many species (Baayen and Niemann, 1989; Tessier *et al.*, 1990; Shi *et al.*, 1991; 1992; Baayen *et al.*, 1996; 1997; Ouellette *et al.*, 1999; 2002; Hall *et al.*, 2011; Pereira *et al.*, 2013). The phenolic nature of these coatings may contribute to chemical inhibition and physical restriction of the fungus and its product within vessels (Shi *et al.*, 1992; Hilaire *et al.*, 2001).

Altogether, our results indicated that resistant accessions developed different mechanisms creating physical and chemical barriers to impede the progressions of fungal cell toward the vascular stele. Interestingly, it showed quantitative rather than qualitative differences between accessions that differed between each other in the intensity and the spatial distribution of the established defence reactions. This study contributes to our understanding of the resistance mechanisms that are efficient to block the infection by *F. oxysporum* and provides important advances in the mechanisms acting in pea against *Fop race 2*. Potentiating the different defence mechanisms detected in these pea accessions in a single resistant cultivar through breeding might contribute to the control of this important pea disease.

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Supplementary Figures

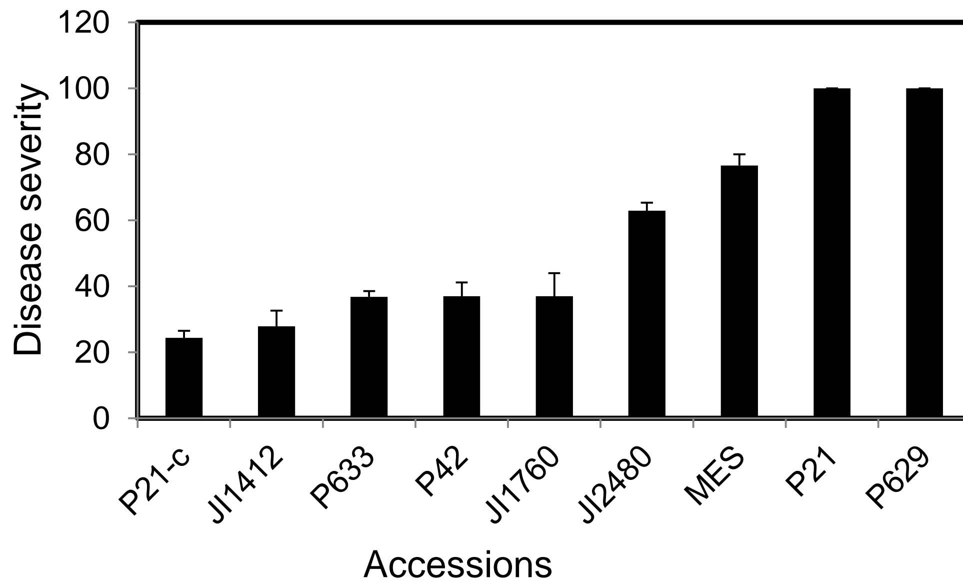


Figure S1. Disease severity of *Fop* race 2 on different pea accessions. Vertical bars are standard errors for n = 3.

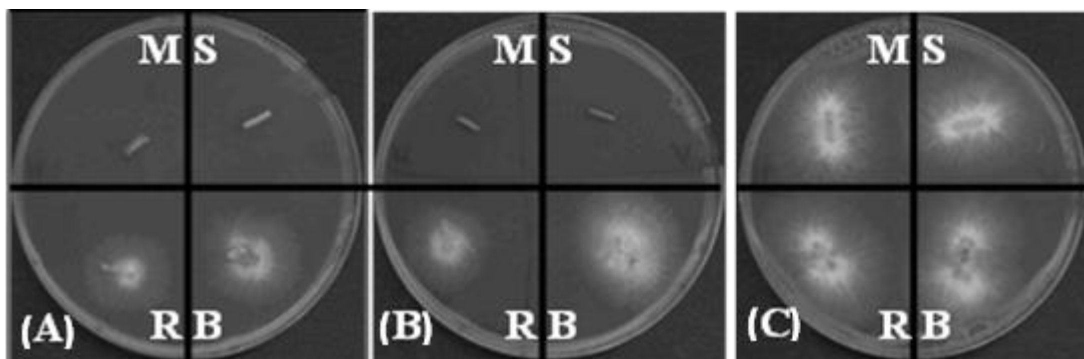
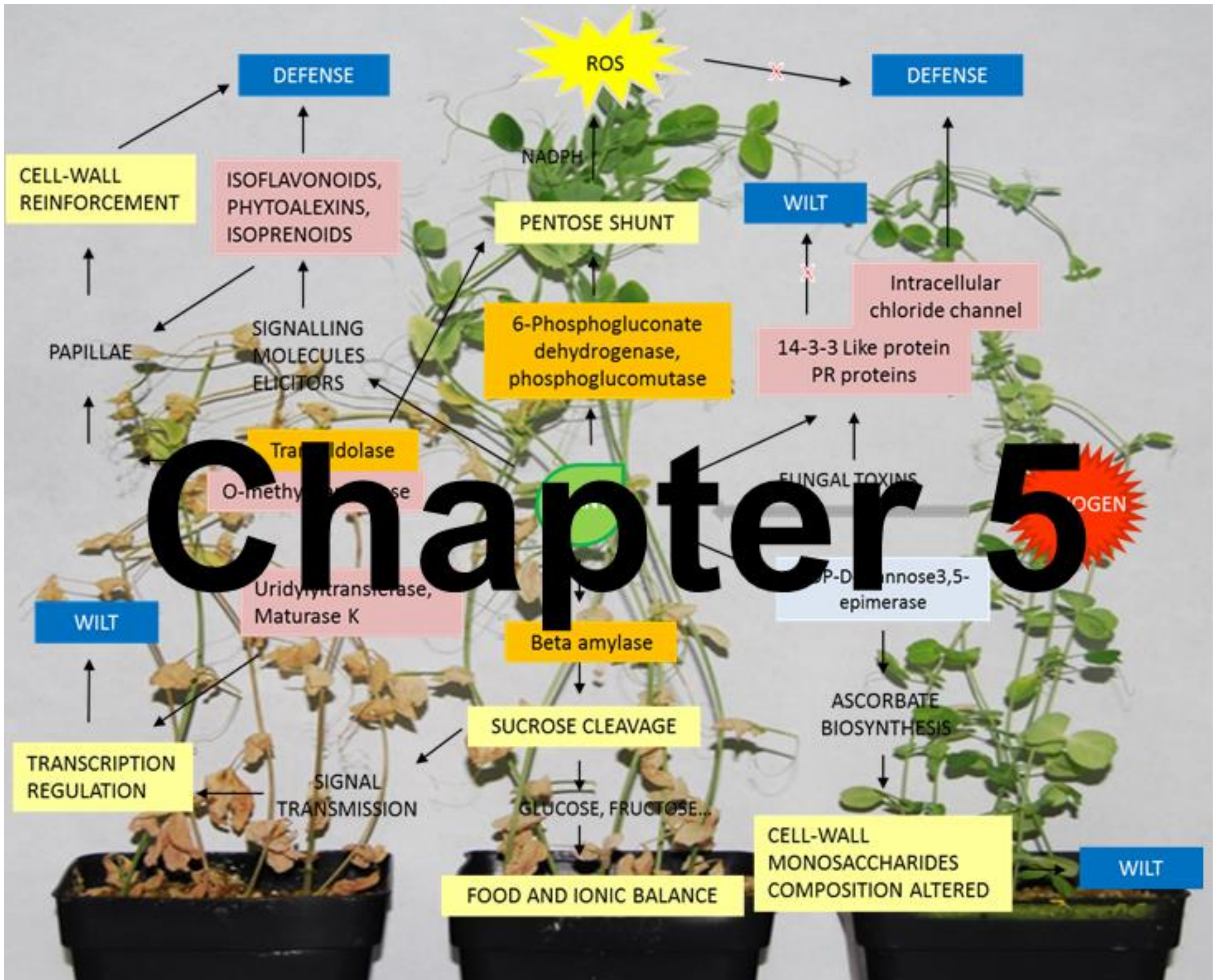


Figure S2. Isolation of *Fop* race 2 colonies from inoculated resistant (A) partially resistant (B) and susceptible plants (C) Photographs compare the extension of *Fop* race 2 colonies out of plant tissues from plants 7 dpi. R, B, M and A stand for root, basal stem, middle stem and apex sections, respectively.

Chapter 5



Chapter 5

Understanding pea resistance mechanisms in response to *Fusarium oxysporum* through proteomic analysis

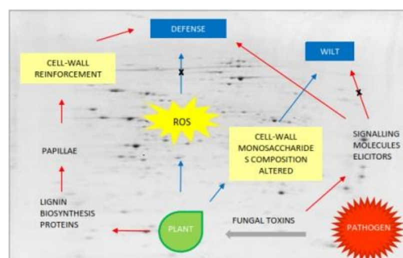
Castillejo María Ángeles, Bani Moustafa, Rubiales Diego

Abstract

Fusarium oxysporum f.sp. *pisi* (*Fop*) is an important and destructive pathogen affecting pea crop (*Pisum sativum*) throughout the world. Control of this disease is achieved mainly by integration of different disease management procedures. However, the constant evolution of the pathogen drives the necessity to broaden the molecular basis of resistance to *Fop*. Our proteomic study was performed on pea with the aim of identifying proteins involved in different resistance mechanisms operating during *F. oxysporum* infection. For such purpose, we used a two-dimensional electrophoresis (2DE) coupled to mass spectrometry (MALDI-TOF/TOF) analysis to study the root proteome of three pea genotypes showing different resistance response to *Fop* race 2. Multivariate statistical analysis identified 132 differential protein spots under the experimental conditions (genotypes/treatments). All of these protein spots were subjected to mass spectrometry analysis to deduce their possible functions. A total of 53 proteins were identified using a combination of peptide mass fingerprinting (PMF) and MSMS fragmentation. The following main functional categories were assigned to the identified proteins: carbohydrate and energy metabolism, nucleotides and amino-acid metabolism, signal transduction and cellular process, folding and degradation, redox and homeostasis, defense, biosynthetic process and transcription/translation. Results obtained in this work suggest that the most susceptible genotypes have increased levels of enzymes involved in the production of reducing power which could then be used as cofactor for enzymes of the redox reactions. This is in concordance with the fact that a oxidative burst occurred in the same genotypes, as well as an increase of PR proteins. Conversely, in the resistant genotype proteins responsible to induce changes in the membrane and cell wall composition related to reinforcement were identified. Results are discussed in terms of the differential response to Fop.

Keywords

Pisum sativum-Fusarium oxysporum interaction, resistance mechanisms, proteomic approach.



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1. Introduction

Fusarium oxysporum is a soil-borne plant fungal pathogen causing vascular wilt disease through roots in a wide variety of plant species. A large number of *formae speciales* has been recognized specialized to infect different species. Out of them, *Fusarium oxysporum* f.sp. *pisi* (*Fop*) is an important and destructive pathogen of field pea (*Pisum sativum*) (Kraft *et al*., 1998). Four different races of *Fop,* races 1, 2, 5 and 6 have been described (Infantino *et al*., 2006). Races 1 and 2 have been reported in every country where pea are grown, while races 5 and 6 are, to date, are only important in western Washington State (Infantino *et al*., 2006).

The infection cycle of *F. oxysporum* is initiated by the germination of spores in the soil in response to an undetermined signal within the host root exudates. Upon germination, infective hyphae adhere to the root surface and penetrate the root epidermis directly without the formation of any distinctive structure (Bishop and Cooper, 1983a; Rodriguez-Galvez and Mendgen, 1995). The mycelium then advances inter- or intracellularly through the root cortex, until it reaches the xylem vessels and enters them through the pits (Bishop and Cooper, 1983b; Beckman, 1987). At this point, the fungus switches to an endophytic mode of host colonization, during which it remains exclusively within the xylem vessels, using them as avenues to rapidly colonize the host. At this stage, the characteristic wilt symptoms appear as a result of severe water stress, which ultimately lead to complete plant death. Upon plant death, the pathogen grows on the plant surface where it produces chlamydospores that are dispersed into the soil for a second cycle of infection (Bishop and Cooper, 1983b).

As for many soilborne pathogenic fungi, the use of fungicides is expensive and not completely effective in controlling fusarium wilt (Sharma *et al*., 2010). As a consequence, control of this disease should be attempted mainly by integration of different disease management procedures including agronomic and farming practice (Navas-Cortes *et al*., 1998), soil disinfestation (Momma *et al*., 2010), biocontrol (Alabouvette *et al*., 2009). However the most economical and effective method for protecting crops is the use of resistant cultivars (Sharma *et al*., 2010).

Resistance to *Fop* race 1, 5 and 6 is conferred by single dominant gene while resistance to race 2 has been recently shown to be quantitative (Bani *et al*., 2012; Mc Phee *et al*., 2012). Nevertheless, the constant evolution of the pathogen drives the necessity to broaden the molecular basis of resistance to *Fop*. Some studies using plant mutants defective in signal transduction and defence responses have provided insights into the function of the network regulating resistance to *F. oxysporum* in

Arabidopsis (Berrocal-Lobo and Molina, 2004; 2008; Zhu *et al.*, 2013). Transcriptome and metabolome analyses have been conducted in *F. graminearum* during the invasion of hosts, sexual development, and conidial germination, in response to azole fungicide and/or in *F. graminearum* mutants as well as in barley and wheat during infection to understand defence responses (reviewed by Kazan *et al.*, 2012).

Proteome analysis of phytopathogenic fungi and their interactions with hosts has increased dramatically over the last years, because of the technical development of "omics" and bioinformatic tools, and the growing number of fungal genomes being sequenced (Yang *et al.*, 2013). Several proteomic investigations in *Fusarium* spp. have been performed so far. Extensive proteomic studies have been conducted in *F. graminearum*-infected wheat, barley, and their wild relatives (Yang *et al.*, 2010; Ding *et al.*, 2011; Shin *et al.*, 2011; Gunnaiah *et al.*, 2012). However, up to our knowledge few proteomic works were performed in legumes. Thus, the antifungal activity against *F. oxysporum* of the proteins found in exudates of germinating white lupin seeds was assessed by Scarafoni *et al.* (2013). In chickpea, Palomares-Rius *et al.* (2011) observed that co-infection of a *Fusarium oxysporum* f.sp. *ciceris*-resistant plant by the fungus and the root-knot nematode *Meloidogyne artiellia* causes breakdown of the resistance and thus limits its efficacy in the control of fusarium wilt. In pea no proteomic study on the response to *F. oxysporum* has been reported so far.

The aim of the current study was to provide a complementary proteomic approach for the investigation of pea plant defence mechanisms involved during *F. oxysporum* f.sp. *pisi* infection. For such purpose, we used a two-dimensional electrophoresis (2-DE) coupled to mass spectrometry (MALDI-TOF/TOF) analysis to study the root proteome of three pea genotypes showing different resistance response to *Fop* race 2, one of the widely distributed *Fop* race and to which polygenic resistance has been found.

2. Materials and methods

2.1. Fungal isolates and cultural conditions

Fusarium oxysporum f.sp. *pisi* (*Fop*) race 2 strain R2F42 was kindly provided by Dr. W. Chen (USDA-ARS, Pullman, USA) for use in this experiment. The fungal strain was stored as microconidial suspensions at -80 ºC in 30% glycerol. For microconidia production, cultures were grown in potato dextrose broth (PDB; Difco, Detroit, MI) at 28 ºC in a shake culture set at 170 rpm (Bani *et al.*, 2012).

2.2. Plant material, growth conditions, and inoculation

Three pea genotypes with different levels of resistance to *Fop* race 2 were used including the susceptible genotype (S) P629, the partially resistant (P) P615 and the resistant genotype (R) P42 (Bani *et al.*, 2012). Pea seeds were surface-sterilized for 20 min in a 20% solution of sodium hypochlorite and then rinsed three times with sterile water. Then the seeds were wrapped in wet filter paper in a petri dish, stratified for two days at 4 °C in the dark and incubated at 26 ± 2 °C until germination. Once germinated, the seedlings were sown in vermiculite and grown in controlled environment under a 16/8 h light-dark photoperiod at 26 ± 2 °C constant temperatures with 200 µmol $m^{-2}s^{-1}$ of illumination. Plants were watered every three days with tap water. Seven days old pea seedlings were inoculated with the dip root technique as described previously (Bani *et al.*, 2012) using microconidial suspensions containing 5 x 10^6 microconidia per ml of water. Control plants were treated in the same way and were immersed in sterile water. Inoculated and control plants were maintained in the same growth condition as above.

For the proteomic assay, three analytical replicates per treatment (inoculated and non-inoculated), genotype and sampling time (24 and 72 hours after inoculation "hai") were performed, consisting each replicate in five roots from different plants. Root tissue was abundantly washed with distilled water and blotted dry with filter paper. The whole roots were cut, immediately frozen in liquid nitrogen, and stored at -70 °C until protein extraction. Simultaneously, five plants per genotype were inoculated and used to evaluate the disease severity symptoms by estimating the percentage of leaves with symptoms such as is described by Bani *et al.* (2012). The evaluation started 10 days post inoculation, taking place every 3 days and finishing 30 days after inoculation.

2.3. Protein Extraction and Two-Dimensional Gel Electrophoresis

Root pea samples (ca. 0.5 g fresh weight) from three independent replicates per treatment, sampling time and genotype were crushed in a pre-cooled mortar with liquid nitrogen until a fine powder was formed and proteins were TCA-phenol extracted (Wang *et al.*, 2006). Following overnight ammonium acetate precipitation, the pellet recovered by centrifugation was rinsed with cold methanol and acetone, dried at room temperature and resuspended in 50 µl of solubilization buffer containing 7 M urea (Merck), 2M thiourea (Sigma Aldrich), 2% (W/V) CHAPS (Sigma Aldrich), 2% (V/V) bio-lyte 3-10 carrier ampholytes (BioRad), 2% (W/V) DTT (Sigma Aldrich) and bromophenol blue traces (Sigma Aldrich). Protein concentration was determined by

Bradford assay (BioRad) and the samples were stored at -20 °C before electrophoresis.

IPG DryStrips (Amersham Biosciences) 18 cm, pH 3-10 non-linear gradients were used. Strips were passively rehydrated for 6 h and actively (50 V) for 6 h more with 300 µl of sample buffer, containing 200 µg protein. Strips were loaded onto a PROTEAN IEF System (BioRad) and focused at 20 °C with increasing linear voltage: 1 h at 500 V, 1 h at 1,000 V, 1 h at 8,000 V and 8,000 V until reaching 50,000 Vh. IEF strips were equilibrated by first soaking them for 15 min in 375 mM tris-HCl buffer pH 8.8, 6MUrea, 2% SDS, 20% glycerol solution containing 2% DTT, and then for 15 min in the same solution containing 2.5% iodoacetamide instead of DTT.

Strips were then transferred onto vertical slab 10% SDS polyacrylamide gels and the electrophoresis was run at 15 °C and 30 V for 30 min, and then at 60 V for about 14 h until the dye front reached the bottom of the gel. Broad molecular range markers (Bio-Rad) containing myosin (200 kDa), β-galactosidase (116.25 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa) and aprotinin (6.5 kDa) were loaded beside the strip. Gels were fixed with methanol: acetic acid (10:7 V/V) solution for 30 min, covered with Sypro Ruby stained overnight and rinsed in methanol: acetic (10:7 V/V) acid for 30 min.

2.4. Image Acquisition and Statistical Analysis of Dataset

Gel images were obtained with a Molecular imager FX ProPlus Multi-imager system (Bio-Rad), using 532 nm laser and 555 nm LP emission filter and analysed with the PDQuest Advanced v.8.0.1 software (Bio-Rad) using a 10-fold over background as the minimum spot presence/absence criterion. Normalized spot volumes (individual spot intensity/normalization factor) calculated for each gel based on total quantity in valid spots were determined for each spot, these values being used to designate the possible differences when images were compared. For further MS analysis, those proteins with more/less than 1.5 fold change on abundance level, high statistical significance ($P < 0.05$), and presence in all the replicated gels were selected.

For statistical treatments and cluster analysis of protein abundance values we used the software Statistix 10 and the web-based NIA array analysis tool (Sharov *et al.*, 2005). After uploading the data table (Supplementary Material Table S1) and indication of biological replications, the data were statistically analyzed using the following settings: error model "max (average, actual)", 0.01 proportion of highest variance

values to be removed before variance averaging, 10° of freedom for the Bayesian error model, 0.05 FDR threshold, and zero permutations. A hierarchical clustering was performed to check the entire dataset, and the results were represented in dendrograms. Then, the entire dataset was analyzed by PCA using the following settings: covariance matrix type, four principal components, 1.5-fold change threshold for clusters, and 0.3 correlation thresholds for clusters. PCA results were represented as a biplot, with proteins which were more abundant in those experimental situations located in the same area of the graph. Protein spot data for this analysis are recorded in the Supplementary Material Table S2. Finally, histograms representing log average protein spot values were downloaded employing the software (Supplementary Material Fig. S1).

2.5. Mass Spectrometry Analysis and Database Searching

Protein spots were automatically excised from gels using an Investigator ProPic (Genomic Solutions) station and the digestion protocol used was that of Shevchenko *et al.* (1996) with minor variations. Gel plugs were distained by incubation (twice for 30 min) with a solution containing 200 mM ammonium bicarbonate in 40% acetonitrile at 37 °C. They were then subjected to three consecutive dehydration/rehydration cycles with pure acetonitrile and 25 mM ammonium bicarbonate in 40% acetonitrile, respectively, and finally dried at room temperature for 10 min. Then, 20 µl of trypsin, at a concentration of 12.5 ng μl^{-1} in 25 mM ammonium bicarbonate, was added to the dry gel pieces and the digestion proceeded at 37 °C for 12 h. Digestion was stopped and peptides were extracted from gel plugs by adding 10 µl of 1% (v/v) trifluoracetic acid (TFA) and incubating for 15 min.

Peptide fragments from digested proteins were then crystallised with α-cyano-4-hydroxycinnamic acid as a matrix and subjected to a MALDI-TOF/TOF (4800 Proteomics Analyzer; Applied Biosystems, Foster City, CA, USA) mass spectrometer in the m/z range 800-4,000, with an accelerating voltage of 20 kV. Spectra were internally calibrated with peptides from trypsin autolysis (M+H+ = 842.509, M+H+ = 2,211.104). The five most abundant peptide ions were then subjected to fragmentation analysis, providing information that can be used to determine the peptide sequence.

A combined peptide mass fingerprinting (PMF) tandem mass spectrometry (MS plus MSMS) search was performed using GPS Explorer™ software v.3.5 (Applied Biosystems) over non redundant NCBInr database employing the MASCOT search engine (Matrix Science, London; http://www.matrixscience.com). The following parameters were allowed: taxonomy restrictions to Viridiplantae, a minimum of two

peptide matches, a maximum of one miscleavage and peptide modifications by carbamidomethylcysteine and methionine oxidation were accepted. The maximum tolerance for peptide mass matching was limited to 20 ppm. Confidence in the PMF matches was based on the score level and confirmed by the accurate overlapping of the matched peptides with the major peaks of the mass spectrum. For a functional characterization of putative and predicted proteins of unknown function, we have used BLAST (Basic Local Alignment Search Tool) to find entries in phylogenetically related organisms by sequence similarity. The MALDI-TOF analysis was carried out in the UCO-SCAI proteomics facility, a member of Carlos III Networked Proteomics Platform, ProteoRed-ISCIII.

3. Results

3.1. Plant symptom evaluation

Disease was evaluated by estimating the percentage of leaves with symptoms and the area under the disease progression curve (AUDPC) as described by Bani *et al.* (2012). Symptoms were also measured from control (non-inoculated) plants to counteract those symptoms due to the inoculation procedure. Control symptoms were calculated as average of the three non-inoculated genotypes. The assessment of susceptible cultivar P629 (S) showed that the initial symptoms appeared in the primary leaves one week after inoculation and sequentially reached the later-formed leaves until the whole plant withers and dies at 21 days post inoculation showing a 100% of disease severity (Fig. 1 and Fig. 2A). At the contrary, the partially resistant P615 (P) and resistant P42 genotypes (R) showed a 48.9% and 22.9% of disease severity respectively, compared to 19.8% showed in the control. Similarly, the AUDPC values calculated from the disease severity at 30 dpi showed a big difference between the three genotypes tested, with values of 1994 for the susceptible, 999 for the partially resistant and 407 for the resistant genotype, while the control showed a value approximately similar to the resistant genotype (409) (Fig. 2B).

The one-way ANOVA performed for disease severity detected statistically significant differences between the three genotypes ($P < 0.001$) and a clear discrimination between the susceptible, partially resistant and resistant genotypes was performed by a mean comparison analysis, while no significant difference was detected between the resistant genotype and control (Fig. 2B).

Figure 1. Fusarium wilt symptoms 21 days after inoculation by the *Fop* race 2 of the three pea accessions: susceptible (S), partially resistant (P) and resistant (R).

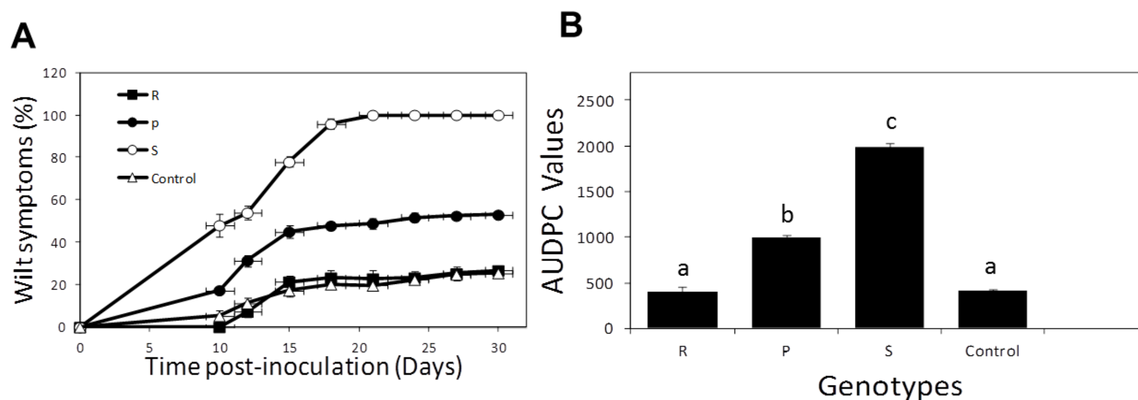

Figure 2. A: Evolution of fusarium wilt symptoms on the susceptible (S), partially resistant (P) and resistant (R) pea accessions induced by the *Fusarium oxysporum* f.sp. *pisi* (*Fop*) race 2. **B:** Distribution of the pea accessions upon inoculation with *Fop* according to AUDPC values calculated from the evaluation of percentage of leaves showing symptoms for each individual plant at 30dpi. Control was calculated as average of the three non-inoculated genotypes. Vertical bars are standard errors for n= 5.

3.2. 2DE and MSMS analysis: statistical treatment of data

As determined following the Sypro Ruby staining of the 2DE gels and the use of the PDQuest software, an average of 332 ± 49 individual protein spots were resolved (Fig. 3A, Table 1). After normalization of protein spots images and manual verification, 132 differential protein spots were detected. The following criteria were used for considering a spot as being variable: (i) consistently present or absent in all three replicates; (ii) display genotypes- or treatment-ratios differing at least 1.5-fold; and (iii) statistically significant differences ($P < 0.05$) among genotypes or treatments. Table 1 summarizes the features of the experiment.

Qualitative changes between the different conditions analysed were represented in Venn diagrams (Supplementary Material Fig. S2). The number of common and

unique constitutive proteins shared between genotypes non-inoculated and inoculated (Supplementary Material Fig. S2A) were very similar on both sampling times. Thus, genotype R at 24 hours after inoculation (hai) shared less proteins with S than P, however at 72 hai the number of proteins shared with R was the same for both.

Table 1. Summary of the features of two-dimensional experiment.

Features	Number of protein spots
Average of total spots detected by 2DE	332±49
Differential protein spots/identified	
Total	132 (40% of the total detected spots)/53
Between genotypes	
- Non inoculated (T1)	48/27
- Non inoculated (T2)	48/28
- Inoculated (T1)	75/47
- Inoculated (T2)	75/47
In response to *F. oxysporum* infection	
- S (T1)	23
- S (T2)	35
- P (T1)	32
- P (T2)	26
- R (T1)	22
- R (T2)	31

The 132 differential proteins were selected using the PDQuest (Bio-Rad) software and statistically analyzed by the NIA array analysis software to be subjected to MALDI-TOF/TOF mass spectrometry analysis. T1: 24 hours after inoculation; T2: 72 hours after inoculation; S: P629 susceptible genotype; P: P615 partially resistant genotype; R: P42 resistant genotype.

In response to *Fop* inoculation (Supplementary Material Fig. S2B) we could see a decrease in the number of unique proteins in all the three genotypes. In the susceptible genotype (S) some of the induced proteins were isomerase (spot 112), caffeoyl-CoA O-methyltransferase (CCoAOMT, spot 2107), D-3-phosphoglycerate dehydrogenase (spot 3609) and diphosphomevelonate decarboxylase (spot 3410). In the inoculated partial resistant genotype (P) we found NAD(P)H-dependent 6'-deoxychalcone synthase (spot 5105) and proteasome subunit alpha (spot 3101). And in the inoculated resistant genotype (R) we found chalcone O-methyltransferase (spot 302), CCoAOMT (spot 2108) and phosphoserine aminotransferase (spot 8307). Abundance data of all of the 132 differential protein spots were analyzed using the web-based NIA array analysis software tool. The hierarchical clustering revealed that experimental conditions could be divided into two main clusters, namely cluster 19 (clustering R genotype) and

cluster 22 (clustering P and S) (Fig. 3B). This clustering indicated that R genotype had protein abundance profiles similar between them but different from those shown by P and S. At the same time the cluster 22 was divided into two clusters, 21 (P genotype clustering closest together) and 20 (S genotype clustering closest together). Also the clustering indicated that the experimental sampling times clustered closest together in each genotype. These results revealed that protein profiles between treatments of the same genotype were quite similar and slightly different between sampling times. The protein dataset was analyzed by PCA (Fig. 3C), thus the first four components accounted for 86 % of the biological variability of the experiment (Fig. 3D).

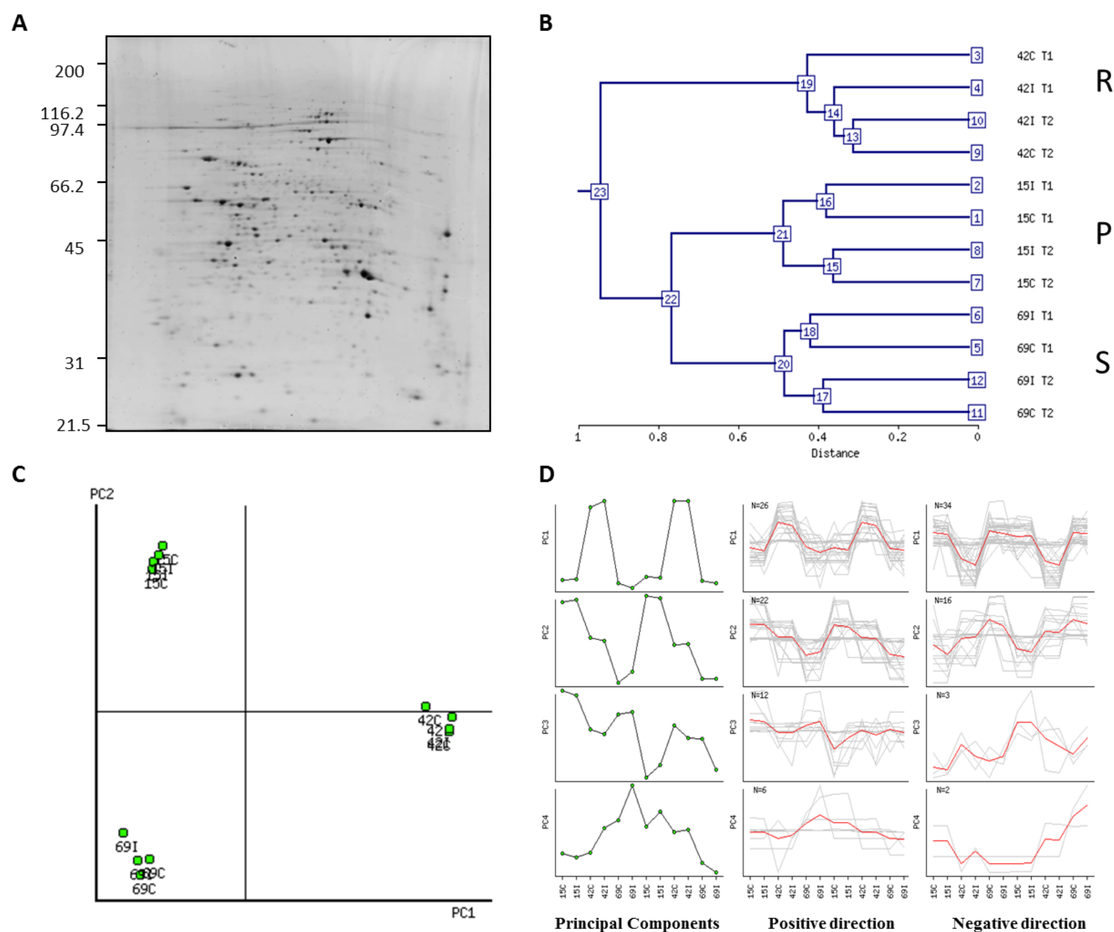

Figure 3. A: Representative Sypro Ruby-stained 2-D protein gel of the resistant (P42) genotype. **B:** Dendrogram showing hierarchical clustering of experimental conditions. The abundance clusters are numbered from 1 to 23. **C:** Two-dimensional biplots showing associations between experimental samples generated by principal component analysis (PCA). A short distance between samples in the component space is indicative of similarity in abundance profiles. **D:** Protein spot abundance clustering based on PCA. For each PC, two clusters of proteins were identified that were positively and negatively correlated with the PC. Protein clustering was performed sequentially starting from the first PC. Proteins that were already clustered with a PC were not included in the clusters associated with subsequent PCs. Protein spots identified in this analysis are recorded in the Supplementary Information Table S2.

3.3. Protein identification and abundance pattern analysis

After MSMS analysis a protein searching against Viridiplantae index of the non-redundant NCBI database was performed. Of the 132 proteins identified 53 were successfully identified with a high probability score (Table 2), 23% of which matched *P. sativum* proteins and almost 74% belonged to legume species. For those proteins that gave an unknown function or which did not match *P. sativum*, a Blast comparison analysis was performed against UniProt database, improving our results in 21% of identifications, and matching most of them with *P. sativum* or *M. truncatula*.

The following main functional categories were assigned to the identified proteins (Supplementary Material Fig. S3): Carbohydrate and energy metabolism (21%), nucleotides and aminoacid metabolism (7%), signal transduction and cellular process (8%), protein folding and degradation (8%), redox and homeostasis (13%), defence related (9%), biosynthetic process (24%) and transcription/translation (4%). The proteins identified and their functions are listed in Table 2, including the number of peptides that hit protein and the sequence coverage, as an indication of the confidence in their identification. In most cases, similar experimental and theoretical *Mr/*p*I* values were observed. In those proteins in which differences between theoretical and observed *Mr/*p*I* were detected, post-transcriptional modifications, different members of the same functional family (small shift in the p*I*), or degradation products (significant differences between theoretical and observed *Mr* values) can be attributed. Five proteins were represented by more than one spot with slightly different *Mr* and p*I* values: 6-phosphogluconate dehydrogenase, gi|603221 (spots 4404 and 4409); Leukotriene-A4 hydrolase-like protein, gi|217074536 (spots 1603 and 1606); Endochitinase A2, gi|1705807 (spots 7105 and 8102); CCoAOMT, gi|30580341 (spots 2107 and 2108); and Isoflavone reductase-like NAD(P)H-dependent oxidoreductase, gi|6525021 (spots 3102 and 4101) (Table 2). Mean log abundance intensities for all 53 protein spots identified are shown in Fig. S1. Considering both sampling times we found in the categories of proteins related with PC1 (positive direction) those constitutively more abundant in S than R genotype, and the opposite was found in PC1 (negative direction). The functional category of proteins falling in PC1 belongs to carbohydrate and aminoacid metabolisms, stress and defence, protein folding, biosynthetic processes and transcription/translation. In PC2 (positive direction) we found proteins constitutively more abundant in S than in P genotypes, mainly belonging to stress and biosynthetic process categories, and the opposite was found for PC2 (negative direction) with proteins falling in the categories of carbohydrate metabolisms and biosynthetic processes.

Table 2. Identified proteins and classification according to their functions.

SSP	Protein Name (% Protein Identity)^{a}	Score	Specie^{a}	Entry from NCBInr /UniProt databases^{a}	*Mr/pI* experimental (theoretical)^{b}	PM^{c}	Covarage	Functional category	More/less abundance change ratio^{d}
Carbohydrate and Energy									
503	Beta-amylase (1,4-alpha-D-glucan maltohydrolase)	287	*Vigna unguiculata* (*M. truncatula*)	gi|3913034/ I3RZQ8	61.4/5.1 (56.5/5.0)	8	19	Polysaccharide catabolic process	0.6P^{1}, ∞P^{2}
1202	Fructokinase-like protein	140	*Cicer arietinum*	gi|20975618	39.0/5.3 (26.0/5.0)	5	19	Sucrose and fructose metabolism	(RlaPlaSlb)^{2} 0.5P^{1}
1402	UTP--glucose-1-phosphate uridylyltransferase (UGPase)	522	*Astragalus penduliflorus*	gi|12585472/ Q9LKG7	55.6/5.4 (51.5/6.0)	16	30	Gluconeogenesis	0.5P^{1}, 0R^{1}, 0.4P^{2}, ∞R^{2}, 0.6S^{2}
2210	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	789	*Daucus carota* (*M. truncatula*)	gi|51703306/ G7J2H2	40.0/5.8 (37.0/7.0)	13	37	Calvin cycle	(RCaSCbPCb)^{1} (RCaSCaPCb)^{2} (RlaSlaPlb)^{1} (RlaSlbPlc)^{2}
3308	Dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase (OGDC-E2)	77	*Ricinus communis*	gi|255578100	48.8/6.1 (51.0/9.5)	7	11	TCA Cycle	(RCaSCbPCb)^{1} (RCaPCabSCb)^{2} 0P^{1}
3407	Formamidase	115	*Lupinus albus*	gi|222840535	51.9/5.9 (50.5/6.0)	6	17	Glyoxylate cycle	(SCaPCbRCc)^{2} (SlaRlbPlb)^{1} (SlaPlbRlb)^{2}
4404	6-phosphogluconate dehydrogenase (6PGD)	388	*Medicago sativa*	gi|603221	53.6/6.3 (54.0/5.5)	15	38	Pentose shunt	(SlaPlaRlb)^{1} (SlaPlaRlb)^{2} 0.6P^{1}
4409	6-phosphogluconate dehydrogenase (6PGD)	491	*Medicago sativa*	gi|603221	54.3/6.5 (54.0/5.5)	16	34	Pentose shunt	(RCaSCbPCb)^{1} (RCaPCabSCb)^{2} (RlaPlbSlb)^{1} (RlaPlabSlb)^{2} 1.75P^{2}
5708	Phosphoglucomutase, cytoplasmic (PGM)	193	*Pisum sativum*	gi|12585296	81.2/6.7 (63.5/5.5)	16	37	Glucose metabolism	(SlaRlabPlb)^{2} 1.5P^{1}, 1.9S^{1}, 0.6S^{2}
5712	NADP dependent malic enzyn	202	*Phaseolus vulgaris*	gi|510876	74.2/6.9 (65.0/6.0)	4	5	Malate metabolic process	(SCaRCabPCb)^{2}
7202	Fructose-bisphosphate aldolase	125	*Arabidopsis thaliana* (*P. sativum*)	gi|255644696 / Q01516	39.4/7.4 (42.5/8.0)	3	12	Glycolisis	(PlaRlaSlb)^{1} 0.4P^{1}, 0.5R^{1}, 1.6S^{1}, 0.5P^{2}, 0R^{2}
Nucleotides and aminoacid metabolism									
202	Adenosine kinase/ copper ion binding (ADK1)	142	*Arabidopsis thaliana*	gi|42572347	39.8/5.3 (33.5/6.0)	5	26	Purine ribonucleoside salvage	0.4P^{1}
3609	D-3-phosphoglycerate dehydrogenase (PHGDH)	79	*Ricinus communis*	gi|255555301	65.0/6.1 (63.5/7.5)	7	13	L-serine biosynthetic process	(RCaSCaPCb)^{2} (RlaSlaPlb)^{1} (RlaSlabPlb)^{2} 0.6P^{1}
5407	Apyrase S-type	806	*Pisum sativum*	gi|11596073	51.1/6.7 (50.5/7.0)	16	42	Transit peptide	(RCaSCabPCb)^{1} (RCaSCabPCb)^{2} (RlaSlabPlb)^{2} 2.0S^{1}, 0.6S^{2}
8307	Phosphoserine aminotransferase (PSAT)	88	*Ricinus communis* (*A. thaliana*)	gi|255561385 / Q1EBU1	43.4/7.9 (48.8/8.5)	6	18	Aminoacid biosynthesis	(RCaSCbPCb)^{1} (RCaPCbSCb)^{2} (RlaPlabSlb)^{1} 0R^{1}
Signal transduction and cellular process									
111	14-3-3-like protein	141	*Pisum sativum*	gi|4850247	32.4/5.0 (29.5/4.5)	6	36	DNA binding	(PlaSlaRlb)^{1} 0.6R^{2}
302	Chalcone O-methyltransferase (ChOMT)	67	*Medicago sativa*	gi|44887779	43.5/5.3 (41.0/5.0)	4	12	protein trafficking and sorting, signal transduction, biosynthesis, metabolism, and gene expression	(RCaPCbSCb)^{1} (RlaPlbSlb)^{2} 0.56P^{1}, ∞R^{1}, 2.5P^{2}
2208	Hypothetical protein OsJ_06593 (89% SNARE-interacting protein KEULE)	74	*Oryza sativa/Zea mays*	gi|222622784 / B6SHD7	39.4/5.6 (75.5/8.0)	14	26	Vesicle trafficking	(RCaSCaPCb)^{1} (RCaSCaPCb)^{2} (RlaSlaPlb)^{1}
3003	Intracellular chloride channel	64	*Medicago truncatula*	gi|92882351	26.1/5.9 (26.5/6.0)	5	23	Cellular processes	(PCaSCaRCb)^{2} (PlaSlaRlb)^{1} (PlaSlaRlb)^{2} 2.5R^{1}, 0.4R^{2}

Table 2. (Continued)

SSP	Protein Name (% Protein Identity)[a]	Score	Specie[a]	Entry from NCBInr /UniProt databases[a]	*Mr/pI* experimental (theoretical)[b]	PM[c]	Covarage	Functional category	More/less abundance change ratio[d]
Folding, sorting and degradation									
1404	Polyubiquitin 1	214	*Phaseolus vulgaris*	gi|33327284	50.9/5.5 (6.5/6.5)	6	64	Proteolysis	(RCaSCabPCb)^{1} (RCaSCaPCb)^{2} (RIaSIaPIb)^{2} 0.6P^{1}, ∞S^{1}, 0.4P^{2}, 0.5S^{2}
2405	Polyubiquitin 2	122	*Phaseolus vulgaris*	gi|33327286	50.4/5.6 (15.5/6.5)	3	38	Proteolysis	(PCaSCaRCb)^{1} (PCaSCaRCb)^{2} (PIaSIaRIb)^{1} (PIaSIaRIb)^{2}
3101	Unknown (100% Proteasome subunit alpha type)	240	*Medicago truncatula*	gi|217072000 / B7FHY1	31.8/5.8 (27.5/5.5)	7	27	Proteolysis	(PCaSCabRCb)^{2} (PIaSIabRIb)^{1} 0P^{1}, 14.4R^{1}, 0.5R^{2}, 0S^{2}
3415	Hypothetical protein SORBIDRAFT_04g035840 (90% Leucine aminopeptidase 2, chloroplastic)	118	*Sorghum bicolor (Triticum urartu)*	gi|242066822 / M7ZYF8	58.9/6.0 (62.0/7.5)	8	22	Proteolysis	(RCaPCbSCb)^{1} (RCaPCbSCb)^{2} (RIaSIbPIb)^{1} (RIaPIbSIb)^{2} (SIaRIabPIb)^{2}
Redox homeostasis									
1104	Short-chain alcohol dehydrogenase SAD-C	559	*Pisum sativum*	gi|6119844	31.9/5.4 (28.0/5.5)	12	53	Oxidoreductase activity	0.4P^{2}, 0.4R^{2}, 0.6S^{2}
1112	NADP-thioredoxin reductase A	368	*Medicago truncatula*	gi|111550245	37.6/5.5 (40.0/7.0)	7	22	Cell redox homeostasis Removal of superoxide radicals	(RIaPIaSIb)^{1}
2005	Chain A, Crystal Structure of Recombinant Ascorbate Peroxidase	941	*Pisum sativum*	gi|1420981	28.4/5.8 (27.0/5.5)	17	66	Hydrogen peroxide catabolic process	0.3P^{1}, 0R^{1}, 0.6S^{2}
2007	Short-chain alcohol dehydrogenase A	255	*Pisum sativum*	gi|37051111	31.3/5.6 (29.5/5.5)	6	29	Oxidoreductase activity	(RIaPIabSIb)^{1} 0.5P^{1}, 0R^{1}, 1.8S^{2}
4204	Aldo/keto reductase (AKR)	219	*Medicago truncatula*	gi|124360836	39.8/6.3 (38.0/6.0)	5	16	Oxidoreductase activity	(PCaSCaRCb)^{1} (PCaSCaRCb)^{2} (PIaSIaRIb)^{2} 0S^{1}
4706	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial (CI-75kD)	104	*Solanum tuberosum*	gi|3122572	89.3/6.5 (80.0/5.9)	6	12	ATP synthesis coupled electron transport Response to oxidative stress	(RCaSCbPCb)^{2} (RIaSIbPIb)^{2}
5304	12-oxophytodienoic acid 10, 11-reductase	171	*Pisum sativum*	gi|13516922	44.6/6.9 (41.5/6.0)	9	23	Flavin mononucleotide binding	0.4S^{1}, 1.7P^{2}, 1.6R^{2},
Defense related									
3507	TIR Disease resistance protein	62	*Medicago truncatula*	gi|124361210	60.5/6.0 (56.5/6.5)	10	20	Defense response	(SIaRIaPIb)^{1} 0S^{1}, 0.6R^{2}
4707	Hsp organizing protein/stress-inducible protein	76	*Dactylis glomerata*	gi|281399029	80.6/6.4 (65.0/6.0)	2	4	Stress response	(PCaRCaSCb)^{1}
7105	Endochitinase A2	539	*Pisum sativum*	gi|1705807	33.2/7.5 (35.5/7.5)	7	29	Defense response	(RIaPIbSIb)^{1} 0.2R^{1}, 0.4S^{2}
7108	Beta-1,3-glucanase	687	*Pisum sativum*	gi|261212	37.4/7.6 (37.5/6.0)	11	36	Defense response	0.5P^{1}, 1.5S^{1}, 0.6P^{2}
8102	Endochitinase A2	78	*Pisum sativum*	gi|1705807	32.9/7.7 (35.5/7.5)	4	16	Defense response	(PCaSCaRCb)^{2} 3.1S^{1}, 0S^{2}

Table 2. (Continued)

SSP	Protein Name (% Protein Identity)[a]	Score	Specie[a]	Entry from NCBInr /UniProt databases[a]	Mr/pI experimental (theoretical)[b]	PM[c]	Covarage	Functional category	More/less abundance change ratio[d]
Biosynthetic process									
112	Unknown (100% Isomerase)	82	*Medicago truncatula*	gi|217072906 / B7FJ84	36.2/5.3 (31.5/5.5)	2	10	Biosynthetic process	(SCaRCaPCb)^{2} (RIaSIaPIb)^{1} (RIaSIaPIb)^{2}
1301	Unknown (99% Transaldolase)	138	*Medicago truncatula*	gi|217075715 / G7KXR2	44.4/5.3 (48.5/6.0)	8	23	Lignin biosynthetic process	(PCaSCabRCb)^{2} (PIaRIbSIc)^{2} 0P^{1}, 0S^{1}, 0.3R^{2}, 1.6S^{2}
1603	Unknown (99% Leukotriene-A4 hydrolase-like protein (LTA4H))	71	*Medicago truncatula*	gi|217074536 /G7IAW9	73.0/5.5 (68.7/5.2)	9	19	Leukotriene biosynthetic process	(SIaPIbRIb)^{1} ∞S^{1}
1606	Unknown (99% Leukotriene-A4 hydrolase-like protein (LTA4H))	91	*Medicago truncatula*	gi|217074536 / G7IAW9	73.8/5.5 (68.7/5.2)	9	23	Leukotriene biosynthetic process	(SIaPIabRIb)^{1} 0.2S^{1}
2107	Caffeoyl-CoA O-methyltransferase (CCoAOMT)	286	*Medicago sativa*	gi|30580341	32.1/5.6 (28.0/5.5)	13	67	Lignin biosynthetic process	0P^{1}
2108	Caffeoyl-CoA O-methyltransferase (CCoAOMT)	109	*Medicago sativa*	gi|30580341	31.8/5.7 (28.0/5.5)	7	36	Lignin biosynthetic process	(RCaPCaSCb)^{1} ∞R^{2}, 0.3S^{2}
3416	GDP-mannose-3',5'-epimerase (GME)	148	*Caragana korshinskii (Glycine max)*	gi|222160334	49.7/6.2 (43.0/5.5)	7	24	Cellular metabolic process	(RCaPCabSCb)^{1} (RIaSIabPIb)^{1} 0S^{1}, 0P^{2}, 0.4S^{2}
3509	Glutamate decarboxylase (GAD)	213	*Arabidopsis thaliana*	gi|21536919	58.5/6.2 (56.0/5.5)	8	17	Glutamate metabolic process	(RCaPCabSCb)^{1} (RIaPIbSIb)^{1} (RIaPIbSIb)^{2}
2104	NADPH: isoflavone oxidoreductase (IFR)	918	*Pisum sativum*	gi|1708427	37.9/5.6 (35.4/5.4)	20	77	Phytoalexin biosynthesis	(SIaPIbRIb)^{2}
3102	Isoflavone reductase-like NAD(P)H-dependent oxidoreductase (IFR)	250	*Medicago sativa*	gi|6525021	36.9/6.0 (34.0/6.6)	5	14	Phytoalexin biosynthesis	(RIaPIabSIb)^{1} (PIaRIaSIb)^{2} 0.5R^{1}
4101	Isoflavone reductase-like NAD(P)H-dependent oxidoreductase (IFR)	445	*Medicago sativa*	gi|6525021	37.1/6.3 (34.0/6.5)	9	21	Phytoalexin biosynthesis	(SIaRIabPIb)^{1} (SIaRIabPIb)^{2} 0.6P^{1}, 0S^{2}
5105	Unknown (99% NAD(P)H-dependent 6'-deoxychalcone synthase)	119	*Medicago truncatula*	gi|217072106 / G7J9I2	36.0/6.7 (35.5/5.5)	7	24	Flavonoid biosynthesis	(PCaRCaSCb)^{1} (PCaRCaSCb)^{2} (PIaRIaSIb)^{1} (RIaPIaSIb)^{2} 1.7S^{1}
3410	Diphosphomevelonate decarboxylase	75	*Hevea brasiliensis (A. thaliana)*	gi|164604978 / F4JCU3	50.0/6.0 (46.5/7.0)	4	11	Isoprenoid biosynthetic process	(SCaPCaRCb)^{2} 0.6R^{2}, ∞S^{2}
Transcription/Translation									
4909	Maturase K	82	*Dionysia aretioides*	gi|49364267	124.4/6.4 (60.5/9.5)	15	19	RNA-binding	∞R^{1}, 0.5R^{2}
5408	DNA binding protein	163	*Trifolium pratense*	gi|84468322	55.0/6.8 (44.5/6.0)	10	20	Nucleic acid binding	(RCaPCabSCb)^{1} (RCaPCabSCb)^{2} (RIaSIabPIb)^{2} 0.6S^{2}
Unknown and others functions									
1111	Predicted protein	75	*Populus trichocarpa*	gi|224061791	32.2/5.4 (41.5/6.5)	11	32	Unknown	(RCaSCaPCb)^{1} (RCaSCaPCb)^{2} 0.1P^{1}, 0R^{1}, 0P^{2}
9108	Predicted protein	356	*Populus trichocarpa*	gi|222838537	34.2/9.2 (9.6/9.5)	3	48	Unknown	0.6P^{1}, 0P^{2}, 2.0R^{2}, 0
9802	Glyoxysomal fatty acid beta-oxidation multifunctional protein MFP-a	131	*Brassica napus*	gi|34922417	86.9/8.8 (79.5/9.0)	11	17	Lipid metabolism	1.5S^{1}, 2.5P^{2}, 0.6S^{2}

[a] Percentage of protein identity, species and UniProt accession number, where appropriate, from Blast comparison are displayed in brackets. [b] Experimental mass (Mr, kDa) and pI were calculated with PDQuest software (BioRad) and standard molecular mass markers. Theoretical values were retrieved from the protein database (NCBInr). The software assigns a standard spot number to each spot. protein (SSP). [c] PM: number of peptides matched (from Peptide Mass Fingerprinting) with the homologous protein from the database. Some of these peptides were automatically MSMS fragmented. [d] The significant ($P < 0.05$) changes (more/less abundant) are given as normalized volume (calculated with PDQuest software) ratios: SC Susceptible Control, non-inoculated), PC (Partially resistant Control, non-inoculated), RC (Resistant Control, non-inoculated), SI (Susceptible Inoculated), PI (Partially resistant Inoculated) and RI (Resistant Inoculated). Single letters mean infected/control ratios. Superscript numbers (1,2) represent hours after inoculation (24 and 72 hai, respectively). Genotypes comparison in shown in bracket.

For PC3 (positive direction), it was observed a decrease of proteins in response to inoculation in S genotype at 24 hai (specifically proteins belonging to carbohydrate metabolisms and stress), and the opposite for PC3 (negative direction) (protein folding). Similarly for 72 hai we could observe that PC4 (positive direction) explained a decrease of proteins in response to inoculation in P genotype (mainly belonged to stress), and the opposite occurred in PC4 (negative direction) (stress and carbohydrate metabolisms).

The different expression dynamics of each identified protein can be distinguished in the hierarchical clustering-heat map of the differentially regulated proteins identified in this study (Fig. 4). The heat map representation shows the protein value according to the level of normalized experiments that are indicated from 0 (no expression; in green) to 1,800 (maximum positive expression; in red). This yielded two heterogeneous main clusters, the first one (**I**) included constitutively less abundant proteins in R genotype. In turn, this cluster could be divided into two groups (**Ia** and **Ib**). Cluster **Ia** included proteins more abundant in P belonging to carbohydrate and aminoacid metabolisms, biosynthetic processes and protein folding. Cluster **Ib** included proteins more abundant in S genotype belonging to carbohydrate and aminoacid metabolisms, stress and defence, biosynthetic processes and transcription/translation. The second main cluster (**II**) was divided into two more groups (**IIa** and **IIb**). The first group **IIa** showed proteins constitutively more abundant in R genotype, comprising stress and defence, metabolic and biosynthetic processes and protein folding, while **IIb** was a small heterogeneous group showing proteins mostly increased in S genotype at 24 hai.

4. Discussion

To our knowledge this is the first comparative proteomic study performed in pea to characterize the resistance mechanisms in response to *F. oxysporum*. We selected three genotypes showing different levels of resistance based on a previous work where a *Pisum* spp. germplasm collection was screened against *Fop* race 2 (Bani *et al.*, 2012). Our results of the disease symptoms evaluation corroborate those obtained by Bani *et al.* (2012), supporting the significant differences in the resistance level observed between these three genotypes. Some molecular studies have been carried out to assess plant responses to *F. oxysporum*. Some transcriptomic and expression studies were performed in banana (Li *et al.*, 2012; Bai *et al.*, 2013), tomato (De Jonge *et al.*, 2012), melon (Sestili *et al.*, 2011) and chickpea (Ashraf *et al.*, 2009). Far less studies have been found using proteomic approach to study such interaction, as in

xylem-sap of tomato (Rep *et al*., 2002), in exudates of germinating lupin seeds (Scarafoni *et al*., 2013) and in chickpea (Palomares-Rius *et al*., 2011).

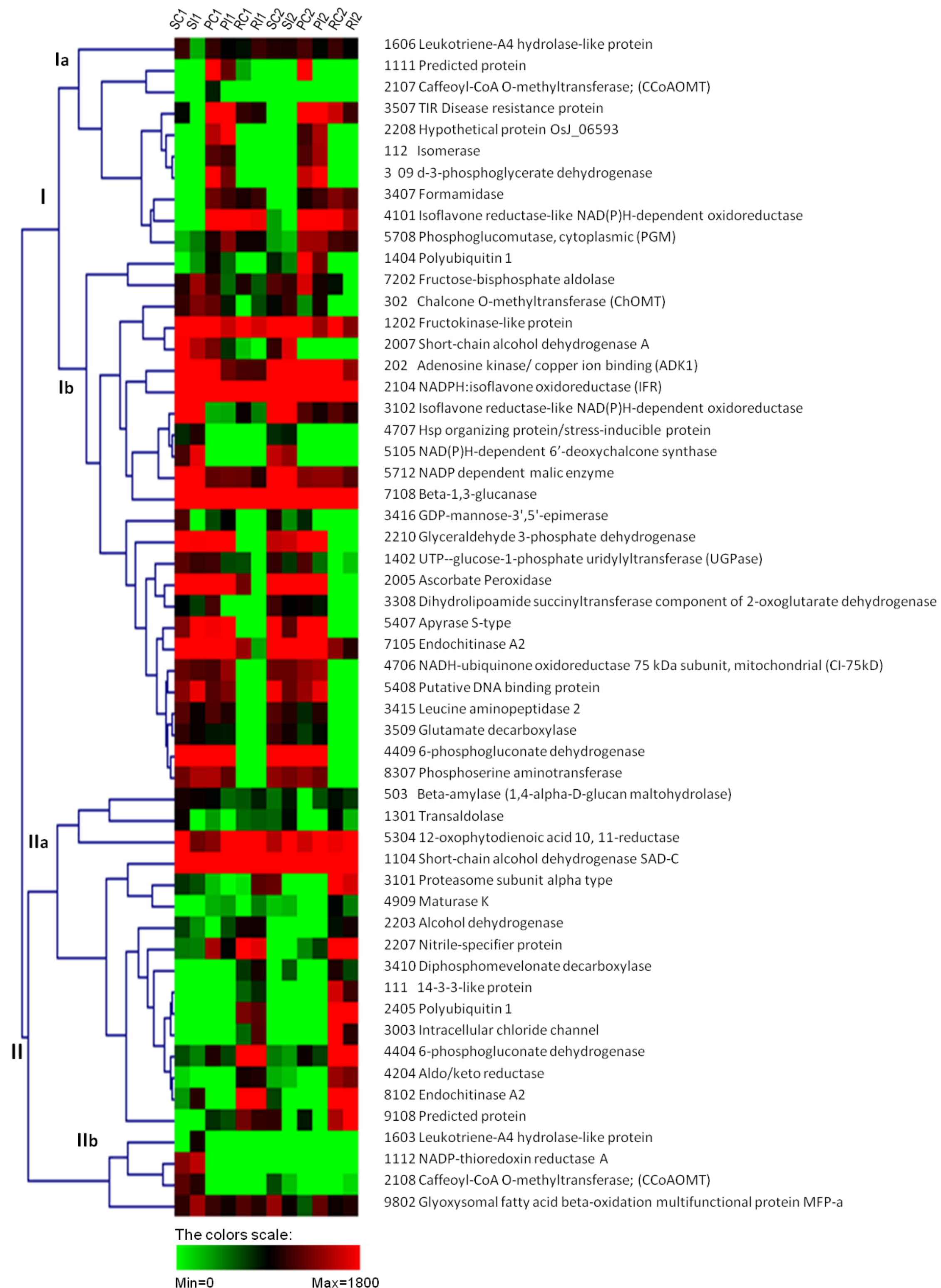

Figure 4. Hierarchical clustering of the differentially regulated proteins identified in this study. Clustering was based on protein abundance levels, where S, P, R represent susceptible, partially resistant and resistant genotypes, C and I represent non-inoculated (control) and infected plants, respectively, and numbers 1 and 2 represent the sampling time, 24 and 72 hai, respectively. Bar color is a scale spanning from 0 (decrease) to 1800 (increase) values.

Our proteomic study was performed on pea roots with the aim of identifying proteins involved in different pea resistance mechanisms operating during *F. oxysporum* infection. Two sampling times were selected based on previous microscopical observations (results not shown), 24 hai as an early time of pea response when the fungus adhere to the root surface and penetrate the root epidermis, and 72 hai as a late time of pea response, when reaches the xylem vessels using them to rapidly colonize the host. Similar observations also were obtained from others pathosystems as *F. oxysporum*-cotton and *F. oxysporum*-tomato, where vessel responses were evident 24 hai and reached a maximum at 72 hai (Shi *et al.*, 1992; Ollivain and Alavouvette, 1999).

PCA of data explained 86% of the main variation of the experiment. We found a similar protein abundance profile in the three replicates from each experimental condition, pointing out the reliability of our data. Fifty-three proteins could be matched against the NCBInr database, so that in most of them theoretical and experimental p*I* and *Mr* were in good agreement, encouraging confidence in protein identifications. Moreover, a Blast comparison analysis for those proteins with unknown function improved our identifications in 21%.

4.1. Functional categorization of identified proteins and their relationship to the study

Proteins were assigned to functional categories based on sequence homology or annotated function (Table 2) and then divided into seven groups. The patterns observed for the identified proteins during the conditions studied (genotype and response to *Fop*) are discussed. Also a schematic diagram hypothesizing the metabolic pathway network in relation to some of the identified proteins has been generated (Fig. 5).

4.1.1. Primary metabolism

Carbohydrate and energy metabolism accounting 21% of the total was one of the main protein groups identified in this work. They belonged to glycolysis, gluconeogenesis, TCA cycle, calvin cycle, pentose shunt, as well as enzymes of the metabolism of sucrose, fructose and maltose. Generally, a decrease of proteins of the carbohydrate metabolism was observed. By contrast, we observed an increase of beta amylase, 6-phosphogluconate dehydrogenase (6PGD) and phosphoglucomutase (PGM) in S and P genotypes in response to infection, enzymes involved in polysaccharides catabolism and pentose phosphate pathway. 6PGD catalyses the decarboxylating reduction of 6-

phosphogluconate into ribulose 5-phosphate in the presence of NADP with the consequent generation of NADPH. PGM catalyzes the conversion of glucose 1-phosphate to glucose 6-phosphate. Sugars are known to efficiently regulate both biotic and abiotic stresses. Sucrose production is regulated by sugar cleaving enzymes like sucrose synthase, beta amylase, invertase, glycoside hydrolases, etc. (Roitsch *et al.*, 2003; Koch, 2004). The sugars possibly helped in generating alarm signals and efficiently transmitted them throughout with the aid of sugar transporters. In addition, they were assumed to activate the wound repair machinery by promoting the synthesis of cell wall polysaccharides (Gupta *et al.*, 2010).

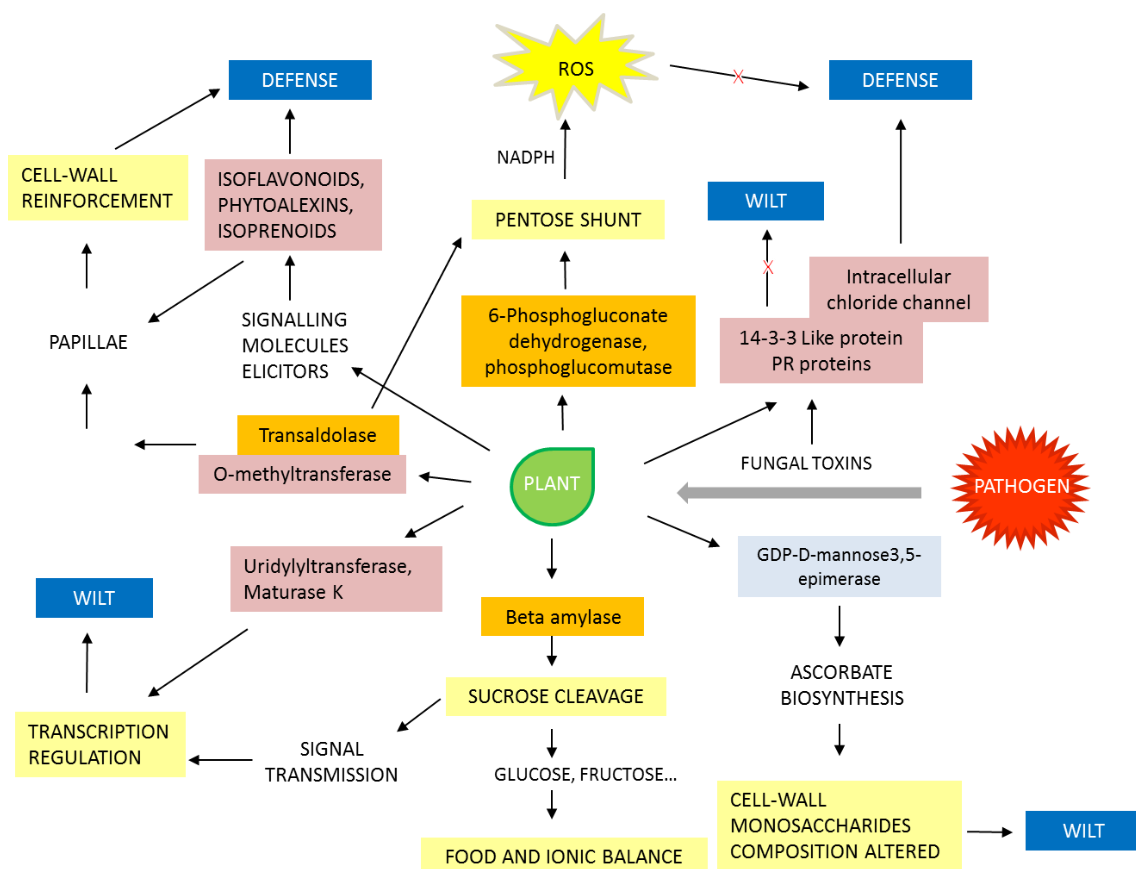

Figure 5: Metabolic pathway network generated by analyzing differential proteins from pea upon *F. oxysporum* attack. Pink box: increased proteins in R genotype in response to *Fop*; Orange box: increased proteins in S genotype in response to *Fop*; Blue box: decreased proteins in S and P genotypes in response to *Fop*; Yellow box: cell process; Deep blue box: the effect; Arrow: expression and regulation.

Glucose 6-phosphate's metabolic fate depends on the needs of the cell at the time it is generated. If the cell is low on energy, glucose 6-phosphate will enter in the glycolytic pathway, eventually yielding two molecules of adenosine triphosphate. If the cell is in need of biosynthetic intermediates, glucose 6-phosphate will enter the pentose

phosphate pathway, where it will undergo a series of reactions to yield ribose and/or NADPH. In response to *Fop* could happen that the machinery of carbohydrate metabolism in S and P genotypes goes toward the production of reducing power in form of NADPH, which will then be used as cofactor for enzymes of the redox reactions, such as been described on previous works (Noel *et al.*, 1978; Tsunezuka *et al.*, 2005).

Nucleotides and amino acid metabolism accounted 7% of the total identified proteins. Four proteins were identified in this functional category: adenosine kinase (ADK1), apyrase S-type, D-3-phosphoglycerate dehydrogenase (PHGDH) and phosphoserine aminotransferase (PSAT). All of them decreased in response to *Fop* infection, being more significant in P. Only apyrase S-type increased significantly in S at 24 hai. Apyrase is a calcium-activated plasma membrane-bound enzyme that catalyses the hydrolysis of ATP to yield AMP and inorganic phosphate, and ADK is a nucleoside kinase that catalyzes the salvage synthesis of 5′-AMP from adenosine and ATP. PHGDH and PSAT are involved in serine biosynthesis via phosphorylated pathway, and seem to be strongly inhibited in the resistant genotypes on the onset of infection.

4.1.2. ROS burst and oxidative stress caused in compatible interaction

This group of proteins accounted 13% of the total. The production and accumulation of reactive oxygen species (ROS) in plants as a defence response to pathogen attack are well documented (Jones *et al.*, 2006; Torres, 2010). The oxidative burst is the earliest typical event in a plant-pathogen interaction (Averyanov, 2009). In our study, seven proteins identified were found to be associated with redox homeostasis (Table 2). Interestingly, four of them showed similar expression pattern being mostly accumulated in S and P genotypes (NADP-thioredoxin reductase A, ascorbate peroxidase, short-chain alcohol dehydrogenase A and NADH-ubiquinone oxidoreductase 75 kDa). After inoculation three proteins showed an increase, especially in S genotype (NADP-thioredoxin reductase A, short-chain alcohol dehydrogenase A and 12-oxophytodienoic acid 10, 11-reductase). It is not strange to find these proteins more represented in S infected plants, with more significance at 72 hai as signal of the fast colonization of the root by *Fop*. Bani *et al.* (2012) described that only seven days after inoculation *Fop* had already colonized the whole plant in the susceptible genotypes. However, we also found two redox homeostasis proteins increased in both partially and resistant genotypes in response to inoculation (aldo/keto reductase and 12-oxophytodienoic acid 10, 11-reductase). This could be explained because *Fop* also colonizes the basal

region of the plant of both genotypes, such as it has been detected in the screening made by Bani *et al.* (2012) for detection of *Fop* within plant tissue.

Another defence related proteins were identified to be more represented in S and P genotypes. This group represented the 9% of the total identified proteins. In response to inoculation again we could observe a tendency to increase in S genotype of the follow proteins, especially at 24 hai: Hsp organizing protein/stress-inducible, beta-1,3-glucanase and endochitinase A2. All of these proteins were directly related previously with resistance in other plant-fungus interactions, thus ascorbate peroxidase, chitinase and beta-1,3-glucanase were identified in chickpea-*F. oxysporum* f.sp. *ciceris* interaction (Palomares-Rius *et al.*, 2011) as well as in *M. truncatula* and pea in response to rust (Castillejo *et al.*, 2010b; Barilli *et al.*, 2012), Hsp was identified in pea in response to *Mycosphaerella pinodes* (Castillejo *et al.*, 2010a), and an expressed gen with high homology to 12-oxophytodienoic acid reductase was also induced by *M. pinodes* in pea (Ishiga *et al.*, 2014).

The fact that we found the typical response to defence and to oxidative stress, mainly in the susceptible genotype (S), can be explained because in this genotype *Fop* extent quickly for fast colonization within the plant. This is in agreement with the observed symptoms for S genotype 21 days after inoculation which represented 100% of disease severity, while P and R genotypes were placed under 50% (Fig. 2), coinciding with the results previously described by Bani *et al.* (2012) for the same genotypes.

In addition of ROS detoxification, some of the identified proteins in our work were related to drought stress tolerance in plant, such as NADP-thioredoxin reductase (NTRs) (Cha *et al.*, 2014), NADH-ubiquinone oxidoreductase (Castillejo *et al.*, 2008) and alcohol dehydrogenase (Ranjan *et al.*, 2012). NTRs are key-regulatory enzymes determining the redox state of the thioredoxin (Trx) system that provides reducing power to peroxidases or oxidoreductases. We identified the cytoplasmic NTRA which was previously shown to be involved in the plant stress response counteracting oxidative and drought stresses (Cha *et al.*, 2014). An explanation of the increase of these proteins may be due to the plant response to a perturbation of the water flux due to the vessel plugging induced by the intensive fungal growth within xylem cells, which will be later the cause of characteristic wilt symptoms.

4.1.3. Activation of signal transduction pathway in response to *Fop*

Signal transduction pathways are activated following the recognition of biotic and abiotic stresses at the cellular level, leading to changes in many metabolic pathways and cellular processes, such as redox homeostasis, cell rescue/defense pathways, and photosynthesis (Cheng *et al.*, 2010). Within this functional category (represents 8% of the total) we identified three proteins increased in R genotype at 24 hai: 14-3-3-like protein, chalcone O-methyltransferase and intracellular chloride channel. In plants, the 14-3-3 proteins bind many effectors that are secreted by pathogens. In a plant-fungus encounter, they have a potential role in acting as receptors of fungal toxins (Ferl, 1996). It has been reported that this protein may play a role in key physiological processes, in particular, abiotic and biotic stress responses, metabolism (especially primary carbon and nitrogen metabolism), as well as various aspects of plant growth and development. 14-3-3s have long been thought to play a role in defence against pathogens (reviewed in Robert *et al.*, 2002). Arabidopsis plants with reduced 14-3-3 expression show an impaired resistance to powdery mildew fungus infection, whereas over-expression of 14-3-3 increases resistance and leads to the plant hypersensitive response (cell death to decrease pathogen spread) (Yang *et al.*, 2009).

In addition, for this protein it has been speculated that its interaction with H^+ATPase provide a satisfactory explanation to the irreversible opening of the stomatal pore resulting in constant nutrient leaching resulting in wilt (Wüertele *et al.*, 2003). However, controversial results were obtained in a more recent work reviewed in Denison *et al.* (2011). The most direct evidence that 14-3-3 protein plays an important role in environmental stress responses comes from the overexpression of Arabidopsis 14-3-3 in cotton. These transgenic plants were more tolerant to drought, as determined by less wilting and visible damage to the leaves. Transpiration and photosynthesis rates are higher than wild-type due to increased opening of the stomata (Yang *et al.*, 2004). One mechanism by which 14-3-3 could act in the regulation of such environmental stress responses is though the regulation of ion channels. In our work we found a 14-3-3 like protein increased in R genotype together with an increase of chloride channel protein in response to infection, fact that may be implicated in wilt suppression. Chloride channel proteins are important for maintaining safe ion concentrations within plant cells (De Angeli *et al.*, 2007). Activation of anion efflux has been mostly described as a component of the early responses induced by plant/pathogen interaction in the context of innate immunity (Garcia-Brugger *et al.*, 2006). In our work this protein together 14-3-3 like protein may be mediating an early response to *Fop*.

O-methyltransferase (OMT) methylates the 2'-hydroxyl of isoliquiritigenin (2',4,4'-trihydroxychalcone) to form 4,4'-dihydroxy-2'-methoxychalcone. Methylation catalyzed by OMT, acylation and glycosylation of secondary metabolites, including phenylpropanoids and various derived phenolic compounds, are fundamental chemical modifications. The control of the production of plant phenolics involves a matrix of potentially overlapping regulatory signals. These include developmental signals, such as during lignification of new growth or the production of anthocyanins during fruit and flower development, and environmental signals for protection against abiotic and biotic stresses (Cheynier *et al.*, 2013). We identified significantly increased levels of ChOMT in both resistant P and R genotypes after *Fop* infection.

Finally in this category we identified a SNARE protein constitutively more abundant in P genotype, with a light tendency to increase in response to infection at 72 hai. The importance of SNARE proteins in plant immunity has been demonstrated. Recent results have highlighted the importance of intracellular vesicle trafficking in resistance and the significance of endocytic and exocytic processes (Robatzek, 2007).

4.1.4. Folding and protein degradation

Four proteins were identified in this category representing 8% of the total: polyubiquitin 1 and 2, proteasome subunit alpha type, leukotriene-A4 hydrolase and leucine aminopeptidase 2. Proteasome activity can control the plant response to stressful conditions including oxidative or biotic stresses and may also control the functioning of key signal transduction cascades (Kurepa and Smalle, 2008). Those proteins involved in recruitment of proteins for ubiquitination and proteasome degradation as polyubiquitin 1 and proteasome alpha type were significantly increased at 24 hai in S and R genotypes, respectively, but decreased at 72 hai in the three genotypes. Leucine aminopeptidase (LAP) are enzymes that preferentially catalyze the hydrolysis of leucine residues at the N-terminus of peptides and proteins. LAP is induced by wounding and bacterial pathogen infection in tomato (Gu *et al.*, 1996). This protein was identified constitutively more represented in S and P genotypes and increased in P in response to infection.

4.1.5. Biosynthetic process

This was the main group of identified proteins with 24% of the total. Three proteins were related to lignin biosynthesis: two CCoAOMT and a transaldolase. CCoAOMT proteins methylate caffeoyl-CoA to feruloyl-CoA and 5-hydroxyferuloyl-CoA to sinapoyl-CoA and play a role in the synthesis of feruloylated polysaccharides. They are involved

in the reinforcement of the plant cell wall and also in the response to wounding or pathogen challenge by the increased formation of cell wall-bound ferulic acid polymers (component of lignin). Transaldolases are enzymes of the non-oxidative phase of the pentose phosphate pathway, although they have also been involved in lignification (Vanholme *et al.*, 2012).

In tomato plants infected with *F. oxysporum* it has been described that continued deposition of material occurred around penetration hyphae with consequent formation of elongated penetration papillae. These were lignified and apparently effective in preventing further hyphal growth, with the same frequency of formation in resistant and susceptible cultivars (Bishop and Cooper, 1983a). In our system we identified a significant increase of proteins involved in lignin biosynthesis in response to the pathogen attack, especially in the resistant genotype. We could associate this increase with the apposition of lignin for cell wall reinforcement and papillae formation within epidermal and cortical cells, such as has been described in other systems (Bishop and Cooper, 1983a; 1983b; Olivain and Alabouvette, 1999; Ouellette *et al.*, 2002).

In addition we also identified six proteins involved in secondary metabolites biosynthesis. A common role of secondary metabolites in plants is defence mechanisms. They are used to fight off herbivores, pests, and pathogens. These were NADPH: isoflavone oxidoreductases (IFR), NAD(P) H-dependent 6'-deoxychalcone synthase (CHS), isomerase and diphosphomevelonate decarboxylase (DPM-DC). Three of them were constitutively more represented in the susceptible genotype (two IFRs and a chalcone synthase) and other three proteins were more represented in the resistant ones (IFR, isomerase and DPM-DC). Chalcone synthase (CHS) is the first committed enzyme in flavonoid biosynthesis. Flavonoids are important plant secondary metabolites that serve various functions in higher plants including antifungal defence. Phytoalexins are anti-microbial compounds that are both synthesized and accumulated in plants after exposure to microorganisms or abiotic agents (Paxton, 1980; VanEtten *et al.*, 1994). Synthesis of phytoalexins during pathogenesis is regulated by the activity of phenylalanine ammonia-lyase (PAL), an enzyme initiating phenylpropanoid metabolism, chalcone synthase (CHS) and chalcone isomerase (CHI) (Morkunas *et al.*, 2013). Pisatin is an isoflavonoid phytoalexin synthesized by pea. Isoflavone reductases (IFR) are involved in the synthesis of this phytoalexin, which catalyzes an intermediate step in pisatin biosynthesis. The ability of some plants to resist colonization depends on their ability to compartmentalize the infection in a restricted area. Such compartmentalization processes comprise vessel occlusion with material usually denoted as gums (gels), rich in phytoalexins, phenolics and eventually lignin (Niemann

et al., 1990). In response to infection we observed an increase of proteins belonged to flavonoids biosynthesis, especially in the susceptible genotype. Perhaps an attempt of the pathogen restriction is given by these plants mediated by phenolic compounds.

Diphosphomevelonate decarboxylase (DPM-DC) enzyme is related to isoprenoid biosynthesis, which serve numerous biochemical functions: as quinones in electron transport chains, as components of membranes, in subcellular targeting and regulation, as photosynthetic pigments, as hormones (gibberellins, brassinosteroids, abscisic acid), and as plant defense compounds (monoterpenes, sesquiterpenes, diterpenes) (Lange *et al.*, 2000). We can think that this protein could be taking part in plant defence compounds biosynthesis or as hormones biosynthesis. ABA has been described as an effective stomatal closing agent, and acts to reduce transpiration and canopy water loss. It plays an important role under soil drought condition acting in the measurement of soil water availability (Davis *et al.*, 2002). We found this protein constitutively more represented in R genotype, so we could relate this protein with the resistance to *Fop*.

We also identified a GDP-D-mannose 3,5-epimerase (GME) significantly decreased in S and P genotypes in response to *Fop* infection. This protein converts GDP-D-mannose to GDP-L-galactose, and is generally considered to be a central enzyme of the major ascorbate biosynthesis pathway in higher plants. The analysis of the cell-wall composition of leaves and developing fruit performed in transgenic tomato lines defective in GME revealed that the cell-wall monosaccharide content was altered in the transgenic lines, especially those directly linked to GME activity, such as mannose and galactose (Gilbert *et al.*, 2009). We could interpret this decrease as part of the pathogenesis mechanism produced by the fungus, resulting in weakening of cell-wall. A glutamate decarboxylase (GAD) was constitutively more represented in S genotype. This enzyme catalyzes the decarboxylation of glutamate to GABA and CO_2. It has been described the possible role of GABA as a signal molecule, as well as in response to stress and carbon:nitrogen metabolism (Bouché and Fromm, 2004). The involvement of this protein in our work is unclear and may be related with the metabolic imbalance produced by the fungus in this genotype.

Finally in this group we identified two leukotriene-A4 hydrolases (LTA4H), enzyme that participates in arachidonic acid metabolism. Arachidonic acid has only rarely been encountered in higher plants. In fungal infections of plants, it is known to elicit the production of plant defense compounds (phytoalexins), probably after conversion to oxygenated metabolites (Savchenko *et al.*, 2010). LTA4H proteins identified in our work showed very close *Mw* and p*I*, so that we can suppose both are

isoforms responding differentially to the infection. While one of them increased significantly in S genotype the other one decreased in response to *Fop* infection.

4.1.6. Transcription/translation

Two proteins were identified in this category: maturase K and DNA binding protein. Maturase K (matK) is a plant plastidial gene. The protein it encodes is an intron maturase, a protein that splices introns. We found this protein significantly increased in R genotype at 24 hai, but decreased at 72 hai. DNA binding was found increased in P at 72 hai. Since these proteins increased in the resistant genotypes in response to infection we could speculate that may play a role in transcriptional activation of genes involved in defense against *Fop*.

5. Conclusions

To the best of our knowledge this is the first comparative proteomic study performed in pea root to characterize the resistance mechanisms in response to *F. oxysporum*. A general decrease of proteins from the carbohydrate metabolism was observed, however an increase of enzymes belongeing to the pentose shunt occurred in the most susceptible genotypes. We hypothesize that these genotypes goes toward the production of reducing power in form of NADPH, which will then be used as cofactor for enzymes of the redox reactions. To support this theory we rely on the fact that a oxidative burst occurred in the same genotypes, as well as an increase of PR proteins may be due to the fast colonization of *Fop* within the plant. However, in this compatible interaction between susceptible plants and *Fop*, the ROS generation failed to prevent fungal colonization or reproduction within the xylem tissues. On the contrary, in the resistant genotype such reaction was not observed. Some proteins responsible to induce changes in the membrane and cell wall composition related to reinforcement were identified. This involves the localization of the fungus in the infected cells by cell wall reinforcement and restriction of fungal growth in roots. Aiming to solve the queries of defence responsive metabolic network we have tried to hypothesize the probable roles of most of identified proteins and its mediation in defence. We found significant coincidences with others very close systems, which demonstrate the utility of our proteomic approach to unravel interesting aspects of this plant-pathogen interaction. Nevertheless, a deeper study of the mechanisms of signal recognition, translation into the cell and a more extensive molecular characterization of genes/proteins related to *Fop* defence should be addressed.

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Supplementary Data

Supplementary Table S1. Dataset containing protein expression intensity values for 132 protein spots selected due to prospective differential behavior. The abundance values were obtained after data normalization using the PDQuest 2D analysis software.

SSP	P615 C-T1	P615 C-T1	P615 C-T1	P615 I-T1	P615 I-T1	P615 I-T1	P42 C-T1	P42 C-T1	P42 C-T1	P42 I-T1	P42 I-T1	P42 I-T1	P629 C-T1	P629 C-T1	P629 C-T1	P629 I-T1	P629 I-T1	P629 I-T1	P615 C-T1	P615 C-T1	P615 C-T1	P615 I-T1	P615 I-T1	P615 I-T1	P42 C-T1	P42 C-T1	P42 C-T1	P42 I-T1	P42 I-T1	P42 I-T1	P629 C-T1	P629 C-T1	P629 C-T1	P629 I-T1	P629 I-T1	P629 I-T1
106	3475,7	2806,1	15,6	1031,2	279,8	694,3	595,5	2067,6	899,6	2043,7	985,4	1285,2	1208,5	881,6	599,3	1443	104,1	2914,3	2984,4	8202,3	1041,7	2669,5	7728,8	6227,3	6217,8	1899,2	22,6	25,6	1640,7	5209,8	538	3786,2	1097	4993,8	783,7	2060
107	143,8	917,1	340	1813,8	944	74,9	706,4	863,9	1164,6	1357,7	1349,3	871,2	0	0	0	0	0	0	726,4	311,2	518,8	127,5	444,2	407,3	1572,3	2683,8	884	1295,5	1336	800,4	0	0	0	0	0	0
110	0	0	0	0	0	0	833,1	49,8	289,9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1116,3	1558,8	730,7	993,2	338,1	531,1	0	0	0	0	0	0
111	0	0	0	0	0	0	199,8	288,1	824,3	424,2	888,7	496	0	0	0	0	0	0	0	0	0	0	0	0	1331,2	2396,5	1102,2	2246,3	451,8	202	0	0	0	0	0	0
112	632,2	2186,8	430,1	988,7	1054,4	840,7	0	0	0	0	0	0	0	0	0	0	0	0	1416,9	1204,3	559,6	1054,2	1653	1556,2	0	0	0	0	0	0	0	0	0	0	0	0
202	3040,7	2917,8	2058,3	1927,9	915,1	670,9	808,6	1335	975,5	672,3	1665	708,9	2450,6	1628,2	2195,9	3291,7	2255,7	2121,4	2812,3	2153,6	3544,4	2015,7	1875	2251,4	1989,5	1890,8	1833,9	1431,1	1607,7	985,3	3266,3	1758,8	3293,4	4617,3	2386,8	1813,8
301	866,7	1333,9	957,1	1435,6	772,5	956,4	836	1303,2	370,6	902,7	1010,6	525,1	622,7	702,8	896,1	1358,4	632	608,9	201,4	159,2	117	689,9	872,3	1110,9	744,7	582,9	421,1	917	1244,7	419,6	617,1	87,7	935,7	321	23,5	549,2
302	1119,4	1555,2	743,2	1107,4	691	115,5	0	0	0	797,1	738	0	1125,7	944,6	772,2	1545,1	1546,4	681,2	427,4	319,1	210,8	464,1	1156,2	818,5	0	0	0	0	0	0	468,3	980,4	642,2	1145,7	1032,5	684,9
503	534,6	1005,2	571,1	380	529,4	379,2	222,7	887,2	305,2	751,3	856,8	277,7	808	907,7	755,3	780,7	948,7	569,8	0	0	0	235,4	741,3	488,35	216,3	1182,8	621,8	229,2	885,3	557,25	202,7	621,3	369,7	594,8	803,8	559,3
602	123,8	4586,6	928,6	6042	5385,7	3874,3	6069,5	6983,1	794,9	7496,3	7191,5	4881,3	1261,4	3516,8	1832,3	1196,6	4364,1	2208,6	3442,9	1373,1	3438	2156,4	4114,5	11328,9	5425,4	2311,7	3398,4	7688,2	5146,4	7990,9	618,8	4731,5	708,4	688,8	1144,1	1900
705	113,3	1869,5	1631,4	2187,1	4015,4	79,9	2781,7	3229,7	912,7	777,3	2292,8	546,6	573,7	470,1	900,7	1129,8	3471,8	611,7	1346	7080,4	1892	945,9	2216,3	2967,8	1814	3416,4	1658,5	705	4778,5	3316,7	621,3	3062,3	369,1	1023	2930,2	1068,6
708	912,6	2438,1	1115,4	983,9	2047,2	1015,9	1040,1	2254,1	693,3	1054	1842,9	699,5	729,4	1535,3	835,8	768,8	1713	684,4	1214,4	2644,7	1823,9	537,6	2355,9	2590,7	905,4	2490,4	1160,9	567,1	3523	2026,1	594,6	2303,6	956,7	1591,2	2107	1075,4
709	962,4	11966,4	3586	11401,7	7286,6	8127,2	11447,2	13819,4	6255,3	12908,2	3207,8	7640,2	3651,8	7428	1562,9	1530,4	5617	3103,7	10839,8	12707,1	10614,9	5859,1	11656,9	9250	16265,1	7760,6	11416	21421,5	7943,1	4873,3	344,8	5164,5	2754,7	1345,4	4584,1	3140,7
910	348,2	957,1	354,1	0	0	0	379,9	522,1	451	402,5	521,3	238,1	314,6	296,4	172,3	397,9	369,5	91,7	375,2	708,5	541,85	0	0	0	286,1	1121,5	703,8	618,6	698,6	778,6	1250,8	768	1733,6	547,35	640,2	454,5
915	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	887,75	1240,3	535,2	403,3	623	226,4
1003	1029,5	121,8	466,6	73,2	750,4	1143	725,2	1001	920	837,7	1382,3	1273	279,8	252,7	1106,6	2205,7	196,3	777,4	0	0	0	1606,3	1026,4	1173,7	1396,9	1316,9	401,4	89,7	2415,6	2449,4	1246,5	1441,4	964,8	4414,8	778,3	32,5
1004	0	0	0	0	0	0	0	0	0	0	0	0	0	835,5	788,6	127,4	380,8	762,4	4402,5	1025,4	1067,2	1288	2325,8	4319,2	0	0	0	0	0	0	0	0	0	0	0	0
1102	1439,7	1448	764,2	0	0	0	0	0	0	0	0	0	321,1	396	471,4	18,2	470	923,7	0	0	0	0	0	0	2287,9	288,9	379,1	1827,1	2695,3	958,9	1155,7	1216,4	262,4	0	0	0
1103	866,3	1839	1033,5	782,2	55,4	691,2	802,6	1053,7	307,9	698,8	904,4	598,3	0	0	0	0	0	0	5354,6	2021,2	3768,5	374	1432,5	590,4	564,9	1206,5	766,7	616,9	904,95	1193	0	0	0	0	0	0
1104	1376,4	3140,2	3665,3	3414,1	2515,9	38	5209,7	6046,8	6623,2	4889	8453,7	3779	3801,2	3206,7	4282,4	4709,4	1740,6	9603,4	17331,3	7866,6	12336,3	3295,8	6860,2	4640,9	21085,3	11005,2	24976,8	6965,1	7729,8	8411,7	6186,8	3172,2	5906,9	1975,7	5722,2	1413,2
1110	3839,2	5258,5	3592,7	3128,4	3268,6	4011,2	394,5	364	334,8	0	0	0	3069,3	3821,3	3677,9	3985,4	2931,2	2230,8	2321,9	3208,9	3874,4	2731,5	4825,8	3814,6	180,6	790,2	1215,8	460,1	739,55	1019	3582,1	1587,8	2181,5	7112,9	2617,8	1370,7
1111	5362	11076,6	9595,3	2261,3	1138	14,7	119,1	241	363,3	0	0	0	0	0	0	0	0	0	8949,6	6205,5	4796,7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1112	0	0	0	0	0	0	0	0	0	0	0	0	1104,3	871,3	1880,8	1280,3	1622,1	1663,9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1113	1464,6	3177	1855,3	189,7	1581,9	1904,6	0	0	0	0	0	0	94,3	569,9	701,4	694,4	1421	2148,9	11068,8	1755,5	3938,7	1026	1925,5	1519,1	0	0	0	0	0	0	558,5	709,2	859,8	1272	1395,5	1683,4
1202	3396,3	3706,8	2118	1485,9	1484	1741,6	582,4	2353,3	2614,8	608,5	2674,8	1609,7	1985,7	3265	5866,1	4987,4	2388,2	3756,6	2129,7	1394,5	2216,2	816,3	1569,2	1715,6	1252,3	2105,3	2327,4	703,1	1625,5	1511,7	3378,7	4043,5	3309,9	3057,4	3768,9	3528,7
1205	1303,7	1912	1241,8	980	1219,2	846,5	760,9	1302,9	757,4	575,8	905	338,8	889,8	761,8	1063,1	1386,3	61,4	1173,6	1007,3	994,5	307,9	755,6	1273,5	1044,9	996,3	1545,2	1200,6	633	1024,3	1842,6	320,2	264,7	211,8	1682,2	1436,4	923,9
1206	7697,6	2618,5	2883,1	7694,7	6938	6183,5	0	0	0	0	0	0	6527,8	638,8	2617,7	5899,9	420,2	1658	685,4	1335,3	6,9	3258,5	821,2	4029,4	0	0	0	0	0	0	3600,8	3755,7	761	4704,8	652,5	2048,9
1301	246,9	273,5	301,7	0	0	0	301,6	495,5	456,1	303,3	722,4	354,7	735,9	512	1057,8	0	0	0	0	0	0	0	0	0	753	691,8	760,2	212,3	243,8	166,1	325,5	191	801,6	600,4	663,3	797,6
1306	0	0	0	0	0	0	944,5	745	330,3	1105,8	743	866,9	0	0	0	0	0	0	0	0	0	0	0	0	985	1381,9	1752,9	145,9	929,9	1399,8	0	0	0	0	0	0
1402	819	899,8	1236,4	156,5	823,6	567	498	379,7	617,3	0	0	0	1139,7	804,4	1380,9	957,6	672,8	1242,4	2007,5	425	740	411,4	481,6	341,1	0	0	0	107,3	151,7	196,1	869,5	810,2	1120,5	642,2	492,4	567,3
1404	630,3	929,5	760,2	583	442	301,5	0	0	0	0	0	0	0	0	0	320,1	289	257,9	3976,2	1996,4	2435,6	1080,7	1091,8	1314	0	0	0	0	0	0	817,3	563,8	487,6	187,9	595,5	241,7
1603	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	730,8	1475,2	182,3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1606	624,1	1400,9	818,8	727,9	719,6	743,6	1075,6	674	273,5	952,9	1337,3	617,6	961	872,4	1050,7	148,2	31,7	488	1871,1	708,8	486,9	357,8	963,4	770,4	995,2	1302,4	576,1	391,8	976,1	765,5	593,3	1093,5	911,3	839,5	901,8	777,2
1909	252,3	2478,7	522,4	1326	2087,5	565,2	823,4	1787	463,8	624,5	2524,6	1278,2	132,9	432,4	149,8	0	0	0	1328,8	2340,3	227,3	274,3	1676,5	581,7	1659,8	1692,4	686,7	1706,8	185,5	949,7	311,6	361,3	261,9	90,8	598,3	204,6
2001	876,8	1058,4	1289,6	3284,1	3274,7	274,9	15414	6676,9	8150	12232,6	7756,9	6344,6	11286	11852,1	9982,9	15360,8	7798,8	7713,7	4834,7	147,7	3365,7	3108,7	3569,9	4634,8	17493,9	9377,3	10744,1	172	10991,7	15942,2	13296,9	4661,1	5990,5	21803,6	7133,2	6360,1
2002	0	0	0	0	0	0	4203,4	3608,3	8158,4	3516,3	7145,2	3806,7	825,7	240,1	1004,3	1220,7	104,1	825,7	1666,4	3424,2	2545,3	469,5	529,2	588,9	8148	6775,6	9283,6	4193,6	6511,5	3887,6	1639,7	2559,6	1270,2	1854,2	1390,9	927,6
2005	12550,6	5161,3	6797,5	4557,1	2113,2	21,7	74,6	1194	2312,5	0	0	0	6745,8	6591,5	2946	7261,3	747	8053,6	8192,3	4297,3	5870,5	6005,8	5387,8	2686,8	0	0	0	0	0	0	8832,9	6546,2	713,3	487,7	1302,7	7718,3
2006	11678,2	9995,8	12280,6	15968,7	4536,7	12056,9	0	0	0	0	0	0	0	0	0	0	0	0	15758,1	9122,1	5337,2	8560,3	8380,8	5356,7	0	0	0	0	0	0	0	0	0	0	0	0
2007	2300,9	998,6	385,6	23,9	867,1	835,6	89,4	166	244,5	0	0	0	1514,6	2789,7	1052,3	1877,2	1185,2	1411	0	0	0	0	0	0	0	0	0	0	0	0	1786,6	1019,4	28,1	2941,2	742,4	1375,1
2101	0	0	0	0	0	0	1450,5	1778,7	3514,9	1371,2	2126,7	910,4	0	0	0	0	0	0	88,3	3345,8	753,8	0	0	0	7903,9	3009,3	12235,1	1816,2	2501,8	3359,5	0	0	0	784,2	606,5	428,8
2102	1267,1	153,7	3310,9	575,3	561,2	0	1646,7	2856,9	1031,7	1558,7	3581,6	1528,6	921,6	1077	1394,7	2289,7	1356	939,3	915,2	341,6	425,5	530,6	561,8	1178,8	1879,5	3582,8	2810,7	1081,7	2735,4	4882,1	1014,4	1573,5	1082,7	1720,6	746,8	360,5
2104	5465,3	4298,7	6088,6	4160	4433,2	3392,4	3175	3276,8	3075,7	1369,8	5406,9	2074,4	4286	4678	4898	7617,1	5227,8	3661,3	6203,1	4020,4	5152,3	3685,9	4054,2	4004,5	5128,3	4750,1	6159	5407,5	5144,4	4622,1	7472,4	7356,5	6029,5	11363,3	7973,7	7047,2
2106	3197,5	2350,7	41,1	2377,4	1802,3	2010,2	1685,1	1001	317,7	0	0	0	1262	2931,5	343,6	1627,5	1980,2	1144,7	3435,9	3670	1506,5	278,2	3396,7	3277,1	0	0	0	0	0	0	3017,4	145,5	2824,5	6114,5	2626,2	3445,4
2107	632	1005,5	259,4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2108	0	0	0	0	0	0	0	0	0	0	0	0	1091,5	652,3	1552,1	1335,8	172,5	1157,2	0	0	0	0	0	0	0	0	0	190,7	106,3	21,9	27,5	465,2	1025,5	303,4	212,1	7,6
2201	457,7	1119,6	789,2	1040	1161,6	802,6	551,9	1225	698,9	690,1	1339,1	766,9	959,5	1030,8	917,2	1088,7	707,3	529,8	985,4	1217,1	373,9	583,1	457,2	1689,5	1168,6	1873,7	1776,4	490,8	1977,5	1085,6	361,7	943,9	284	701,4	1044,6	460,9
2203	0	0	0	504	320,2	688,5	1990,4	285,4	135,2	1133,9	1240,1	0	534,3	521,9	546,7	203,8	309,1	414,4	0	0	0	0	0	0	621,5	1243,7	136,5	295,6	803,45	1311,3	0	0	0	0	0	0
2204	1673,3	2768,1	2854,6	3226,4	2832,1	2982,4	4164,4	2255,6	1992,9	2627,6	3062,8	1714,7	4893,5	4754,7	1180,9	6196,3	2366,6	996,4	2499,4	1553,9	987,9	1457,3	1705,6	1581,45	3261,7	2324,6	1924,7	2398,7	2616,1	2984,1	4503,2	2269,9	3151,6	5589	887,6	4489,7
2207	876,5	772,3	2614,5	479,5	797,4	832,4	3422,8	2005,7	1640,2	1840,1	2799,1	426,8	229,5	517,9	373	438,1	354	269,9	300,6	329,6	358,6	359	320,7	956,5	1926	3606,6	1680,6	1312,2	2399,6	2489,8	0	0	0	0	0	0
2208	1744,6	1478,2	1242,2	2721,5	734,5	2197,2	0	0	0	0	0	0	0	0	0	0	0	0	1520,7	398,8	1029,3	746,6	275,2	3302,5	0	0	0	0	0	0	0	0	0	0	0	0
2209	1841,5	1559,6	3984,8	2143,1	1159,5	938,8	0	0	0	668	979,3	358,9	773,9	777,1	801	822,4	785,4	538,9	934,8	1182,6	985,4	1204,8	740,9	1213,4	989	1130,3	816,8	978	1621,4	1161,7	0	0	0	0	0	0
2210	3522,2	3422,1	4687,2	5466,7	2859,8	4397,3	0	0	0	0	0	0	2112,7	3612,3	2412	2080,4	2082,1	1222,4	2355,3	3213,4	1688,4	1912,4	2548,1	2853,5	0	0	0	0	0	0	1732	1705,2	1152,2	1467,2	1588,3	1684,9
2301	13972,7	9068,1	8275,8	4601,2	5286,6	10070,7	4898,2	7911,6	8204,2	4937,9	11401,2	4385,9	14109,4	11864,2	8560,4	14803,6	8470,2	7993,2	7776,3	4347,7	6681,1	7714,4	4718,4	3020,9	6165,6	6154,1	7467,5	5702,9	7450,4	8851,2	8623,5	7802,4	14186,9	12324,3	8639,7	11711,8
2403	1765,2	2400,6	1092,1	1742,1	1908,5	1653,7	1938,7	1825,3	1050,7	2046,9	2014,7	1417,1	2212	2505,9	2157,5	2163,4	2123,3	1752,2	1507,6	1359,4	1037,8	1472	1287	1848,4	2224	2698,9	1366,9	1335,9	1938,3	1746,8	1412,7	1981,1	1400,2	2311	1694,2	1669,3
2405	0	0	0	0	0	0	1438,1	964	1279,5	1433,4	1193,5	605	0	0	0	0	0	0	0	0	0	0	0	0	3960,9	2780,9	3042,6	1443,2	2532,7	2705,2	0	0	0	0	0	0
2410	1212	1241,9	476,3	1200,4	1371,9	478,6	0	0	0	1437,4	978,3	576,2	0	0	0	1271,5	1976,2	566,8	670,8	996,1	463,8	1482	1099,5	957,1	0	0	0	651,3	433,3	215,3	1814,9	1171,9	528,8	0	0	0
2703	2040	3254,1	1098,5	1569,3	1480,8	2805,3	4099,8	3872,6	1137,4	3505,3	1305,1	874	1066,7	202,9	974,3	588,2	796	831,2	1981,4	2880,3	2015,2	2701,4	671,5	3719,1	3206,3	1274,3	2403,5	1283,5	2433,4	2311,8	1425,4	677,4	485,1	1117,05	1477,6	756,5
2706	1633	2337	307,2	2789,5	2530,7	1181,4	1589,5	1977,6	775,9	1253,6	879,4	1458,4	0	0	0	0	0	0	1849,3	2226,4	1404,6	2079,8	2306,2	2598,1	1860,1	3244,4	2112,1	2129,9	3087,5	1179,9	0	0	0	0	0	0
2707	2187,2	2561,5	2218,5	3316,7	3943,7	1191,9	1372,4	1620,9	501,4	1094,2	1839,6	622,8	1924,4	2537,5	1095,4	1908,3	3813,3	990	1146,5	2312,5	943,9	2376,9	1319,9	2195,2	1453,6	1141,5	846,8	1278,4	824,9	1140,6	998,4	2782,6	1615	995	2690	1143,3
3001	9351	7963,3	7022	11476,2	4875	8243,8	713,8	3017,5	5098,6	5532,6	5283	3504,3	7686,5	7472,3	1255,6	8712,4	5181,2	5843,9	7597,3	4004,7	4634,3	6717,8	6231,8	3896,7	8288	3814	5035,9	5766,9	3622,6	4424,2	6973,6	806,6	2753,6	8096,8	3474,8	213,8
3003	0	0	0	0	0	0	935,4	310,3	32,3	834,7	1430,3	906	0	0	0	0	0	0	0	0	0	0	0	0	2339,6	2671,3	2022,7	882,4	1039,7	775	0	0	0	0	0	0
3101	181	192,9	170,9	0	0	0	21,2	131	76	860,5	1510,2	915,5	4,9	32	1707,8	76,7	488,1	899,5	0	0	0	0	0	0	3129	3700,8	3156,8	24,4	2405,9	2473,1	213,4	2874,5	358,2	0	0	0
3102	241,6	374,5	49,6	41,6	252,9	440,9	508,7	902,2	851,9	279,6	435,9	357,75	3788,6	895,7	6299,2	4053	4143,5	2629,1	996	449,3	1757,3	956,3	370,5	1145,9	817,7	1757	693,1	365,9	1402,3	786	3531	3760	5019,5	3759,4	3880,4	1693,2
3202	0	0	0	294,2	320	347,6	2675,3	2393,4	1061,7	1426,3	3078	598,3	389,8	782,7	492	0	0	0	260,8	151,4	206,1	0	0	0	3023,8	2291	2138,3	2183,2	3975,8	1671,9	13,7	582,1	408,3	410,6	723,9	419
3203	0	0	0	0	0	0	851,2	1002,7	608,8	673	1363,3	579,8	0	0	0	0	0	0	0	0	0	0	0	0	669,2	1901,5	619,7	417,7	1835,3	677,7	0	0	0	0	0	0

Supplementary Table S1 (continued)

	P615	P615	P615	P615	P615	P615	P42	P42	P42	P42	P42	P42	P629	P629	P629	P629	P629	P629	P615	P615	P615	P615	P615	P615	P42	P42	P42	P42	P42	P42	P629	P629	P629	P629	P629	P629
SSP	C-T1	C-T1	C-T1	I-T1	I-T1	I-T1	C-T1	C-T1	C-T1	I-T1	I-T1	I-T1	C-T1	C-T1	C-T1	I-T1	I-T1	I-T1	C-T2	C-T2	C-T2	I-T2	I-T2	I-T2	C-T2	C-T2	C-T2	I-T2	I-T2	I-T2	C-T2	C-T2	C-T2	I-T2	I-T2	I-T2
3304	5454,2	2638,2	3875,3	2574,4	3417,2	5719,9	4694,1	1744,8	1909,6	3269,4	4649,2	1780,9	4534,7	4237,6	4327,1	4288,2	2866,6	2121,8	3420,7	2269,4	735,7	2740,1	2669,7	1443,3	6661,6	3799,4	2498,7	5145,6	829,9	2595,9	3667,1	2544	2411	4904,1	1316,5	90
3308	1022,4	637,1	1400,1	0	0	0	0	0	0	0	0	0	654,7	873,4	621,8	427,6	952,8	261,6	408,6	1183,5	681,2	543,6	773,4	658,5	0	0	0	0	0	0	560,3	1408,9	999,2	316,3	1154,6	714
3404	1374,5	1427,2	531,7	1071	1074,2	753	1318,8	1207,2	690,2	1511,1	978,8	945,9	1543,4	644,1	628,1	1223,4	1464,8	208,9	938,2	1344	867,4	1402,1	1530,5	1296	1226,7	1523,2	461,9	1416,1	1887,6	1158,1	541,5	1047,3	560	797,9	1336,9	843,2
3407	1375	1453,8	736,5	1048,1	891,3	1003,5	792,3	913	798,1	1051,8	1253,6	590,2	0	0	0	0	0	0	726,9	1050	690,7	1005,5	1016	934	1330,9	1353,3	1085	800,4	1417,4	1005,5	0	0	0	0	0	0
3410	0	0	0	0	0	0	373,1	810,6	504,7	679,7	1547,7	214,7	0	0	0	0	0	0	0	0	0	0	0	0	781,4	1382,1	367,5	166,8	966	428,1	0	0	0	492,3	440	466,15
3415	1281,2	843	1048,4	895,7	689	990,7	0	0	0	0	0	0	1573,4	954,2	717,6	819,9	861	606	534,4	756,6	531,6	1248,6	731,8	650,5	0	0	0	0	0	0	758,8	1076,9	1353,2	829,9	770	1032,5
3416	405,6	762,8	345,1	1053,2	597,4	527,2	0	0	0	0	0	0	619	1297,9	1223,3	0	0	0	255	962,1	608,55	0,0	0	0	0	0	0	0	0	0	431,7	1258,8	877,7	0	317,15	634,3
3504	2248,6	2066	2326,6	2141,2	2178,8	1215,1	2031,9	1697,3	881,5	1428,3	2784,5	1094,1	2988,1	1738	1404,1	2222,5	2306,1	1099,8	1245,2	1481,4	591,5	1496,3	908,4	1426	3246,6	2213,1	1871	1362	2350,5	1362,4	559,6	2042	1218,1	1511,1	4264,8	3023,9
3507	3250,4	3446,2	2068,8	2735,7	3222,7	2420,7	1288,8	932	576	930	1137	451,2	728,2	689	651,1	0	0	0	2390,9	2563,8	2477,35	2242,1	2399	4201,1	1511,1	2430	809,7	659,7	1731,2	542,2	0	0	0	0	0	0
3508	0	0	0	0	0	0	2500,8	1709	919,4	1966,9	2395,2	1216,7	1425,3	1499,5	1819,4	1055,7	2027	919,2	0	0	0	0	0	0	2705,8	1372	1053,5	1054,4	2748,4	1798,8	1355	1752	1269,2	1173,9	1899,4	426,1
3509	984,8	647,5	353,4	723,5	699,2	511,5	0	0	0	0	0	0	961,9	968,9	843,4	563,9	1112,5	603,8	551,3	730,7	371,9	876,9	669,8	524,1	0	0	0	0	0	0	978,85	1026,8	930,9	753,1	965,4	666,4
3606	1349,1	2293,5	414,5	1149,1	1337	172,8	2293,8	885,7	827,7	335,1	1545,3	703,2	1487,2	1326,8	1060,7	1145,5	1253,3	606,6	1308,5	2765,1	1662,7	929,6	2100,2	1779,4	4167	2985,4	1792,3	1392,9	2015,4	1516,5	1088,9	586	1448,9	1054,4	2414,4	927,3
3609	1536,5	3158,9	1437,4	1015	577,8	2004,9	0	0	0	0	0	0	0	0	0	0	0	0	1360,9	1527	1772,2	2190	2285,8	1139,3	0	0	0	0	0	0	0	0	0	0	0	0
3610	0	0	0	0	0	0	0	0	0	0	0	0	536,8	1825,7	1198	906,6	2733,9	852,7	0	0	0	0	0	0	0	0	0	0	0	0	1290,1	2065,9	1443	884,3	3112,1	1447,1
3611	442,2	686,1	292,9	331,8	821	359,5	538,5	493	449,2	150,9	671,2	410	1033,3	1474,8	1554,1	1111	1502,2	973,4	0	0	0	0	0	0	251,5	793,7	522,6	0	0	0	764,7	661,7	1137,3	571,4	1503,4	879,2
4101	6465,2	4639,5	881,4	3120,9	1646,6	2583,5	1006,4	3919,5	2888	1009,2	3006,2	1217,1	0	0	0	0	0	0	2928,5	3451,5	3161,9	1677,8	3227,2	2879,5	1569,3	2706,6	1098,9	178,2	2418,5	1651,3	229	294,3	261,7	0	0	0
4102	0	0	0	0	0	0	326,9	1197,3	1256,2	507,4	1709	1108	187,5	871,1	527,7	0	0	0	0	0	0	0	0	0	730,9	941,1	1303	1565,75	1362,1	1769,4	0	0	0	0	0	0
4204	0	0	0	0	0	0	742,3	1109,6	603,2	1001,4	1435,7	117,4	32	260,4	44,8	0	0	0	0	0	0	0	0	0	1260,5	1978,5	859,6	671,5	1760,4	1235,3	140,4	214,5	319,9	54	249,3	194,9
4304	409,3	772,1	578,3	701,8	365,3	585,9	1003,5	725	447,8	1323,9	737,1	594,3	1070,8	308,4	922,8	715	624,7	505	147,9	437,9	669,8	652,2	425,6	628,1	1148,6	988,7	599,7	694,9	1020,4	775,3	608,9	504,8	704,6	708,4	500,5	695,6
4404	612	751,1	1180,3	897,1	419,8	234,8	2129,4	3334,6	1919,5	2754,7	3189,1	1528,8	454,7	492,4	535,1	293,9	507	243	880,2	747,1	675,2	424,3	719,5	459,7	4568,8	3994	2882,2	2842,6	3396,9	2642,6	220,7	513,5	369,9	245,2	251	338,4
4407	1701,3	709,6	569	3080,9	1256,2	911,7	1882,8	1074,9	433,6	808,6	878,3	279,7	1830,7	1476,6	829,6	1606,1	1587	659,7	493,3	1118,2	1063,4	388,1	904,8	347,9	1499,7	1622,7	542,7	472,5	645,5	706,9	1088,7	1054,1	988,9	1275,9	1232	599,6
4409	2216,4	2994,7	1761,7	3534,5	2361,6	2681,4	0	0	0	0	0	0	2497,8	1322,4	2659,8	2521,1	4099,1	1977,9	1364,4	3675,6	1380	2109,2	3267,8	356,3	0	0	0	0	0	0	2565,5	3187	2267,2	2316,2	4186,5	2205,7
4410	0	0	0	399,7	274,2	266	0	0	0	0	0	0	1114,5	990	802,7	0	0	0	0	0	0	230,9	499	364,95	0	0	0	0	0	0	944,7	955,6	933,8	1006,1	886,1	727,6
4503	487,3	913,8	438,5	572,3	266,8	745,6	1519,2	919,7	716,7	1341,8	813,9	639,2	506,7	988,5	789,6	445,2	653,7	436,8	606,7	1138,4	895,5	1298,1	942	965,7	1512,7	1751,1	833,5	505,2	995,3	1032,1	359,9	660,7	89,7	246,9	530,2	597,4
4702	461,4	1649,7	548,7	1141,9	1575,3	760,3	1023,5	1471	267,9	1073	1549,8	349,7	662,4	852,7	471,3	583,8	1511,6	291,2	932,7	1567,6	1230,7	1762,2	1356,4	763	1542,2	989	455,1	596,4	1501,6	882,1	381,3	1064	560,1	417,9	757,6	557,3
4704	942,5	1208,6	833,5	1655,8	1581,2	753,3	2543,6	2474,4	420,9	1396,5	1769,9	297,8	0	0	0	359,35	546,8	171,9	1841,8	2615	490	966,7	1661,3	1235,4	1867,5	1669,8	1135,4	1283,7	1722,5	1039,8	0	0	0	0	0	0
4706	852,4	1476,9	749,4	937,9	2276,7	880,7	0	0	0	0	0	0	1158	1410,1	907,4	455,8	1854	873	877,2	1643	1445,7	1284,1	1662,7	1272	0	0	0	0	0	0	818,3	1606,2	1076,2	680,1	1760	1035,9
4707	0	0	0	0	0	0	0	0	0	0	0	0	356,5	874,5	581,4	527,6	1731,6	347,2	0	0	0	0	0	0	0	0	0	0	0	0	110,3	1135,9	534,7	206,7	1327,3	376,8
4806	339,7	633,5	286,3	229,6	243	277	1314	1907,5	601,1	1155,3	2128,1	501,1	520	617,2	424,1	483,7	627,4	340	394,7	569,8	285,5	530,7	536,6	412,5	1688,7	2369,1	1054	488,5	2232,5	1162,2	485,5	580	391	497,55	633,4	361,7
4909	127,2	369,3	151,2	79,5	622,8	122,2	0	0	0	344,1	386,3	365	0	0	0	0	0	0	0	0	0	0,0	0	0	647,4	1138,2	316,7	230,4	526,1	333,2	139,1	210,2	136,6	154,4	310,2	142,6
5105	0	0	0	0	0	0	0	0	0	0	0	0	1074,3	1523,2	465,8	1921,5	2588	628,9	0	0	0	0	0	0	0	0	0	0	0	0	1192,9	2287,3	1131,2	788,8	2231,3	1027,6
5304	1074,5	1431,7	1392,8	1989,2	1532,2	1879,9	3417,7	4641,9	1545,9	5514,2	1846,8	1672,8	5225,3	838,2	2026	167,2	1030,3	2362,9	1829,5	1363,7	1606,8	4121	1949,2	2174,4	1728,2	2062,4	1386,5	1721,9	3416,1	3382,4	1692,6	1335,4	1402	2773,9	4820,1	2028,9
5402	1277,8	441,2	383,4	1219,1	900,5	1233	1115,3	1442,4	849,4	1095	1366,1	581,7	1859,1	907,2	1034,5	1438,8	1626,9	730,6	1152	1591,6	871,1	1761,3	1190,7	972,1	705,1	1155,6	855	418,9	378,9	887,9	1131,2	828,8	1037,1	466,8	727,3	717,4
5406	1920,7	621,7	359,6	1271,5	823,1	783,2	805,4	1380,9	698,5	918,7	1219,9	520,2	592,6	873,1	645,1	462,1	927,7	519,1	894,8	466	773,7	437,4	717,1	445,3	642,2	964,9	886,4	457,9	1021,2	1146,5	781,9	815,5	849	534,9	771,6	632,3
5407	2176	1496,1	1619,7	4007	1471,9	703	0	0	0	0	0	0	413,4	1812,9	1551,2	3749	2649,3	1120,6	1625,4	4331,5	2500,1	3172,9	1915	1252,7	0	0	0	0	0	0	2272,1	1211,3	2150,7	567,7	1539,8	1150,5
5408	1552	1128,2	563,6	2247,2	1072,1	415,4	0	0	0	0	0	0	1889,5	1267,2	744,7	2144,5	2358,7	690,4	851,2	1973,3	1284,9	1868,3	2156,7	1139,4	0	0	0	0	0	0	1617,9	2447,6	1246,3	478,7	2018,8	935,2
5702	3210,9	3654,4	2890,6	4771,2	5204,5	1704,5	6028,6	6214,2	1164,1	5669,8	4301,6	905,1	54,5	1326,4	71,8	479,9	2209,6	548,2	2230,4	6081,8	5489,9	3841,9	5447,8	5332,3	4858,9	762,2	1770,6	3087,4	8132,6	3191	801,6	1245,7	974,1	609,2	1515,4	559,2
5707	336,6	499,1	237,5	466,6	271,9	295,5	1146,5	1690,1	93,5	282,7	472,7	377	2527	4055,7	999,9	1657,9	5131,5	999,2	271,2	1395,3	607,6	200	1156,4	774,7	689,3	1591,2	447,4	259,4	928,9	400,6	2709	4003,7	2304,3	1426,2	4316,7	1352,5
5708	613,6	1338,9	580,7	903,2	2116,6	870,4	857,8	1059,1	426,7	730,9	1196,9	419,9	108,7	311,5	146,1	136	840,1	103,3	642,6	2052,9	1535,5	1116,7	1680,9	1389,3	719,3	1578,8	745,9	329,4	1409,3	1070,5	154,2	376,1	273,8	112,9	192,6	209,7
5709	311,6	500,3	405,95	325,1	535,8	266,5	121,2	638,4	198,3	455,8	652,3	553	335,6	500,7	285,6	193,3	762,4	186,4	240	562,3	401,15	436,7	531	342,4	163,2	1074,6	372,7	363,3	657,9	348,4	396,1	416,1	330,5	159,9	697,7	197,2
5712	1371,1	1270,9	762,5	871,4	2022	627,7	719,7	2075,4	666,4	739,1	1506,1	347,2	2220	2630,6	1415,1	1640,4	3402	1416,5	531,9	1683,3	1613,4	1870,2	1380,5	727,4	1118,6	1604,3	1254,9	544,1	1548,9	1146,1	2717,1	4389,4	3336,7	923,8	3329,9	1818
6107	2917,4	3526,2	3638,8	3482,1	3043,8	3498,4	1997,3	5032,2	1900	2077,4	2842,3	1317,1	3418,3	2939,8	2870,3	2985,1	4909,5	2361,5	1880,4	1930,6	3338	2042,7	3908,2	3539,8	1024,8	2471,7	2879,1	490,1	3888	3593,2	2674,8	2955,4	3061,4	695,9	3370,9	41,1
6306	6838,8	1626,3	1517,9	5475,6	1986,7	3318,4	5910,1	1010,8	2024,6	6366,4	2188,8	1314	1801	2057,9	1545,3	3101	1972,5	5749,4	1276,2	2525,8	1585,9	2045,4	2528,8	1208,2	1054,4	1893,5	1874,4	958,7	1255,5	2445,3	4880,9	327,5	2386	1375,1	2435,8	2030,9
6401	2029,5	2081,2	1091,1	2997,6	1002,7	1897,8	1923,6	2642,3	1457	2181,5	1655,6	1015,1	2169,5	2037,4	1680,2	2712,2	2454,3	1405,1	1074,6	1565,1	2629,3	2148	2736,9	2076,4	886,8	1461,3	1666,4	614,6	1915,3	2658,1	2479,7	1683,2	2119,8	947	1869,4	1880
6409	0	0	0	0	0	0	98,9	172,1	216,9	126,7	602,2	524,4	0	0	0	0	0	0	0	0	0	0	0	0	350,8	386,8	587,2	0	0	0	0	0	0	0	0	0
6607	212,7	655,9	465,5	858,6	800,3	521,7	0	0	0	0	0	0	29,1	1722,6	430,1	319,5	2527,1	235,6	0	0	0	0	0	0	36,8	216,35	395,9	0	0	0	0	0	0	0	0	0
7105	19840,2	10576,9	438,3	9566,6	6611,7	6315,4	191,5	1716,2	2165,9	202,2	293,8	221,8	6521,3	10155,9	8056,3	4074	10279,6	11106,8	15191,2	16441,2	9958,2	6686,7	19001,3	6171,8	109,1	236,7	3540,1	239,9	1560,9	848,4	8889,1	18321,1	14768,1	1700,3	1641,6	11876,5
7106	10742,5	10219,1	4649,5	15792,4	9470,5	12750,8	11477,5	16744,6	8191,7	14695	8860,6	3013,9	11355,7	9932,2	4571,5	10673,4	12706,4	4452,4	6589,5	14325,8	5925,3	8890,3	16090,6	7068,4	6269	6701,2	5678,3	2687,4	17189,9	7746,3	8020,7	8511,8	4928,3	2103,2	8884,3	9311
7108	9914,8	7805,8	5464,2	3448,1	3373,9	3792,7	572,9	8334,9	4967,2	2256,6	3843,3	5072,6	3500,2	2944,8	7847,9	4532,4	7481,3	9878,7	2150,9	4800,6	8307,4	2375	6383,4	716	867,5	1519,2	7677,6	779,8	6598,9	4545,9	7326,8	8888,8	10621,8	718,5	7635,5	9568,7
7202	1782,7	647,6	410,8	645,9	402,1	206,2	581,7	1679,6	239,3	494,9	494,1	384	611,6	1500,5	512,6	887	1894,8	1372,9	2174,2	1592,9	1145,5	450,2	1415,6	585,6	422,6	670,45	918,3	0	0	0	998,7	663,4	1555,2	69,6	1586,7	1145,3
7203	14873,2	582,2	9766,7	27338,3	14069,7	8528,5	24899	21976,9	8301,1	32610,6	17261,3	12205,1	16832,7	10953,1	12935	21178,7	16879,8	752,9	5617,5	15010,9	9685,6	883,8	17157,7	1700,9	10921,6	6323,1	10288,1	7523,7	23230	27440,3	15653,4	90,3	15199,6	6307,1	10997	16760,6
7204	7470,2	10821,9	9553,5	16590,3	2670,7	12597,9	14407,1	15444,9	3677	20602,3	1272,9	7971,4	4214,1	5939	6858,1	9387,4	9598,8	4880,1	5447,7	7180,8	7820,9	4263,5	10626,2	8078,3	7029,6	5196,4	8481,4	6178,9	15918,9	22973,2	5044,7	5267,8	6484,7	2323,5	5775,3	10024
7205	1203,3	449,2	1092,3	546,1	759,4	1099,1	458,8	1754	790,6	634,3	1003,2	307,8	1286,8	1776,7	1447,4	1767,7	2215,1	1839	225,6	1002,3	1050,7	723,1	1171,1	671,8	297,8	385,1	1147,7	834,9	1102,3	567,5	1793,8	2086,1	1627,3	132,8	68,1	1777,9
7304	9563,7	2443,6	1148,1	7741,4	3441,9	4801,7	4142,9	7071,2	4127,5	4323,4	3758,8	2958,9	8232,8	4693,1	4776	9534,3	8443	5629,1	5218,7	4910,2	5385,9	3747,7	5867,1	3941,4	937,2	2152	4975,4	1739,5	4236,6	3007,3	8308,6	6493,2	10281,7	2013,6	6125,8	5040,9
7309	1799	1350	1357,3	1493,1	515,5	841,7	885,8	1118	608,8	792,5	1046,5	1396,9	1681,8	1849,7	1634,1	2532,4	2753,5	835,2	1105,1	1297	2142,8	1460,9	2467,2	1209,5	757,7	526,9	1404	984,5	2556,7	1417	2823,7	85	1800,9	892,5	556	711,8
7404	1787,1	2182	657	1565,3	1297,2	1028	1350,5	2442,9	1371,1	1313,8	2257,9	1016,3	1560,5	1697,2	1808,8	1525,9	2586,8	1705,6	739,2	1310,4	1612,1	1904,9	2004,6	1611,6	892,3	1692,7	1802,7	277,1	1928,6	1818,7	1736,9	2211,1	1911,3	506,8	1672,4	2010,3
7603	1581,5	1763,1	1094	1046,2	2078,7	682,6	467,9	1616,2	576,5	599,7	849,1	541,3	985,5	1425,5	1577,4	1121,9	2407,1	1150,6	2906,9	1903,1	1712,8	1571,0	1823,2	1318,8	592,7	678,5	764,3	360,5	1374,9	632	1754,8	2445,2	2302,5	1851,15	1794,5	1907,8
7707	615,6	1019,3	609,2	258,3	1405,7	609,9	66,4	1059,1	176	404,3	1082,9	743	317,1	685,7	432,9	225,2	1300,6	423,5	545,5	1340,9	717,7	804,6	807,3	801,9	808,5	999,6	489,9	598,9	693,1	504,7	325,3	818,1	853,5	132,8	732,3	846
7708	447,7	735,9	546,6	149,4	968,5	474,2	508,1	883,6	334,1	514	1059,3	428,1	313	964,1	764,2	224,3	838,7	565,7	338,8	398,9	679,8	355	661,4	647,7	307,2	677,3	452,6	335,6	453	479,9	453,6	865,5	819,5	161,8	1116,5	892,2
8102	0	0	0	0	0	0	890,2	6075,8	3712,4	670,9	5801,8	2330,8	80,6	499,3	289	509,9	1263,7	886,8	0	0	0	0	0	0	1934,5	5466,4	6267,2	2166,8	8011,9	3447,7	77,4	964,7	521,1	0	0	0
8201	2271,9	2161,5	1622,6	2227,3	464,5	1812,3	2774,3	3086,4	1110,7	3392,2	1596,6	1392,7	3652,3	2574	1496,7	2127,2	2956	1500,1	1666,4	2330,4	2495,3	1979,6	1943,1	2747,6	1465,2	1743,3	1487,4	997,6	2429,7	2297,3	2981,6	1079,8	1783,1	1801	1196,8	2218,2
8307	1182,1	1928,1	1227,3	1457,6	731,5	1157,4	0	0	0	0	0	0	1512,4	1186,6	876,6	1319,6	2166,3	840,2	625,4	1542,6	1789,9	1000,9	1409,8	1106,9	0	0	0	0	0	0	1302,1	1343,6	1314,3	1220,3	1174,9	1265,7
9103	910	857	963,2	2592,9	453,6	286,3	101,4	637,6	20,1	442,2	689,5	0	0	0	0	0	0	0	234	1570,2	924,8	1306,3	703,3	1909,2	7,9	1220,7	2234,2	13980,1	4318,2	2800,9	0	0	0	53,5	188,05	322,6
9105	189,4	2770,2	537,9	508	830,1	826,9	1054,9	2160,7	1371,4	761,7	1064,5	548,8	1345,2	897,3	662,5	273,6	1226,3	2485,2	559,7	694,2	987,2	74,5	1626	680,1	220,9	1888	7688,2	1283	1323,5	1397,1	974,9	1779	294,5	238,8	1252,6	1292,2
9108	123,8	1026,7	584,5	377,7	493,05	608,4	1459,4	1429,2	721,3	865,5	991,4	928	0	0	0	0	0	0	11,1	1285,8	648,45	0,0	0	0	1439,7	1871,9	1007,5	4797,3	2157,1	1860,5	603,2	1222,3	912,8	0	0	0
9109	0	0	0	0	0	0	573	2480,4	452,4	514,4	1787,8	1150	0	0	0	0	0	0	0	0	0	0	0	0	1044,9	1186,3	903,5	2083,6	2360	1807,2	0	0	0	0	0	0
9802	551,5	1505,6	822,3	445,6	1048,9	897,6	832,8	1959,8	387,2	1130	940,8	561,5	989,5	658,6	1094,8	646,8	2615	922,1	573,4	119,3	689,8	971,2	1378,4	1073,8	467,5	922,6	924,8	527,6	916,1	1137,3	888	1852,8	1340,5	263,2	1340,1	695,1

Supplementary Table S2. PCA analysis was performed as described by Sharov *et al.* (2005). This analysis allows for generation of associations between proteins and experimental conditions at 24 and 72 hai.

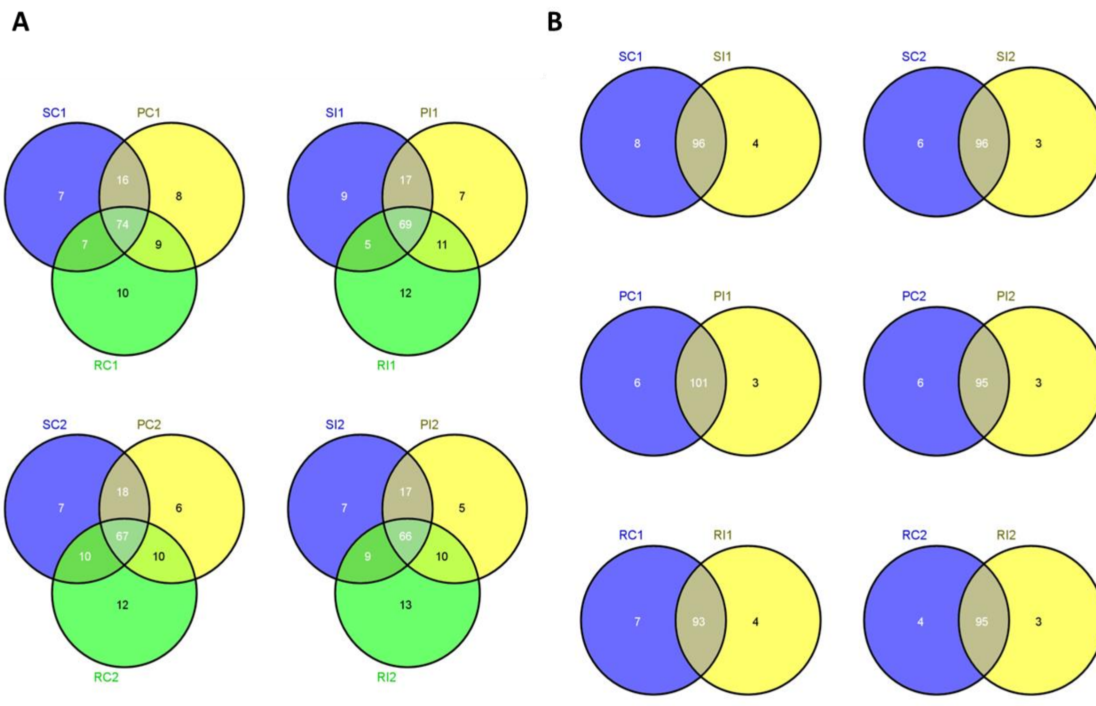
SSP	Log10Change	Correlation	PCnumber	Direction	SC	SI	PC	PI	RC	RI
2005	8 164	0.682	1	positive	37 058	35 468	38 813	27 734	27 713	-10
3509	14 383	0.989	1	positive	29 651	28 595	27 843	28 043	-10	-10
5407	14 774	0.989	1	positive	30 218	33 488	32 407	32 059	-10	-10
4706	14 553	0.988	1	positive	30 569	2 956	29 916	30 914	-10	-10
2007	7 908	0.689	1	positive	3 216	31 656	29 825	24 128	21 866	-10
1202	0.339	0.812	1	positive	35 267	35 502	34 753	31 948	31 848	31 394
2106	7 611	0.651	1	positive	30 347	3 189	2.83	33 117	29 097	-10
1404	10 284	0.677	1	positive	-10	24 592	28 829	26 301	-10	-10
4409	14 972	0.988	1	positive	33 146	34 368	3 356	3.45	-10	-10
1206	15 023	0.984	1	positive	3 346	32 046	35 881	38 396	-10	-10
5408	14 631	0.99	1	positive	30 837	3 181	29 981	30 001	-10	-10
7105	1 327	0.952	1	positive	39 091	38 892	36 546	38 672	29 508	23 733
2707	0.319	0.891	1	positive	32 428	32 859	33 648	33 976	30 158	30 327
1402	7 545	0.656	1	positive	30 341	29 678	29 865	26 212	26 891	-10
5712	0.299	0.769	1	positive	33 057	32 993	30 411	30 146	29 993	28 624
3001	0.311	0.675	1	positive	36 193	38 071	39 061	3 888	33 469	36 701
3415	14 482	0.988	1	positive	30 108	28 771	30 179	29 288	-10	-10
7309	0.224	0.692	1	positive	32 354	32 551	31 727	29 371	29 268	30 213
3416	9 419	0.611	1	positive	29 975	-10	26 762	28 403	-10	-10
3308	11 791	0.765	1	positive	28 503	26 759	29 867	-10	-10	-10
6607	14 154	0.988	1	positive	24 445	27 598	26 042	28 515	-10	-10
503	0.177	0.612	1	positive	29 145	2 875	28 289	26 275	25 934	27 508
7205	0.293	0.687	1	positive	31 732	32 858	29 237	28 863	29 345	2 764
4407	0.232	0.715	1	positive	31 169	30 752	29 455	31 825	29 811	2 766
1110	8 292	0.693	1	positive	3 545	3 472	36 202	35 377	25 606	-10
1113	14 491	0.987	1	positive	25 254	31 088	33 121	2 919	-10	-10
2301	0.183	0.714	1	positive	40 521	40 003	40 069	37 964	38 341	37 975
7603	0.319	0.971	1	positive	31 152	31 641	31 615	30 572	28 798	28 134
202	0.366	0.792	1	positive	33 142	33 991	34 205	30 244	30 075	29 665
2210	15 014	0.985	1	positive	34 217	32 413	3 584	36 124	-10	-10
2104	0.264	0.929	1	positive	3 664	37 212	37 185	35 988	35 017	33 955
7108	0.264	0.709	1	positive	3 636	38 417	38 754	35 482	34 584	35 478
8307	14 646	0.989	1	positive	30 656	31 267	31 488	30 304	-10	-10
1102	11 586	0.763	1	positive	25 926	22 992	30 674	-10	-10	-10
2207	-0.583	-0.812	1	negative	25 489	25 406	30 826	28 343	33 505	3 114
1301	-7 329	-0.512	1	negative	28 668	-10	24 364	-10	26 112	26 302
4102	-10 641	-0.69	1	negative	26 452	-10	-10	-10	28 971	29 942
602	-0.394	-0.56	1	negative	33 033	3 354	29 073	37 002	35 092	38 067
4404	-0.789	-0.961	1	negative	26 928	25 196	29 115	26 489	33 782	3 376
106	-0.262	-0.778	1	negative	29 351	28 804	27 274	27 672	30 148	31 377
9109	-14 506	-0.988	1	negative	-10	-10	-10	-10	29 361	30 081
1306	-14 393	-0.989	1	negative	-10	-10	-10	-10	27 888	29 509
4806	-0.476	-0.847	1	negative	27 113	26 711	25 966	23 964	30 593	30 301
4204	-10 737	-0.715	1	negative	18 574	-10	-10	-10	28 987	27 424
3410	-14 265	-0.988	1	negative	-10	-10	-10	-10	27 279	27 846
3003	-14 175	-0.989	1	negative	-10	-10	-10	-10	2 324	30 114
5304	-0.366	-0.766	1	negative	3 316	28 699	31 103	32 527	34 632	34 104
709	-0.362	-0.72	1	negative	35 424	34 754	35 386	39 431	39 985	38 334
2405	-14 584	-0.988	1	negative	-10	-10	-10	-10	3 083	3 005
4503	-0.243	-0.846	1	negative	28 657	27 014	27 635	26 854	30 002	2 948
7203	-0.363	-0.65	1	negative	41 258	3.81	36 424	4 172	42 191	4 279
6409	-13 827	-0.989	1	negative	-10	-10	-10	-10	21 891	25 341
111	-14 157	-0.989	1	negative	-10	-10	-10	-10	25 587	27 573
1003	-0.347	-0.798	1	negative	26 311	28 424	25 891	25 993	29 416	30 562
3203	-14 432	-0.988	1	negative	-10	-10	-10	-10	29 051	29 086
2101	-14 791	-0.988	1	negative	-10	-10	-10	-10	33 192	31 413
2102	-0.291	-0.653	1	negative	30 471	31 549	29 365	27 545	32 287	33 104
3102	1 277	0.948	2	positive	34 433	35 483	22 173	22 221	28 641	25 465
3508	14 439	0.921	2	positive	31 966	30 979	-10	-10	31 981	32 528
1004	11 975	0.792	2	positive	29 093	25 227	-10	-10	-10	-10
8102	13 968	0.902	2	positive	23 552	2 919	-10	-10	34 342	33 192
5707	0.77	0.869	2	positive	33 369	33 098	25 337	25 246	27 527	25 674
2001	1 003	0.94	2	positive	40 419	39 886	30 259	31 568	39 745	39 265
2108	12 155	0.792	2	positive	30 145	28 086	-10	-10	-10	-10
4707	12 036	0.791	2	positive	27 528	28 338	-10	-10	-10	-10
7202	0.254	0.572	2	positive	28 908	3 121	2 892	25 762	27 896	26 576
2203	6.71	0.561	2	positive	27 277	24 722	-10	26 818	26 285	3 074
910	6 864	0.582	2	positive	2 402	23 766	26 906	-10	26 505	25 662
3610	12 293	0.791	2	positive	30 232	31 083	-10	-10	-10	-10
7304	0.218	0.657	2	positive	37 554	38 854	34 762	37 023	36 942	35 607
5105	12 287	0.791	2	positive	29 607	3 165	-10	-10	-10	-10
3101	6 811	0.588	2	positive	18 093	25 091	22 586	-10	17 748	30 251
1112	12 355	0.791	2	positive	30 859	31 795	-10	-10	-10	-10
3611	0.411	0.731	2	positive	31 248	30 702	26 496	26 635	26 922	25 394
2002	14 109	0.897	2	positive	27 664	26 736	-10	-10	36 975	36 602
1104	0.561	0.705	2	positive	35 726	3 632	33 999	28 379	37 731	37 312
2107	-6 791	-0.567	2	negative	-10	-10	2 739	-10	-10	-10
9103	-12 226	-0.812	2	negative	-10	-10	29 586	28 424	20 379	2 742
2706	-12 402	-0.794	2	negative	-10	-10	3 023	33 071	31 291	30 687
4909	-12 636	-0.81	2	negative	-10	-10	22 838	22 606	-10	2 562
107	-11 826	-0.776	2	negative	-10	-10	25 506	27 027	29 506	30 677
2703	-0.427	-0.723	2	negative	27 747	28 634	32 876	32 714	34 189	32 006
2208	-14 474	-0.923	2	negative	-10	-10	31 685	32 142	-10	-10
2006	-15 377	-0.923	2	negative	-10	-10	40 521	39 804	-10	-10
9108	-11 851	-0.776	2	negative	-10	-10	26 236	26 847	30 591	2 967
3407	-12 267	-0.795	2	negative	-10	-10	3 056	29 906	29 205	29 637
1205	-0.28	-0.677	2	negative	29 526	26 665	31 636	30 016	29 585	2 749
1111	-13 372	-0.818	2	negative	-10	-10	39 186	25 259	23 394	-10
4101	-12 652	-0.797	2	negative	-10	-10	34 741	33 744	33 522	31 891
5708	-0.673	-0.871	2	negative	22 314	23 573	28 929	30 737	28 628	2 855
112	-14 212	-0.923	2	negative	-10	-10	29 247	29 809	-10	-10
5702	-0.91	-0.748	2	negative	22 384	29 215	35 101	35 422	35 465	3 448
3609	-14 426	-0.923	2	negative	-10	-10	32 812	30 234	-10	-10
1103	-12 012	-0.789	2	negative	-10	-10	30 722	2 492	28 052	28 592
1606	0.48	0.559	3	positive	29 816	21 201	29 516	28 635	27 658	29 653
1909	8.25	0.604	3	positive	23 116	-10	2 838	30 648	29 447	31 014
3507	8 683	0.623	3	positive	2 838	-10	3 455	34 431	29 467	28 929
4410	11 659	0.682	3	positive	29 824	-10	-10	24 882	-10	-10
3202	10 702	0.617	3	positive	27 255	-10	-10	2 505	32 775	31 398
4704	-8 355	-0.608	3	negative	-10	25 095	29 925	30 983	3 141	29 556
2410	-13 864	-0.796	3	negative	-10	30 512	29 518	29 655	-10	29 695
1603	-8 582	-0.634	3	negative	-10	27 645	-10	-10	-10	-10
2209	9 505	0.67	4	positive	28 943	28 472	33 529	31 226	-10	27 902
7707	0.308	0.635	4	positive	26 579	26 978	28 608	27 818	23 642	28 374
5709	0.259	0.84	4	positive	25 604	24 796	26 004	25 556	23 952	27 386
302	9 474	0.672	4	positive	29 714	30 705	30 373	26 488	-10	28 848
9105	-0.201	-0.597	4	negative	29 676	29 737	28 169	28 475	3 165	28 828
7204	-0.204	-0.8	4	negative	37 449	38 811	39 626	39 156	3 971	37 734
110	-9 217	-0.684	4	negative	-10	-10	-10	-10	23 601	-10
705	-0.326	-0.65	4	negative	27 952	31 267	28 462	29 487	33 046	29 962

A: 24 hai

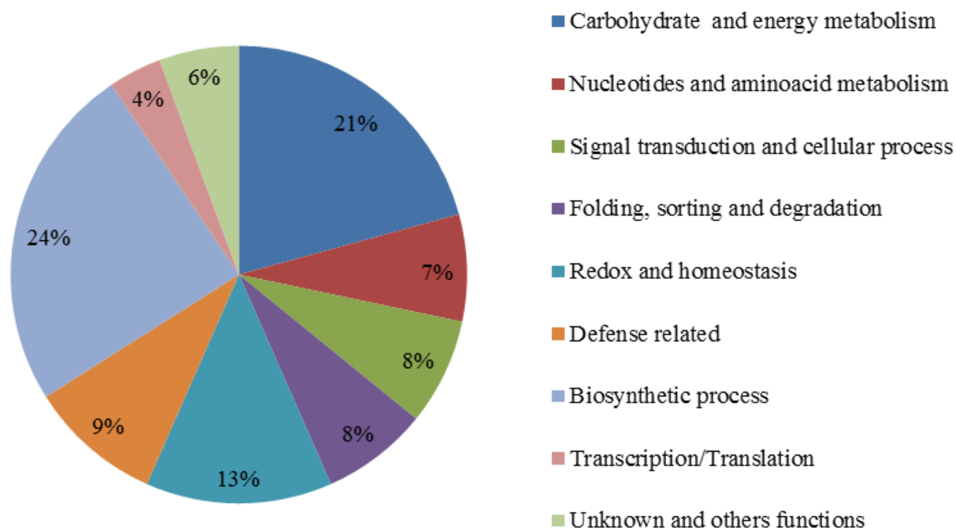
Supplementary Figure S1. A: Mean log abundance intensities for protein spots identified by PCA and pairwise comparisons at 24 hours after inoculation (24 hai). (PC1+) Protein spots positively correlated with PC1. (PC1-) Protein spots negatively correlated with PC1. (PC2+) Protein spots positively correlated with PC2. (PC2-) Protein spots negatively correlated with PC2. (PC3+) Protein spots positively correlated with PC3. (PC3-) Protein spots negatively correlated with PC3. (PC4+) Protein spots positively correlated with PC4. (PC4-) Protein spots negatively correlated with PC4. Legend for graphs: the mean log intensity values were calculated from the sample replications. SC Susceptible Control), PC (Partially resistant Control), RC (Resistant Control), SI (Susceptible Inoculated), PI (Partially resistant Inoculated) and RI (Resistant Inoculated).

B: 72 hai

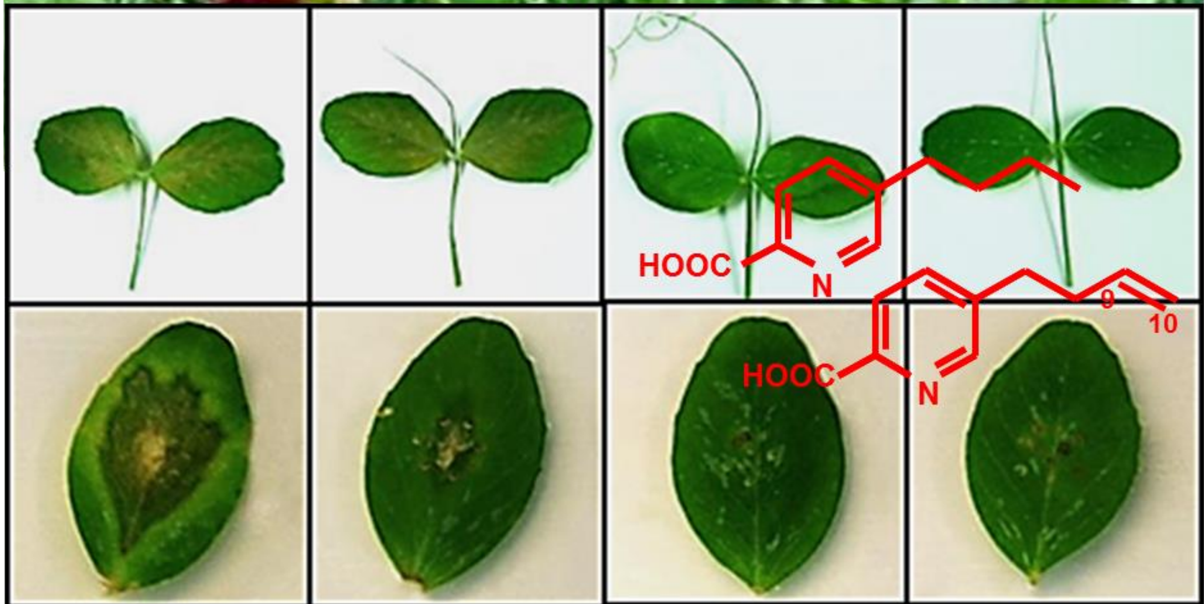
Supplementary Figure S1. B: Mean log abundance intensities for protein spots identified by PCA and pairwise comparisons at 72 hours after inoculation (72 hai). (PC1+) Protein spots positively correlated with PC1. (PC1-) Protein spots negatively correlated with PC1. (PC2+) Protein spots positively correlated with PC2. (PC2-) Protein spots negatively correlated with PC2. (PC3+) Protein spots positively correlated with PC3. (PC3-) Protein spots negatively correlated with PC3. (PC4+) Protein spots positively correlated with PC4. (PC4-) Protein spots negatively correlated with PC4. Legend for graphs: the mean log intensity values were calculated from the sample replications. SC Susceptible Control), PC (Partially resistant Control), RC (Resistant Control), SI (Susceptible Inoculated), PI (Partially resistant Inoculated) and RI (Resistant Inoculated).



Supplementary Figure S2. Venn diagram showing the shared proteins between enotypes/treatments.



Supplementary Figure S3. Functional categorization of identified proteins. Relative proportions are expressed as percentage values.


Chapter 6
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Chapter 6

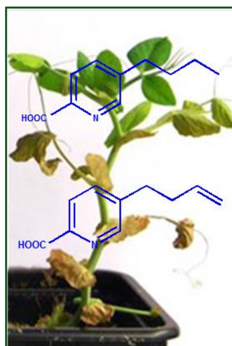
Identification of the main toxins isolated from *Fusarium oxysporum* f.sp. *pisi* race 2 and their relation with isolates Pathogenicity

Moustafa Bani, Nicolas Rispail, Antonio Evidente, Diego Rubiales, Alessio Cimmino

Abstract

Fusarium oxysporum f.sp. *pisi* (*Fop*) is a pathogen of field pea inducing severe vascular wilt worldwide. Plant resistance to race 1, 5 and 6, producing wilt symptoms, is conferred by single dominant gene, while resistance to race 2, producing near-wilt symptoms, have been recently showed to be quantitative. Among several virulence factors reported to play a role in the infection process, toxin production is one of the best studied. In this view, five race 2 isolates have been investigated for toxins production in vitro and on their relation with isolates pathogenicity. All the isolates produce different amounts of fusaric and 9,10-dehydrofusaric acids. The content of the two toxins has been quantified and correlated with the pathogenicity and aggressiveness of isolates on field pea. Results suggest that toxin production is an important determinant of *Fop* race 2 pathogenicity.

Keywords
Fusarium oxysporum f.sp. *pisi*; *Pisum sativum*; near wilt; phytotoxins; fusaric acids.



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1. Introduction

Fusarium oxysporum f.sp. *pisi* (Fop) is an important pathogen causing vascular wilt of field pea (*Pisum sativum*) worldwide (Kraft and Pfleger, 2001). Four different races of *Fop*, races 1, 2, 5 and 6 have been described (Infantino *et al.*, 2006). Races 1 and 2 have been reported in every country where pea are grown, while races 5 and 6 are, to date, only important in western Washington State (Infantino *et al.*, 2006). Plants infected with race 2 are most often scattered throughout the field rather than being concentrated in specific areas as with the other races leading to its description as near wilt (Kraft and Pfleger, 2001). Resistance to *Fop* race 1, 5 and 6 is conferred by single dominant gene while resistance to race 2 have been recently shown to be quantitative (Infantino *et al.*, 2006; Bani *et al.*, 2012)

Several virulence factors have been reported to play a role at different stages of the infection process to induce disease and counteract the plant defence reaction in several *formae specialis* of *F. oxysporum* (Roncero *et al.*, 2003). However, these studies have not targeted the virulence factors of *Fop*. One of the best studied virulence factors is the fusaric acid, 1 (Fig. 1).

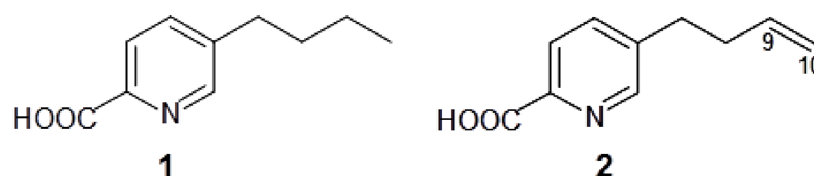

Figure 1. Structures of fusaric, **1**, and 9,10-dehydrofusaric, **2**, acids.

FA is a non-specific toxin produce by many *Fusarium* species (Bacon *et al.*, 1996; Abouzeid *et al.*, *2004*). At high concentration it induces many physiological responses in plant cells including alteration of cell growth, mitochondrial activity and membrane permeability while at lower concentration it could trigger plant defence reactions and programmed cell death (Bouizgarne *et al.*, 2006; Jiao *et al.*, 2013). FA was also shown to induce wilt symptoms on pepper and cucumber (Sarhan and Hegazi, 1988; Wang *et al.*, 2012). Thus it is thought to participate to *F. oxysporum* pathogenicity by reducing plant cell viability. However, FA was also detected within plant tissue colonized by non-pathogenic isolates which questions the exact importance of FA during the infection process (Bouizgarne *et al.*, 2006). Thus the role of FA in *F. oxysporum* pathogenicity is still under debate. Apart from FA, some *F. oxysporum* isolates have been shown to produce additional toxins such as beauvericin, enniantin B, bikaverin, moniliformin, fumonisin and trichothecenes (Mirocha *et al.*, 1989; Irzykowska *et al.*, 2012) that can also contribute to their pathogenicity.

Toxin produced by *Fop* and their potential function in *Fop* pathogenicity is still unknown. To improve our understanding on *Fop* pathogenicity, the main toxins of several isolates of *Fop* race 2 were identified and quantified.

2. Materials and methods

2.1. General experimental procedures

IR spectra were recorded as deposit glass film on a Perkin-Elmer (Norwalk, CT) Spectrum One FT-IR spectrometer and UV spectra were measured in MeCN on a Perkin-Elmer Lambda 23 UV/Vis spectrophotometer. ^1H NMR spectra were recorded at 600 or 400 MHz, in CD_3OD, on Bruker (Kalsruhe, Germany) spectrometers. The same solvent was used as internal standard. ESI and APCI MS spectra were recorded on Agilent Technologies (Milan, Italy) 6120 Quadrupole LC/MS instrument. Analytical and HPLC grade solvents for chromatographic uses were purchased from Carlo Erba (Milan, Italy). All other analytical grade chemicals were purchased from Merck (Darmstadt, Germany). Analytical and preparative thin layer chromatography (TLC) were performed on silica gel (Kieselgel 60, F_{254}, 0.25 and 0.5 mm respectively, Merck, Darmstadt, Germany) or reverse phase (Whatman, KC18 F_{254}, 0.20 mm, Maidstone, UK) plates. The spots were visualized by exposure to UV radiation (254 nm), or by spraying first with 10% H_2SO_4 in MeOH and then with 5% phosphomolybdic acid in EtOH, followed by heating at 110 °C for 10 min, or by exposure to iodine vapours. The HPLC system (Shimadzu, Tokyo, Japan) consisted of a Series LC-10AdVP pump, FCV-10AlVP valves, SPD-10AVVP spectrophotometric detector and DGU-14A degasser. The HPLC separations were performed using a Macherey-Nagel (Duren, Germany) high-density reversed-phase Nucleosil 100-5 C_{18} HD column (250x4.6 mm i.d.; 5 μm) provided with an in-line guard column from Alltech (Sedriano, Italy). Water was HPLC quality, purified in a Milli-Q system (Millipore, Bedford, MA, USA). Disposable syringe filters, Anotop 10-0,2 μm, were purchased from Whatman (Springfield Mill, Maidstone, Kent, UK). Fusaric acid was purchased from Sigma (St. Louis, MO, USA) whilst 9,10-dehydrofusaric acid (DFA, **2,** Fig. 1) was purified and identified from the culture filtrates of *Fusarium nygamai* as previously described (Capasso *et al.*, 1996). The methyl esters of **1** and **2** were prepared by diazotation of the corresponding acids as previously reported (Amalfitano *et al.*, 2002).

2.2. Fungal strain, culture medium and growth conditions

The *Fusarium oxysporum* f.sp. *pisi* race 2 isolates F42 and F69 were kindly provided by Dr. W. Chen (USDA-ARS, Pullman, USA). The *F. oxysporum* f.sp. *pisi* strain CBS

127.73 NRRL36628 (*Fop*1) was provided by CBS-KNAW Fungal Biodiversity Center Utrecht, The Netherlands. In addition, the strain Pt1 and Arg3 were isolated from wilted pea plants collected at Alvaiázere, Portugal and Setif, Algeria respectively. Isolation of fungal colonies on surface-sterilized wilted pea fragments was performed as described previously (Bani *et al.*, 2012) and maintained as single-spore colony. They have been deposited in the collection of Institute for Sustainable Agriculture, IAS-CSIC, Córdoba, Spain. The fungal strain was stored as microconidial suspensions at -80 ºC in 30% glycerol. For microconidia production, cultures were grown in potato dextrose broth (PDB; Difco, Detroit, MI) at 28 ºC in a shake culture set at 170 rpm (Di Pietro and Roncero, 1998). For toxin production, Erlenmeyer flasks (500 ml) containing 200 ml of Czapek-Dox medium (5% glucose, 0.1% yeast extract, 0.05% K_2HPO_4, 0.2% $NaNO_3$, 0.05% MgSO4•7H_2O and 0.001% FeSO4•7H_2O), were inoculated with 200 µl of fresh *Fop* microconidia at 10^9 conidia/mL and incubated under shaking at 28 °C for 7 days. The content of the flask was filtered on cheesecloth and centrifuged at 7,000 rpm for 10 min. The supernatant containing the secreted *Fop* toxin was then frozen at -80 ºC and lyophilized before further analysis. Morphological characterisation of each strain was performed at X 40 and X 63 magnification with a bright light microscope with Nomarsky filter.

2.3. DNA extraction, PCR amplifications and sequencing

Genomic DNA was extracted from *F. oxysporum* mycelium following previously reported protocol (Raeder and Broda, 1985). Molecular characterization of each *Fop* strain was performed by analysis of the internal transcribed spacers (ITS) and the 5' intron-rich portion of elongation factor alpha (EF-a). ITS and EF-alpha sequences were obtained by PCR amplification with primers ITS-1/ ITS-4 (Waalwijk *et al.*, 1996) and EF-1/EF2 (O'donnell *et al.*, 2009) respectively. Each 50 µl reaction mixture contained 50 ng of template DNA, 2 units of BioTaq DNA polymerase (BIOLINE), 1× PCR buffer, 2 mM $MgCl_2$, 200 µM dNTPs, and 0.3 µM of each primer. The PCR amplifications were performed on a MyCycler (BIORAD) thermocycler as follows: (i) for ITS, 94 °C for 2 min, 40 cycles at 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 2,5 min followed by a final step at 72 °C for 10 min; ii) for EF-a, 94 °C for 5 min, 30 cycles at 94 °C for 35 s, 52 °C for 35 s, and 72 °C for 1 min followed by a final step at 72 °C for 10 min. All amplifications were purified with the PCR cleanup kit of QIAgen and cloned in pGEMT vector (Promega). Two positive clones per amplicon were sequenced by STABVida (Setubal, Portugal) using the pGEMT vector specific primers SP6 and T7.

2.4. Sequence data analysis

Reads of each sequence were assembled and manually corrected with Chromaspro 1.7.5 (Technelysium). Sequences were used in BLAST (Altschul *et al.*, 1990) searches against the GenBank (http://www.ncbi.nlm.nih.gov/BLAST) and Mycobank (http://www.mycobank.org/BioloMICSSequences.aspx?expandparm=f&file=all) databases to identify the most similar sequences available in the databases. Pair-wise alignments were performed with the Neddleman-Wunsh algorithm (Needle method) implemented at EMBL-EBI webserver (http://www.ebi.ac.uk/Tools/psa/emboss_needle/nucleotide.html). All DNA sequences have been deposited in GenBank (http://www.ncbi.nlm.nih.gov/) under accession numbers KF913723 - KF913732 (Table 1).

Table 1. Comparison of ITS and EF-a sequence of the *Fop* isolates with the race 2 reference strain of *Fop* F42.

Fop isolates	ITS			EF-a		
	Genbank number	Length (bp)	Identity (%)	Genbank number	Length (bp)	Identity (%)
F42	KF913723	544	100	KF913728	713	100
F69	KF913724	544	100	KF913729	713	100
Arg3	KF913725	544	99.8	KF913730	714	99.0
Pt1	KF913726	545	99.8	KF913731	712	98.6
Fop1	KF913727	544	100	KF913732	714	99.0

2.5. Plant materials

Assignment of the different strains to a specific race of *F. oxysporum* f.sp. *pisi* was performed by testing the pathogenicity profile of these isolates on a set of differential pea lines (Table 2) (Kraft and Pfleger, 2001; Bani *et al.*, 2012). The comparison of the aggressiveness of each strain was performed on *P. sativum* cv. Messire. To determine the effect of fungal culture filtrates or their corresponding organic extracts, seven pea accessions with a wide range of response to *Fop* race 2 were used including the susceptible accessions JI1213, Messire and P629, the partially resistant accessions JI2480 and P615 and the resistant accessions P42 and P633.

For all experiments, germinated pea seedlings were sown in vermiculite and grown in controlled environment under a 16/8 h light-dark photoperiod at 26 ± 2 °C constant temperatures with 200 µmol m^{-2} s^{-1} of illumination. Plants were watered every three days with tap water.

Table 2. List of the pea differential lines for *F. oxysporum* f.sp. *pisi* and their susceptibility response to the new *Fop* isolates.

Accessions	Expected[a]				Observed[b]		
	R1	R2	R5	R6	F42	Arg3	Pt1
Dark Skin Perfection	R	S	S	S	S	S	S
Mini	S	R	S	S	PR	PR	R
New Era	R	R	S	S	nd	R	R
New Season	R	R	S	R	R	R	R
74SN5	R	R	R	R	R	R	R
P629	R	S	nd	nd	S	S	S
JI1412	R	R	nd	nd	R	R	R
Messire	R	S	nd	nd	S	S	S

[a] Accessions response to each *Fop* races as described in Kraft and Pfleger, 2001 and Bani *et al.* (2012). R stand for resistant reaction and S susceptible. [b] Accessions response to the inoculation with the reference R2 strain F42 and the isolates from Algeria and Portugal obtained in the present study

2.6. Pathogenicity test

To determine the pathogenicity of each *Fop* isolates, seven days old pea seedlings were inoculated with the dip root technique as described previously (Bani *et al.*, 2012) and maintained in the same growth condition as above. Disease was then evaluated every three days by estimating the percentage of leaves with symptoms and the area under the disease progression curve (AUDPC) (Bani *et al.*, 2012). Five plants were used per accessions and each experiment was repeated twice independently.

2.7. Extraction and purification of fusaric and 9,10-dehydrofusaric acids from fungal culture filtrates

Lyophilized *Fop* culture filtrates (200 ml) were re-dissolved in 1/10 of the initial volume with distilled water. The solutions were adjusted to pH 2.5 with 1M HCl and extracted with EtOAc (3 X 20 ml). The acidic organic extracts were combined, dried (Na_2SO_4) and evaporated under reduced pressure yielding brown oily residues (38.9, 101.7, 36.4, 42.7, 29.5 mg for F42, F69, *Fop1,*Arg3 and Pt1 respectively). The five acidic organic extracts were analyzed by TLC on silica gel [eluant EtOAc-MeOH-H_2O (8.5:2:1, v/v/v) and on reverse phase [eluent CH_3CN-H_2O (1:1, v/v)], in comparison with authentic standard samples of **1**, **2**, and their methyl esters. To confirm the presence of **1** and **2**, the residue of organic extract of F42, was purified by TLC eluted with CH_3CN-H_2O (1:1, v/v) yielding two pure solid compounds [**1**, R_f 0.50, eluent EtOAc-MeOH-H_2O (8.5:2:1, v/v/v), R_f 0.51 eluent CH_3CN-H_2O (1:1, v/v) and [**2,** R_f 0.40, eluent EtOAc-MeOH-H_2O (8.5:2:1, v/v/v), R_f 0.58, eluent CH_3CN-H_2O (1:1, v/v)] which were identified as fusaric and 9,10-dehydrofusaric acid as described below.

Fusaric acid (**1**). IR, UV, and ^1H NMR spectra were very similar to data reported ESIMS (+) *m/z*: 381 $[2M+Na]^+$, 202 $[M+Na]^+$: ESIMS (-) *m/z*: 178 $[M-H]^-$; APCIMS (+) *m/z*: 180 $[M+H]^+$ (Capasso *et al.*, 1996). Dehydrofusaric acid (**2**). IR, UV, and ^1H NMR spectra were very similar to data reported. ESIMS (+) *m/z*: 200 $[M+Na]^+$; ESIMS (-) *m/z*: 176 $[M-H]^-$ (Capasso *et al.*, 1996).

2.8. HPLC analysis of acidic organic extracts

A method previously reported (Amalfitano *et al.*, 2002), was optimized and used for analysis. The mobile phases employed were MeOH (eluent A) and 1% K_2HPO_4 adjusted to pH 7.35 with concentrate H_3PO_4 (eluent B). Elution was initially with A:B (50:50) which was transformed to a linear gradient over 20 minutes to A:B (75:25); the initial conditions were restored according to a linear gradient over 5 minutes, and the column was re-equilibrated under these conditions for 10 minutes before the next run was initiated. The flow rate was 1 ml/min and 20 µl aliquots of the samples were injected for analysis. Detection was performed at 268 nm, corresponding to the maximum of absorption of **1** and **2**. The HPLC calibration curves (the relevant data are in Table 3) for quantitative determination of **1** and **2** were performed with absolute amounts of standards dissolved in methanol in the range between 0.2 and 20 µg for each compound, in triplicate for each concentration. A HPLC linear regression curves (absolute amount against chromatographic peak area) for **1** and **2**, were obtained based on weighted values calculated from seven amounts of the standards in the above range. The samples were dissolved in methanol, passed through disposable filters and aliquots (20 µl) were injected into the HPLC instrument. Each sample was assayed in triplicate. The quantitative determination of **1** and **2** was calculated interpolating the mean area of their chromatographic peaks with the data of the calibration curves.

Table 3. Analytical characteristics of calibration curve[a] for FA (1) and DFA (2)

Compound	R_t (min)	Range (µg)	Slope	Intercept	r^2	Number of data point	LOD(µg) [b]
1	7.80 ± 0.5	0.5-20	8015,9	- 1700,5	0.999	6	0.5
2	5.20 ± 0.5	0.2-20	6449,5	- 1243,7	0.998	7	0.2

[a]Calculated in the form y=a+bx where y=chromatographic peak area and x=µg of metabolite injected. [b]Limit of detection

2.9. Recovery studies

Recovery studies were performed using the best producer isolate F69. Pure fusaric and 9,10-dehydrofusaric acids were added to the culture filtrate from 0.3 to 2.0 mg/L. The samples were prepared as described above and the extracts analysed by HPLC to

determine recovery. Three replicate injections were performed for each concentration. The recovery throughout the range was higher than 96 ± 2%.

2.10. Biological assays

2.10.1. Leaf absorption assay

The toxicity effect of fungal culture filtrate of isolate F42 of *Fop* race 2 was assayed by incubating fully expanded leaves in F42 culture filtrate. For this, the leaf petiole was immersed into an eppendorf tube containing 1 ml of one week old fungal culture filtrate before or after autoclaving at 121 ºC for 20 min and incubated at room temperature for 24 and 48 h. Four fully developed leaves were used for each pea accessions.

2.10.2. Leaf puncture assay

A leaf-puncture bioassay on pea leaves was performed to evaluate the toxic effect of fungal culture filtrates. Fully expanded leaves from pea plants were placed on Petri dishes containing water-agar medium and punctured by a sterile needle on the upper surface. Droplets (10 µl) of the culture filtrate or corresponding organic extract in 1% MeOH were applied on the wounded leaves. Plates were then incubated at room temperature under darkness. After 3 days of incubation, the area (mm^2) of the necrotic lesions was measured. Droplets of pure standard of FA and DFA were used as positive control reactions while droplets of sterile water, Czapek Dox medium or 1% MeOH were used as negative control. The experiments were performed with four replicates for each treatment.

2.11. Statistical analysis

Analyses of variance (ANOVA) were carried out for phytotoxicity records, with the different tested solutions and metabolites as fixed factors of their respective bioassays. One way ANOVA was also performed to test significance of aggressiveness difference between each strains. Phytotoxicity and percentage of symptoms values were transformed using the square root transformation in order to increase the normality of their distribution. Whenever the ANOVA test was statistically significant ($p \leq 0.05$), a Duncan's multiple range test assessing the differences of the means between each treatment was performed. All statistical analyses were performed using Genstat release 11.1 software (VSN International Ltd., Hemel Hempstead, UK).

3. Results and discussion

F. oxysporum species complex is composed by many species very closely related morphologically which make it difficult to identify. To ensure that all fungal strains corresponded to isolates of the race 2 of *F. oxysporum* f.sp. *pisi* these isolates were characterized at morphological and molecular levels before further analysis. As expected, all fungal strains showed morphological characteristics of *F. oxysporum* complex species. Amplification of ITS and EF-a gave sequences ranging from 544-545 nt and 710-714 nt long respectively according to the isolate (Table 1). These sequences showed between 99-100% identity to *F. oxysporum* sequences from different *formae speciales* including ff.spp. *pisi, ciceri, medicaginis* and *lycopersicii* according to the BLAST comparison performed. In addition they share 99.8-100% and 98.6-100% identity to the reference *Fop* race 2 strain F42 respectively (Table 1). This clearly identified them as *F. oxysporum* although it was not possible to identify the *forma specialis* to which they belong by these methods, hampered by the potential polyphyletic origin of the isolates and potential horizontal transfer of host-specificity genes (O'donnell *et al.*, 2009). Nevertheless, the *in planta* pathogenicity test indicated that they corresponded to *F. oxysoprum* f.sp. *pisi.* Indeed, all these strains were pathogenic on the susceptible pea cultivar Messire (Fig. 2). Slight differences in aggressiveness were observed between isolates as determined by their AUDPC value ($p < 0.001$). The strain F69 was the most aggressive reaching AUDPC values of 2388 while the isolates *Fop1* and Arg3 were the lesser aggressive reaching AUDPC of only 1678 and 1778.5 respectively (Fig. 2A). This difference was rooted to a faster disease development induced by the strain F69 that lead to complete plant death as early as 12 dpi while the Arg3 and *Fop 1* strain required 25 days to induce a similar effect (Fig. 2B). This was confirmed by the ANOVA that revealed significant differences between isolates only up to 15 dpi ($p < 0.001$) while at later stages the differences were not significant ($p > 0.5$). In addition, the inoculation of a set of differential lines (Kraft and Pfleger, 2001; Bani *et al.*, 2012) allowed assigning the new isolates Arg3 and Pt1 to the race 2 of *Fop* since accession response to these isolates followed a similar pattern as for the reference race 2 strain F42. Indeed, no or only mild symptoms were detected on the race 2 resistant accessions while the susceptible accessions Dark Skin Perfection, P629 and Messire developed characteristic wilt symptoms (Table 2). By contrast, *Fop*1 showed near wilt symptoms on most accessions with symptoms not developing further than leaf yellowing which impeded a clear classification of this strain to a specific race (data not shown). However, the very close molecular relationship between this isolate and the Algerian (Arg3) isolate as shown by the analysis of EF-a sequence (Table 1)

together with the near wilt symptoms detected would suggest that this isolate also belong to the race 2 of *Fop.*

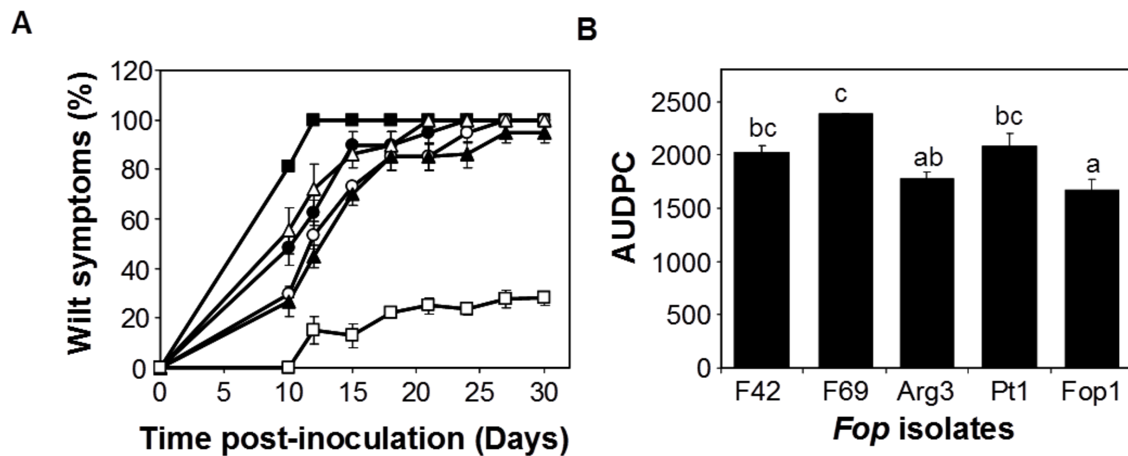

Figure 2. Pathogenicity of the *F. oxysporum* f.sp. *pisi* race 2 isolates on the susceptible pea cultivar Messire. **A**, evolution of fusarium wilt symptoms on the susceptible pea cultivar Messire induced by the *Fop* race 2 isolates F42 (●), F69 (■), Arg3 (○), Pt1 (△) and *Fop1* (▲) respectively, compared to control plants treated with water (□). Disease progression was estimated as percentage of leaf with symptoms over time. **B**, Comparison of AUDPC values calculated from the periodic assessment of fusarium wilt symptoms development. Different letters between each histograms indicates significant difference between value according to Duncan Multiple Range Test at α = 0.05.Vertical Bars are standard error for n = 5.

To characterize further the mechanisms of pathogenicity of *Fop* race 2 isolates that remains largely unknown we evaluated the effect of one week old fungal culture filtrates were tested on pea leaves. Incubation of pea leaves in F42 culture filtrate for 24 h induced leaf withering followed by leaf discoloration that initiate at the central vein and progress to cover the whole leaf and petiole as early as 48 h of incubation (Fig. 3A). Similar effects were detected on all pea accessions tested including on resistant accessions (Fig. 3B). Leaf puncture assay also showed a progressive spreading of necrosis and leaf discoloration after treatment with fungal filtrate while control remained symptomless (Fig. 3C). Broad effect of *F. oxysporum* culture filtrates was previously detected for *F. oxysporum* f.sp. *orthoceras* and *F. oxysporum* f.sp. *albedinis* which extract induced wilting on both host and non-host species (Dor *et al.*, 2007; Sedra and Lazrek, 2011). This supported the presence of non-specific toxin within these filtrates. In addition it indicated that resistance of the pea accessions tested was not based on toxin detoxification. This was also detected in *F. oxysporum* ff.spp. *melonis* (Megnegneau and Branchard, 1991) and *cubense* (Morpurgo *et al.*, 1994). However, only three resistant *Pisum* spp. accessions were tested here. Thus, it could not be discarded that the resistance mechanisms of other resistant pea accessions to *Fop* race 2 is not based on toxin detoxification as detected previously in near-isogenic lines of tomato differing in their susceptibility to *F. oxysporum* f.sp. *lycopersicii* (Sutherland and Pegg, 1995).

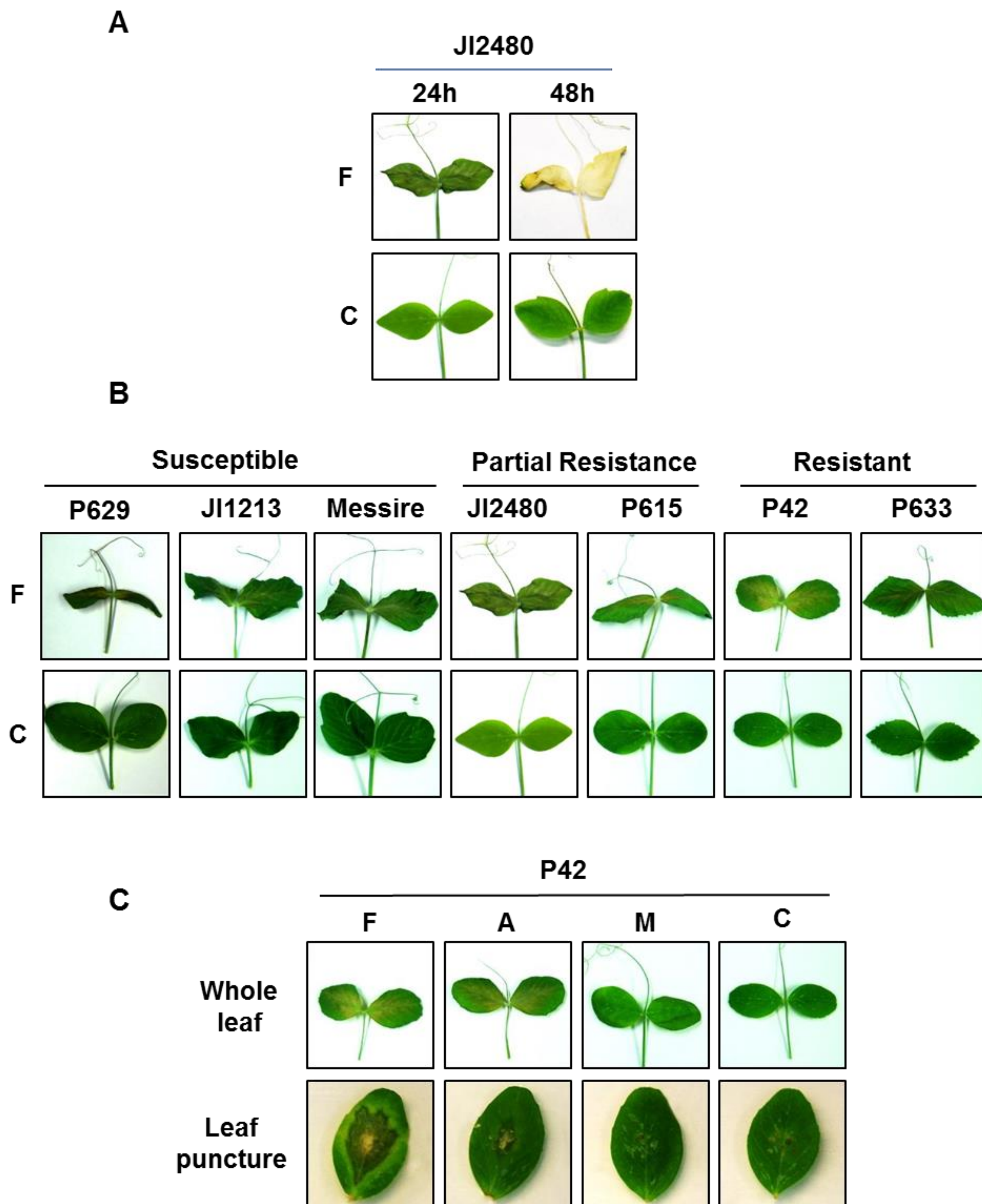

Figure 3. Effect of the culture filtrates of *F. oxysporum* f.sp. *pisi* race 2 isolate F42 on pea leaves. **A**, progression of wilting symptoms induced on the partially resistant accession JI2480 by the culture filtrates after 24 and 48h of treatment (F) compare to control with sterile water (C). **B**, Effect of the culture filtrates after 24h of treatment on leaves of seven pea accessions differing in their susceptibility to *Fop* race 2. **C**, Comparison of the leave response of the resistant pea accession P42 to culture filtrates (**F**), autoclaved culture filtrates (**A**), sterile Czapek Dox medium (**M**) and sterile water (**C**) evaluated with the leaf absorption and the leaf puncture assays.

Interestingly, organic extracts of all *Fop* race 2 isolates culture filtrates tested were able to induce leaf necrosis on Messire leaves when evaluated with the leaf puncture assay after 3 days of incubation (Fig. 4A). However, difference in lesion size could be detected between the strains ($p < 0.001$) well in agreement with the difference in aggressiveness of these strains on this pea cultivar (Fig. 4B). The lesion size ranged

from 16.8 mm^2 for Arg3 to 42.4 and 58.4 mm^2 for F42 and F69 culture filtrate respectively (Fig. 4B).

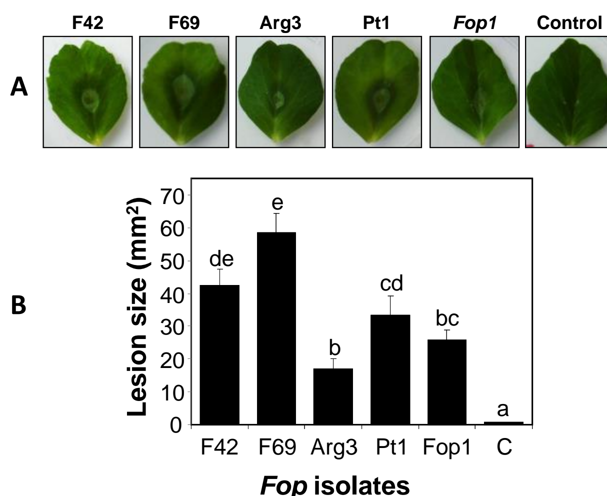

Figure 4. Effect of culture filtrates of the different *F. oxysporum* f.sp. *pisi* race 2 isolates on leaves of the susceptible pea cultivar Messire. **A**, Comparison of Messire leaves response to culture filtrates of each *Fop* race 2 isolates or sterile Czapek Dox medium (Control). **B**, Comparison of the lesion size induced by each isolate or by sterile Czapek Dox medium (C). Different letters between each histograms indicates significant difference between value according to Duncan multiple rangetTest at α = 0.05.Vertical bars represent standard errors for n = 4.

Various pathogenicity factors including cell-wall degrading enzyme, phytoalexin-detoxifying enzymes and toxins have been shown to mediate *F. oxysporum* virulence (Roncero *et al.,* 2003). By contrast to previous study that showed the complete loss of toxic activity after autoclaving culture filtrates (Sutherland and Pegg, 1995), autoclaving only slightly attenuated the toxicity of the filtrate on leaf absorption and puncture assays (Fig. 3C). This ruled out that the toxicity is due to proteins and suggest the presence of heat-stable metabolite as already found for other isolates of *Fop* (Sharma *et al*., 2010) and human pathogenic strains of *F. oxysporum* (Hernandes *et al*., 2012).

To identify the heat-stable metabolite(s) responsible for the toxicity of *Fop* race 2 culture filtrates, the organic extract of culture filtrates obtained from the isolate F42 was fractionated and further analyzed. TLC analysis of these fractions showed that this strain produced two toxins, identified as FA (**1**) and DFA (**2**) by comparison with standard samples. Their corresponding methyl esters were not detected. These results were confirmed by purification of **1** and **2** from this organic extract. ^{1}H NMR spectrum was identical to those previously reported (Capasso *et al*., 1996). The data obtained from ESI MS spectra further supported the isolation of **1** and **2**. The ESI MS recording in positive ion mode showed the sodiated dimeric form $[2M+Na]^+$ and the sodium cluster $[M+Na]^+$ at *m/z* 381 and 202, respectively. In APCI MS it showed the pseudomolecular ion $[M+H]^+$ at *m/z* 180. When the ESI MS was recorded in negative

ion mode, it showed the pseudomolecular ion $[M-H]^-$ at 178. The ESI MS spectrum of **2** recorded in positive and negative ion mode showed the sodium cluster $[M+Na]^+$ and the pseudomolecular ion $[M-H]^-$ at *m/z* 200 and 176, respectively. Further studies indicated that all *Fop* race 2 isolates investigated produced **1** and **2** but not their related methyl esters. Both toxins have been already identified from cultures of *F. oxysporum* pathogenic to the parasitic plants *Striga hermonthica* (Abraham and Hanssen, 1992; Savard *et al.*, 1997; Amalfitano *et al.*, 2002). However, it is the first time that **2** is reported from *F. oxysporum* isolates pathogenic to crops. No other toxins could be detected in any of these isolates. Production of toxins showed large qualitative and quantitative variation according to growth condition including growth medium, temperature and ambient pH between others (Loffler and Mouris, 1992; Amalfitano *et al.*, 2002; Dor *et al.*, 2007). Thus, it could not be ruled out that these strains may produce additional toxins as described for other *F. oxysporum* strains (Mirocha *et al.*, 1989; Irzykowska *et al.*, 2012).

Testing these acids with the leaf puncture assay indicated that both **1** and **2** possessed phytotoxic activity and induced necrotic lesion similar to that induced by the F42 culture filtrates (Fig. 5B). Comparison of lesion size indicated that **1** (55.8 mm^2) was 2.5 time more phytotoxic than **2** (22.6 mm^2). Many studies demonstrated the function of **1** in pathogenicity, although in several instance no correlation was detected between **1** content and fungal virulence (Bacon *et al.,* 1996; Gapillout *et al.*, 1996; Bouizgarne *et al.*, 2006). **1** was shown to alter membrane permeability, inhibit O_2 uptake, decrease mitochondrial activity, decrease transpiration rate and stomatal conductance, inhibit ATP synthesis and induce cell depolarization suggesting that **1** participate in *F. oxysporum* pathogenicity by decreasing plant cell viability (Bouizgarne *et al.*, 2006). By contrast, nearly nothing is known on the action of **2** (Capasso *et al.*, 1996). This compound was previously shown to induce tomato leaves chlorosis and inhibit root elongation to similar extent than **1** (Capasso *et al.*, 1996). Here we demonstrated that DFA was also phytotoxic on pea leaves (Fig. 4) indicating that it may also contribute to the pathogenicity of *F. oxysporum.*

To confirm the function of **1** and **2** in *Fop* race 2 pathogenicity, we quantified the production of both acids by all *Fop* race2 isolates tested and related it to their level of aggressiveness. For the quantification, we slightly modified a previously reported HPLC method (Amalfitano *et al.*, 2002). The characteristics of the calibration curves, the absolute range and the detection limits (LOD) of **1** and **2** are summarized in Table 3. Regression analysis suggests that the calibration curves are linear. A representative HPLC chromatogram of the ethyl acetate extract of the culture filtrates of *Fop* F42 is presented in Fig. 5A. The metabolite chromatographic peaks (*a*) and (*b*) in the sample

was coincident to the 7.80 min and 5.20 min retention times of **1** and **2** standards (Fig. 5A). The retention times were highly reproducible, varying less than 0.50 min. For all strains matrix substances absorbing at 268 nm were eluted within the first 20 minutes. **1** could be quantitatively and reproducibly detected from 0.5 μg, and **2** from 0.2 μg, with lower amounts having poor reproducibility.

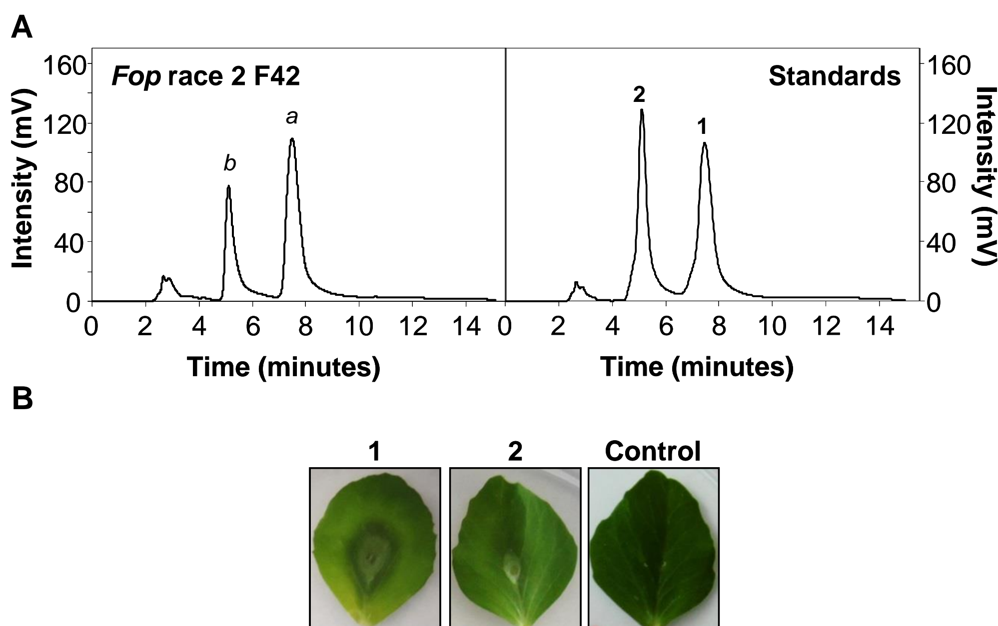

Figure 5. Characteristics of the main toxins produced by the *F. oxysporum* f.sp. *pisi* race 2 isolates. **A**, HPLC profile of the organic extract of the *Fop* race 2 isolate F42 (left) and fusaric and 9,10-dehydrofusaric acids (**1** and **2**) standards (right). **B**, Comparison of the leaf response of the susceptible pea cultivar Messire to droplets of pure **1** and **2.**

This method allowed detecting quantitative differences between *Fop* race 2 isolates ($p < 0.001$) for both toxins. F69 was the best producer strain for both **1** and **2** (258.49 and 243.36 mg/L, respectively) whereas the lesser producer strains were Arg3 for **1** (9.94 mg/L) and *Fop*1 for **2** (21.44 mg/L) (Fig. 6).

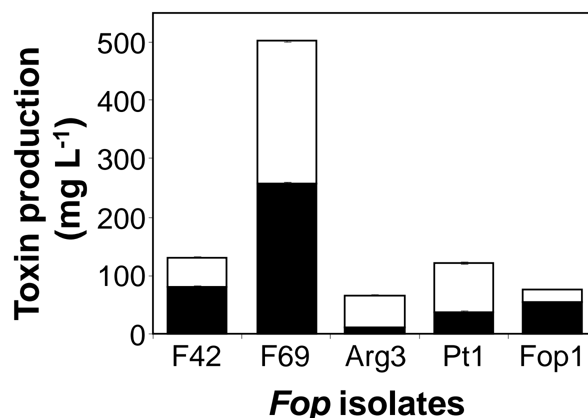

Figure 6. Quantification of **1** and **2** amounts produced by the differents isolates of *F. oxysporum* f.sp. *pisi* race 2. The histograms show the cumulative production of **1** (black column) and **2** (white column) for each *Fop* race 2 isolates. Vertical bars are standard error for n = 3.

The capacity to produce and secrete **1** was significantly and positively correlated with the leaf lesion size (r^2 = 0.83; Fig. 7A) and slightly correlated with *in planta* pathogenicity (r^2 = 0.66; Fig. 7B). Production of **2** was significantly correlated with *in planta* pathogenicity (r^2 = 0.8; Fig. 7D) and to a lesser extent with lesion size (r^2 = 0.66; Fig. 7C). Interestingly, the total toxin production was significantly correlated with both virulence parameters (r^2 = 0.78 and 0.76 for leaf lesion size and *in planta* pathogenicity respectively; Fig. 7E and F). This reinforced the important role of **1** in *F. oxysporum* pathogenicity as it was already demonstrated for *F. oxysporum* ff.spp. *carthami* (Chakrabarti and Chaudhary, 1980), *lycopersici* (Barna *et al.*, 1983; Toyoda *et al.*, 1988), *melonis* (Megnegneau and Branchard, 1988) and *gladioli* (Remotti an Loffler, 1996) between others. It also demonstrated that **2** is an important pathogenicity factor acting in synergy with **1** which was not described before for *F. oxysporum* (Fig. 7).

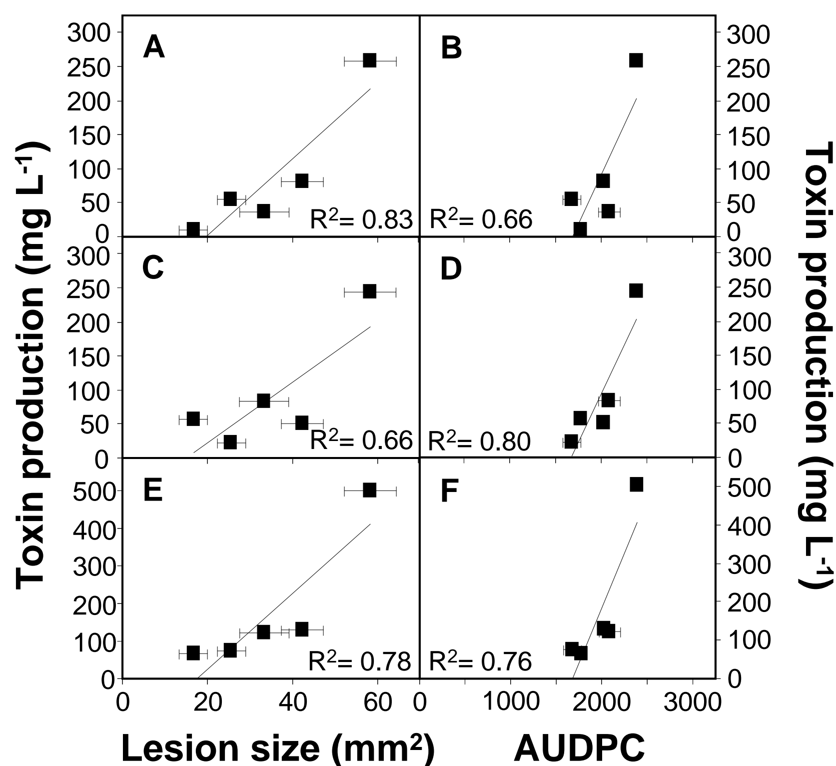

Figure 7. Relationship between the toxins production and phytotoxicity. The graphics represent the linear correlation calculated for **1** (A, B), **2** (C, D) and the sum of both toxins (E, F) production by each *Fop* race 2 isolates with the lesion size induced by their corresponding culture filtrate (A, C, E) or their overal aggressiveness on the susceptible pea cutivar Messire estimated by their AUDPC values (B, D, F). Horizontal bar are standard error for lesion size area or AUDPC values with n = 4 and n = 5 respectively while vertical bars are standard error bars of toxin content calculated with n = 3.

In conclusion, it has been demonstrated that *Fop* race 2 isolates mainly produced two toxins that were identified as FA and DFA. Both toxins showed high phytotoxic activity on pea when tested on whole leaves and leaf puncture assay. In addition, the amount of both toxins within culture filtrates correlated with strain aggressiveness which indicates a role of these toxins during *Fop* pathogenicity. Although the

importance of FA in the pathogenicity of *F. oxysporum* is still under debates, the results obtained in the present study strongly indicate that toxin production is an important determinant of *Fop* race 2 pathogenicity.

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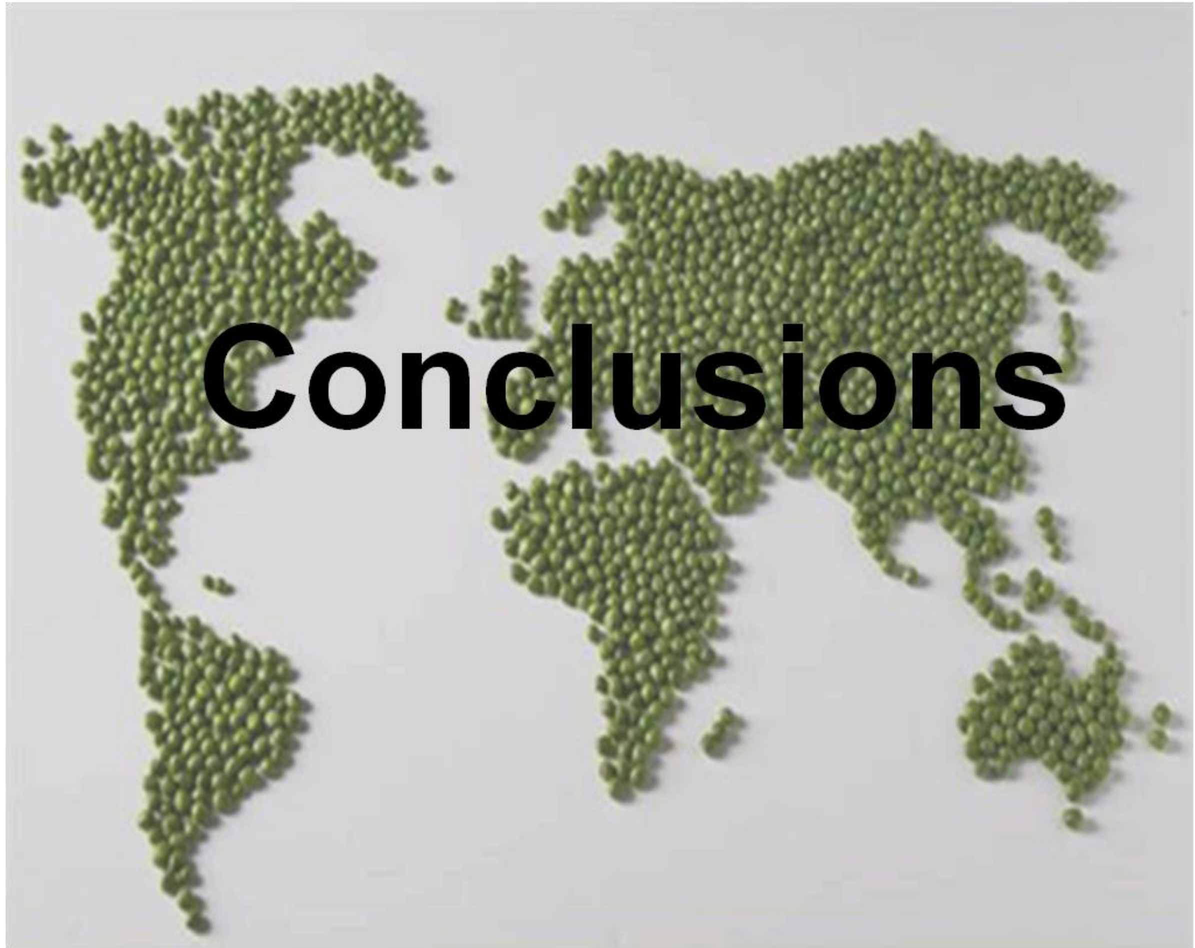
Conclusions

Conclusions

Chapter 1:

1- *Medicago truncatula* A17 genotype is partially resistant to *Fome* regardless of the experimental conditions (plant age, substrate and inoculation method).

2- *M. truncatula* response to *Fome* is not dependent on plant age at inoculation time.

3- Disease development is not affected by inorganic substrate type.

4- The root dipping method is the most adequate method of inoculation of *F. oxysporum* since it induces faster and stronger symptom development, and it allows clear discrimination between *M. truncatula* accessions.

5- Superficial leaf temperature assessment using infra-red imaging might be useful not only to check for *Fome* infection but also to screen for resistance against *F. oxysporum*.

Chapter 2:

1- A wide range of responses to *Fop* race 2 was identified in the *Pisum* spp. collection studied. Responses of accessions varied from high resistance to susceptibility in a quantitative manner.

2- Eleven accessions were identified showing a very high level of resistance to *Fop* race 2. They may be useful for breeding.

3- The methodology developed and applied to test for resistance to *Fop* in *Pisum* is robust and reproducible.

4- Disease severity assessment is sufficient to discern different categories of responses. AUDPC appears as the most accurate and precise discriminating parameter. However, it is more laborious and time consuming.

5- The main resistance mechanisms acted in the resistant accessions at root and crown level.

Chapter 3:

1- *Fop* spore germination is stimulated by root exudates from both host (pea) and non-host species confirming that the stimulating signal is not host dependent.

2- Genetic variation has been identified in pea germplasms for induction of *Fop* spores germination by root exudates, with 3 accessions whose root exudates inhibited germination.

3- The amount of pisatin in the root exudates of the *P. sativum* ssp. *sativum* accessions was negatively correlated with the level of *Fop* germination induced by these root exudates.

4- The constitutive amount of pisatin accumulated in root exudates play an important role in this pre-penetration resistance mechanism.

5- Beside pisatin, additional exuded compounds contribute to the inhibition of *Fop* germination.

Chapter 4:

1- Resistant accessions develop several barriers at the epidermis, exodermis, cortex, endodermis and vascular stele efficiently impeding fungal progression.

2- The main detectable differences between accessions concern the spatial expression of the plant defence reactions and their intensity.

3- The main components of these different barriers are the accumulation of phenolic compounds, lignin and carbohydrates.

4- Three barriers at the epidermis, exodermis and cortex were very effective in restricting transverse centripetal progression of *Fop* in the outermost root tissues of resistant accessions.

5- The strengthening of the endodermis is the main mechanism preventing the spreading of *Fop* to the vascular stele in the partially resistant accessions.

6- Vascular occlusion, cell wall strengthening and the accumulation of different substances around infected vessels were very effective in restricting the vertical progression of *Fop* towards the upper section of the plants and the horizontal colonization of the rest of the cells from the initially infected vessel.

7- These responses could also be detected in susceptible accessions, but they appeared later and at lower levels, being ineffective to block pathogen progression.

8- The cell degradation surrounding the endodermis in susceptible accession support the important role of cell wall degrading enzymes (CWDEs) in *F. oxysporum* pathogenicity.

9- The resistant accessions develop chemical mechanisms to inhibit and/or restrain the activity of CWDEs.

Chapter 5:

1- A total of 132 proteins were differentially accumulated in response to *Fop* infection in the three contrasting pea accessions studied of which 53 were successfully identified.

2- Principal component analysis and clustering analysis revealed that the protein profile clustered according to the pea accessions while only discrete differences were detected between treatments.

3- The protein differentially expressed in response to the pathogen were related to carbohydrate and energy metabolism, nucleotide and amino-acid metabolism, signal transduction and cellular process, protein folding and degradation, redox and homeostasis, defence, biosynthetic process and transcription/translation.

4- An important depletion in enzymes related with the carbohydrate and energy metabolism have been detected in all three accessions upon *Fop* infection

5- Susceptible and partially resistant accessions mainly accumulated protein related with the redox homeostasis including ascorbate peroxidase and NADP-thioredoxin reductase.

6- An increase of the pentose shunt enzymes allowing the production of NADPH was also detected in the susceptible and partially resistant accessions.

7- The protein changes detected in the susceptible and partially resistant accessions indicate a change in the metabolic fluxes toward the detoxification of reactive oxygen species in these accessions.

8- By contrast, the resistant accessions accumulated mainly proteins related to the formation of phenolic compounds and cell wall strengthening.

Chapter 6:

1- *Fop* race 2 isolates mainly produced two toxins that were identified as fusaric acid and 9,10-dehydrofusaric acid.

2- Both toxins showed high phytotoxic activity on pea when tested on whole leaves and leaf puncture assays.

3- The amount of both toxins within culture filtrates correlated with strain aggressiveness which indicates an important role of these toxins in *Fop* pathogenicity.

Annex

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BSPP

A detailed evaluation method to identify sources of quantitative resistance to *Fusarium oxysporum* f. sp. *pisi* race 2 within a *Pisum* spp. germplasm collection

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Fusarium oxysporum f. sp. *pisi* (*Fop*) is an important pathogen of field pea (*Pisum sativum*) worldwide. The constant evolution of the pathogen drives the necessity to broaden the genetic basis of resistance to *Fop*. To achieve this, it is important to have a large germplasm collection available and an accurate and efficient method for disease assessment. Here, a detailed evaluation method coupling disease incidence, disease rating over time and its related area under the disease progression curve (AUDPC) was established and used to screen a *Pisum* spp. germplasm collection against one isolate of *Fop* race 2. A large variation in the disease response of specific pea accessions ranging from highly resistant to susceptible was observed within the collection, indicating the quantitative expression of the resistance. The repetition of the inoculation experiments on a subset of 19 accessions, including two susceptible accessions, indicated that the scoring method was robust and reproducible and confirmed the highly resistant phenotypes of 11 accessions. To initiate the characterization of resistance mechanisms within these accessions, the external and internal stem symptoms were compared between these selected pea accessions, together with the extent of fungal colonization within plants. All these tests indicated that, in all resistant accessions, the resistance mechanisms efficiently stopped pathogen progression at the crown. Incorporation of these sources of resistance to breeding programmes will contribute to improved *Fop* resistance in pea cultivars.

Keywords: *Fusarium oxysporum* f. sp. *pisi*, *Pisum sativum*, quantitative resistance, resistance mechanisms, screening method

Introduction

Fusarium wilts are among the most important diseases affecting grain legumes throughout the world (Kraft *et al.*, 1998). *Fusarium oxysporum* f. sp. *pisi* (*Fop*) is an important and destructive pathogen of field pea (*Pisum sativum*). It has been reported in every country where pea is grown (Kraft & Pfleger, 2001). This soilborne pathogen can survive as thick-walled chlamydospores, which remain viable in the soil for more than 10 years (Kraft, 1994). The infection cycle of *F. oxysporum* is initiated by the germination of spores in the soil in response to an undetermined signal within the host root exudates (Di Pietro *et al.*, 2003). Upon germination, infective hyphae adhere to the root surface and penetrate the root epidermis directly without the formation of any distinctive structure (Bishop & Cooper, 1983a; Rodriguez Galvez & Mendgen, 1995). The mycelium then advances inter- or intracellularly through the root cortex, until it reaches the xylem vessels and enters them through the pits (Bishop & Cooper, 1983b; Beckman, 1987). At this point, the fungus switches to an endophytic mode of host colonization, during which it remains exclusively within the xylem vessels, using them as avenues to rapidly colonize the host (Bishop & Cooper, 1983b). At this stage, the characteristic wilt symptoms appear as a result of severe water stress, which ultimately lead to complete plant death. Upon plant death, pathogenic hyphae grow outward from the vascular tissue and begin to intensely sporulate on the plant surface (Di Pietro *et al.*, 2003).

Characterization of *Fop* isolates according to their capacity to induce disease in a set of differential lines, their assignment to specific vegetative compatibility groups and the establishment of their molecular fingerprint profiles allowed the identification of four different races of *Fop* (races 1, 2, 5 and 6) (Haglund & Kraft, 1979; Correll *et al.*, 1987; Grajal-Martin *et al.*, 1993). Races 1 and 2 occur worldwide, while races 5 and 6 are, to date, only important in western Washington State, USA (Infantino *et al.*, 2006). In addition, *Fop* is continually evolving, with new variants of the pathogen emerging (Bodker *et al.*, 1993; Kraft & Pfleger, 2001). As for many soilborne pathogenic fungi, the use of fungicides is not necessarily effective in controlling fusarium wilt (Sharma *et al.*, 2010). As a consequence, control of this disease is achieved mainly by integration of different disease management procedures including agronomic and farming practices (Navas-Cortes *et al.*, 1998), soil disinfestation

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(Momma *et al.*, 2010), biocontrol (Alabouvette *et al.*, 2009) and breeding for resistance (Sharma *et al.*, 2010). Among these methods, the use of resistant cultivars is widely recognized as the safest, most economical and effective method for protecting crops from this disease. Fortunately, resistance to *Fop* in pea is conferred by single race-specific genes that have been successfully transferred to pea cultivars (Infantino *et al.*, 2006). Although the use of these resistant pea cultivars has proven effective in controlling this disease, there is a constant risk of resistance breakdown, because monogenic resistance can be easily overcome by the emergence of new pathogen variants. A continuous search for novel resistance sources to complement and strengthen the resistance of elite cultivars is thus essential, with an emphasis on resistance sources based on quantitative and polygenic mechanisms. However, sources of fusarium wilt resistance in pea are limited (Ali *et al.*, 1994). To broaden the genetic basis of resistance it is important to evaluate large and diverse germplasm collections, including wild species, and to use precise and accurate screening techniques (Infantino *et al.*, 2006).

Different screening methods for *Fop* resistance have been described although most of them only consider the disease incidence (DI) or the proportion of symptomless plants to classify accessions as resistant or susceptible (Haglund, 1989; McPhee *et al.*, 1999; Sharma *et al.*, 2010). This disease scoring method may not be adequate for quantitative resistance, where there is a continuous gradient of symptom severity (Russell, 1978). Here, a more detailed disease scoring method that considers not only DI, but also the progression of disease symptoms, was established and tested on a set of differential lines and a *Pisum* spp. germplasm collection for resistance to *Fop* race 2. This screening method detected the existence of quantitative resistance to the pathogen within this collection and identified several *Pisum* accessions with high resistance that have a good potential for improving pea resistance to race 2 of *Fop*.

Materials and methods

Fungal isolates and cultural conditions

Fusarium oxysporum f. sp. *pisi* race 2 strain R2F42 was kindly provided by Dr W. Chen (USDA-ARS, Pullman, USA) for use in all the experiments. The fungal strain was stored as microconidial suspensions at −80°C in 30% glycerol. For microconidia production, cultures were grown in potato dextrose broth (PDB; Difco) at 28°C in a shake culture set at 170 rpm (Di Pietro & Roncero, 1998). To determine the extent of *Fop* colonization, the fungus was reisolated from the root and the basal, middle and apical stem regions of three inoculated pea plants of four susceptible and 13 resistant accessions 7 days post-inoculation (dpi), following the method described by Lichtenzveig *et al.* (2006), except that the 1 cm long fragments were plated on PDA containing 0·1 mg mL^{-1} kanamycin and incubated at 28°C for 3 days. With this method, typical colonies of *Fop* can be easily detected as white filaments emerging from the plant tissue and colonizing the PDA medium.

Plant material and growing conditions

A collection of 80 accessions of *Pisum* spp. of diverse origin was used in this study. The collection was composed of seven *P. sativum* cultivars from the USDA core collection of the differential set for the four races of *Fop* (Table 1) and 73 accessions from the John Innes pea collection that had been obtained from different countries.

Pea seeds were surface-sterilized for 20 min in a 20% solution of sodium hypochlorite and then rinsed three times with sterile water. The seeds were wrapped in wet filter paper in a Petri dish, stratified for 2 days at 4°C in the dark and incubated at 26 ± 2°C until germination. Once germinated, the seedlings were transferred to pots (6 × 6 × 8 cm) containing sterile vermiculite (1–3 mm diameter) and grown in a controlled environmental chamber under a 16/8 h light-dark photoperiod at 26 ± 2°C temperature regime with 200 μmol m^{-2} s^{-1} of illumination. Plants were watered every 3 days with tap water.

Inoculation and disease assessment

Seven-day-old *Pisum* spp. seedlings (2–3 node stage) were inoculated following a modified version of the dip technique described by Haglund (1989). For this procedure, vermiculite was removed from the roots which were trimmed by a third and immersed for 5 min in a suspension containing 5×10^6 microconidia mL^{-1} of water. Control plants were treated in the same way and were immersed in sterile water. Seedlings were planted in individual pots containing sterile vermiculite and maintained in the same growth chamber.

Screening of the whole collection including the differential lines was first performed on five seedlings per *Pisum* accession. The 15 most resistant accessions were then tested twice in independent experiments along with two partially resistant and two susceptible accessions, with five seedlings per accession and per experiment.

Disease symptoms were assessed every 3 days from 10 to 30 dpi. Two different leaf symptom-based approaches were used to estimate the disease symptom rate (DR) at the leaf and the whole plant level. At the whole plant level, DR was evaluated as the percentage of leaves showing symptoms for each individual plant (PSL) (Fig. 1a). At the leaf level, evaluation of DR was established by assigning a visual index ranging from 1 (healthy leaf) to 5 (dead leaf) to each leaf within a plant and reporting these values for each individual plant by calculating the mean value of the visual index (MVI) of all its leaves (Fig. 1b). These data were used to calculate the area under the disease progression curve (AUDPC) using the formula.

$$\text{AUDPC} = \Sigma[(x_i + x_{i+1})/2] * (t_{i+1} - t)$$

where x_i = estimated proportion of disease severity at date i, x_{i+1} = estimated proportion of disease severity at

Table 1 Disease ratings of differential lines of *Pisum sativum* to *Fusarium oxysporum* f. sp. *pisi* (*Fop*) race 2

Accession	*Fop* 2	DI (%)	Disease ratings[a]			
			Plant mean disease index		Leaves showing symptoms (%)	
			MVI	AUDPC	PSL	AUDPC
Little Marvel	S	100	5·0 ± 0·00 b	84·6 ± 5·40 b	100·0 ± 0·00 b	2250 ± 0·0 b
Dark Skin Perfection	S	100	5·0 ± 0·00 b	57·9 ± 8·92 b	100·0 ± 0·00 b	1519 ± 20·6 b
New Season	R	0	2·3 ± 0·24 a	13·2 ± 2·56 a	43·4 ± 6·18 a	514 ± 74·7 a
Mini	R	20	3·1 ± 0·52 a	12·1 ± 4·79 a	61·3 ± 10·57 a	438 ± 137·1 a
Mini 93	R	40	3·4 ± 0·73 a	23·3 ± 8·42 a	65·0 ± 15·00 a	741 ± 184·9 a
Wsu31	R	20	3·0 ± 0·52 a	24·7 ± 4·31 a	59·9 ± 10·78 a	846 ± 215·7 a
74SN5	R	20	3·0 ± 0·62 a	18·6 ± 2·73 a	66·9 ± 12·52 a	622 ± 97·1 a

[a]Data are means of five replicates; different letters indicate significant difference between values according to Duncan's multiple range test at α = 0·05. DI, disease incidence; MVI, mean value of visual index for all leaves on an individual plant; PSL, percentage of leaves showing symptoms for each individual plant.

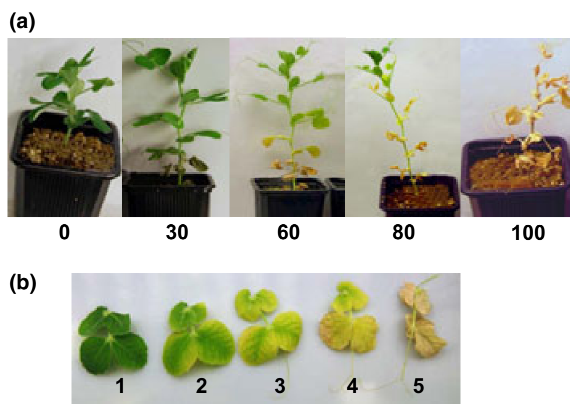

Figure 1 Evolution of disease symptoms induced by *Fusarium oxysporum* f. sp. *pisi* (*Fop*) race 2 on susceptible pea accessions. (a) The photographs represent the evolution of fusarium wilt symptoms at whole plant level of susceptible pea accessions inoculated with one isolate of *Fop* race 2. Numbers below each photograph represent their respective disease ratings, estimated as the percentage of leaves showing symptoms. (b) Typical progression of fusarium wilt disease of susceptible pea accessions inoculated with *Fop* race 2 at leaf level. Numbers under each leaf indicate its respective disease ratings value based on a disease index scale ranging from 1 (healthy leaf) to 5 (dead leaf).

date i+1, and $t_{i+1}-t_i$ = number of days between scoring dates i and i+1. Disease incidence (DI), determined as the proportion of dead plants, was also scored at 30 dpi.

To classify accessions as resistant or susceptible, their disease symptoms were compared to those of accessions P627 and P21 used as resistant and susceptible controls, respectively. In addition, the differential lines New Season (resistant) and Little Marvel (susceptible) were included in order to confirm their response to the infection.

Whole plant staining

To detect the extent of fungal colonization, three plants of the 12 most resistant accessions and the susceptible accession P21 were harvested 30 dpi, washed with sterile water to remove any unadhered *Fop* microconidia, cleared with 2·5% KOH at 90°C for 1 h, rinsed twice with deionized water and incubated overnight at room temperature in a solution of 1% HCl. The root and stem regions were then stained in a 1% Parker blue Quink ink aqueous solution for 30 min at 60°C and destained 16 h at room temperature in lactoglycerol. The resulting stained tissues were then stored at room temperature in 100% glycerol until observation under a binocular microscope. Following this treatment stained fungal structures were clearly visible.

Detection of internal symptoms

A red–brown discoloration within plant tissue has been previously shown to be associated with *F. oxysporum* infection in field peas (Kraft & Pfleger, 2001). In order to observe this red–brown discoloration within pea plant tissue, the basal and middle part of the stem and the upper part of the root system of three plants from the 12 most resistant accessions were harvested at 30 dpi, longitudinally cut with a razor blade and observed under a Nikon SMZ1000 binocular microscope (Nikon Europe B.V.). For comparison, three plants of the susceptible accession P21 were harvested at 10 dpi and treated in the same way.

Statistical analysis

To analyse the significance of the differences in DR and DI between the different pea accessions to *Fop*, all data obtained from the DI, MVI, PSL and AUDPC values were subjected to an analysis of variance (one-way ANOVA). Percentage PSL and DI data were subjected to an angular transformation to normalize the data and stabilize the variances before being subjected to the analysis of variance (Baird *et al.*, 2002). Whenever the ANOVA was statistically significant ($P \leq 0{\cdot}05$) for a specific variable, a Duncan's multiple range test was conducted to assess the differences of the means between each accession. The coefficient of correlation existing between the different disease parameters was calculated using the non-parametric Spearman's rank correlation coefficient analysis.

All statistical analyses were performed with the GENSTAT release 11·1 software (VSN International Ltd.).

Results

Disease development

The assessment of susceptible cultivars including Little Marvel and Dark Skin Perfection differential lines showed that the initial symptoms appeared on the primary leaves around 10–15 dpi and sequentially reached the later-formed leaves until the whole plant withered and died (Fig. 1a). At the leaf level, the disease symptoms initiated at the leaf margins, which yellowed and/or curled downward. Leaf yellowing was associated with necrosis until the whole leaf wilted and became dry and brittle (Fig. 1b). These observations allowed the development of the two scales to estimate disease rate (DR).

Validation of the scoring method on differential lines of *P. sativum*

Before applying these screening methods to the *Pisum* spp. germplasm collection, they were tested on seven well-described differential lines. As expected, Little Marvel and Dark Skin Perfection lines were highly susceptible to the isolate of *Fop* race 2, showing a DI of 100% and DR values at 30 dpi of 5 and 100% according to the MVI and PSL evaluation methods, respectively (Table 1). Similarly, the resistant differential cultivars (Haglund & Kraft, 1979) were all resistant, with DI values ranging from 0 for New Season to 40% for Mini93. DR scores at 30 dpi ranged from 2·3 for New Season to 3·4 for Mini93 for the MVI scale, and from 43·4% for New Season to 66·9% for 74SN5 according to the PSL scale. The AUDPC values calculated from the DR data sets ranged from 12·1/438 (AUDPC MVI/AUDPC PSL) for Mini to 24·7/846·4 for Wsu31 over the whole experiment (Table 1). The statistical analysis performed on both DR scales and their associated AUDPC values indicated significant differences between race differential cultivars ($P < 0{\cdot}001$) and a clear discrimination between the susceptible and resistant genotypes (Table 1).

Screening of wild *Pisum* spp. collection against *Fop* race 2

The same scoring parameters were used to screen a collection of 73 *Pisum* spp. accessions to identify new sources of resistance to *Fop* race 2 (Tables 2 & 3). Large variation in the disease response was detected among the *Pisum* spp. accessions for all the parameters monitored (Fig. 2, Table 2). Thus, DI ranged from 0 to 100%, DR ranged from 1·3/20% to 5/100% according to MVI/PSL parameters and from 4·0/125 to 90·0/2220 for their respective AUDPC values (Table 2). The continuous distribution of the pea accessions for the different parameters monitored, as shown for their AUDPC values, indicated that resistance in this germplasm collection is quantitative (Fig. 2). The one-way ANOVA performed for all parameters detected statistically significant differences between accessions. Mean comparison analysis performed for the DR and AUPDC parameters failed to separate them in discrete groups except for the most resistant and susceptible accessions. To simplify classification, the accessions were separated into three main groups according to the result of the mean comparison test of their AUDPC values. All accessions not significantly different to the resistant control P627, according to the Duncan's multiple range test for both AUDPC parameters, were considered resistant. Accessions not significantly different from the susceptible control P21 were considered susceptible and the rest of the accessions were considered partially resistant. As expected, the differential lines New Season and Little Marvel were classified within the resistant and susceptible groups, respectively (Table 2). According to this classification, 18 accessions were categorized as resistant (24·7% of the collection), 25 accessions as partially resistant (34·3%) and 30 as susceptible (41%) (Fig. 2).

The independent repetitions of the assessment of 17 resistant and partially resistant accessions confirmed their low incidence and severity scores compared to the highly susceptible accessions P21 and P662 (Table 3). As expected, P21 and P662 showed a DI value of 100% and DR values of 5 and 100% for MVI and PSL measurements, respectively. Conversely, the DI of the resistant accessions ranged from 0 to 53% while MVI and PSL values varied from 1·8 and 30·0% for P633 to 3·5 and 68·8% for P18 (Table 3). Similarly, the AUDPC values of resistant accessions remained relatively low, ranging from 9·7/343·2 for the highly resistant P23 to 37·1/1264·6 for the partially resistant P316 according on the MVI and PSL scales, compared to 73·9/2028 to 83·5/2274 for the susceptible accessions P662 and P21, respectively (Table 3). Statistical analysis confirmed the differences between genotypes at $P < 0{\cdot}001$ for all parameters evaluated. In addition, the mean comparison tests allowed separation of the different accessions into three groups for all parameters, confirming the classification. The results obtained largely supported the data of the initial screening with very few exceptions. For instance, 11 out of the originally 15 resistant accessions were confirmed as resistant while the remaining four were classified as partially resistant along with P18 and P316 (Table 3).

To determine the most adaptable and easiest method of disease scoring for future screening of pea resistance to *Fop*, the correlation between the different parameters evaluated was examined using a non-parametric Spearman's correlation rank analysis. The highest and significant correlations were obtained between both AUDPC values ($r = 0{\cdot}942$; $P < 0{\cdot}001$) and both DR measurements ($r = 0{\cdot}925$; $P < 0{\cdot}001$). Similarly, a good positive correlation was observed between the AUDPC values and their respective DR evaluations at 30 dpi with Spearman's rank correlation of $r = 0{\cdot}803$ and 0·716 ($P < 0{\cdot}001$) for MVI and PSL, respectively (Table 4). By contrast, only a low correlation with $r \leq 0{\cdot}524$ was observed between DI and any of the other parameters (Table 3).

Table 2 Disease ratings of *Pisum* spp. accessions to *Fusarium oxysporum* f. sp. *pisi* (*Fop*) race 2

			Disease ratings[a]			
			Plant mean disease index		Leaves showing symptoms (%)	
Accession	Species	DI (%)	MVI	AUDPC	PSL	AUDPC
New Season (R)	*Pisum sativum*	0	2·3 ± 0·24 a–h	13·2 ± 2·56 a–e	43·4 ± 6·18 a–f	514 ± 74·7 a–c
P627	*P. sativum* ssp. *arvense*	0	1·8 ± 0·79 a–d	4·0 ± 3·51 a	24·0 ± 19·39 ab	118 ± 84·5 a
P656	*Pisum fulvum*	40	2·9 ± 0·79 b–j	5·2 ± 1·96 a–c	53·3 ± 18·50 b–h	244 ± 82·9 ab
P23	*P. sativum* ssp. *elatius*	0	2·12 ± 0·72 a–f	5·4 ± 3·16 a–c	48·8 ± 13·25 a–g	279 ± 75·8 ab
JI1760	*P. sativum* (cv. Consort-*af*)	20	1·9 ± 0·40 a–d	5·0 ± 2·54 ab	37·0 ± 12·93 a–e	309 ± 141·0 ab
P633	*P. sativum* ssp. *arvense*	0	1·4 ± 0·09 ab	6·5 ± 1·85 a–c	22·3 ± 1·72 a–c	311 ± 63·9 ab
JI1559	*P. sativum* (cv. Mexique 4)	0	1·8 ± 0·14 ac	6·9 ± 1·91 a–c	30·4 ± 3·88 a–d	475 ± 124·4 a–c
JI1412	*P. sativum* (cv. Marlin)	0	2·1 ± 0·19 a–e	12·5 ± 0·67 a–g	35·3 ± 4·38 a–e	485 ± 57·2 a–c
P669	*P. fulvum*	20	2·5 ± 0·45 a–g	8·6 ± 1·68 a–d	67·3 ± 13·01 d–l	529 ± 84·0 a–d
P42	*P. sativum* ssp. *arvense*	0	1·3 ± 0·18 a	8·1 ± 1·95 a–d	20·0 ± 9·35 a	536 ± 106·7 a–e
P638	*P. sativum* ssp. *arvense*	0	2·8 ± 0·09 a–h	10·7 ± 1·30 a–f	53·9 ± 4·82 a–g	577 ± 67·8 a–f
P632	*P. sativum* ssp. *arvense*	0	2·5 ± 0·26 a–g	17·0 ± 3·45 a–j	43·4 ± 9·00 a–e	649 ± 103·8 a–g
P613	*P. sativum* ssp. *tibetanicum*	20	2·2 ± 0·64 a–f	21·0 ± 17·26 a–k	48·2 ± 11·90 a–g	687 ± 393·2 a–h
P650	*P. sativum* ssp. *jormadi*	0	1·9 ± 0·32 a–d	12·6 ± 4·16 a–g	34·1 ± 10·18 a–f	712 ± 149·3 a–i
P615	*P. sativum* ssp. *elatius*	40	3·3 ± 0·75 c–l	14·1 ± 4·96 a–i	84·0 ± 16·00 h–n	716 ± 215·0 a–i
P614	*P. sativum* ssp. *elatius*	0	2·0 ± 0·18 a–d	13·1 ± 2·10 a–h	38·0 ± 4·18 a–e	731 ± 87·3 a–j
JI1766	*P. sativum* (cv. Barton-*af,st)*	100	5·0 ± 0·00 m	30·0 ± 0·00 a–o	100·0 ± 0·00 n	750 ± 0·0 a–j
P639	*P. sativum* ssp. *arvense*	0	1·9 ± 0·78 a–d	19·4 ± 14·02 a–k	28·0 ± 19·60 a–c	752 ± 395·7 a–j
JI1566	*P. sativum* (cv. Almota)	0	3·1 ± 0·26 c–k	25·1 ± 2·23 a–n	61·4 ± 8·09 b–j	840 ± 56·1 b–k
P634	*P. sativum* ssp. *arvense*	20	2·4 ± 0·70 a–g	19·6 ± 10·18 a–k	37·3 ± 16·81 a–e	841 ± 275·7 b–k
JI1747	*P. sativum* (cv. Almires)	40	3·6 ± 0·61 f–m	26·0 ± 4·24 a–n	70·7 ± 14·39 e–n	888 ± 98·8 b–l
P641	*P. sativum* ssp. *arvense*	0	3·0 ± 0·14 c–j	21·5 ± 1·44 a–l	53·9 ± 3·05 a–g	890 ± 41·9 b–l
P640	*P. sativum* ssp. *arvense*	0	2·9 ± 0·26 b–j	23·5 ± 3·61 a–m	62·8 ± 6·97 c–k	996 ± 126·8 c–m
P18	*P. sativum* ssp. *elatius*	40	3·3 ± 0·83 c–l	36·4 ± 20·12 d–p	57·6 ± 20·95 c–j	998 ± 514·5 c–m
P628	*P. sativum* ssp. *arvense*	40	3·6 ± 0·51 f–m	30·4 ± 6·69 a–o	72·2 ± 10·37 e–n	1090 ± 136·5 c–n
JI502	*P. sativum* (cv. Rondo)	80	4·7 ± 0·31 m–o	37·1 ± 4·33 e–p	92·5 ± 6·71 l–n	1165 ± 114·9 d–o
P621	*P. sativum* ssp. *jormadi*	20	2·7 ± 0·55 a–h	33·8 ± 7·66 c–o	55·4 ± 10·48 b–j	1181 ± 181·6 e–o
P637	*P. sativum* ssp. *arvense*	40	3·6 ± 0·59 e–m	31·7 ± 6·62 a–o	71·1 ± 10·59 e–n	1186 ± 124·6 f–o
P645	*P. sativum* ssp. *arvense*	20	2·9 ± 0·50 b–i	33·3 ± 8·77 b–o	55·6 ± 11·86 b–i	1193 ± 229·5 f–o
P636	*P. sativum* ssp. *arvense*	40	3·3 ± 0·71 d–l	32·5 ± 12·97 a–o	73·4 ± 9·81 e–n	1200 ± 316·5 f–o
JI2480	*P. sativum* (CGN 3352)	40	3·8 ± 0·44 g–m	28·3 ± 5·22 a–o	83·3 ± 9·43 f–n	1223 ± 165·1 f–o
JI2302	*P. sativum* (B76-197)	60	4·1 ± 0·56 h–m	36·2 ± 8·48 d–p	84·3 ± 10·20 g–n	1225 ± 189·1 f–o
P619	*P. fulvum*	80	4·4 ± 0·40 i–m	41·3 ± 14·80 h–r	93·3 ± 6·67 l–n	1232 ± 334·5 g–o
P316	*P. fulvum*	60	4·4 ± 0·60 i–m	39·0 ± 10·26 f–q	88·0 ± 12·00 j–n	1296 ± 201·1 g–p
P731	*P. sativum* ssp. *arvense*	80	3·8 ± 0·62 g–m	41·8 ± 15·66 i–r	96·7 ± 3·33 l–n	1331 ± 334·7 h–p
JI210	*P. sativum* (cv. Lucknow Boniya)	100	5·0 ± 0·00 m	45·7 ± 13·99 k–s	100·0 ± 0·00 n	1352 ± 315·2 i–q
P649	*P. sativum* ssp. *jormadi*	40	3·0 ± 0·74 c–j	38·0 ± 16·01 e–p	63·0 ± 13·97 d–l	1378 ± 361·6 j–r
P635	*P. sativum* ssp. *arvense*	60	4·6 ± 0·34 k–m	39·4 ± 9·32 g–q	93·3 ± 6·67 l–m	1418 ± 279·4 k–s
P11	*P. sativum*	40	3·7 ± 0·62 g–m	43·5 ± 11·02 j–s	72·0 ± 11·38 e–n	1429 ± 281·6 k–s
P68	*P. sativum* ssp. *elatius*	40	2·8 ± 0·92 a–h	40·7 ± 15·44 g–r	44·0 ± 23·15 a–e	1443 ± 329·8 k–s
P630	*P. sativum* ssp. *arvense*	80	4·5 ± 0·47 k–m	47·2 ± 9·44 k–u	93·3 ± 6·67 l–n	1471 ± 235·2 k–s
P626	*P. sativum* ssp. *arvense*	100	5·0 ± 0·00 m	50·2 ± 3·19 l–w	100·0 ± 0·00 n	1532 ± 106·3 l–t
P54	*P. sativum* ssp. *arvense*	80	4·6 ± 0·40l m	44·4 ± 4·19 j–s	93·3 ± 6·67 l–m	1563 ± 177·0 m–u
P631	*P. sativum* ssp. *arvense*	80	4·7 ± 0·33l m	50·2 ± 6·84 m–w	93·3 ± 6·67 l–n	1601 ± 103·6 m–v
P617	*P. sativum* ssp. *thebaicum*	100	5·0 ± 0·00 m	55·71 ± 11·47 o–y	100·0 ± 0·00 n	1656 ± 189·8 n–v
P643	*P. sativum* ssp. *arvense*	80	3·6 ± 0·34 e–m	53·0 ± 5·63 n–x	71·1 ± 7·00 d–m	1659 ± 123·8 n–v
P19	*P. sativum* ssp. *elatius*	100	5·0 ± 0·00 m	47·8 ± 5·04 k–v	100·0 ± 0·00 n	1684 ± 136·8 n–v
P670	*P. fulvum*	40	3·3 ± 0·69 c–l	46·2 ± 12·26 k–t	65·4 ± 14·49 d–l	1686 ± 177·9 n–v
P642	*P. sativum* ssp. *arvense*	80	4·6 ± 0·40 lm	63·4 ± 16·45 p–z	91·1 ± 8·89 l–n	1748 ± 310·9 n–v
P647	*P. sativum* ssp. *arvense*	100	5·0 ± 0·00 m	66·1 ± 9·42 q–z	100·0 ± 0·00 n	1778 ± 194·1 o–v
P648	*P. sativum* ssp. *arvense*	100	5·0 ± 0·00 m	63·9 ± 7·07 p–z	100·0 ± 0·00 n	1920 ± 105·1 p–v
P651	*P. sativum* ssp. *elatius*	80	4·4 ± 0·57 i–m	73·3 ± 16·66 t–z	86·7 ± 11·93 i–n	1942 ± 308·0 p–v
JI82	*P. sativum*	100	5·0 ± 0·00 m	67·9 ± 11·42 r–z	100·0 ± 0·00 n	1991 ± 216·7 q–v
P24	*P. sativum* ssp. *elatius*	80	4·4 ± 0·51 j–m	74·2 ± 12·37 u–z	90·0 ± 8·94 k–n	1999 ± 251·0 q–v
JI1951	*P. sativum*	100	5·0 ± 0·00 m	74·5 ± 3·00 u–z	100·0 ± 0·00 n	2021 ± 71·2 r–v
P691	*P. sativum* ssp. *elatius*	100	5·0 ± 0·00 m	69·8 ± 6·44 s–z	100·0 ± 0·00 n	2022 ± 140·0 r–v
P14	*P. sativum* ssp. *abysinicum*	100	5·0 ± 0·00 m	75·2 ± 6·37 v–z	100·0 ± 0·00 n	2063 ± 145·2 s–v
P629	*P. sativum* ssp. *arvense*	100	5·0 ± 0·00 m	75·2 ± 3·94 v–z	100·0 ± 0·00 n	2152 ± 70·1 t–v

Table 2 (Continued)

			Disease ratings[a]			
			Plant mean disease index		Leaves showing symptoms (%)	
Accession	Species	DI (%)	MVI	AUDPC	PSL	AUDPC
JI1213	*P. sativum* (cv. erylis)	100	5·0 ± 0·00 m	79·0 ± 5·15 x–z	100·0 ± 0·00 n	2153 ± 97·5 t–v
JI1210	*P. sativum* (cv. Erygel)	100	5·0 ± 0·00 m	77·7 ± 3·11 w–z	100·0 ± 0·00 n	2184 ± 40·7 uv
P667	*P. fulvum*	100	5·0 ± 0·00 m	86·5 ± 3·50 z	100·0 ± 0·00 n	2190 ± 60·0 uv
P666	*P. fulvum*	100	5·0 ± 0·00 m	86·0 ± 4·00 z	100·0 ± 0·00 n	2200 ± 50·0 uv
JI2840	*P. sativum* (RIL 15x399_68)	100	5·0 ± 0·00 m	82·5 ± 4·69 yz	100·0 ± 0·00 n	2250 ± 0·0 v
P21	*P. sativum* ssp. *elatius*	100	5·0 ± 0·00 m	90·0 ± 0·00 z	100·0 ± 0·00 n	2250 ± 0·0 v
P312	*P. sativum* ssp. *elatius*	100	5·0 ± 0·00 m	90·0 ± 0·00 z	100·0 ± 0·00 n	2250 ± 0·0 v
P623	*P. sativum* ssp. *transcaucasicum*	100	5·0 ± 0·00 m	90·0 ± 0·00 z	100·0 ± 0·00 q	2250 ± 0·0 v
P657	*P. fulvum*	100	5·0 ± 0·00 m	90·0 ± 0·00 z	100·0 ± 0·00 n	2250 ± 0·0 v
P659	*P. fulvum*	100	5·0 ± 0·00 m	90·0 ± 0·00 z	100·0 ± 0·00 n	2250 ± 0·0 v
P661	*P. fulvum*	100	5·0 ± 0·00 m	90·0 ± 0·00 z	100·0 ± 0·00 n	2250 ± 0·0 v
P662	*P. fulvum*	100	5·0 ± 0·00 m	90·0 ± 0·00 z	100·0 ± 0·00 n	2250 ± 0·0 v
P671	*P. sativum* ssp. *elatius*	100	5·0 ± 0·00 m	90·0 ± 0·00 z	100·0 ± 0·00 n	2250 ± 0·0 v
P675	*P. sativum* ssp. *elatius*	100	5·0 ± 0·00 m	90·0 ± 0·00 z	100·0 ± 0·00 n	2250 ± 0·0 v
Little Marvel (S)	*P. sativum*	100	5·0 ± 0·00 m	84·6 ± 5·40 z	100·0 ± 0·00 n	2250 ± 0·0 v

[a]Data are means of five replicates; different letters indicate significant difference between values according to Duncan's multiple range test at α = 0·05. DI, disease incidence; MVI, mean value of visual index for all leaves on an individual plant; PSL, percentage of leaves showing symptoms for each individual plant.

Detection of *Fop* within plant tissue

To detect the extent of *Fop* colonization within plants, the fungi were reisolated from the different parts (root and basal, middle and apical stem regions) of inoculated plants of 15 of the selected accessions. In the susceptible genotype P21 and the partially resistant accession P316, *Fop* colonies were detected at both extremities of all plated plant segments indicating that as early as 7 dpi, *Fop* had already colonized the whole plant in these accessions (Fig. 3). By contrast, *Fop* colonies were recovered only from root and basal stem segments of the other resistant and partially resistant *Pisum* accessions monitored (Fig. 3). While *Fop* colonies were detected on each

Table 3 Disease ratings of selected accessions to *Fusarium oxysporum* f. sp. *pisi* (*Fop*) race 2

			Disease ratings[a]			
			Plant mean disease index		Leaves showing symptoms (%)	
Accession	Species	DI (%)	MVI	AUDPC	PSL	AUDPC
P23	*Pisum sativum* ssp. *elatius*	20 ± 0·0 bc	2·3 ± 0·42 ab	9·7 ± 4·65 a	45·8 ± 8·45 a	343 ± 106·4 a
JI1412	*P. sativum* ssp. *sativum*	0 ± 0·0 a	2·1 ± 0·08 ab	12·2 ± 1·03 a	34·6 ± 2·83 a	440 ± 20·6 ab
JI1760	*P. sativum* ssp. *sativum*	7 ± 6·7 ab	2·4 ± 0·30 ab	10·4 ± 1·86 a	43·9 ± 7·48 a	477 ± 62·4 ab
P627	*P. sativum* ssp. *arvense*	7 ± 6·7 ab	2·1 ± 0·36 ab	15·2 ± 3·69 a	31·3 ± 8·93 a	479 ± 113·9 ab
P656	*P. fulvum*	33 ± 6·7 cd	3·6 ± 0·50 cd	12·8 ± 3·72 a	68·3 ± 11·76 b–d	505 ± 109·1 ab
P633	*P. sativum* ssp. *arvense*	0 ± 0·0 a	1·8 ± 0·12 a	11·7 ± 1·85 a	30·0 ± 2·78 a	507 ± 60·4 ab
P42	*P. sativum* ssp. *arvense*	0 ± 0·0 a	1·8 ± 0·14 a	12·2 ± 1·61 a	32·3 ± 3·33 a	571 ± 66·4 ab
JI1559	*P. sativum* ssp. *sativum*	0 ± 0·0 a	2·1 ± 0·12 ab	15·1 ± 2·69 a	32·8 ± 2·30 a	634 ± 79·9 a–c
P614	*P. sativum* ssp. *elatius*	0 ± 0·0 a	2·1 ± 0·12 ab	14·1 ± 1·30 a	35·6 ± 2·84 a	660 ± 49·0 a–c
P639	*P. sativum* ssp. *arvense*	7 ± 6·7 ab	2·1 ± 0·35 ab	18·4 ± 6·07 ab	34·3 ± 8·82 a	684 ± 172·1 a–d
P650	*P. sativum* ssp. *jormadi*	0 ± 0·0 a	1·9 ± 0·22 a	12·9 ± 2·29 a	34·0 ± 6·86 a	694 ± 97·4 a–d
P632	*P. sativum* ssp. *arvense*	0 ± 0·0 a	2·5 ± 0·11 ab	20·8 ± 1·89 ab	42·2 ± 4·06 a	715 ± 50·5 b–d
P638	*P. sativum* ssp. *arvense*	13 ± 6·7 a–c	2·9 ± 0·22 bc	19·3 ± 3·38 ab	54·5 ± 5·44 ab	755 ± 67··7 b–d
P669	*P. sativum* ssp. *elatius*	40 ± 20·0 cd	3·4 ± 0·40 cd	20·3 ± 4·06 ab	76·5 ± 7·63 cd	756 ± 84·6 b–d
P18	*P. sativum* ssp. *elatius*	53 ± 6·7 de	3·5 ± 0·50 cd	34·3 ± 9·78 c	68·9 ± 11·24 bc	965 ± 245·3 c–e
P615	*P. sativum* ssp. *elatius*	53 ± 6·7 de	3·8 ± 0·45 cd	29·9 ± 6·41 bc	83·8 ± 9·03 c–e	1010 ± 163·2 de
P316	*P. sativum* ssp. *arvense*	73 ± 0·7 e	4·1 ± 0·41 d	37·1 ± 6·25 c	88·6 ± 6·68 de	1265 ± 129·0 e
P662	*P. fulvum*	100 ± 0·0 f	5·0 ± 0·00 e	73·9 ± 0·43 d	100·0 ± 0·00 e	2028 ± 64·2 f
P21	*P. sativum* ssp. *elatius*	100 ± 0·0 f	5·0 ± 0·00 e	83·5 ± 2·91 d	100·0 ± 0·00 e	2274 ± 23·0 f

[a]Data are means of five replicates; different letters indicate significant difference between values according to Duncan's multiple range test at α = 0·05. DI, disease incidence; MVI, mean value of visual index for all leaves on an individual plant; PSL, percentage of leaves showing symptoms for each individual plant.

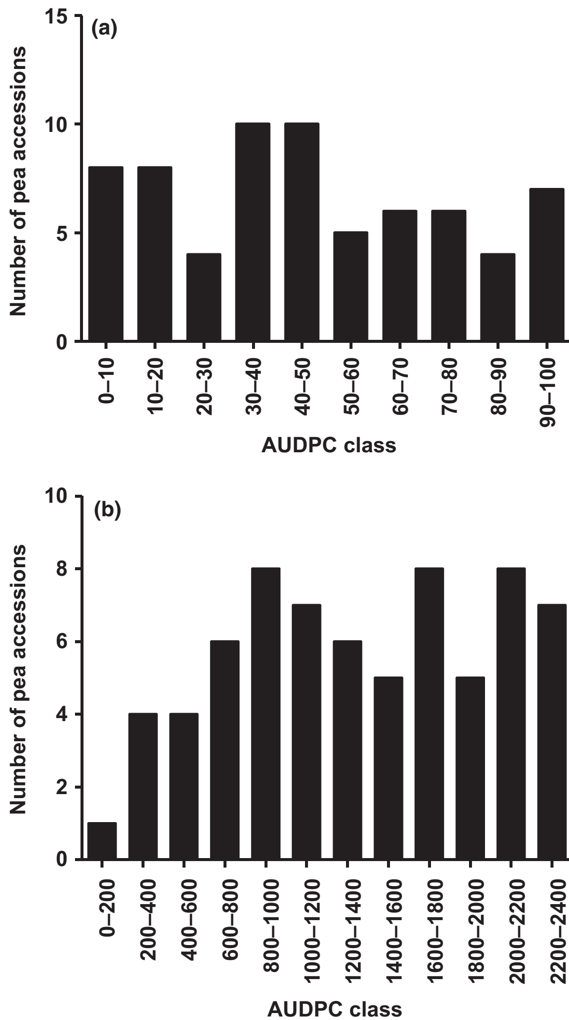

Figure 2 Distribution of the pea accessions upon inoculation with one isolate of *Fusarium oxysporum* f. sp. *pisi* race 2 according to AUDPC values calculated from the evaluation of disease ratings for mean value of visual index for all leaves on an individual plant, MVI (a) and percentage of leaves showing symptoms for each individual plant, PSL (b).

extremity of the basal segment in nine resistant and partially resistant genotypes including P23, P42, P614, P615, P627, P632, P633, P650 and JI1760, they were detected only at the lowest extremity in the other four resistant accessions P638, P639, P656 and P669 (Fig. 3).

Detection of external and internal symptoms

At the end of the experiment, plants from the selected accessions were removed from vermiculite and visually compared. Interestingly, the external area of the upper root system, the crown and the basal shoot region of resistant and partially resistant genotypes were black–brown in colour. The extent of this discoloration varied according to the genotype, the largest coloured area being observed in the highly resistant accession P42 (Fig. 4). This black–brown external discoloration was not

Table 4 Correlation of the different parameters assessed calculated according to Spearman's rank correlation

	DI	MVI	AUDPC (MVI)	PSL	AUDPC (PSL)
DI	1·000				
MVI	0·483**	1·000			
AUDPC (MVI)	0·312*	0·803**	1·000		
PSL	0·524**	0·925**	0·757**	1·000	
AUDPC (PSL)	0·298*	0·727**	0·942**	0·716**	1·000

*, **Statistical significance at $P < 0·01$ and $P < 0·001$, respectively. DI, disease incidence; MVI, mean value of visual index for all leaves on an individual plant; PSL, percentage of leaves showing symptoms for each individual plant.

detected on the susceptible accessions, suggesting that it might play a role in the resistance to *Fop* (Fig. 4). Observation under a binocular microscope of longitudinal sections of resistant accessions showed that the discolored root and shoot tissues were still healthy albeit brown, suggesting that this discoloration is due to an accumulation of pigmented substances within cells rather than cell death (Fig. 5). These sections also revealed the extent of differences in the typical vascular discoloration between accessions. As is characteristic for *Fop* race 2 infection, the vascular tissue of the whole plant of susceptible accessions such as P21 turned dark red (Fig. 5). By contrast, this vascular discoloration did not extend further than the plant crown in the resistant and partially resistant accessions, as shown for P633 in Figure 5.

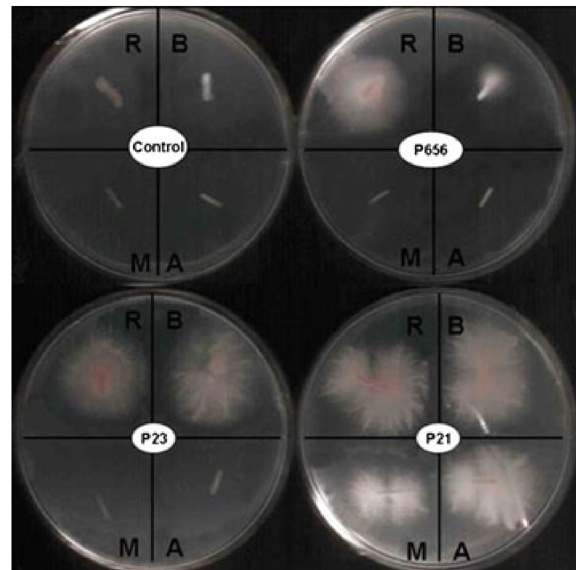

Figure 3 Isolation of *Fusarium oxysporum* f. sp. *pisi* (*Fop*) race 2 colonies from inoculated plants. Photographs compare the extension of *Fop* race 2 colonies out of plant tissues from control non-inoculated plants, and plants 7 dpi of the susceptible accession P21, the partially resistant accession P656 and the resistant accession P23. R, B, M and A stand for root, basal stem, middle stem and apex sections, respectively.

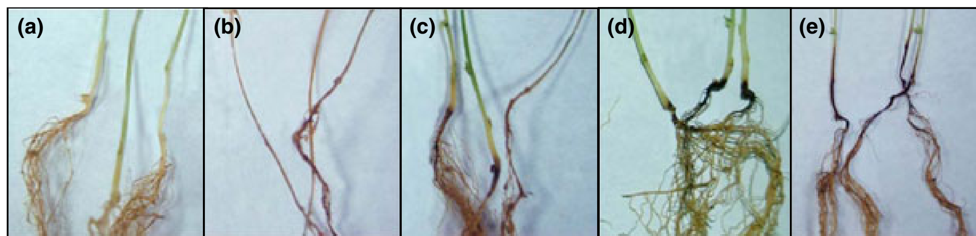

Figure 4 Comparison of *Fusarium oxysporum* f. sp. *pisi* -induced superficial crown darkening on different susceptible and resistant accessions. (a) Control plants of the susceptible accession P21, non-inoculated. (b) Susceptible accession P21 at 30 dpi. (c) Partially resistant accession P656 at 30 dpi. (d) Resistant accession P23 at 30 dpi. (e) Resistant accession P42 at 30 dpi.

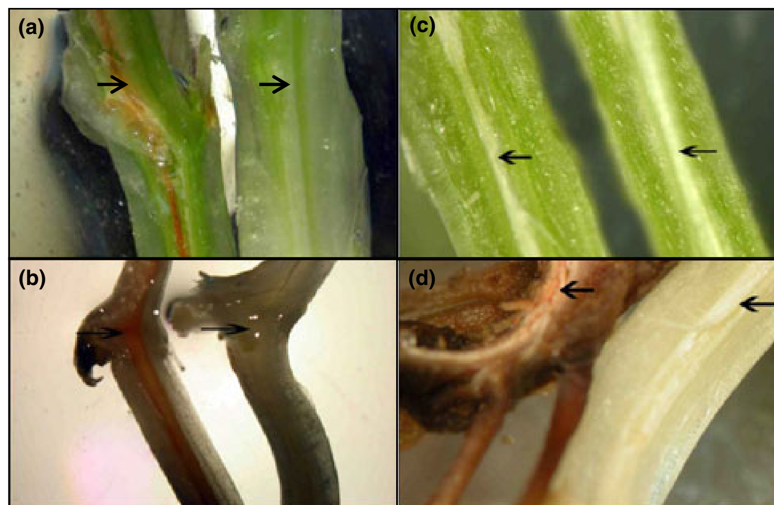

Figure 5 Comparison of the extension of the typical *Fusarium oxysporum* f. sp. *pisi* -induced vascular discoloration between susceptible and resistant pea accessions. Each picture shows hand-made longitudinal sections of stem (a, c) and crown (b, d) for inoculated (left) and non-inoculated control (right) plants. (a, b) Susceptible accession P21 showing the typical dark red vascular discoloration in crown and stem 30 dpi with *Fop* race 2. (c, d) Resistant accession P633 showing the typical dark red vascular discoloration only within crown section. Black arrows indicate vascular tissue.

Staining with the commercial Parker blue Quink ink at 30 dpi supported this observation. With this staining method, the whole shoot of the susceptible accessions, such as P21, appeared blue (Fig. 6a,b), indicating the presence of fungi in the entire shoot of susceptible plants. In these genotypes, some patches of more intense staining could be detected within the surface of the shoot that corresponded to fungal colonies growing out of the susceptible plant tissues (Fig. 6a). By contrast, the blue staining was only observed in the crown and basal shoot section of most resistant accessions while the rest of the shoot remained clear (Fig. 6c,d). Altogether, these findings indicated that in most cases the fungal progression was efficiently stopped at crown level and suggested that the most discriminating defence mechanism may be acting in the crown (Figs 3–6).

Discussion

Fusarium oxysporum f. sp. *pisi* is a recurring problem causing important yield losses wherever pea is grown. In this study, different methods to accurately evaluate fusarium wilt disease in a controlled environment were assessed and used to screen a *Pisum* spp. collection to identify new sources of resistance to *Fop* race 2. The different methods of disease evaluation (DI, DR and AUDPC) revealed large variability in the response of the different accessions to *Fop* race 2 ranging from resistant to susceptible, and including many partially resistant accessions. Such a range of responses suggests that resistance to this *Fop* isolate in this *Pisum* spp. collection is mainly of a quantitative nature. As a result of this study, 11 new sources of resistance were identified and confirmed (JI1412, JI1559, JI1760, P23, P42, P614, P627, P633, P639, P650 and P656) showing a very high level of resistance to *Fop* race 2 that may be useful for a breeding programme.

Fusarium wilt disease causes a series of external symptoms including vein clearing, leaf epinasty, wilting, chlorosis, necrosis, and abscission leading to complete plant wilting and death (MacHardy & Beckman, 1983). Under the conditions of this study, the *Fop* race 2 isolate used was highly pathogenic causing fusarium wilt symptoms on susceptible and partially resistant

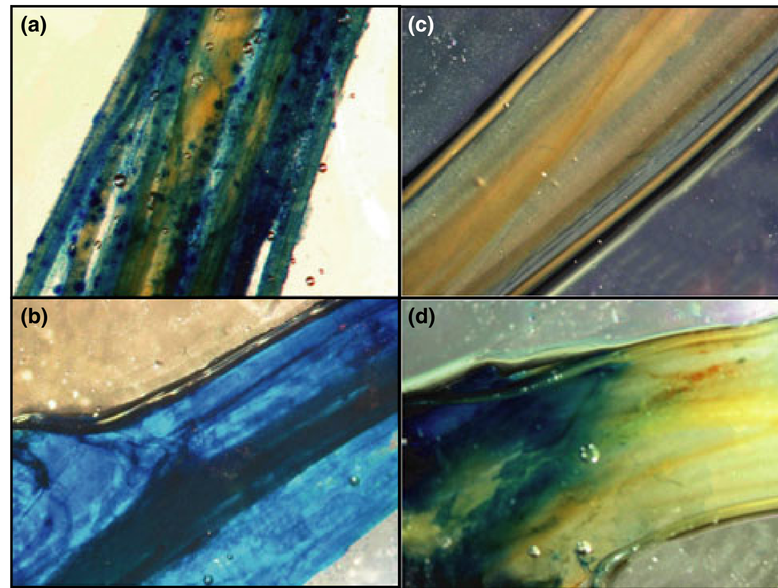

Figure 6 Comparison of the extension of *Fusarium oxysporum* f. sp. *pisi* race 2 colonization within plant tissues between susceptible and resistant pea accessions. Each picture shows a section of stem (a, c) and crown (b, d) of inoculated plants superficially stained with the commercial ink Parker Blue Quink 30 dpi, indicating fungal presence as a blue coloration. (a, b) Susceptible accession P21 showing fungal presence over the whole crown and stem surface. (c, d) Resistant accession P23 showing fungal presence only within the crown section.

accessions which allowed the development of a methodology based on leaf symptoms to evaluate the *Pisum* spp. collection.

Previous studies described pea lines as resistant (no symptoms) or susceptible (dead plants) to specific *Fop* isolates and ignored any variation in their symptom severity (Haglund & Kraft, 1979; Bodker *et al.*, 1993). As a consequence, most previous studies on the identification of resistance sources to this pathogen were based on the sole evaluation of DI (Haglund & Kraft, 1979; Haglund, 1989; McPhee *et al.*, 1999; Sharma *et al.*, 2010). In the case of *Fop* race 2, variations in the DI values of pea accessions have often been detected, hampering their classification within the resistant or susceptible group and the establishment of clear segregation ratios within populations (Hare *et al.*, 1949; McPhee *et al.*, 1999). In the present study, similar variation for DI was detected between experiments. In addition, DI values did not always agree with the resistance reactions in the collection, because accessions such as P11 and P670 had severe fusarium wilt symptoms, but DI values of only 40%. This highlights the need to evaluate additional parameters to accurately estimate disease reaction to *Fop* race 2 in pea. The evaluation of disease severity is often a good method to assess quantitative resistance mechanisms (Russell, 1978), but only a limited number of studies have used a disease severity index to evaluate pea resistance to fusarium wilt (Charchar & Kraft, 1989; Lebeda & Svabova, 1997; Neumann & Xue, 2003; Lebeda *et al.*, 2010). In these studies, the disease scoring was based on a 0–5 (Charchar & Kraft, 1989; Neumann & Xue, 2003) or 0–3 (Lebeda & Svabova, 1997; Lebeda *et al.*, 2010) rating system of the whole plant which appears inadequate to accurately assign plant symptoms to a specific disease index. Instead, in the present study, two different methods considering leaf symptoms of the whole plant to assess DR were tested on a series of differential lines and used to evaluate disease reactions of a *Pisum* spp. germplasm collection. Results obtained from both methods were highly similar, clearly discriminating between the resistant and susceptible genotypes and detecting intermediate reactions. The high correlation between both methods indicates that only one of them is required to determine the disease reaction of pea accessions. The proportion of leaves showing symptoms is the fastest method and would therefore be the method of choice to evaluate DR in future screening of pea germplasm to *Fop* race 2. Fusarium wilt disease development requires several weeks from plant infection to plant death, thus AUDPC (Teng & James, 2002) that considers severity over time was also calculated. Although more time-consuming, this method appears more reliable and reproducible for accurately estimating the disease response of pea accessions, supported by the high correlation between both AUDPC measurements and between AUDPC and the DR values. By contrast, the low correlation coefficient obtained when comparing DI measurements with any other disease parameters confirmed that this parameter alone is not adequate to describe disease response to *Fop* race 2 in pea.

Despite the limited number of accessions used in this study, this *Pisum* spp. collection contained sufficient genetic variation to detect a wide range of responses to *Fop* race 2 from highly resistant to susceptible genotypes. Among the 73 accessions screened, 59% of the collection showed some resistance, with 24·7% genotypes presenting a high level of resistance to this isolate, even more

than the resistant differential cultivars. Nevertheless, it should be noted that only phenotypes of the selected accessions have repeatedly shown consistent reaction to the pathogen and that other accessions may need repeated testing to confirm their relative resistance. Of the 11 highly resistant accessions, only three belong to *P. sativum* ssp. *sativum*, whereas eight belong to other subspecies of *P. sativum* including two *P. sativum* ssp. *elatius*, four *P. sativum* ssp. *arvense* and one *P. sativum* ssp. *jormadi* accession and one belonging to *Pisum fulvum*. While screening for resistance to all four races of *Fop* has been extensively performed, few studies have described very high levels of resistance in collections of *Pisum* spp. (Lebeda & Svabova, 1997). Indeed, only one previous study specifically dealt with wild species and subspecies and although the authors reported a wide variety of responses to *Fop*, they failed to identify complete resistance (Lebeda & Svabova, 1997). On the other hand, the screening of 452 pea accessions from the USDA core collection revealed that 14% of the whole collection (62 accessions) were resistant to races 1 or 2 of *Fop* (McPhee *et al.*, 1999). Interestingly, one *P. sativum* ssp. *abyssinicum* and one *P. sativum* ssp. *elatius* accession were also identified as resistant in this study (McPhee *et al.*, 1999). The *P. sativum* ssp. *elatius* accession PI344012 was also included in the present study (P24); however under these experimental conditions, this accession was highly susceptible to the *Fop* race 2 isolate, showing a disease incidence of 80%.

Resistance to all of the *Fop* races in pea has been considered qualitative with a monogenic inheritance (Infantino *et al.*, 2006). For race 2 of *Fop*, most previous studies identified only one genetic factor controlling resistance to this race (Hare *et al.*, 1949; Haglund, 1989; McPhee *et al.*, 1999). However, mixed phenotypes were often observed within accessions even when considering the set of differential lines used to characterize *Fop* isolates (Haglund, 1989; McPhee *et al.*, 1999). In these studies, these authors attributed the variability to heterogeneity of the seed population, excessive root trimming prior to inoculation, or the presence of another fungal pathogen (McPhee *et al.*, 1999). Interestingly, subsequent studies by the same authors reported intermediate resistance reactions in response to *Fop* race 2, questioning the reaction of these lines to this race (McPhee *et al.*, 2004). In the present study, a continuity of responses was detected from highly resistant to susceptible. A previous screening of another collection of *Pisum* spp. for resistance to *Fop* race 2 also detected large variation in the responses of individual accessions (Lebeda & Svabova, 1997). Such continuity would support the existence of quantitative resistance mechanisms in the *Pisum* spp. collection, which also can be seen in the differential lines in which little variation was detected. Thus in accordance with the observation of McPhee *et al.* (2004), the results suggest the existence of additional genetic factors that control *Fop* race 2 resistance, although genotypic analysis of progenies of these accessions would be needed to confirm this hypothesis.

As a result of the present study, 43 accessions with quantitative resistance were identified, of which 11 developed only very mild symptoms. As a next step it will be important to characterize the resistance mechanisms responsible for their phenotype to ease selection in breeding programmes. Many studies on *Solanum lycopersicum*, *Arabidopsis thaliana* and *P. sativum* have indicated that resistant plants to *F. oxysporum* display a wide and complex array of anatomical and biochemical responses to counteract pathogen infection (Beckman, 1987; Kraft, 1994; Michielse & Rep, 2009). However, the actual resistance mechanisms acting in a specific resistant accession are still unclear (Zvirin *et al.*, 2010). As a starting point to characterize the resistance mechanisms, a detailed observation of internal and external symptoms was undertaken. These initial studies suggested that the strongest resistance response acted at the crown level. Indeed, fungal colonies were only isolated from roots and the basal part of the stem in resistant and most partially resistant accessions, while *Fop* was present in the whole plant in susceptible accessions as early as 7 dpi. In addition, observation of external and internal symptoms indicated clear differences between susceptible and resistant genotypes. Pea infection by *Fop* is usually associated with a discoloration of vascular tissue which turns orange or dark red (Kraft & Pfleger, 2001). In agreement with these observations, vascular tissue of most accessions screened in the present study turned dark red. However, this discoloration was restricted to the root and basal stem section in the resistant and partially resistant accessions, while it reached the shoot apex in susceptible accessions. This agreed with the observation that *F. oxysporum* colonization of resistant host and non-host was limited to the root and basal part of the stem of different plant species (Beckman, 1987; Charchar & Kraft, 1989). Interestingly, the restriction of the extent of vascular discoloration observed in resistant and partially resistant accessions was accompanied by a blackening of the cortical and epidemical cells around the crown region. Previous studies indicated that pea infection by *Fop* race 2 was often associated with secondary cortical decay (Hagedorn, 1984; Kraft & Pfleger, 2001). However, observations on the blackened regions in this study did not suggest any decay of these tissues, but rather a cortical hardening as the blackened cells appeared to be still alive. Further studies are now underway to determine the mechanisms acting in these resistant accessions at the cellular and molecular levels. In the meantime, the incorporation of these resistant accessions in breeding programmes of elite pea cultivars together with the application of a simplified scoring method derived from the present study is expected to improve the resistance status of pea to *Fop* race 2 in the near future.

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Article

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Identification of the Main Toxins Isolated from *Fusarium oxysporum* f. sp. *pisi* Race 2 and Their Relation with Isolates' Pathogenicity

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Supporting Information

ABSTRACT: *Fusarium oxysporum* f. sp. *pisi* (*Fop*) is a pathogen of field pea inducing severe vascular wilt worldwide. Plant resistance to races 1, 5, and 6, producing wilt symptoms, is conferred by a single dominant gene, while resistance to race 2, which gives near-wilt symptoms, have been recently showed to be quantitative. Among the virulence factors reported to play a role in the infection process, toxin production is one of the best studied. Thus, five race 2 isolates have been investigated for toxin production in vitro and their relation to isolates' pathogenicity. All the isolates produced different amounts of fusaric and 9,10-dehydrofusaric acids. The content of the two toxins has been quantitated and correlated with the pathogenicity and aggressiveness of isolates on field pea. Results suggested that toxin production is an important determinant of *Fop* race 2 pathogenicity.

KEYWORDS: *Fusarium oxysporum, Pisum sativum, near wilt, phytotoxins, fusaric acids*

INTRODUCTION

Fusarium oxysporum f. sp. *pisi* W.C. Snyder & H.N. Hansen. (*Fop*) is an important pathogen causing vascular wilt of field pea (*Pisum sativum* L.) worldwide.[1] Four different races of *Fop,* races 1, 2, 5, and 6, have been described.[2] Races 1 and 2 have been reported in every country where peas are grown, while races 5 and 6 are, to date, only important in western Washington State.[2] Plants infected with race 2 are most often scattered throughout the field rather than being concentrated in specific areas as observed with the other races, which is described as *near wilt*.[1] Resistance to *Fop* race 1, 5, and 6 is conferred by a single dominant gene while resistance to race 2 has been recently shown to be quantitative.[2,3]

Several virulence factors have been reported to play a role at different stages of the infection process to induce disease and counteract the plant defense reaction in several formae speciales (ff. spp.) of *Fusarium oxysporum*.[4] However, these studies have not targeted the virulence factors of *Fop*. One of the best studied virulence factors is fusaric acid, **1** (Figure 1).

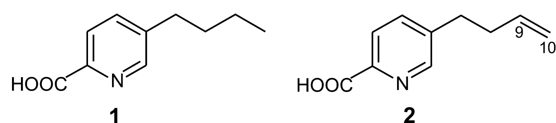

Figure 1. Structures of fusaric, **1**, and 9,10-dehydrofusaric, **2**, acids.

Fusaric acid is a nonspecific toxin produced by many *Fusarium* species.[5,6] At high concentration it induces many physiological responses in plant cells including alteration of cell growth, mitochondrial activity, and membrane permeability while at lower concentration it can trigger plant defense reactions and programmed cell death.[7,8] Fusaric acid was also shown to induce wilt symptoms on pepper and cucumber.[9,10] Thus **1** is considered to participate in *F. oxysporum* pathogenicity by reducing plant cell viability. However, **1** was also detected within plant tissue colonized by nonpathogenic isolates, which questions the exact importance of **1** during the infection process.[7] Thus, the role of **1** in *F. oxysporum* pathogenicity is still under debate. Apart from fusaric acid, some *F. oxysporum* isolates can produce additional toxins such as beauvericin, enniantin B, bikaverin, moniliformin, fumonisin, and trichothecenes[11−14] that can also contribute to their pathogenicity. Toxins produced by *Fop* and their potential function in *Fop* pathogenicity are still unknown. To improve understanding on *Fop* pathogenicity, the main toxins of several isolates of *Fop* race 2 were identified and quantitated.

MATERIALS AND METHODS

General Experimental Procedures. IR spectra were recorded as deposited glass film on a Perkin-Elmer (Norwalk, CT) Spectrum One FT-IR spectrometer, and UV spectra were measured in MeCN on a Perkin-Elmer Lambda 23 UV/vis spectrophotometer. ^{1}H NMR spectra were recorded at 600 or 400 MHz, in CD_3OD, on Bruker (Kalsruhe, Germany) spectrometers. The same solvent was used as internal standard. ESI and APCI MS spectra were recorded on an Agilent Technologies (Milan, Italy) 6120 Quadrupole LC/MS instrument. Analytical and HPLC grade solvents for chromatography were purchased from Carlo Erba (Milan, Italy). All other analytical grade chemicals were purchased from Merck (Darmstadt, Germany). Analytical and preparative thin layer chromatography (TLC) were performed on silica gel (Kieselgel 60, F_{254}, 0.25 and 0.5 mm respectively) (Merck, Darmstadt, Germany) or reverse phase (KC18 F_{254}, 0.20 mm) (Whatman, Maidstone, U.K.) plates. The spots were

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visualized by exposure to UV radiation (254 nm), or by spraying first with 10% H_2SO_4 in MeOH and then with 5% phosphomolybdic acid in EtOH, followed by heating at 110 °C for 10 min, or by exposure to iodine vapors. The HPLC system (Shimadzu, Tokyo, Japan) consisted of a series LC-10AdVP pump, FCV-10AlVP valves, SPD-10AVVP spectrophotometric detector, and DGU-14A degasser. The HPLC column used was 250 mm × 4.6 mm i.d., 5 μm, high-density Nucleosil 100-5 RP18 (Macherey-Nagel, Duren, Germany) with a 7.5 mm × 4 mm i.d. guard column of the same material (Alltech, Sedriano, Italy). Water was HPLC quality, purified in a Milli-Q system (Millipore, Bedford, MA, USA). Disposable syringe filters, Anotop 0.2 μm, were purchased from Whatman (Maidstone, U.K.). Fusaric acid was purchased from Sigma (St. Louis, MO, USA).

The methyl esters of **1** and **2** were prepared by diazotization of the corresponding acids as previously reported.[16]

Purification of 9,10-Dehydrofusaric Acid. 9,10-Dehydrofusaric acid, **2** (Figure 1), was purified from fungal culture filtrates of *Fusarium nygamai* as previously described by Capasso et al., 1996.[15] Briefly, culture filtrates were acidified up to pH 2 and exhaustively extracted with EtOAc. The organic extract was purified by combination of column and TLC on silica gel and reverse phase yielding **2** as a homogeneous amorphous solid (121.6 mg/L).

Fungal Strains, Culture Medium, and Growth Conditions. The *Fusarium oxysporum* f. sp. *pisi* race 2 isolates F42 and F69 were kindly provided by Dr. W. Chen (USDA-ARS, Pullman, WA, USA). The *F. oxysporum* f. sp. *pisi* strain CBS 127.73 NRRL36628 (*Fop*1) was provided by CBS-KNAW Fungal Biodiversity Center (Utrecht, The Netherlands). In addition, the strains Pt1 and Arg3 were isolated from wilted pea plants collected at Alvaiázere, Portugal, and Setif, Algeria, respectively. Isolation of fungal colonies on surface-sterilized wilted pea fragments was performed as described previously[3] and maintained as a single-spore colony. They have been deposited in the collection of Institute for Sustainable Agriculture, IAS-CSIC (Córdoba, Spain). The fungal strains were stored as microconidial suspensions at −80 °C in 30% glycerol. For microconidia production, cultures were grown in potato dextrose broth (PDB) (Difco, Detroit, MI) at 28 °C in a shake culture set at 170 rpm.[17] For toxin production, Erlenmeyer flasks (500 mL) containing 200 mL of Czapek−Dox medium (5% glucose, 0.1% yeast extract, 0.05% K_2HPO_4, 0.2% $NaNO_3$, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.001% $FeSO_4 \cdot 7H_2O$), were inoculated with 200 μL of fresh *Fop* microconidia at 10^9 conidia/mL and incubated under shaking at 28 °C for 7 days. The content of the flask was filtered on cheesecloth and centrifuged at 7,000 rpm for 10 min. The supernatant containing the secreted *Fop* toxins was then frozen at −80 °C and lyophilized before further analysis. Morphological characterization of each strain was performed at X40 and X63 magnification with a bright light microscope with Nomarsky filter.

DNA Extraction, PCR Amplifications, and Sequencing. Genomic DNA was extracted from *F. oxysporum* mycelium following a previously reported protocol.[18] Molecular characterization of each *Fop* strain was performed by analysis of the internal transcribed spacers (ITS) and the 5′ intron-rich portion of the elongation factor alpha (EF-alpha). ITS and EF-alpha sequences were obtained by PCR amplification with primers ITS-1/ITS-4[19] and EF-1/EF-2[20] respectively. Each 50 μL reaction mixture contained 50 ng of template DNA, 2 units of BioTaq DNA polymerase (Bioline, London, U.K.), 1× PCR buffer, 2 mM $MgCl_2$, 200 μM dNTPs, and 0.3 μM of each primer. The PCR amplifications were performed on a MyCycler (Biorad, Hercules, CA) thermocycler as follows: (i) for ITS, 94 °C for 2 min, 40 cycles at 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 2.5 min followed by a final step at 72 °C for 10 min; (ii) for EF-alpha, 94 °C for 5 min, 30 cycles at 94 °C for 35 s, 52 °C for 35 s, and 72 °C for 1 min followed by a final step at 72 °C for 10 min. All amplifications were purified with the PCR cleanup kit of QIAgen and cloned in pGEMT vector (Promega, Madison, WI). Two positive clones per amplicon were sequenced by STABVida (Setubal, Portugal) using the pGEMT vector specific primers SP6 and T7.

Sequence Data Analysis. Reads of each sequence were assembled and manually corrected with Chromaspro 1.7.5 (Technelysium Pty Ltd., South Brisbane, Australia). Sequences were used in BLAST[21] searches against the GenBank[22] and Mycobank[23] databases to identify the most similar sequences available in the databases. Pairwise alignments were performed with the Neddleman−Wunsh algorithm (Needle method) implemented at EMBL-EBI webserver.[24] All DNA sequences have been deposited in GenBank (Table S1 in the Supporting Information).

Plant Materials. Assignment of the different strains to a specific race of *F. oxysporum* f. sp. *pisi* was performed by testing the pathogenicity profile of these isolates on a set of differential pea lines (Table 1).[1,3] The comparison of the aggressiveness of each strain was performed on *P. sativum* cv. 'Messire'. To determine the effect of fungal culture filtrates or their corresponding organic extracts, seven pea accessions with a wide range of response to *Fop* race 2 were used including the susceptible accessions JI1213, 'Messire', and P629, the partially resistant accessions JI2480 and P615, and the resistant accessions P42 and P633.

Table 1. List of the Pea Differential Lines for *F. oxysporum* f. sp. *pisi* and Their Susceptibility Response to the New *Fop* Isolates

accessions	expected[a]				observed[b]		
	R1	R2	R5	R6	F42	Arg3	Pt1
'Dark Skin Perfection'	R	S	S	S	S	S	S
'Mini'	S	R	S	S	PR	PR	R
'New Era'	R	R	S	S	nd	R	R
'New Season'	R	R	S	R	R	R	R
'74SN5'	R	R	R	R	R	R	R
P629	R	S	nd	nd	S	S	S
JI1412	R	R	nd	nd	R	R	R
'Messire'	R	S	nd	nd	S	S	S

[a]Accessions' response to each *Fop* race as described in Kraft and Pfleger[1] and Bani et al.[3] R stand for resistant reaction and S susceptible. [b]Accessions' response to the inoculation with the reference R2 strain F42 and the isolates from Algeria and Portugal obtained in the present study.

For all experiments, germinated pea seedlings were sown in vermiculite and grown in a controlled environment under a 16/8 h light−dark photoperiod at 26 ± 2 °C with 200 μmol/m²/s of illumination. Plants were watered every three days with tap water.

Pathogenicity Test. To determine the pathogenicity of each *Fop* isolate, seven day old pea seedlings were inoculated with the dip root technique as described previously[3] and maintained in the same growth conditions as above. Disease was then evaluated every three days by estimating the percentage of leaves with symptoms and the area under the disease progression curve (AUDPC).[3] Five plants were used per accession, and each experiment was repeated twice independently.

Extraction and Purification of Fusaric and 9,10-Dehydrofusaric Acids from Fungal Culture Filtrates. Lyophilized *Fop* culture filtrates (200 mL) were redissolved in 1/10 of the initial volume with distilled water. The pH of solutions were adjusted to 2.5 with 1N HCl and extracted with EtOAc (3 × 20 mL). The acidic organic extracts were combined, dried (Na_2SO_4), and evaporated under reduced pressure, yielding brown oily residues (38.9, 101.7, 36.4, 42.7, and 29.5 mg for F42, F69, *Fop1*, Arg3, and Pt1 respectively). The five acidic organic extracts were analyzed by TLC on silica gel [eluent EtOAc−MeOH−H_2O (8.5:2:1, v/v/v)] and on reverse phase [eluent CH_3CN-H_2O (1:1, v/v)], in comparison with authentic standard samples of **1**, **2**, and their methyl esters. To confirm the presence of **1** and **2**, the residue of organic extract of F42 was purified by TLC eluted with CH_3CN-H_2O (1:1, v/v), yielding two pure solid compounds: **1**, R_f 0.50, eluent EtOAc−MeOH−H_2O (8.5:2:1, v/v/v), R_f 0.51 eluent CH_3CN-H_2O (1:1, v/v), and **2**, R_f 0.40, eluent EtOAc−MeOH−H_2O (8.5:2:1, v/v/v), R_f 0.58, eluent CH_3CN-H_2O (1:1, v/v), which were identified as fusaric acid and 9,10-dehydrofusaric acid as described below.

Fusaric acid (1). IR, UV, and ^1H NMR spectra were very similar to data reported.[15] ESIMS (+) m/z: 381 $[2M + Na]^+$, 202 $[M + Na]^+$. ESIMS (−) m/z: 178 $[M - H]^-$. APCIMS (+) m/z: 180 $[M + H]^+$.

9,10-Dehydrofusaric acid (2). IR, UV, and ^1H NMR spectra were very similar to data reported.[15] ESIMS (+) m/z: 200 $[M + Na]^+$. ESIMS (−) m/z: 176 $[M - H]^-$.

HPLC Analysis of Acidic Organic Extracts. A method previously reported[16] was optimized and used for analysis. The mobile phases employed were MeOH (eluent A) and 1% K_2HPO_4 adjusted to pH 7.35 with concentrated H_3PO_4 (eluent B). Elution was initially with A:B (50:50), which was transformed using a linear gradient over 20 min to A:B (75:25); the initial conditions were restored using a linear gradient over 5 min, and the column was re-equilibrated under these conditions for 10 min before the next run was initiated. The flow rate was 1 mL/min, and 20 μL aliquots of the samples were injected for analysis. Detection was performed at 268 nm, corresponding to the maximum of absorption of **1** and **2**. The HPLC calibration curves for quantitative determination of **1** and **2** were performed with absolute amounts of standards dissolved in MeOH in the range between 0.2 and 20 μg for each compound, in triplicate for each concentration. HPLC linear regression curves (absolute amount against chromatographic peak area) for **1** and **2** were obtained based on weighted values calculated from seven amounts of the standards in the above range. The samples were dissolved in MeOH and passed through disposable filters, and aliquots (20 μL) were injected into the HPLC instrument. Each sample was assayed in triplicate. The quantitative determination of **1** and **2** was calculated by interpolating the mean area of their chromatographic peaks with the data from the calibration curves.

Recovery Studies. Recovery studies were performed using the best producer isolate F69. Pure fusaric and 9,10-dehydrofusaric acids were added to the culture filtrate from 0.3 to 2.0 mg/L. The samples were prepared as described above and the extracts analyzed by HPLC to determine recovery. Three replicate injections were performed for each concentration. The recovery throughout the range of concentration was higher than 96 ± 2%.

Biological Assays. *Leaf Absorption Assay.* The toxicity of the culture filtrate of *Fop* race 2 isolate F42 was assayed by incubating fully expanded leaves in an F42 culture filtrate. For this, the leaf petiole was immersed into an Eppendorf tube containing 1 mL of one week old fungal culture filtrate before or after autoclaving at 121 °C for 20 min and incubated at room temperature for 24 and 48 h. Four fully developed leaves were used for each pea accession.

Leaf Puncture Assay. A leaf puncture bioassay on pea leaves was performed to evaluate the toxic effect of culture filtrates from all *Fop* race 2 isolates tested. Fully expanded leaves from pea plants were placed on Petri dishes containing water−agar medium and punctured by a sterile needle on the upper surface. Droplets (10 μL) of the culture filtrate or corresponding organic extract in 1% MeOH were applied on the wounded leaves at 2 mg/mL. Plates were then incubated at room temperature under darkness. After 3 days of incubation, the area (mm^2) of the necrotic lesions was measured. Droplets of a pure standard of **1** and **2** at 2 mg/mL were used as positive control reactions while droplets of sterile water, Czapek−Dox medium, or 1% MeOH were used as negative control. The experiments were performed with four replicates for each treatment.

Statistical Analysis. Analyses of variance (ANOVA) were carried out for phytotoxicity records, with the different tested solutions and metabolites as fixed factors of their respective bioassays. One way ANOVA was also performed to test the significance of aggressiveness differences between each strain. Phytotoxicity and percentage of symptoms values were transformed using the square root transformation in order to increase the normality of their distribution. Whenever the ANOVA test was statistically significant ($p \leq 0.05$), a Duncan multiple range test assessing the differences of the means between each treatment was performed. All statistical analyses were performed using Genstat release 11.1 software (VSN International Ltd., Hemel Hempstead, U.K.).

RESULTS AND DISCUSSION

F. oxysporum species complex is composed of many species very closely related morphologically, which makes them difficult to identify. To ensure that all fungal strains corresponded to isolates of the race 2 of *F. oxysporum* f. sp. *pisi*, these isolates were characterized at morphological and molecular levels before further analysis. As expected, all fungal strains showed morphological characteristics of *F. oxysporum* complex species. Amplification of ITS and EF-alpha gave sequences ranging from 544 to 545 bp and 710 to 714 bp long respectively according to the isolate (Table S1 in the Supporting Information). These sequences showed between 99 and 100% identity to *F. oxysporum* sequences from different formae speciales including ff. spp. *pisi*, *ciceri*, *medicaginis*, and *lycopersici* according to the BLAST comparison performed. In addition they share 99.8−100% and 98.6−100% identity to the reference *Fop* race 2 strain F42 respectively (Table S1 in the Supporting Information). This clearly identified them as *F. oxysporum* although it was not possible to identify the ff. spp. to which they belong by these methods, hampered by the potential polyphyletic origin of the isolates and potential horizontal transfer of host-specificity genes.[20] Nevertheless, the in planta pathogenicity test indicated that they corresponded to *F. oxysoprum* f. sp. *pisi*. Indeed, all these strains were pathogenic on the susceptible pea cultivar 'Messire' (Figure 2). Slight differences in aggressiveness were observed between isolates as determined by their AUDPC value ($p < 0.001$). The strain F69 was the most aggressive, reaching AUDPC values of 2388, while the isolates *Fop1* and Arg3 were the least aggressive, reaching AUDPC of only 1678 and 1778.5 respectively (Figure 2A). This difference was rooted to a faster disease development induced by strain F69, which led to complete plant death as early as 12 days post inoculation (dpi) while the Arg3 and *Fop 1* strains required 25 days to induce a similar effect (Figure 2B). This was confirmed by the ANOVA, which revealed significant differences between isolates only up to 15 days post inoculation ($p < 0.001$) while at later stages the differences were not significant ($p > 0.5$). In addition, the inoculation of a set of differential lines[1,3] allowed assigning the new isolates Arg3 and Pt1 to race 2 of *Fop* since accession's response to these isolates followed a similar pattern as for the reference race 2 strain F42. Indeed, no or only mild symptoms were detected on the race 2 resistant accessions while the susceptible accessions 'Dark Skin perfection', P629, and 'Messire' developed characteristic wilt symptoms (Table 1). By contrast, *Fop*1 showed *near wilt* (leaf yellowing) symptoms on most accessions, which impeded a clear classification of this strain to a specific race (data not shown). However, the very close molecular relationship between this isolate and the Algerian (Arg3) isolate as shown by the analysis of EF-alpha sequence (Table S1 in the Supporting Information) together with the *near wilt* symptoms detected would suggest that this isolate also belongs to race 2 of *Fop*.

To characterize further the mechanisms of pathogenicity of *Fop* race 2 isolates that remain largely unknown, the effect of one week old *Fop* culture filtrates was evaluated on pea leaves. Incubation of pea leaves in F42 culture filtrate for 24 h induced leaf withering followed by leaf discoloration that initiated at the central vein and progressed to cover the whole leaf and petiole as early as 48 h of incubation (Figure 3A). Similar effects were detected on all pea accessions tested including on resistant accessions (Figure 3B). The leaf puncture assay also showed a

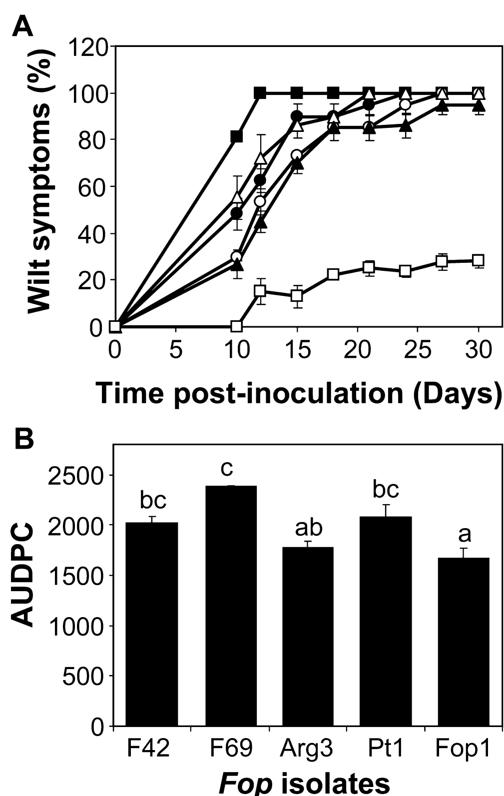

Figure 2. Pathogenicity of the *F. oxysporum* f. sp. *pisi* race 2 isolates on the susceptible pea cultivar 'Messire'. (A) Evolution of *Fusarium* wilt symptoms on the susceptible pea cultivar 'Messire' induced by the *Fop* race 2 isolates F42 (●), F69 (■), Arg3 (○), Pt1 (△), and *Fop1* (▲) respectively, compared to control plants treated with water (□). Disease progression was estimated as percentage of leaf with symptoms over time. (B) Comparison of AUDPC values calculated from the periodic assessment of development of *Fusarium* wilt symptoms. Different letters between each histogram indicate significant difference between the values according to the Duncan multiple range test at $\alpha = 0.05$. Vertical bars are standard error for $n = 5$.

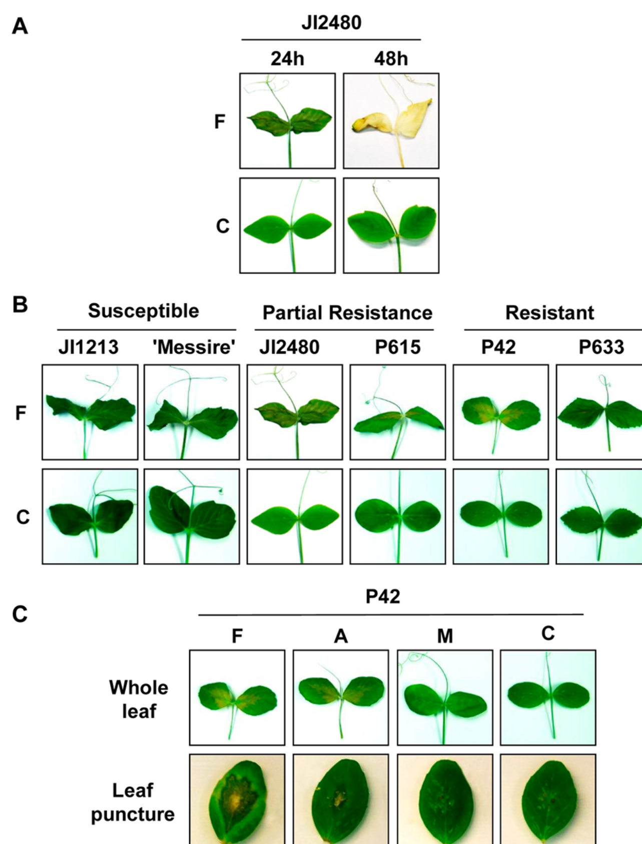

Figure 3. Effect of the culture filtrates of *F. oxysporum* f. sp. *pisi* race 2 isolate F42 on pea leaves. (A) Progression of wilting symptoms induced on the partially resistant accession JI2480 by the culture filtrates after 24 and 48 h of treatment (F) compared to control with sterile water (C). (B) Effect of the culture filtrates after 24 h of treatment on leaves of seven pea accessions differing in their susceptibility to *Fop* race 2. (C) Comparison of the leaf response of the resistant pea accession P42 to culture filtrates (F), autoclaved culture filtrates (A), sterile Czapek−Dox medium (M), and sterile water (C) evaluated with the leaf absorption and the leaf puncture assays.

progressive spreading of necrosis and leaf discoloration after treatment with the fungal culture filtrates while control remained symptomless (Figure 3C). A broad effect of *F. oxysporum* culture filtrates was previously detected for *F. oxysporum* f. sp. *orthoceras* and *F. oxysporum* f. sp. *albedinis*, which extract induced wilting on both host and nonhost species.[25,26] This supports the presence of a nonspecific toxin within these filtrates. In addition it indicated that resistance of the pea accessions tested was not based on toxin detoxification. This was also detected in *F. oxysporum* ff. spp. *melonis*[27] and *cubense*.[28] However, in the present study, only three resistant *Pisum* spp. accessions have been tested. Thus, it may still be possible that the resistance mechanisms of other resistant pea accessions are based on toxin detoxification as detected previously in near-isogenic lines of tomato differing in their susceptibility to *F. oxysporum* f. sp. *lycopersici*.[29] Interestingly, organic extracts of the culture filtrates from all *Fop* race 2 isolates tested were able to induce leaf necrosis on 'Messire' leaves when evaluated with the leaf puncture assay after 3 days of incubation (Figure 4A). However, difference in lesion size could be detected between the strains ($p < 0.001$), in agreement with the difference in aggressiveness of these strains on this pea cultivar (Figure 4B). The lesion size ranged from 16.8 mm^2 for Arg3 to 42.4 and 58.4 mm^2 for F42 and F69 culture filtrate respectively (Figure 4B).

Various pathogenicity factors including cell-wall degrading enzymes, phytoalexin-detoxifying enzymes, and toxins have been shown to mediate *F. oxysporum* virulence.[4] In contrast to a previous study that showed the complete loss of toxic activity after autoclaving culture filtrates,[29] autoclaving only slightly attenuated the toxicity of the filtrate on leaf absorption and puncture assays (Figure 3C). This ruled out that the toxicity is due to proteins and suggested the presence of heat-stable metabolites as found for other isolates of *Fop*[30] and human pathogenic strains of *F. oxysporum*.[31]

To identify the heat-stable metabolite(s) responsible for the toxicity of *Fop* race 2 culture filtrates, the organic extract of culture filtrates obtained from the isolate F42 was fractionated and further analyzed. TLC analysis of these fractions showed that this strain produced two toxins, identified as fusaric acid, **1**, and dehydrofusaric acid, **2**, by comparison with standard samples. Their corresponding methyl esters were not detected. These results were confirmed by purification of **1** and **2** from this organic extract. The ^1H NMR spectrum was very similar to those previously reported.[15] The data obtained from ESI MS

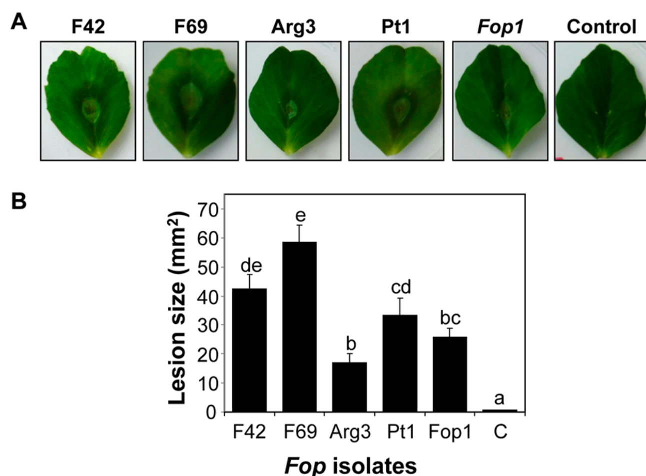

Figure 4. Effect of culture filtrates of the different *F. oxysporum* f. sp. *pisi* race 2 isolates on leaves of the susceptible pea cultivar 'Messire'. (A) Comparison of 'Messire' leaf response to culture filtrates of each *Fop* race 2 isolate or sterile Czapek−Dox medium (Control). (B) Comparison of the lesion size induced by each isolate or by sterile Czapek−Dox medium (C). Different letters between each histogram indicate significant difference between the values according to the Duncan multiple range test at α = 0.05. Vertical bars represent standard errors for n = 4.

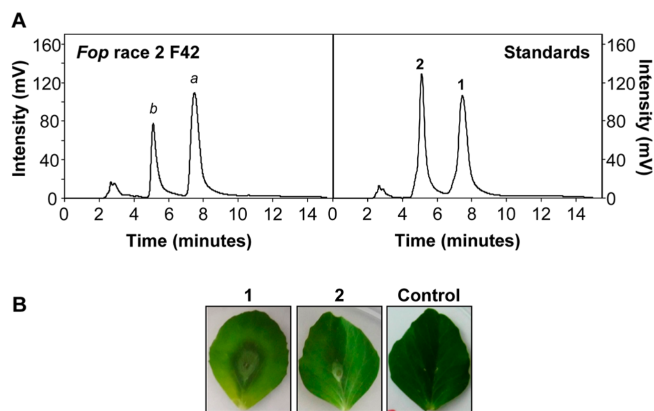

Figure 5. Characteristics of the main toxins produced by the *F. oxysporum* f. sp. *pisi* race 2 isolates. (A) HPLC profile of the organic extract of the *Fop* race 2 isolate F42 (left) and fusaric and 9,10-dehydrofusaric acids (**1** and **2**) standards (right). (B) Comparison of the leaf response of the susceptible pea cultivar 'Messire' to droplets of pure **1** and **2**.

spectra further supported the isolation of **1** and **2**. The ESI MS recorded in positive ion mode showed the sodiated dimeric form $[2M + Na]^+$ and the sodium cluster $[M + Na]^+$ at m/z 381 and 202, respectively. In APCI MS it showed the pseudomolecular ion $[M + H]^+$ at m/z 180. When the ESI MS was recorded in negative ion mode, it showed the pseudomolecular ion $[M - H]^-$ at 178. The ESI MS spectrum of **2** recorded in positive and negative ion mode showed the sodium cluster $[M + Na]^+$ and the pseudomolecular ion $[M - H]^-$ at m/z 200 and 176, respectively. Further studies indicated that all *Fop* race 2 isolates investigated produced **1** and **2** but not their related methyl esters. Both toxins have been already identified from cultures of *F. oxysporum* pathogenic to the parasitic plants *Striga hermonthica*.[16,32,33] However, it is the first time that **2** is reported from *F. oxysporum* isolates pathogenic to crops. Other toxins were not found in any of these isolates. Production of toxins showed large qualitative and quantitative variation according to growth conditions including growth medium, temperature, and ambient pH among others.[16,25,34] Thus, it could not be ruled out that these strains may produce additional toxins as described for other *F. oxysporum* strains.[11−14]

Testing these acids with the leaf puncture assay indicated that both **1** and **2** possessed phytotoxic activity and induced necrotic lesion similar to that induced by the F42 culture filtrates (Figure 5B). Comparison of lesion size indicated that **1** (55.8 mm^2) was 2.5 time more phytotoxic than **2** (22.6 mm^2). Many studies demonstrated the function of **1** in pathogenicity, although in several instance no correlation was detected between the concentration of **1** and fungal virulence.[5,7,35] **1** has been shown to alter membrane permeability, inhibit O_2 uptake and ATP synthesis, decrease mitochondrial activity, transpiration rate, and stomatal conductance, and induce cell depolarization,[7,10] suggesting that **1** participates in *F. oxysporum* pathogenicity by decreasing plant cell viability.[7] By contrast, nearly nothing is known about the action of **2**.[15] This compound was previously shown to induce tomato leaf chlorosis and inhibit root elongation to a similar extent as **1**.[15] Here we demonstrated that dehydrofusaric acid was also phytotoxic on pea leaves (Figure 4), indicating that it may also contribute to the pathogenicity of *F. oxysporum*.

To confirm the function of **1** and **2** in *Fop* race 2 pathogenicity, we quantitated the production of both acids by all *Fop* race 2 isolates tested and related it to their level of aggressiveness. For the quantitation, we slightly modified a previously reported HPLC method.[16] The characteristics of the calibration curves, the absolute range, and the detection limits (LOD) of **1** and **2** are summarized in Table S2 in the Supporting Information. Regression analysis suggests that the calibration curves are linear. A representative HPLC chromatogram of the ethyl acetate extract of the culture filtrates of *Fop* F42 is presented in Figure 5A. The metabolite chromatographic peaks (*a* and *b*) in the sample were coincident with the 7.80 and 5.20 min retention times of **1** and **2** standards (Figure 5A). The retention times were highly reproducible, varying less than 0.50 min. For all strains matrix substances absorbing at 268 nm were eluted within the first 20 min. **1** could be quantitatively and reproducibly detected from 0.5 μg, and **2** from 0.2 μg, with lower amounts having poor reproducibility.

Although **1** and **2** were secreted by all isolates of *Fop* race 2 tested, this method allowed detecting quantitative differences between these isolates ($p < 0.001$) for both toxins. Of the strain tested, F69 produced the highest amount of **1** and **2** (258.49 and 243.36 mg/L, respectively) whereas strains Arg 3 and *Fop1* produced the least **1** (9.94 mg/L) and **2** (21.44 mg/L) respectively (Figure 6). The capacity to produce and secrete **1** was significantly and positively correlated with the leaf lesion size (r^2 = 0.83; Figure 7A) and slightly correlated with in planta pathogenicity (r^2 = 0.66; Figure 7B). Production of **2** was significantly correlated with in planta pathogenicity (r^2 = 0.8; Figure 7D) and to a lesser extent with lesion size (r^2 = 0.66; Figure 7C). Interestingly, the total toxin production was significantly correlated with both virulence parameters (r^2 = 0.78 and 0.76 for leaf lesion size and in planta pathogenicity respectively) (Figures 7E and 7F). This reinforced the important role of **1** in *F. oxysporum* pathogenicity as it was already demonstrated for *F. oxysporum* ff. spp. *carthami*,[36] *lycopersici*,[37,38] *melonis*,[39] and *gladioli*,[40] among others. It also demonstrated that **2** is an important pathogenicity factor acting

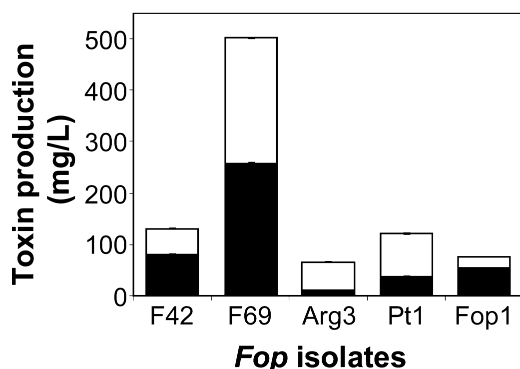

Figure 6. Quantitation of the amount of **1** and **2** produced by the different isolates of *F. oxysporum* f. sp. *pisi* race 2. The histograms show the cumulative production of **1** (black column) and **2** (white column) for each *Fop* race 2 isolate. Vertical bars are standard error for $n = 3$.

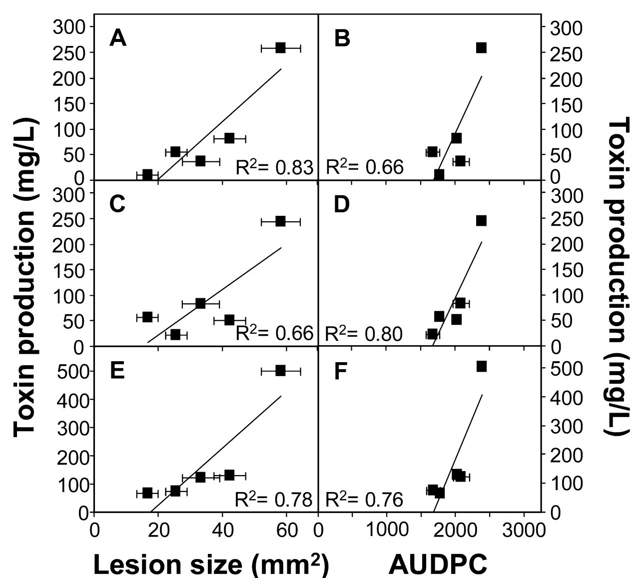

Figure 7. Relationship between toxin production and phytotoxicity. The graphics represent the linear correlation calculated for **1** (A, B), **2** (C, D), and the sum of both toxins (E, F) production by each *Fop* race 2 isolate with the lesion size induced by their corresponding culture filtrate (A, C, E) or their overal aggressiveness on the susceptible pea cultivar 'Messire' estimated by their AUDPC values (B, D, F). Horizontal bars are standard errors for lesion size area or AUDPC values with $n = 4$ and $n = 5$ respectively while vertical bars are standard error bars of toxin content calculated with $n = 3$.

in synergy with **1**, which was not described before for *F. oxysporum* (Figure 7).

In conclusion, it has been demonstrated that *Fop* race 2 isolates produced mainly two toxins that were identified as fusaric and 9,10-dehydrofusaric acids. Both toxins showed high phytotoxic activity on pea when tested on whole leaves and leaf puncture assay. In addition, the amount of both toxins within culture filtrates correlated with strain aggressiveness, which indicates a role of these toxins during *Fop* pathogenicity. Although the importance of fusaric acid in the pathogenicity of *F. oxysporum* is still under debate, the results obtained in the present study strongly indicate that toxin production is an important determinant of *Fop* race 2 pathogenicity.

■ ASSOCIATED CONTENT

Supporting Information

Table S1, comparison of ITS and EF-alpha sequence of the *Fop* isolates with the race 2 reference strain of *Fop* F42, and Table S2, analytical characteristics of calibration curves for **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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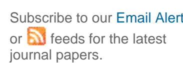

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Resistance reaction of Medicago truncatula genotypes to Fusarium oxysporum: effect of plant age, substrate and inoculation method

Nicolas Rispail, Moustafa Bani, Diego Rubiales

Abstract

Fusarium wilt caused by several formae speciales of Fusarium oxysporum is an important disease of most crop and pasture legumes including pea, chickpea, alfalfa and barrel medics. Medicago truncatula is an important pasture legume and a model legume species. Thus it can be used to increase our knowledge on the resistance mechanisms efficient to block F. oxysporum infection provided that its response to the disease is characterized. Here we evaluated the physiological and susceptibility response to the disease of two contrasting M. truncatula genotypes, and the effect of several cultural conditions known to affect the disease incidence such as plant age at infection time, growth substrate and the method of inoculation. Our results indicated that the A17 accession harbours a moderate level of resistance to the disease. We also showed that the method of inoculation strongly affected fusarium wilt disease development in this model species while it was not significantly altered by the plant age or the inorganic growth substrate tested. In addition we describe a rapid change in leaf temperature after infection that can be used as indirect parameter to confirm fungal infection at a very early stage of the interaction.

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Asunto: Crop & Pasture Science - Decision on Manuscript CP14216.R1

17-Nov-2014

Dear Dr Rispail:

Thank you for sending the revised version of this paper (Resistance reaction of Medicago truncatula genotypes to Fusarium oxysporum: effect of plant age, substrate and inoculation method) and for dealing so thoroughly with the referees' comments. I have now had the opportunity to examine your revised manuscript and I am pleased to accept it for publication in Crop & Pasture Science.

Thank you for your excellent contribution. On behalf of the Editors of Crop & Pasture Science, we look forward to your continued contributions to the Journal.

Sincerely,

Dr Marta Santalla
Associate Editor, Crop & Pasture Science editorial.cp@csiro.au

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Bani Moustafa, 2015