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TESIS DOCTORAL

Diversificación de cultivos para el control de estreses bióticos en leguminosas

Ahmed Amarna

Córdoba, julio de 2023

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En concreto, el alumno ha llevado a cabo 23 ensayos de campo, 11 para el estudio del oidio en guisante, 8 para el estudio de la roya en habas, y 4 para el estudio de las malas hierbas en habas. Estos ensayos se han completado con experimentos en cámara en condiciones controladas encaminados fundamentalmente a la determinación de mecanismos que expliquen los resultados obtenidos en campo. El alumno ha completado satisfacotiramente las actividades formativas requeridas, y ha adquirido importantes conocimientos en la realización de experimento, su análisis y exposición.

Existen ya dos publicaciones derivadas de estos trabajos, incluidas en los dos primeros cuartiles de JCR:

Villegas-Fernandez, A.M.; Amarna, A.A.; Moral, J.; Rubiales, D. Crop Diversification to Control Powdery Mildew in Pea. Agronomy 2021, 11. (IF: 3,7). Q1 (Agronomy)
Villegas-Fernandez, A.M.; Amarna, A.A.; Moral, J.; Rubiales, D. Crop Diversification to Control Rust in Faba Bean Caused by Uromyces viciae-fabae. Journal of Fungi 2023, 9 (IF: 4,7). Q2 (Microbiology)

Además, la Introducción y el tercer capítulo de la tesis han sido enviados a las revistas Horticulturae y European Journal of Agronomy, respectivamente.

Consideramos, pues, que la tesis doctoral está concluida y es apta para su defensa.

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Resumen

Los monocultivos intensivos, aunque muy productivos, conllevan una pérdida de biodiversidad que dan lugar a una serie de problemas a largo plazo para la agricultura y los ecosistemas, como son, entre otros, pérdida de fertilidad del suelo o proliferación de plagas y enfermedades. La diversificación de cultivos se presenta como una alternativa necesaria para hacer frente a estas situaciones.

Las leguminosas son unos cultivos de gran importancia para la alimentación humana y animal que, por su capacidad de fijar el nitrógeno atmosférico, juegan un papel fundamental en el marco de una agricultura sostenible. Se ven, sin embargo, sometidas a estreses biológicos importantes, como son las enfermedades o las malas hierbes. El control químico de los mismos conlleva elevados costes tanto para los agricultores como para el medio ambiente.

El objetivo general de este estudio ha sido investigar si la diversificación de cultivos puede ser de utilidad para el control de enfermedades y malas hierbas en las leguminosas. Para ello se plantean los casos del oidio en guisante, la roya y las malas hierbas en las habas. Se emplean dos herramientas de diversificación como son la mezcla de cultivos (*intercropping*) y la mezcla de variedades.

Se estudió el efecto del *intercropping* sobre el odio de guisante en seis ensayos de campo en diferentes ambientes. El guisante se mezcló con tres cultivos: habas, trigo o cebada. Del análisis global de todos los ensayos se concluye que la combinación con cebada y con habas redujo la severidad de la enfermedad, 44% y 32% respectivamente. En el caso de la cebada, el efecto barrera parecía ser importante para la explicación de esta disminución. Además, la cebada produjo más biomasa y creció más alto que el resto de cultivos. Esto se estudió en ensayos en cámara en condiciones controladas, confirmando la existencia de este efecto barrera.

Las mezclas en diferentes proporciones de las variedades de guisante Messire, susceptible a oidio, con Eritreo, resistente, dieron lugar a reducciones de la enfermedad en Messire, tanto mayores cuanto mayor era la proporción de Eritreo en la mezcla. Esta estrategia puede ser de utilidad para prolongar la duración de la resistencia a oidio.

Igualmente se estudió el efecto del intercropping sobre la roya de las habas, con cuatro ensayos de campo en diferentes ambientes. El análisis puso de manifiesto que sólo la combinación de habas con cebada conseguía reducir la roya, con una reducción global del 22% en los cuatro ensayos. Nuevamente el efecto barrera de la cebada parece fundamental para esta disminución, lo que se vio confirmado mediante ensayos en cámara en condiciones controladas.

También se investigó la mezcla de variedades sobre la roya. En este caso, se mezclaron en proporciones diferentes la variedad Baraca, susceptible a roya, con Joya, resistente a la misma. Nuevamente la severidad en Baraca disminuyó a medida que aumentaba la proporción de Eritreo. Esta variedad, además de ser resistente a roya, producía más biomasa y alcanzaba mayor altura que Baraca, lo que apunta a un posible papel del efecto barrera, más allá de otros como puede ser la dilución del inóculo. Un estudio en cámara en condiciones controladas demostró la importancia del efecto barrera en la reducción de roya en la mezcla de estas dos variedades.

El control de malas hierbas en habas mediante *intercropping* se estudió en cuatro ensayos de campo. Las habas se mezclaron con guisante, trigo o cebada. Se probaron dos sistemas diferentes: alterno con reemplazo y alterno con adición. En el caso de reemplazo no se obtuvo reducción de la presión de malas hierbas en el monocultivo de habas en comparación con las mezclas. En cambio, en el sistema de adición, la mezcla de las habas con cebada consiguió reducción tanto de la cobertura como la biomasa de las malas hierbas (92,7% and 76,6% respectivamente). La diversidad de malas hierbas no se vio alterada por ninguno de los tratamientos estudiados, tanto de mezclas como de monocultivos.

El posible efecto de alelopatía por parte de la cebada en el control de malas hierbas se estudió en un ensayo en cámara en condiciones controladas. Se hizo crecer cebada en macetas, y se sembraron cuatro especies de malas hierbas (*Polypogon monspeliensis*, *Matricaria camomilla*, *Sinapis arvensis* y *Medicago truncatula*), habiendo extraído en unos casos las plantas de habas, mientras que en otros se las dejaron creciendo junto a las malas hierbas. Tanto el número como la biomasa de las malas hierbas se vio sustancialmente reducido en los casos en que había crecido la cebada con anterioridad. No se observaron diferencias importantes entre los casos en que se extrajo la cebada y en los que permaneció con las malas hierbas. Esto confirma la importancia del efecto alelopático de la cebada frente a las malas hierbas.

Los trabajos futuros deberán centrarse en la identificación de más mecanismos que expliquen los efectos de la diversificación de cultivos tanto en las enfermedades como en las malas hierbas. Asimismo, sería conveniente desarrollar programas de mejora encaminados a la obtención de variedades adaptadas a la diversificación, de modo que se pudieran maximizar los beneficios de ésta.

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Introduction

1. Introduction

Monocropping, that is, growing one single crop in an area in a given time period, is the most common form of agriculture practiced since long over the world [1]. It is a simple method for farmers who have extensive experience in cultivation of a single crop and broad knowledge of all the associated practices relating to cultivation, irrigation, fertilization, disease control, and harvesting in every growth stage [2]. Only a type of equipment and tools is required in monoculture, both during sowing and harvesting stages, and there exist agrochemicals specific for each particular crop [3].

However, the practice of planting the same crop in the same plot of land each year has a number of disadvantages, including reducing the soil fertility because of the continued consumption of nutrients by the crop [3]. Therefore, the farmers become obliged to employ chemical fertilizers to make up for this nutrient deficiency in the soil, which entails negative consequences such as higher economic costs and environmental damage [4]. Additionally, monocropping may enhance the presence of diseases, and pests, since the abundance of host plants in an area facilitates their development, reproduction and distribution. The same can be said of weeds since those more competitive with the crop will thrive in its continuous presence. All this will force the use of pesticides for their control, which again will have negative effects on the environment and the farmers' economy [5].

The term *crop diversification* can be defined as the conscious inclusion in agricultural systems of functional biodiversity at the temporal and/or spatial levels to improve productivity and stability of ecosystem services [6]. It is usually done by growing more than one crop in a given area, employing different strategies and designs for plant species distribution in both time and space. Crop diversification may provide numerous advantages, such as increasing crop yields, lowering pest damage, controlling weeds and improving the land, soil, and water use efficiency [7,8]. It is also a climate-smart method that helps farming to become more resilient to the variability of the weather that is associated with climate change [9,10]. Indeed, resistance of plants to the environmental variations and extreme weather conditions such as floods, droughts, and very high or low temperatures grows with crop diversification [11]. In addition, agricultural diversification can prolong the period of agricultural production and distribute it over the longest possible period throughout the year, which means extending crop production to all seasons of the year. Furthermore, it reduces the economic risk that is inherent in relying on a single type of crop, by diversifying the investments among the different cultivated crops, and ensuring that some profits may be reaped [12]. For all this, crop diversification is one of the most important factors in achieving food security and land sustainability [13].

There are equally some challenges in introducing crop diversification in agricultural systems. The design and the chosen crops must be adapted to the local situation in order to achieve the best results. If it is not properly done, the effects may sometimes be the opposite of those desired. In some cases, the risk of occurrence of diseases may increase [14]. Moreover, because most of the agricultural machines and equipment are designed for monoculture, it may be challenging to find machines and equipment that are good for the wide range of the crops involved in crop diversification. Consequently, it is necessary to design appropriate agricultural machinery for the planting, maintenance, and harvesting of the crops involved in the crop diversification systems [15].

Crop Diversification Types

There are a high variety of crop diversification strategies depending on the crops involved and their distribution in time and space. Crop rotation and cover crops imply growing different crops in the same area but not at the same time. When the crops coincide in time in the same area, the most common types are intercropping, agroforestry, and cultivar mixtures.

Crop rotation is the practice of growing different crops in succession in the same land. In the context of crop diversification, it is considered as one of the important environmental agricultural practices for prevention of crop pests and preservation of the productive capacity of the soil [16]. The cover crop is a specific crop that is not grown for yield, but for the benefits it entails, such as: grown primarily to protect soil from erosion, improve soil fertility, increase water availability for plants, control pests and diseases, increase agricultural productivity, preserve and improve the microbial biodiversity in the soils and conserve and sustain overall ecosystem biodiversity [17]. Generally, the cover crops can be cereals or legumes and they are usually grown in the off-season period, before growing the cash crop [18].

Agroforestry is the practice of integrating trees and livestock with crops in the same area at the same time. There are interactions in agroforestry between the trees and crops from ecological and economic standpoints. It was estimated that at the beginning of the 21st century about 0.6 billion hectares of land are covered by agroforestry systems, which have direct impacts on 1.2 billion people globally [18,19].

Intercropping is the agricultural practice of cultivating two or more crops in the same place at the same time, taking advantage of the synergies generated in the combination of plants [20]. It can be used to increase crop yields and control pests [21].

Cultivar mixture is another important type of crop diversification. It corresponds to cultivation of two or more cultivars of the same species at the same time in the same land. The aim of this practice is to take advantage of the positive characteristics of different cultivars of the same crop [22].

Role of legumes in crop diversification

Legume crops are of major importance in the development of a sustainable agriculture. The most relevant characteristic is their ability to fix atmospheric nitrogen, so enriching the soil and reducing the need for synthetic fertilizers. Additionally, legume seeds are rich in proteins and other nutrients, which makes them very interesting for animal feed and human consumption. Because of all this, legume crops are common elements of a diversified agriculture, and partner with other crops, especially cereals, in many intercrop combinations. However, the study of cereal/legumes intercropping has preponderantly focused on the effects for the cereals, disregarding those for legumes.

One of the main challenges for the cultivation of legumes is the wide array of fungal diseases they are susceptible to. These pathogens may, in some cases, compromise yields, so farmers are compelled to employ expensive fungicides, increasing production costs, not to mention the environmental effects. Weeds are equally a problem in legumes, which are not good competitors against them, so herbicides must be often used.

In this work we will review the available knowledge about the employment of the two main crop diversification strategies where crops are grown simultaneously (intercropping and cultivar mixtures) for the control of fungal diseases and weeds in legumes.

2. Intercropping

Intercropping has been part of traditional agriculture all over the world for centuries and farmers have featured various intercropping techniques. Intercropping was practiced in early civilizations: evidence of intercropping can be traced back to the Indus civilization, which thrived between approximately 2600 and 1900 B.C. [23] and Greece (around 300 B.C.) [1].

Intercropping has been traditionally circumscribed to small farming. In large-scale farms in which the farmers make the best use of agricultural machinery, intensive monocropping is less difficult than it is for smallholder farmers who practice farming for subsistence solely and lack regular access to markets. Livelihood of these farmers is ensured through intercropping. As a result, small farms are the places where intercropping is most commonly practiced [23]. Within this context, intercropping has the reputation of producing stable yields from varied crops with reduced reliance on inputs for plant nutrition and crop protection, thus ensuring production of adequate amounts of food in a stable environment. However, in recent times a change of paradigm is taking place, by which intercropping may play a bigger role in global agriculture. There has been a growing interest for intercropping in the last decades, which is reflected in the increasing number of publications of scientific research about the topic. According to the statistics of the Web of Science database, the total number of published works when search is made introducing "intercropping" in the field *topic* during the period 1940-2020 and in the field *title* during the period 1950-2020 has been increasing since the 70s, with a sharp rise in the last two decades (Figure 1).

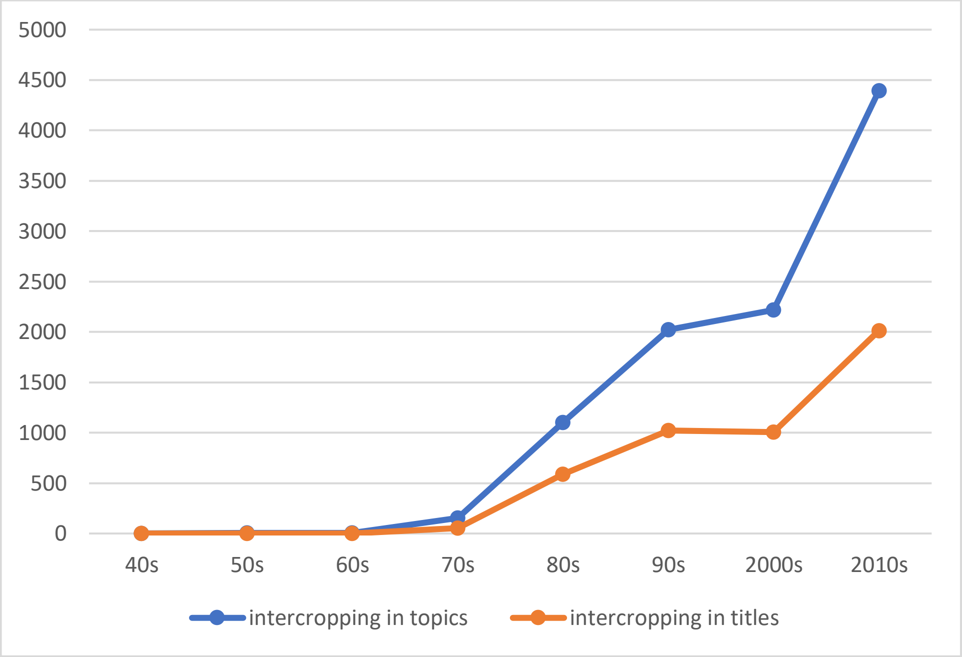

Figure 1. Total number of published research works when "intercropping" is introduced in the *topic* and *title* fields during the period 1940-2020 in the Web of Science database.

The intercropping methods vary widely and depend on a number of factors, including the number of intercropped plant species, how those plants are organized (e.g., in mixed rows, alternate rows, or additional rows), and the percentage of each crop species in the land parcel [23]. According to arrangement of the plants in the field, there are several types of intercropping [1,23] like mixed intercropping and alternate intercropping. In mixed intercropping, the plants are mixed without any defined arrangement, while in alternate intercropping the rows of each crop are alternated with the rows of the other crop(s), one by one, without mixing (Figure 2). A particular case of alternate intercropping is strip intercropping in which a certain number of rows of one crop are alternated with a number of rows of another crop.

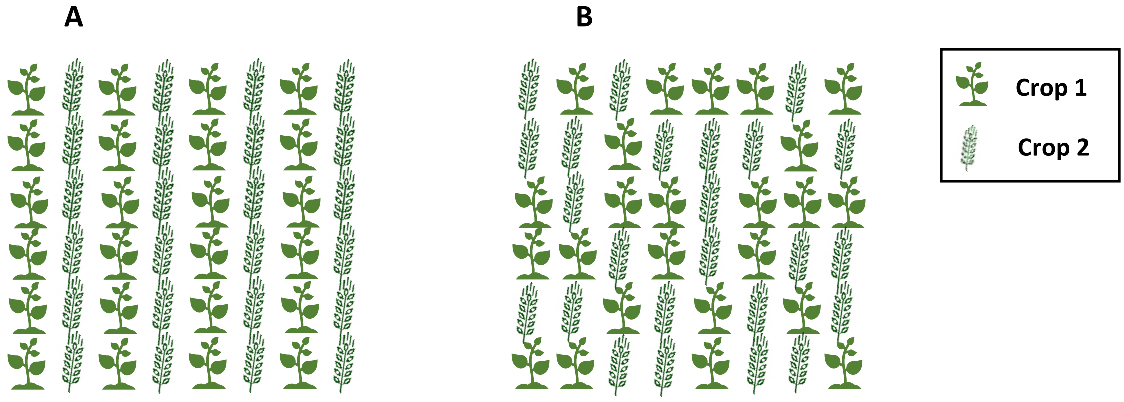

Figure 2. Representation of alternate intercropping (A) and mixed intercropping (B).

The intercropping systems may also be classified by the density of sowing of the mixed crops. Two types are accordingly known: additional intercropping and replacement intercropping. The highest sowing density is concomitant to additional intercropping, which is a cropping system in which the sowing densities of the original crops are maintained. In replacement intercropping, however, a certain proportion of one of the crops is removed and replaced by an equal proportion of the new, or substitute, crop. There may be many other intercropping combinations, depending on the different sowing densities of the existing and new, or added, crops.

Intercropping can play an important role in agricultural sustainability since it corresponds to efficient use of resources above and below the ground like sunlight, soil water, and nutrients [24,25]. It has been reported to increase crop yield and yield stability in many cases and to increase the economic return for the farmer [23]. Especially appealing is the use of intercropping as a means of improving soil fertility when legumes are used as a component of the crop mix, given its established role in biological nitrogen fixation [3].

This, and the fact that it reduces the spread of pests, diseases, and weeds, makes intercropping especially advantageous in organic farming [26]. This effect on diseases has been also the subject of a growing interest in recent decades, as reflects the statistics of scientific publications involving these topics (Figure 3).

One of the most interesting features of intercropping is its ability to reduce crop epidemics, making it a good alternative or complement to pesticides in the control of plant diseases, insects and weeds [1,27]. Again, the effect of intercropping on diseases has gained increased attention in the scientific field in the last decades (Figure 2).

It has been reported that intercropping can reduce the incidence of diseases by as much as 45% in the case of many diseases such as yellow rust of wheat, powdery mildew of wheat, chocolate spot and fusarium wilt of faba bean, yellow rust in barley, and rust in faba bean [28]. A review by Boudreau [27] showed that intercropping reduced disease severity in 73% of the studies involving fungal pathogens and in 70% of the studies relating to virus diseases. In accordance with this, Eshetu et al. [29] stressed that intercropping is an effective method for controlling pests and diseases, especially when the other intercropped plant is not host for the pest or infected with the disease(s) to control.

Despite all its benefits, intercropping faces some challenges. For instance, reduction of yields in mixed cropping systems below the yields in monocropping systems has been reported in some cases [24,30,31]. This reduction can, at least in part, be ascribed to the types of crops or crop cultivars employed in the intercropping, which stresses the importance of choosing the right crops to combine since not all crops have symbiotic relationships with one another. One step further is choosing the cultivars of each crop that can successfully grow in mixtures. Selecting and breeding the optimal cultivars for intercropping is critical for success of the intercropping systems [1]. Another challenge is the limited availability of pesticides that are compatible with the mixed crops, especially herbicides. Equally challenging is the lack of harvesting equipment adapted to intercropping. These difficulties may force the agricultural producers to further rely on the human labor for crop production operations, which can increase the costs of production [24].

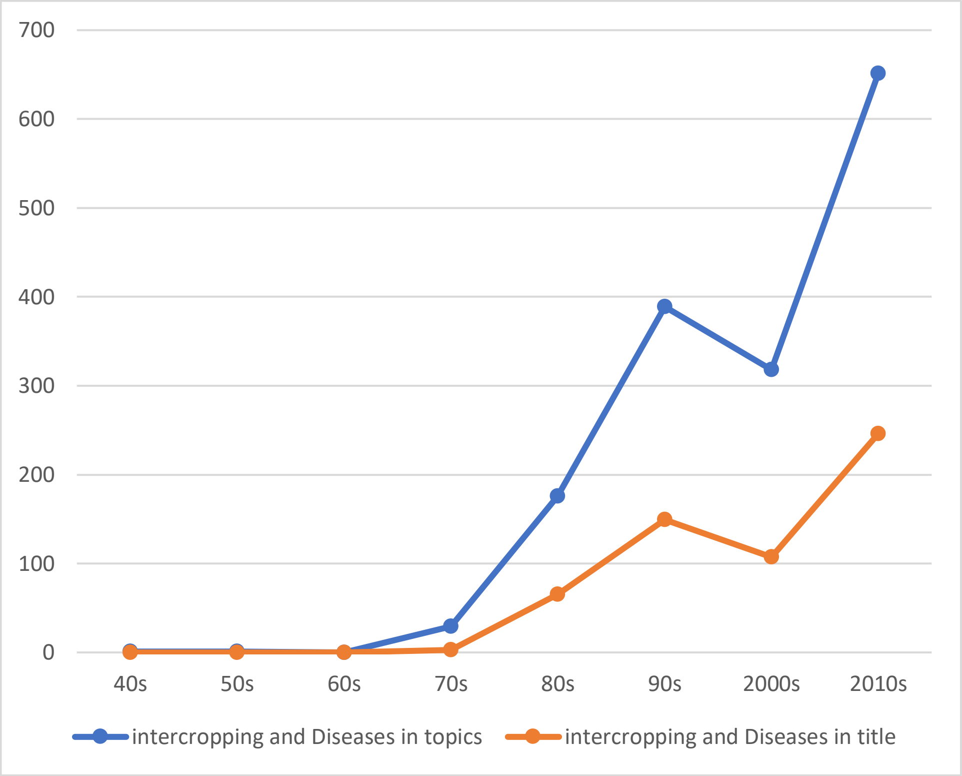

Figure 3. Total number of published research works when "intercropping and diseases" is introduced in the *topic* and *title* fields during the period 1940-2020 in the Web of Science database.

3. Intercropping to control diseases in legumes.

As it happens in other crops, intercropping has proved successful in controlling diseases in legumes. This includes some of the major diseases that affect these crops.

Foliar diseases: biotrophic fungi

Several studies support that mixing faba bean with cereals may help in the control of rust (caused by the pathogen *Uromyces viciae-fabae*). This reduction, however, depends on the accompanying cereal and the intercropping system used. Experiments conducted in Egypt and Palestine for two years [32]] showed that rust severity was reduced in faba bean in mixed intercropping with barley, durum wheat, oat, or triticale, with the highest reduction of 35% in the combination with barley. Eshetu et al. [29] also reported a reduction of 20% of rust in faba bean in Ethiopia in mixed intercropping with wheat, barley, maize, and oat. Equally, in an experiment performed in China, faba bean rust was reduced by 39% when it was intercropped with barley or wheat in strip intercropping, with two rows of faba bean and six rows of wheat [28]. Villegas et al. [33] found a decrease in the severity of rust of up to 22% in faba bean in alternate intercropping with barley in the South of Spain, but not with wheat. This is in accordance with Kamalongo and Cannon [34] who did not obtain any level of control for rust in faba bean when grown in alternate rows with wheat. Intercropping faba bean with a non-cereal crop such as pea has not proven useful for the control of rust [33].

Guo et al. [35] found that when faba bean is intercropped with wheat, its yield increased significantly over the average yield obtained when it is monocropped. The area under the disease progress curve (AUDPC) for faba bean rust decreased by 22.3-54.7%.

The combination of beans and maize on bean rust was assessed in Ethiopia under mix and alternate intercropping, which attained 50% and 35% reductions in disease level [36]. Boudreau et al. [37], however, obtained unconclusive results on the effect of intercropping on rust of beans when they were partnered with maize. Disease decreased in only one of two experiments performed in Oregon (USA). In this case, the system tested was alternate intercropping (one row of maize per two rows of beans). Lemessa [38] reported no control of rust in the mixture of beans and maize under alternate intercropping with addition.

The effect of intercropping on powdery mildew diseases in legumes has been little investigated. Zivanov [39] studied the effect of mixed intercropping of pea and oat on powdery mildew, but got no clears results: they reported a reduction of disease of 20-30% on pea leaves, but no effect on global disease in plants. Villegas et al. [40] conducted several experiments over multiple years and locations in the South of Spain, where pea was mixed in alternate intercropping with one of these crops: faba bean, wheat, and barley. In this case, the results indicated that intercropping pea with barley or faba bean led to a clear reduction in powdery mildew severity on the pea plants (44% and 32%, respectively). However, intercropping with wheat or faba bean did not show the same positive effect on powdery mildew management.

Foliar diseases: necrotrophic fungi

The effect of intercropping faba bean with different cereals on chocolate spot disease (caused by the fungus *Botrytis fabae*) has also been the subject of several studies. In a multi-site experiment in Egypt, the Palestinian Territories, Spain, and Tunisia faba bean was combined either with barley, oat, triticale or wheat in a replacement mixed intercropping design. The results showed a significant decrease in the severity of chocolate spot on faba bean intercropped with barley and oat (with average reductions around 30%), but not with the other two cereals [41]. Sahile et al. [42] reported small reductions of disease in faba bean in replacement alternate intercropping (3 rows of faba bean and 1 row of the companion crop) with both barley and maize in Ethiopia. On the contrary, replacement alternate intercropping (but 1:1, i.e., one row of faba bean and one row of the other crop) of faba bean with barley showed no decrease in chocolate spot severity in Ethiopia [29], but when the alternating cereal was maize, reductions of disease severity of around 25% were obtained, also in Ethiopia [43]. A meta-analysis by Zhang et al. [28], with trials from China, found that strip intercropping of faba bean with barley or wheat resulted in an average reduction of the disease incidence of 33%. The work of Guo et al. [44] also confirmed the decrease of disease in strip intercropping with wheat in China.

In the few cases in which the companion crop of faba bean is other than a cereal, no control of the disease was found, as was seen with pea and common vetch in Spain [41] and with pea in Ethiopia [42].

In Nepal, it was found that the combination of chickpea with mustard at different densities attains a reduction in disease severity of up to 50% in grey mold (caused by *Botrytis cinerea*) when the density of mustard is double than that of chickpea [45].

Replacement mixed intercropping of pea with faba bean, tricale, barley and oat in Spain and Tunisia reduced the severity of pea aschochyta blight (causal agent: *Dydimella pinodes*) in different degrees [46]. The greatest reductions were obtained with faba bean and tricale, with decreases ranging 40-80%, while oat, barley and wheat provided lower reductions and less consistent across experiments. These inconsistent results were also observed by Kinane and Lyngkjaer [47] with pea and barley, also in replacement mixed intercropping. On the contrary, replacement alternate intercropping of pea and barley in France reduced ascochyta blight by 25-30% [48].

Marzani [49] reported that the combination of chickpea with wheat, faba bean or oilseed rape in Irak in alternate intercropping resulted in reductions of ascochyta blight disease of 25% to 40% (with wheat or both faba bean or oilseed rape, respectively).

In the case of lentil, its combination with linseed in Australia under different intercropping strategies has obtained control levels of disease from 50% to 90% [50].

The disease angular leaf spot of beans (caused by the pathogen *Phaeoisariopsis griseola*) has presented reductions in severity of up to 30% with alternate intercropping of beans and maize at different densities in Kenya [51]. Lemessa [38] in Ethiopia, on the contrary, did not find disease reductions with a similar intercropping of beans and maize neither for angular leaf spot and floury leaf spot (caused by *Mycovellosiella phaseoli*).

Soil diseases

Fusarium wilt disease is a soil disease of faba beans caused by the fungal pathogen *Fusarium spp*. Strip intercropping of faba bean in China (with two rows of faba bean alternated with six rows of wheat) has proved to control the disease by as much as 40-80% as compared to the monocrop [52] In other respects, Zhang et al. underlined that intercropping faba bean with cereals can reduce the odds ratio of Fusarium wilt in faba bean by 72% [28]. A controlled-condition experiment equally found the reduction of *Fusarium oxysporum* in the soil by as much as 60% when peanut was mixed with the medicinal herb *Atractylodes lancea* [53]. Chickpea is also a host for *Fusarium oxysporum*, and it has been reported that in India alternate intercropping with mustard, onion or garlic achieved reductions in disease level: 24%, 31% and 28.5% respectively [54].

Experiments in Egypt also found that addition alternate intercropping may reduce root diseases in faba bean caused by soil fungus *Rizoctonia solani* and *Fusarium solani*: the combinations with garlic, lupin and fenugreek increased faba bean survival by 80%, 40% and 60% respectively [55]. These diseases were also reduced in lentil when it was combined with cumin, onion, garlic and anise (20%, 31%, 50% and 59% disease reduction, respectively) in experiments in Egypt [56].

The combination of soybean with corn has proved successful in reducing the levels of root diseases, as is the case of phytophtora blight (caused by *Phytophthora soja*), with reductions of 30% in alternate replacement [28], red crown rot (caused by *Cylindrocladium parasiticum*) with reductions as high as 50% [57] and Fusarium wilt, with reductions of 18-37% under strip intercropping. All these investigations were conducted in China.

Bacterial diseases

The impact of intercropping on common bacterial blight of beans, caused by the pathogen *Xantomonas campestris* pv. *Phaseoli*, has been the subject of research in Ethiopia. In one study, the combination of beans with maize in mixed intercropping reduced disease incidence by 23% [36]. In another study, the combination of beans with maize under the same system got as much as 66% reduction in disease incidence, while the combination with sorghum rendered 57% reduction [58].

Mechanism of intercropping to control diseases in legumes

There are various theories about the mechanism by which intercropping reduces disease incidence, the way in which plants of one species support plants of another species in their fight with pathogens. Knowledge of how some plants reduce the damage to other plants by producing specific chemicals or developing resistance to certain pests or diseases helps in reducing the severity of those pests and/or diseases [59].

Several mechanisms of intercropping have been postulated, some are related to the changes in density of the host crop, and others pertain to the companion crops, by affecting the dispersal of the pathogen, altering the microclimate, inhibiting the pathogen by allelochemicals, or modifying the host itself [27]. In general, as density of the host plant increases, severity of the disease increases. Therefore, the relatively lower plant density of the host in intercropping than in monoculture reduces the level of infestation [59]. As for dispersal of the pathogen, the non-host crop may act as a barrier to the movement of fungal spores. The vector effect is another factor by which many diseases such as the viral, bacterial, and fungal diseases are spread, which are transmitted by different types of insects. The intercropping system directly affects those vectors in different ways, such as reducing the chance for transmission of disease, repelling those insects, or acting as a barrier that reduces movement of those vectors [60]. Even some plants act as traps for pests and harmful insects since certain plants are preferable for pests than others [61]. Legumes in particular play a vital role in the intercropping systems, fixing atmospheric nitrogen in the soil and, thus, benefitting the neighboring plants of another species. This makes the soil rich with fixed N for use of the neighboring plants [27].

As for the diseases in legumes, not many of the studies cited above have suggested which mechanisms might be responsible for the decrease in the level of infection. In the case of chocolate spot of faba beans, Fernández-Aparicio et al. [41] found that relative humidity was higher in the intercrops than in the monocrop, which in principle would be in contrast with the reduction of disease in the mixtures. But they also observed a stronger decline of humidity in the morning in the canopy of monocropped faba bean than in the canopy of the intercrop and hypothesized that it could favor spore release from botrytis conidiophores, so facilitating the spread of the disease. On the contrary, Guo et al. [44] reported a higher relative humidity in the monocrop than in the intercrop, which would clearly promote the development of the disease. It is important to remember that in the first case it was mixed intercropping, while in the second case strip intercropping was chosen.

For rust in faba bean, a clear barrier effect by barley in alternate intercropping appears as responsible of at least some part of the decrease of the disease [33]; barley, with a higher height and vegetative development than the other assessed crops (wheat and pea) was more effective as a barrier. It is remarkable that in this study crop density was disregarded as having an important effect on the development of disease. For rust in beans, the presence of maize has been suggested to alter microclimate thus favoring the reduction of disease [37].

A barrier effect by barley and faba bean also seems to be responsible for part of the reduction of powdery mildew in pea [40]. Again, crop density does not seem to influence importantly the disease.

In the case of root diseases, several studies support that an important contribution to the control comes from the release by the companion crops of root exudates that are toxic for the fungal pathogens, such as phenolic compounds or others [53,54,56,57].

4. **Intercropping to control weeds in legumes.**

Weeds bring about significant crop and financial losses for the agricultural production worldwide, losses that have been estimated of about 10% per year [62]. Chemical control is the most widely used method to control weeds, being the best and most effective method of weed control, but after excessive use of herbicides, many problems start to appear, such as the development of weed resistance to the herbicide molecules and damage of the environment, including the plants, soil, water, and microorganisms. Additionally, herbicides are costly for the farmers and using them poses financial pressures on them [63]. According to pesticide producers, the total expenditure on pesticides worldwide was nearly US$56 billion in 2012. The largest proportion of that expenditure (about 45%) was spent on herbicides, followed by insecticides, fungicides, and other pesticides [64]. Intercropping has proven effective in the control of weeds in many cases, especially in cereals, and has been proposed as an alternative or complement to herbicides [65]. There are also studies which focus on the reduction of weeds in the legume crops.

A quite successful combination for the control of weeds is that of pea and barley. Trials in several countries have obtained different degrees of reduction in weed biomass as compared to pea monocrop under different inercropping systems: Argentina, 30% [66], Denmark, 50% [67], Poland, 60% [68] or a research carried out across Germany, United Kingdom and Italy, with reductions of 66% [69].

Intercropping faba beans with cereals has equally resulted in a reduction of weed infestation, as was shown by studies in different countries. In a two-year study in Greece, faba bean was intercropped with barley in two crop arrangements, alternate rows and mixed. The results indicated that this combination of crops leads to effective suppression of weeds in faba bean, especially the corn poppy [70]. A study was conducted in Iran during two years in which wheat was intercropped with faba bean according to three intercropping patterns: alternate-row intercropping, within-row intercropping, and mixed intercropping. It was found that intercropping faba bean with wheat produced important decreases in weed presence (measured as weed biomass) of around 70% [71]. Another two-year research carried out in Morocco obtained suppression of weeds in faba bean in alternate addition intercropping with oat, wheat or both cereals simultaneously: the reduction in weed pressure was around 50% [72].

In a two-year experiment in Germany, lentil was combined by mixed replacement intercropping with barley, oat, wheat, linseed or buckwheat, being successful only the first three combinations, with levels of control as high as 80% in the case of oat, and around 60% for what and 50% for barley, always in comparison with the monocrop of lentil [73]. Even with the same crops, results may not always be consistent enough. In a three-year experiment in North Dakota (USA), with lentil mixed with wheat in addition alternate and addition mixed intercropping, there was no control of weeds in the first year, probably due to water stress that prevented wheat and lentil to develop a proper canopy to hamper weed development, while in the two next years, weed control reached 96% and 68% in weed biomass respectively [74]. Inconsistent results were also obtained in another two-year experiment in Minnesota (USA): the mixture of lentil in addition alternate intercropping with different crops (wheat, oat, radish, rye and brassica) yielded no clear weed suppression [75].

Intercropping strategies to control weeds in chickpea have been the subject of several studies in Iran, trying different companion crops. The mixture of chickpea with barley in alternate replacement intercropping got biomass reductions near to 90% [76]. When it was combined with wheat under replacement mixed and replacement alternate strategies, the decrease in weed biomass reached 70-80% [77]. On the contrary, alternate replacement intercropping with saffron only obtained a 35% weed biomass reduction. Finally, addition intercropping with an endemic plant of the area such as dragon's head (*Lallemantia iberica* Fish. et Mey) got weed biomass reductions ranging 40-65% [78]. In India, alternate replacement intercropping of chickpea and wheat attained a modest reduction in weed biomass of 35% [79]. In India, a two-year experiment found weed biomass reductions of 25% in the combination of chickpea and mustard in addition intercropping [80].

Wortmann and Workayehu [81] reported reductions in weed biomass in a three-year experiment in Ethiopia testing the combination of beans and maize in addition with replacement intercropping. Strip intercropping in Poland of beans with either wheat or maize obtained reductions ranging 30-50%. [82].

The combination of soybean and maize to control weeds has been investigated in different countries and situations. In China, Su et al. [83] reported reductions of 40% in weed biomass referred to soybean monocrop when soybean and maize were grown under alternate intercropping with replacement. Addition alternate intercropping also obtained reductions of 45-50% in weed biomass in Poland [84]. In Michigan (USA) replacement intercropping got 40% reduction levels and addition intercropping reached 65% [85]. A two-year experiment in Ghana with alternate addition intercropping of soybean and maize presented a 45% of weed biomass reduction on average [86]. In Iran, the mixture of soybean and maize in alternate replacement system was not very successful, with a limited 15% of reduction in weed biomass; but when marshmallow was added to the combination (i.e., soybean – maize – marshmallow), the decrease reached 30% [87]. It was in Iran also where the combinations by mixed replacement intercropping of soybean with basil or borage were assessed, obtaining weed biomass reductions of up to 60-70% [88]. A two-year experiment in France researched the effect on weeds of the combinations of soybean with lentil, sorghum, buckwheat or sunflower under mixed or alternate intercropping, both with replacement. Results showed no weed control when lentil was the companion crop, but reductions of weed biomass in the range of 30-80% in the combinations with sorghum, buckwheat and sunflower [89]. Reductions in weed biomass as compared to soybean monocrop were reported for mixed intercropping with addition of soybean and three *Medicago* spp. in two trials in Minnesota, USA [90].

However, using intercropping to control weeds in soybean has not always been successful. The mixture of soybean and cotton in alternate intercropping with addition got a reduction in weed biomass of 33% for grasses, but none for broad-leaf weeds [91]. De la Fuente [92] reported no weed control with alternate intercropping with replacement of soybean and sunflower in Argentina.

Modest reductions (around 8%) of weed biomass in cowpea intercropped with maize under different systems (alternate with replacement, alternate with addition and mixed) were found in an experiment carried out in Iran too [93]. But in two field experiments in Greece, the combination of cowpea and maize in addition alternate intercropping reach a decrease of weed biomass of around 50% [94]. In these experiments the combination of beans and maize was also tested, reaching similar levels of weed suppression. Intercropping also controls weeds in a forage legume such as subclover, when mixed with durum wheat, as was found in a research carried out in Italy [95]. In this case, three different types of intercropping were tested, two alternate (with different distances between rows) and one mixed. The highest reductions of weed biomass (around 80%) as compared with subclover monocrop were obtained with the alternate intercropping with shorter distance between the rows of the crops (10 cm); the lowest reductions (around 40%) were observed in the alternate system but with a wider distance between rows (17.5 cm).

However, using intercropping does not always guarantee a reduction of weed pressure on legumes. Choudhary et al. [96] reported a decrease of weed infestation in maize in Himalayan India when intercropped with cowpea, french bean or black gram under different systems (alternate in addition or replacement with various proportions), but the same was not true for the legumes: in these crops, weed presence was lower in monocrop than when intercropped with maize.

A parasitic weed like broomrape (*Orobanche crenata*) has also been controlled by the use of intercropping. Fernández-Aparicio et al. [97] found that combining faba bean or pea with oat in mixed intercropping attained a reduction of the number of broomrapes of 50-90% in the south of Spain; mixing with triticale, however, did not attain any significant reduction of broomrape. Broomrape presence was also reduced in faba bean in mixed intercropping with fenugreek by 30-40% in Egypt [98].

Mechanism of intercropping to control weeds in legumes

Different mechanisms have been proposed to explain weed suppression and the reduction of weed density in the intercropping systems such as allelopathy, the competition of the companion crops with weeds for resources (such as light, water of nutrients) or alterations in humidity and temperature [6]. Allelopathy corresponds to stimulatory or inhibitory effect of one plant on development of neighboring plants through the release of one or more biochemicals that affect the survival, germination, growth, and reproduction of neighboring plants [99]. It is associated with many crops, such as barley, sorghum, corn, oat, pearl millet, sesame, soybean and sunflower, which have proved to be effective for weed suppression when any of them is intercropped with cereals, legumes, and oilseeds [100]. Intercropping, then, may facilitate allelopathic interaction that results in effective weed control [101,102] .

In the case of legumes, the investigations about the mechanisms of suppression of weeds have been mainly focused on the competition for light and nitrogen. Su et al., [83] studying the intercrop of soybean and maize, found that weed biomass was directly correlated with the light transmittance below the crop canopy. The lower light transmittance in the intercrop in comparison with soybean monocrop might then account for the reduced weed biomass in the mixture of maize and soybean. Poggio [66] reported that, in the intercrop of pea with barley, the amount of photosynthetically active radition (PAR) that reached the weeds in pea monocrop was higher than in the mixture. In this case, it was also found that barley was more competitive for nitrogen than pea, son in the intercrops there was less available nitrogen for weeds. Similar results for nitrogen dynamics were reported by Hauggaard-Nielsen et al. [67].

As for broomrape in faba bean and pea, it has been suggested that cereals might inhibit the germination of the seeds of broomrape [97]. As for the decrease of broomrape in faba bean when mixed with fenugreek, a molecule exudated by the roots of fenugreek, trigoxazonane, has been proposed as responsible for the inhibition of the germination of broomrape seeds [97,103].

5. Cultivar mixtures to control diseases in legumes.

Cultivar mixtures have been reported to be effective in the control of diseases and pests [104]. This approach involves mixing cultivars with contrasting responses to the pathogens in terms of susceptibility and resistance, which provides an acceptable control of the disease. One of the challenges when using resistant varieties is the possibility that the resistance might be overcome by the pathogen. This is more likely as the exposure of the genes is greater, as is the case of monocrops of resistant cultivars grown year after year in the same place. The point in cultivar mixture is to get enough presence of the resistance genes to get control of the disease, but not so high that the resistance might be endangered. In this method, it is very important to specify the suitable proportions and arrangement of the varieties to minimize crop losses and maximize their yields in a right balance compatible with sustainable agriculture [105].

There are few studies to determine the role of cultivar mixtures in controlling diseases of legume plants. One of these studies was implemented in two sites in Ethiopia by mixing susceptible and resistant bean cultivars to evaluate the effect of this varietal mixture on the spread of the bean rust. Incidence of this disease was reduced when the proportion of the susceptible cultivar in the mixture was reduced [106]. Lemessa [38] tested mixing two bean cultivars in different proportions in Ethiopia, one susceptible to angular leaf spot and rust and the other one resistant to both diseases. Reductions of angular leaf spot and rust were observed in the susceptible cultivar with increasing proportions of the resistant one (as much as 30% for angular leaf spot and 28% for rust with the highest proportions of the resistant cultivar). Another study in Nepal, in which four bean cultivars were mixed, showed that infection by anthracnose was reduced in the cultivar mixtures as compared to plots with just one cultivar [107]. In these previous cases, cultivars were mixed inside the rows of the plots. On the contrary, Villegas-Fernández et al. [40] tested in the South of Spain the mixture of two cultivars of pea, one susceptible and the other one resistant, in alternate rows at different proportions to examine the effect on powdery mildew of pea. The disease decreased in the susceptible cultivars as the proportion of the resistant one increased: from 38% to 49% of disease severity as the proportion of the resistant cultivar increases from 25% to 75%. The same type of experiment was carried out for rust in faba bean [33], mixing two cultivars of faba bean (one resistant and the other one susceptible to rust) in alternate rows at different proportions. The results also presented a reduction in rust severity in the susceptible cultivar as the proportion of the resistant one was higher: 20% to 83% as the proportion of the resistant cultivar varied from 25% to 75%.

Mechanism of cultivar mixtures to control diseases in legumes

Finck et al. [22] suggested that the main mechanisms of crop disease control in the cultivar mixtures are frequency effects (i.e., dilution of inoculum), the barrier effect and induced resistance. The presence of the resistant cultivar hampers the multiplication of the pathogen, which leads to the dilution of the inoculum, dilution that will be higher as higher is the proportion of the resistant cultivar. Equally, the plants of the resistant cultivar act as a barrier to the dispersal of the pathogen, interrupting its expansion. Finally, the induced resistance comes from the stimulation of the mechanisms that promote the recognition of the pathogen.

In the case of cultivar mixtures of legumes, Villegas-Fernández et al. [40] suggested that for powdery mildew in pea the barrier effect might play a major role, since introducing just one row of the resistant cultivar produced a drop in disease severity of as much as 38% in plants placed three rows away. These authors also stressed the relevance of the barrier effect in cultivar mixtures for rust in faba bean. In this case, an experiment under controlled conditions endorsed this hypothesis [33].

6. Conclusions

We have reviewed a total of 76 articles, 41 about diseases, and 35 for weeds. In two cases, a paper included studies about two different diseases, totaling 78 studies for different biological stresses (Tables 1 and 2). Most of the studies about diseases are related to faba bean, with 16 publications. Not all important diseases have been assessed either (as *Ascochyta fabae* of faba bean or rust in pea). This is an issue that, although it has received considerable interest in the last years, still offers a great field of research.

The first conclusion is that intercropping and cultivar mixtures are useful for the control of diseases and weeds. In an overwhelming majority of the works considered, a considerable decrease in disease incidence or weed pressure was obtained. Only in a very few studies no positive or little relevant results were observed. The level of suppression attained is in some cases high enough not to need to resort to any other means of control, and in other cases some complementary action may be needed.

Table 1. Number of studies about intercropping and control of different biological stresses (diseases and weeds) in legume crops. One publication deals with two types of stresses.

Crop	Nº of studies	Biological stress				
		Fungal foliar biotrophic pathogens	Fungal foliar necrotrophic pathogens	Fungal soil pathogens	Bacterial foliar pathogens	Weeds
Faba bean	21	6	6	4		5
Pea	8	1	3			4
Lentil	5		1	1		3
Chickpea	9		2	1		6
Bean	10	3	2		2	3
Soybean	14			3		11
Others	4					4
Total	71	10	14	9	2	35

Table 2. Number of studies about cultivar mixtures and control of different biological stresses (diseases and weeds) in legume crops. One publication deals with two types of stresses.

Crop	Nº of studies	Biological stress		
		Fungal foliar biotrophic pathogens	Fungal foliar necrotrophic pathogens	Bacterial foliar pathogens
Faba bean	1	1		
Pea	1	1		
Bean	5	3	1	1
Total	7	5	1	1

It is important to bear in mind that intercropping and cultivar mixtures present many options for their implementation: crops or cultivars chosen, their number, spatial arrangement of the mixtures or density of plants. This means that a series of parameters must be established and that, depending on how it is done, the result may be one or another. This is true for many of the reviewed studies, where some combinations resulted in disease or weed reductions, while others did not. So, it would be necessary to assess the effect of one or other type of intercropping and cultivar mixtures on diseases as weeds, so as to optimize the best system for each given situation. It is quite likely, then, that a higher level of control will be achieved once the diversification systems are finetuned (testing the effect of the different combination types, distance between rows, plant density and many others).

There is very little information about the mechanisms underlying the effects of intercropping and cultivar mixtures on diseases and weeds of legumes. Most of the few studies that address this issue move in the field of the hypothesis rather than in that of certainties. But it is of high importance to determine these mechanisms since from their understanding it will be possible to better design the diversification strategies adapted to each situation. For instance, if the barrier effect plays a major role in a certain case, it will be important to choose a crop (or a cultivar) that outstands in height and vegetative development.

This leads to the necessity of choosing not only the right crop (in the case of intercropping) but also the cultivars more suited for each situation. It would be very useful to have a database of a set of characteristics of each cultivar that may be relevant for the diversification, so it will be easier to choose those expected to perform better. A step further is the development of breeding for diversification, so that genotypes are selected for their performance under different diversification scenarios, such as intercropping or cultivar mixtures. So far there has been little work in this direction, so there is a great potential here to improve the benefits of diversification in the control of diseases and weeds.

It can be concluded, then, that there is a great potential in crop diversification for the control of diseases and weeds, helping reduce the use of pesticides and herbicides in the frame of a sustainable agriculture. Much more work on different crops and systems will be needed to develop all the possibilities that these strategies offer.

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Objectives

After consideration of the previous background, and taking into account the reality of the legume crops in the Guadalquivir valley area, the following objectives were established for our work:

1. Determine if it is possible to control powdery mildew in pea by intercropping with other crops (wheat, barley and faba bean), with experiments in different environments.
2. Determine if it is possible to control powdery mildew in pea by cultivar mixtures (one cultivar of pea susceptible to the disease and the other one resistant), with experiments in different environments.
3. Assess the possible mechanisms behind the effects of intercropping and cultivar mixtures on powdery mildew of pea.
4. Determine if it is possible to control rust in faba bean by intercropping with other crops (wheat, barley and pea), with experiments in different environments.
5. Determine if it is possible to control rust in faba bean by cultivar mixtures (one cultivar of faba bean susceptible to the disease and the other one resistant), with experiments in different environments.
6. Assess the possible mechanisms behind the effects of intercropping and cultivar mixtures on rust of faba bean.
7. Determine if it is possible to control weeds in faba bean by intercropping with other crops (pea, what and barley), with experiments in different environments, establishing which intercropping method may be more effective.
8. Investigate the mechanisms explaining the effects of intercropping in weeds, especially allelopathy by barley.

Chapter I

Crop diversification to control powdery mildew in pea

1. Introduction

Pea (Pisum sativum L.) is a major temperate grain legume widely cultivated worldwide[1]. Its high protein content makes pea highly suitable for animal feed and a good alternative to meat as a source of proteins for human diets [2]. Like other legumes, pea fixes atmospheric nitrogen, enriching soils and contributing towards sustainability. However, as any crop, pea production can be constrained by a number of pests and diseases [3]. Powdery mildew is an air-borne fungal disease, caused by the biotrophic pathogen Erysiphe pisi DC. Infection affects all aerial parts of the plant, with losses that may reach 50% of yield [4]. Although methods such as planting early in the season or using early-maturing cultivars can be implemented for promoting escape from the disease, effective control is really achieved by using chemical fungicides or resistant cultivars. The problem with chemicals is that their use entails damage to the ecosystems and increases the economic costs of the farmers. As for resistant cultivars, only a limited number of them are available, and with a resistance that relies on a narrow genetic basis. Only three genes for resistance have been described, namely er1, er2 and Er3, with only the first one being widely used in breeding programs all over the world [5]. The risk of the pathogen overcoming these resistances is high, especially when they occur in large areas of genetically homogeneous plants [5]. Evenmore, in addition to *E. pisi*, other species such as *E. trifolii* Grev. or E. baeumleri U.Braun & S.Takam. Magnus might also affect pea under certain conditions and are reported to overcome er1 resistance[6-8].

Crop diversification, either by intercropping, i.e, mixing two or more crops, or by mixtures of genotypes of the same crop, has proven useful to reduce disease pressure in several crops [9,10]. The objective is to modify the traditional monocrop environment in such a way that it hampers the process of infection and the extension of the pathogen, or that it provides additional strengths to the host crop to fight the infection. This effect on diseases adds to other known advantages of diversification, such as an increase of yield and reduction of fertilizers, or the positive effects on beneficial insects and pollination [11-13].

Legumes are popular components in diversification strategies, given the ecological services they provide to other crops and to the environment as a whole. In particular, cereal-legume intercrops are of great interest because of the synergies they deliver and have been the subject of several studies, including their effects on disease reduction [14-16]. On the contrary, mixtures of legume cultivars have been less studied, with only some studies on common bean available so far.

Intercropping pea with cereals can reduce pea diseases such as ascochyta blight (*Peyronellaea pinodes* [Berk. & A. Bloxam] Avesk., Gruy. & Verkl.) [17-19] and broomrape (*Orobanche crenata* Forsk.) [20]. As for powdery mildew, only one experiment has been reported, which presented unclear effects of pea/oat mixtures on disease reduction [21].

With the objective to assess benefits of diversification in the control of powdery mildew in pea we established a series of experiments, first to identify the most efficient intercrop, and second to determine the optimal proportion of resistant and susceptible pea cultivars in a mixture.

2. Materials and methods

2.1. Field trials

Six field trials were carried out in two different locations in the South of Spain (Córdoba and Almodóvar del Río) from 2015 to 2019 (Table 1) to study the effect of various intercrops on pea powdery mildew infection.

Table 1. Field trials carried out for the study of the effect of intercropping on the pathosystem pea/powdery mildew.

Trial	Cord1-15(i)	Cord1-16(i)	Cord1-17(i)	Alm1-19(i)	Cord1-19(i)	Cord2-19(i)
Location	Córdoba	Córdoba	Córdoba	Almodóvar	Córdoba	Córdoba
Max. T (ºC)	35.3	31.7	32.2	35.2	35.7	35.7
Min. T (ºC)	-3.3	-2.3	-3.4	-0.8	-2.8	-2.8
Mean T (ºC)	12.3	12.6	14.3	14.2	12.5	12.5
Precipitation (ml)	150	336	143.8	107.2	110.4	110

Each trial consisted of monocrops of pea, wheat, barley and faba bean (cvs. Messire, Califa, Henley and Brocal, respectively), intercrops of pea with each one of the other crops; and a monocrop of pea at 50% density (doubling the distance between rows). Each plot consisted of eight 3-m long rows (except for the plots with pea at 50% density, with only four rows), with 35 cm between rows. Sowing densities were 30 seeds/row for legumes, and 200 seeds/row for cereals. Intercropping system was alternate with replacement, at 50%. This means that a row of each crop is alternatively sown, so ending up with a rate of 50/50 in the plot (Fig. 1). A randomized complete block design with four replications was used.

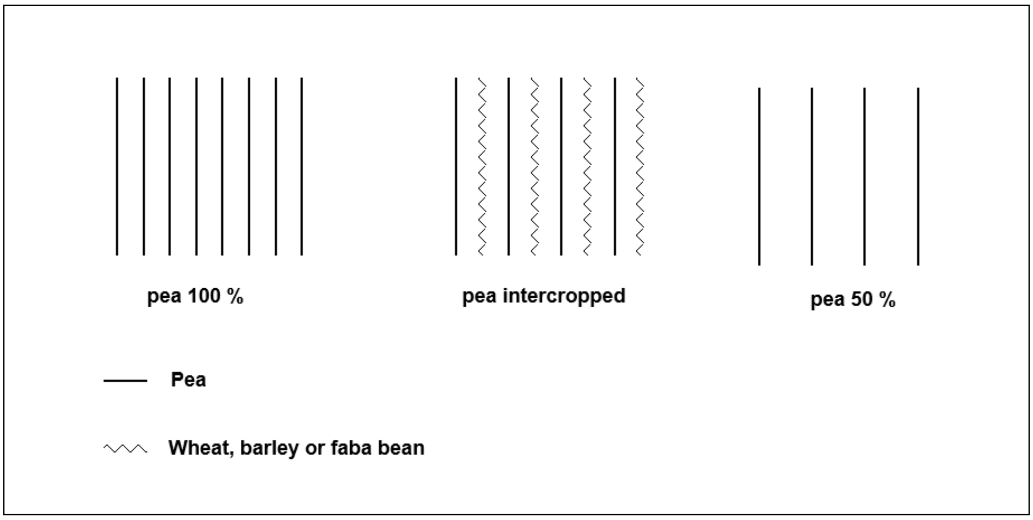

Figure 1. Experimental design for the intercropping experiments.

Disease severity (DS) was visually estimated as the percentage of whole plant canopy covered by powdery mildew. A single severity value was assigned to each plot as the overall DS on the two middle rows (discarding those plants at the extremes of the rows). Evaluations were made one week after first symptoms started and repeated every 7-10 days till plant senescence. The area under disease progress curve (AUDPC) was subsequently calculated with the formula:

$$\mathrm{AUDPC} = \sum_{i=1}^{i=n-1} \tfrac{1}{2} \{(y_{i+1} + y_i)(x_{i+1} - x_i)\}$$

y_i = value of evaluated parameter at day 1
x_i = time (days)
n = total number of observations

Crop height was assessed at full maturity of crops in trials Cord1-18(i), Alm1-19(i) and Cord2-19(i). Five plants of each crop per plot were measured from the ground to the top of plants, not stretching them, with the help of a ruler. Differences between the height of the companion crops and of pea in the intercropped plots were then calculated. The two central rows of each crop in each plot were harvested. Plants were dried in an oven at 60ºC for 3 days and then plant biomass was assessed by weighing dry plants from each row. After this, plants were threshed and seeds were weighed. Biomass data are available for trials Alm1-19(i), Cord1-19(i) and Cord2-19(i), and grain yield data are available for trials Alm1-19(i) and Cord2-19(i).

The Land Equivalent Ratios (LER) of grain and biomass yields values were calculated as follows [22]: $LER_{px} = Y_{ip}/Y_{mp} \quad Y_{ix}/Y_{mx}$

Where LERpx represents the LER value (either for grain yield or biomass yield) of a given combination of pea and other crop "x" (faba bean, wheat or barley). Y_{ip} and Y_{mp} are the yields of pea intercropped and in monocrop, respectively; Y_{ix} and Y_{mx} are the yields of the other crops in intercrop or monocrop, respectively.

Additional field trials were performed over four seasons (Table 2) in which the powdery mildew susceptible pea cv. Messire and its resistant isoline Eritreo [8] were mixed at different ratios (100/0, 75/25, 50/50, 25/75 and 0/100). Mixtures were made by alternating different rows of each cultivar (Figure 2). Disease severity (DS) in cv. Messire was evaluated as described above for the intercropping trials.

Table 2. Field trials carried out for the study of the effect of cultivar mixtures on the pathosystem pea/powdery mildew.

Trial	**Cord1-15(c)**	**Cord1-16(c)**	**Alm1-19(c)**	**Cord1-19(c)**	**Cord2-19(c)**
Location	Cordoba	Cordoba	Almodovar	Cordoba	Cordoba
Max. T (ºC)	35.3	31.7	35.2	31.2	31.2
Min. T (ºC)	-2.2	-2.3	-0.8	-2.8	-0.7
Mean T (ºC)	13.8	12.9	13.5	12.0	12.9
Precipitation (ml)	121.8	281.8	107.8	110	109.4

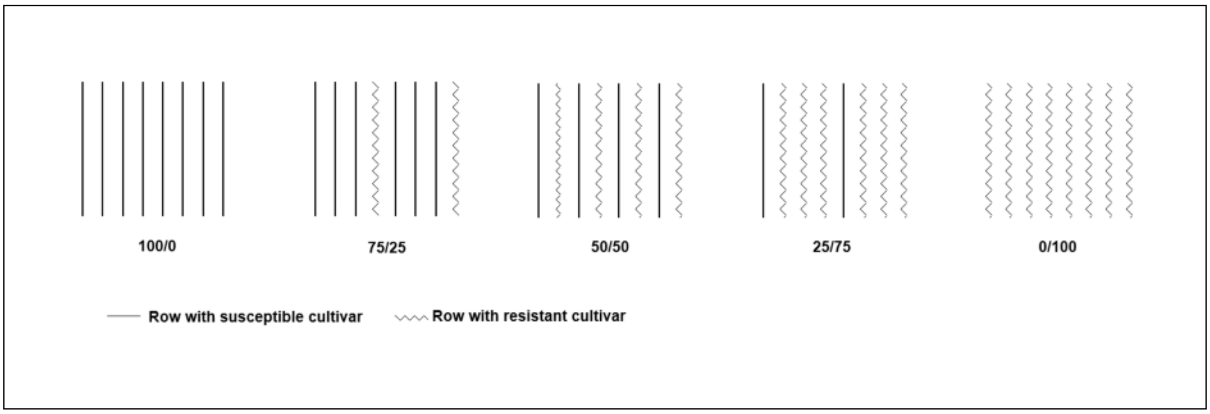

Figure 2. Experimental design for cultivar mixture experiments. Proportions are for Messire/Eritreo cultivars.

2.2. Controlled-conditions experiment

An experiment was performed on seedlings under controlled conditions to investigate possible barrier effects of cereals on powdery mildew dispersal. Seeds were planted in 4 rows separated 7 cm in polystyrene boxes (34x55x16 cm, width:length:height) filled with a mixture of sand and peat at 1:3 rate (v:v) following three treatments: pea monocrop, pea/wheat intercrop and pea/barley intercrop. The number of seeds per row were 18 for pea and 150 for the cereals. Treatments were replicated five times. In the intercrops, the first row was always a cereal, with pea in the second and fourth rows (Figure 3).

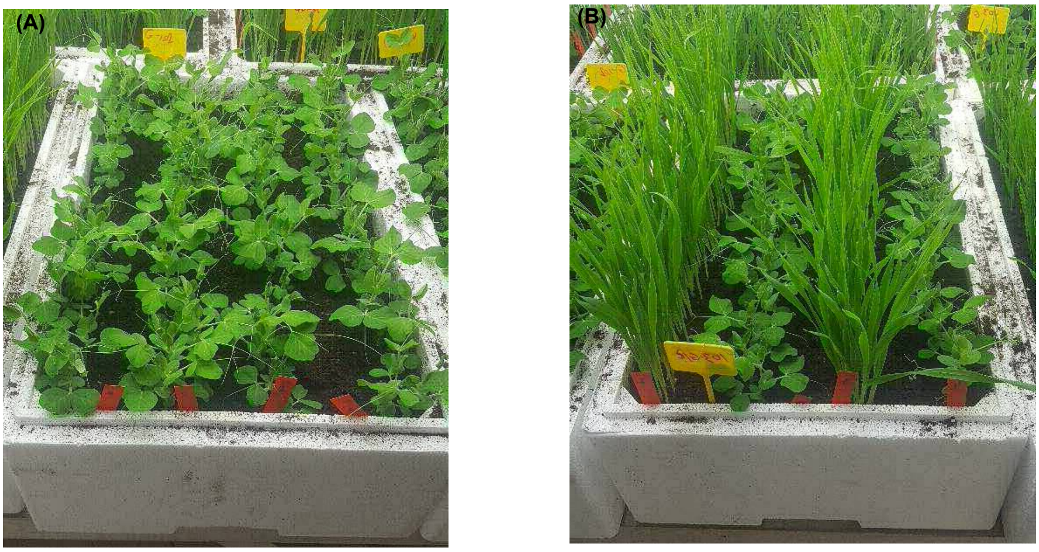

Figure 3. Pea monocrop (A) and pea intercropped with barley (B) in the experiment under controlled conditions.

The fifteen boxes were placed in a growth chamber with a photoperiod of 12 h of visible light (150 µmol m^{-2} s^{-1} photon flux density) at 25 ºC, and 12 h of darkness at 20 ºC. Seventeen days after sowing, seedlings were inoculated with *Erysiphe pisi* isolate CO-07. The isolate originated from infected pea crop in Córdoba and was maintained on living pea plants at IAS-CSIC. *E. pisi* spores from infected leaves were blown with the help of an air compressor in perpendicular direction towards the first row of each box, distributing them homogenously along the line of the row. Number of leaves of cereal plants at the time of inoculation were counted. Ten days after inoculation, pea plants on the second and fourth row of each box were evaluated for severity of powdery mildew. Then, dry biomass of cereal plants was measured as described above for the field trials.

2.3. Statistical analysis

For the intercropping trials, area Under the Disease Progress Curves (AUDPC) were calculated using data of powdery mildew severity by trapezoidal integration. Data of AUDPC were standardized (SAUDPC) considering the length of the evaluation period [23]. The effect of the treatment (pea monocrops and the different combinations) on the dependent variable (Area Under Disease Progress Curve) was examined for each environment (i.e., year × location) using Dunn's Test, while Friedman's Test was used to compare the effect of the treatment for the set of environments. Both tests were used because the data did not satisfy the requirements of parametric tests regarding normality, homogeneity of variance, or sphericity. The O'Brien's Test was used to study the homogeneity of the variances; while the Shapiro-Wilk Test was used to examine whether data conforms to a normal distribution. The means were compared using Dunn's test with a Bonferroni adjustment at P = 0.05 [24].

The effects of treatment and environment on LER values (grain and biomass yields) were subject to two-way analysis of variance (ANOVA) because these data satisfied the normality and homogeneity of variance requirements of ANOVA. To test whether the LER value of each treatment differs from the hypothesized value one ($\mu \neq 1$), the confidential Interval (C.I.) for the mean of each treatment was calculated, and One-Sample T-Test was performed. Factorial ANOVAs were also carried out for biomass of the companion crops and height differences.

The effect of the percentage of sowed resistant cultivar (cv. Eritreo) with respect to susceptible one (cv. Messire) was studied employing the exponential equation of Kiyosawa and Shiyomi [25]:

$$\partial y/(\partial r)=-by$$

Where "y" represents the severity of symptoms on the susceptible cultivar (SAUDPC), "r" represents the ratio of resistant cultivar, and "b is the rate of decrease in disease per unit increase in the resistant cultivar (i.e., slope). Overall as higher disease severity, higher effect due to the resistant cultivar. The previous equation was linearized as $Lny = a-b\times r$, in which "a" is a constant. For each ambient, the linearized equation fit the data well based on the coefficient of determination ($R^2 > 0.620$), P-Value ($P < 0.001$), and the pattern of residuals [26]. Subsequently, we linearized the selected model and compared the regression lines for each ambient based on their homogeneity of variances, slopes, and intercepts.

In the experiment conducted under controlled conditions, the effect of the treatment on the disease severity on pea leaves was subject to ANOVA. Previously, the data were arcsin-transformed to satisfy the normality and homogeneity of variance. Because we were more interested to compare the treatments, the sowing rows were used as blocks. Treatment and environment means were compared using Tukey's HSD test at $P = 0.05$. Biomass and number of leaves of barley and wheat were compared by ANOVA. All the data were analyzed using the software Statistix 10 (Tallahassee, FL, USA).

3. Results

3.1. Intercropping in the pathosystem pea/powdery mildew

Field trials

Powdery mildew disease was present in pea in all trials, with a wide range of incidence across them (Table 3).

Table 3. Final powdery mildew severity (DS) for each treatment of the different intercropping trials carried out (SE: standard error).

	Cord1-15(i)	**Cord1-16(i)**	**Cord1-17(i)**	**Alm1-19(i)**	**Cord1-19(i)**	**Cord2-19(i)**
Pea 100%	29.3	65.7	64.9	30.0	69.9	69.5
Pea/barley	13.8	47.7	53.3	5.1	59.4	43.8
Pea/faba bean	15.0	33.7	66.6	15.0	63.3	51.0
Pea/wheat	17.3	59.2	59.2	10.4	64.1	57.1
Pea 50%	12.0	53.6	48.8	30.0	69.2	49.4
SE	5.5	6.0	6.0	4.2	11.9	5.8

The global analysis revealed significant differences ($p<0.05$) among treatments for SAUDPC (Figure 4). Powdery mildew infection on pea was reduced when pea was intercropped with barley or with faba bean (a decrease of 44% and 32% in SAUDPC, respectively). Powdery mildew was not significantly reduced on pea intercropped with wheat or when grown in monocrop at 50% density.

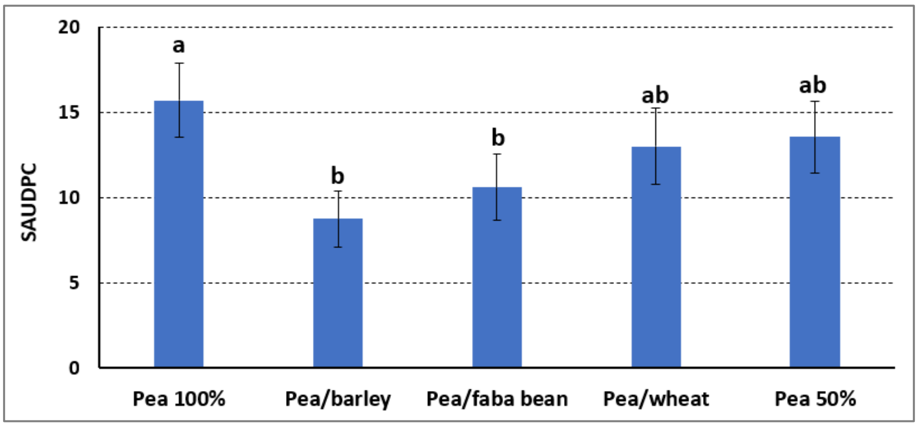

Figure 4. Standardized area under disease progress curve (SAUDPC) for severity of powdery mildew in pea in the different treatments evaluated across the six intercropping field trials. Different letters mean significant differences (Dunn's test corrected by Bonferroni, $p<0.05$). Bars for standard errors are shown.

Pea/barley intercrops presented LER values slightly higher than pea/wheat ones, but differences were not significant. Likewise, no LER value significantly deviated from 1 (Table 4).

Table 4. LER values for grain yield (trials Alm1-19(i) and Cord2-19(i)) and biomass (trials Alm1-19(i), Cord1-19(i) and Cord2-19(i)).No significant differences between any of them in each case was detected, and they did not significantly deviated from 1.

	LER grain yield	LER biomass
Pea/barley	1.06	1.07
Pea/faba bean	1.03	0.92
Pea/wheat	0.85	0.96

The factorial analysis for biomass of the companion crops, with crop and cultivation system (intercrop or monocrop) as fixed factors, revealed a significant interaction between factors ($p<0.05$): barley biomass was higher than that of the other crops, but even higher when intercropped with pea, which did not occur with the others (Figure 5). ANOVA for plant height differences also detected significant differences between crops ($p<0.05$), with barley showing a higher height difference with pea than wheat or faba bean (Table 5).

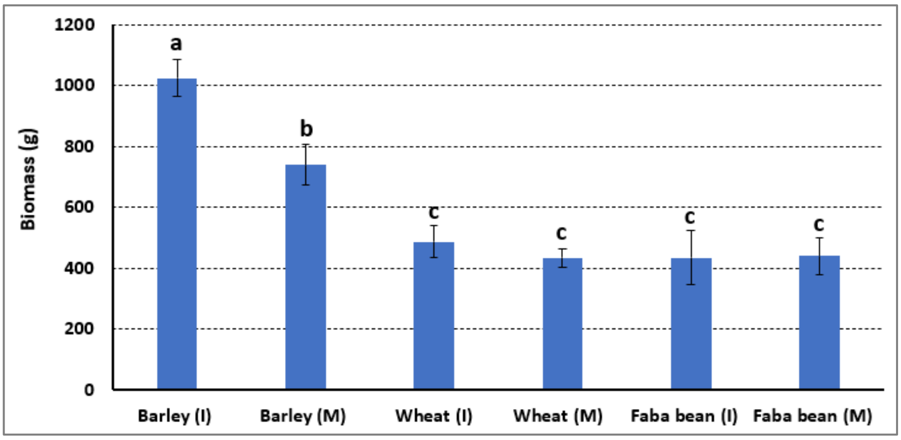

Figure 5. Biomass for the different companion crops (I: intercrop; M: monocrop) in trials Alm1-19(i), Cord1-19(i) and Cord2-19(i). Different letters mean significant differences (Tukey's test, $p < 0.05$). Bars for standard errors are shown.

Table 5. Height differences between the companion crops and pea in each intercropped plot in trials Cord1-18(i), Alm1-19(i) and Cord2-19(i). Different letters on the same crop mean significant differences (LSD test, $p < 0.05$).

	Height difference with pea (cm)
Barley	20.0 a
Wheat	12.9 b
Faba bean	12.3 b

Controlled-conditions experiment

Factorial analysis showed significant differences ($p<0.05$) for powdery mildew severity for factors treatment and row (distance to the focus of inoculum), with no interaction among them. Powdery mildew severity was highest in pea monocrop, followed by pea intercropped with wheat, and lowest in pea intercropped with barley (Table 6). In all treatments powdery mildew severity decreased with distance to the inoculation point, being lower in fourth than in second pea row. Dried biomass and number of leaves of barley was significantly higher ($p<0.05$) than that of wheat (Fig. 6).

Table 6. Powdery mildew severity (%) on pea seedlings grown in rows 2 and 4 of the boxes. Different letters per treatment (pea monocrop, pea/wheat and pea/barley intercrops) mean significant differences (Tukey test, $p<0.05$).

	Row 2	Row 4
Pea	33.5 a	24.1 b
Pea/wheat	11.9 c	10.5 d
Pea/barley	5.2 e	3.6 f

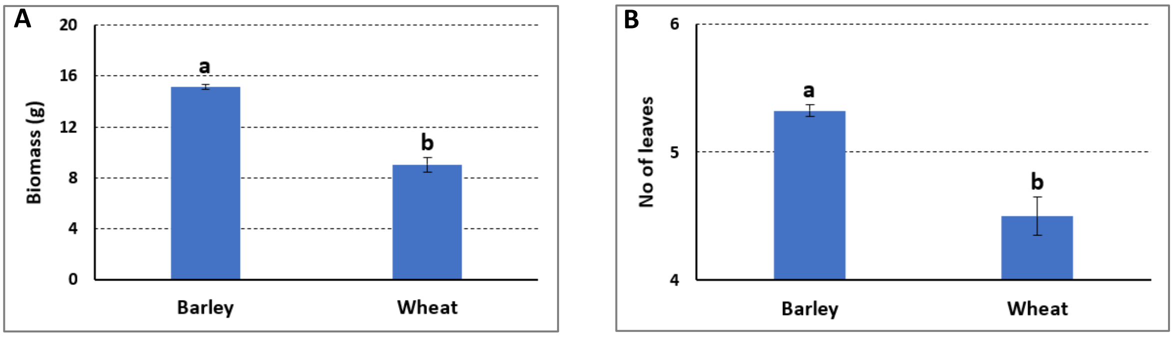

Figure 6. Dry biomass (A) and number of leaves (B) of barley and wheat in the experiments under controlled conditions. Different letters mean significant differences (Tukey test, p<0.05). Bars for standard errors are shown.

3.2. Cultivar mixtures in the pathosystem pea/powdery mildew

Wide range of disease severity was found (Table 7). In all trials, powdery mildew decreased in susceptible cv. Messire as the proportion of the resistant cv. Eritreo increased in the mixture. No powdery mildew was recorded in the plots with 100% of Eritreo. The results adjusted to a non-linear regression curve, with a pseudo R^2 of 0.5829 (Figure 7).

Table 7. Final powdery mildew severity in cultivar Messire for each treatment of the different cultivar-mixtures trials carried out (S/R: proportions of susceptible cultivar Messire and resistant cultivar Eritreo; SE: standard error).

S/R in mixture (%)	Cord1-15(c)	Cord1-16(c)	Alm1-19(c)	Cord1-19(c)	Cord2-19(c)
100/0	19.0	66.7	28.6	54.5	41.6
75/25	10.8	52.6	30.4	34.6	37.1
50/50	7.3	45.1	18.8	18.0	9.6
25/75	6.5	33.9	16.1	18.9	17.0
SE	2.8	1.7	4.7	2.7	5.3

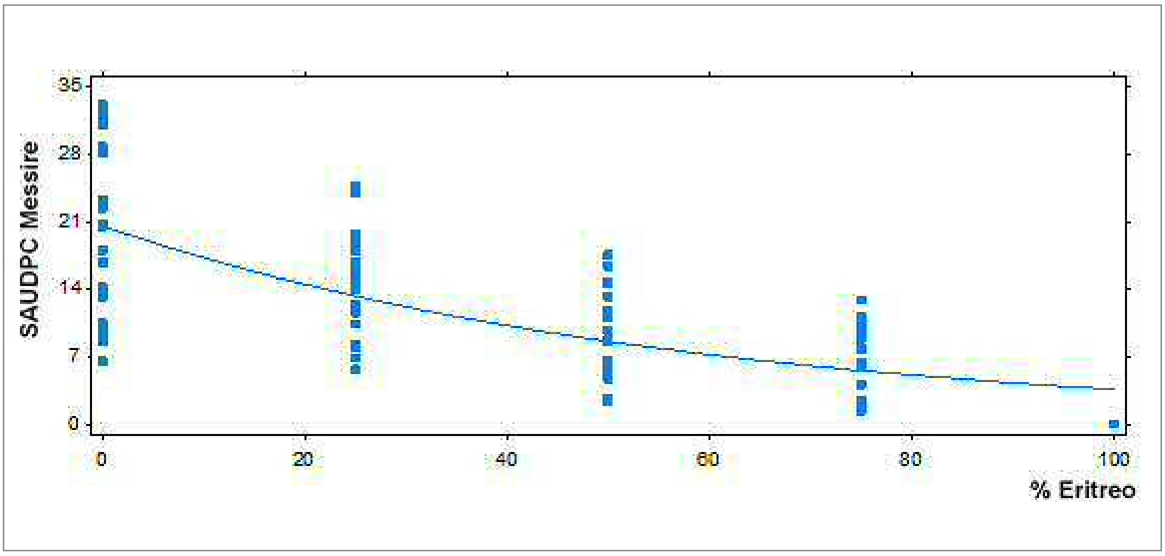

Figure 7. Nonlinear regression fitted curve for powdery mildew SAUDPC in susceptible cultivar Messire with varying proportions of resistant cultivar Eritreo across five different experiments.

4. Discussion

Crop diversification, consisting of growing different crops or cultivars simultaneously in the same piece of land is considered to increase crop resilience [27]. Cereal-legume intercropping might be particularly interesting in low-input systems by reducing the requirements for fossil-based fertilizer N [14,28]. A number of reports have shown reduction of pests and diseases [10,29-32]. However, these effects are only quantitative and influenced by environmental factors, therefore needing monitoring and case by case adjustments. In this work we quantified the reduction of powdery mildew on pea under different cropping systems, either intercropped with barley, wheat or faba bean, monocropped at reduced plant density, or in mixtures of resistant and susceptible pea cultivars. We opted for alternate-row, replacement intercropping at 50% proportion. Addition intercropping system (i.e., introducing rows of the second crop in between the rows of the first crop, so in practice halving the distance between rows) was not studied, but we speculate that doubling plant density would reduce aeration and increase relative humidity around leaves, thus favoring the infection and proliferation of the pathogen [10].

Our results show a significant reduction of powdery mildew on pea when intercropped with barley or with faba bean (44% and 32% SAUDPC reduction, respectively). This tendency was consistent across six field trials, in two different sites within a time span of four years, under an ample range of disease pressure. It is the first time that such a wide study on the effect of intercropping on pea powdery mildew is reported. Zivanov et al. carried out one field trial of pea intercropped with oat, with unconclusive effects on powdery mildew: they reported 20-30% disease reduction in pea leaves, but no effect on global disease on pea plants [21]. Our results are in line with what has been found for other diseases in grain-legume intercropping systems, with disease reductions in the range of 20-50% [10]. It is also similar to the described reduction of Ascochyta blight on pea when intercropped with barley [19], although Fernández-Aparicio et al. reported higher reductions in mixtures with triticale and faba bean [18].

Numerous mechanisms have been suggested to explain the effect on intercropping on plant diseases [10,33]: morphological and physiological changes in host, reduced density of the host crop (dilution effect), barrier effect to spore dispersion, alteration of the microclimate or inhibition of the pathogen by allelochemicals. One or more of these mechanisms may be present in a particular intercropping system. From our field experiments it is difficult to elucidate the mechanisms behind the powdery mildew reduction. We might speculate on dilution effect as host plants are reduced to 50% in the mixture, so the production of secondary inoculum would be reduced. Additionally, pea rows are at twice the distance from one another, so it is more difficult for the fungal spores to travel to produce new infections. However, the fact that there were no significant differences in pea monocrops at 100% and 50% density does not support this dilution effect. Moreover, the better aeration in pea monocrop at 50% density did not result in a reduction of powdery mildew.

The barrier effect by the non-host crop, then, may play an important role in hampering the spread of the disease, especially with this alternate-row design. The non-host crop acts as a physical barrier to spore movement from row to row, hindering the development of successive cycles of infection. In this case, barley and faba bean were more effective than wheat in reducing powdery mildew on pea. Barley produced more biomass than wheat and faba bean, making it a denser barrier; it also produced more biomass in intercrop with pea than as monocrop, which may indicate that barley benefits from the synergy with pea, but also benefits from the lower sowing density, with less barley plants competing with each other from resources in the same space. As for plant height, the difference between barley and pea is higher than those of wheat and faba bean. All this points to a strong barrier effect by barley in the decrease of powdery mildew. The role of faba bean, on the other hand, seems more complex to clarify but it is also likely that the barrier effect plays an important role, as has been described for the reduction of diseases in wheat intercropped with faba bean [16]. The barrier effect of barley was further supported by the results of the experiment under controlled conditions. This effect could be assessed independently by controlling the direction of the flow of spores through the rows of the cereal before reaching the pea plants. Evaluated symptoms were those originated from the primary infection, avoiding the complexities of second cycles of infection that would be accumulative. Results of biomass and number of leaves in this experiment confirm a faster and greater development of barley over that of wheat even at seedling stage, which may account for its higher efficiency as a barrier despite the similarities between both crops.

It has been reported in many cases that the combination of pea with cereals confers benefits in terms of LER (grain and forage), although it is not always so [12,34]. Combining pea and faba bean is less common, but again, positive and negative effects on yield have been described [35,36]. In our experiments no grain or biomass yield advantage or disadvantage has been found. This neutral effect of intercropping on yield facilitates its use in the control of powdery mildew in pea.

The use of varietal mixtures offers a different approach to biodiversity when it is not desired to grow different crops in the same field. The employment of resistant cultivars is an efficient and sustainable strategy to control diseases, but they pose some drawbacks if they are not properly managed. One of the main problems is the overcoming of resistance by the pathogen. The chance of this happening is higher with the multiplication in space and time of the resistant variety: the resistant genes are repeatedly exposed to the pathogen, which by competitive selection may finally find the way to surmount the resistance [37]. The rationale behind the utilization of cultivar mixtures is to have in the field a sufficient “amount” of resistance genes to prevent the disease from causing important damage, but not so many as to exert a too high selection pressure on the pathogen that might finally lead to the overcoming of the resistance. For these mixtures to be effective, it is important that there exist contrasting resistance levels to the disease [38]. Cultivar Eritreo is a near-isogenic line of cultivar Messire carrying gene *Er3*, which confers hypersensitive resistance to powdery mildew [8]. Given that it is a monogenic resistance, there exists a high risk of being overcome by the pathogen, so the mixture with another variety appears as a good strategy to safeguard the resistance [9]. The results show that the SAUDPC values adjust to a non-linear regression with the percentage of resistant variety, so disease symptoms in the susceptible variety decrease as the proportion of the resistant one increases. The decrease is bigger at the beginning, and smaller when the proportion of the resistant variety is high. This means that with a not-so-high percentage of the resistant variety it is possible to get a remarkable reduction of disease without seriously compromising the stability of resistance, as previously described for septoria tritici blotch in wheat [39,40]. This significant disease decrease with the introduction of just one row of the resistant variety may also point to an importance of the barrier effect, which has been observed in other cultivar mixtures in which the resistant cultivar hampers the movement of spores to other rows of susceptible cultivars [41]. Determining the final optimal proportions of the components of the mixtures may be a complex task that takes into account different factors [34,42], although the expected levels of disease in the area may condition the proportion of the resistant variety required.

In conclusion, in this work it has been established for the first time that diversification is a good tool for the control of powdery mildew in pea, whether it is by mixing pea with another crop, or by mixing two cultivars of pea. This adds up to the known advantages of diversification for agriculture, which is of great importance in the context of sustainable agriculture and especially when it comes to organic farming, where the use of fungicides is not accepted. Future work should focus on other diseases of pea, such as rust, and on identifying the best options to simultaneously face different biological stresses, including weeds.

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Chapter II

Crop Diversification to Control Rust in Faba Bean Caused by *Uromyces viciae-fabae*

1. Introduction

Faba bean (*Vicia faba* L.) is one of the major legumes grown worldwide [1] both for animal feed and human consumption. Like most cultivated legumes, it is a crop with great potential as a component of sustainable agriculture given its ability to fix atmospheric nitrogen and so become a "natural fertilizer" for the soil or other crops that alternate with it, either in time, space or both [2]. However, the productivity of faba bean may be limited due to its susceptibility to a wide range of phytopathogenic fungi, including the necrotrophic *Botrytis fabae* Sard. (causal agent of Chocolate spot), Ascochyta fabae Speg. (causal agent of ascochyta blight) and the biotrophic *Uromyces viciae-fabae* (Pers.) J. Schrot (causal agent of rust) [3]. *U. viciae-fabae* is an autoecious macrocyclic species not requiring an alternate host to complete its lifecycle, and sexual reproduction is not commonly observed in temperate regions. Urediospores are dispersed mainly by wind and infect the aerial parts of the plant (leaves, stems and pods). Yield losses due to rust may reach a 20% yield reduction [4], although it has been reported in some cases to reach even 70% [5]. The use of fungicides may achieve effective rust control, although it is expensive for the farmer and not environmentally desirable [6]. As for genetic resistance, some sources of resistance have been described [7–9], but few commercial varieties with complete resistance have been released so far [5].

Crop diversification has proven to be helpful in controlling fungal pathogens in different crops. This has been shown by mixing different crops in the same field or by growing together cultivars of the same crop with differential responses to the pathogen. Reductions in disease severity of 20–40% have been described for both strategies in several crops [10,11], although the level of control may differ from one situation to another, depending on a wide range of factors such as crops to combine, intercrop pattern, crop cultivars or weather conditions. Different mechanisms have been suggested to explain this impact of diversification on fungal pathogens, ranging from barrier effects or microclimatic modifications to enhancement of the crops due to more efficient use of resources [10,12]. In addition, nitrogen nutrition may also play a role in disease suppression by diversification in some cases, although its mechanisms are not clear yet [13,14].

With regard to faba bean, reports of disease suppression by intercropping with cereals include chocolate spot [10,13,15], fusarium wilt [16,17], rhizoctonia [18] and rust [19–21]. The use of cultivar mixtures for reducing disease incidence in faba bean has been less studied, with no reports available so far.

The main objective of this work was to assess the reduction in rust in faba bean either by alternate intercropping with different crops (pea, wheat and barley) or using a mixture of two cultivars of faba bean, one susceptible and one resistant to the disease. Additionally, the mechanisms behind the different interactions in these mixtures, including the effect of nitrogen nutrition, were investigated through several experiments under controlled conditions.

2. Materials and Methods

2.1. Field Trials

From 2015 to 2019, four field trials aiming to study the effect of intercropping on faba bean rust were conducted in two sites (Córdoba and Almodóvar del Rio) located in the South of Spain (Table 1). Faba bean cv. Baraca was intercropped with either pea (cv. Messire), durum wheat (cv. Califa) or barley (cv. Henley). Monocrops of these four cultivars were also included, plus a monocrop of faba bean at 50% of sowing density (doubling the distance between rows). Plot size was 2.8 m × 3 m (8 rows per plot at 35 cm distance between them). Legumes were sown at a density of 80 seeds/m^2 and cereals at 200 seeds/m^2. Alternate intercropping with replacement at 50% was used, i.e., a row of each crop is alternatively sown to a final rate of 50/50 (Figure 1). The experiment design was a randomized complete block with four replications.

Table 1. Field trials conducted for the study of the effect of intercropping on the system faba bean-*Uromyces viciae-fabae*.

Trial	IC-Crd-16	IC-Crd-18	IC-Alm-18	IC-Crd-19
Location	Córdoba	Córdoba	Almodóvar	Córdoba
Season	2015/16	2017/18	2017/18	2018/19
Max. T (°C)	31.7	29.7	30.6	35.7
Min. T (°C)	−2.3	−3.4	−3.2	−2.8
Mean T (°C)	12.6	12.0	12.7	12.5
Rain (ml)	336	444.6	422	110.4

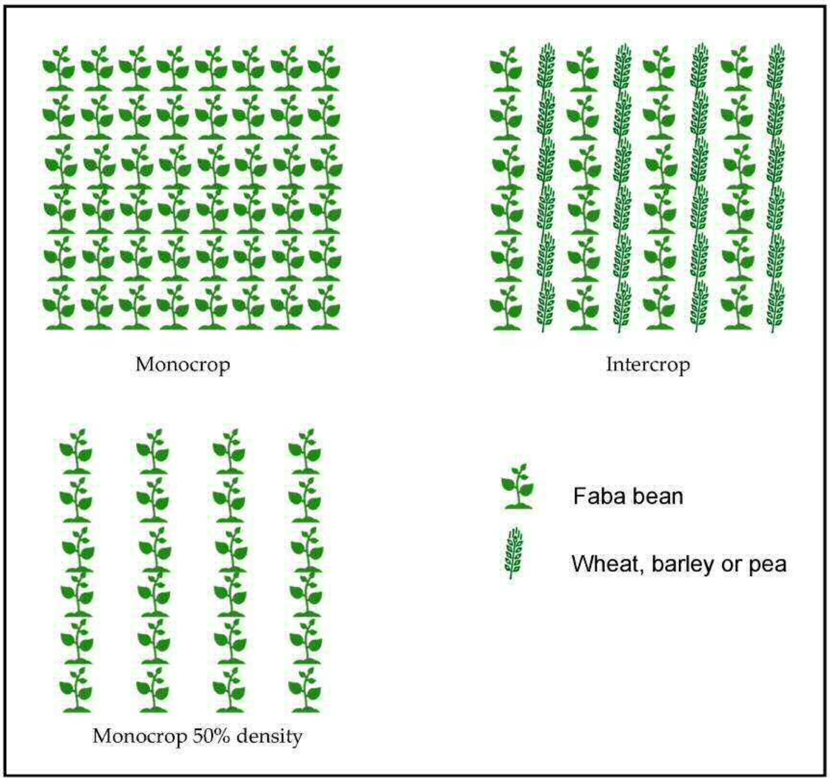

Figure 1. Experimental design for the intercropping experiments.

Rust disease severity (DS) was visually estimated as the percentage of the whole plant canopy showing rust pustules. The severity value for each plot was that of the overall DS on the two middle rows (discarding those plants at the extremes of the rows), so to avoid any edge effect. Evaluations started one week after the appearance of the first symptoms and were repeated every 7–10 days until plant senescence.

Yield and biomass were obtained by harvesting two rows of each crop per plot. However, not all crops could be harvested in all trials due to different problems (e.g., bird damage on the cereals). Grain yield for faba bean was determined in trials IC-Crd-16, IC-Crd-18 and IC-Crd-19; for wheat and barley in trial IC-Crd-19 (where cereal plants were covered with a mesh at maturity to avoid bird damage) and for pea in trials IC-Crd-16 and IC-Crd-18. Crop biomass was determined for trial IC-Crd-19 by drying plants in an oven at 60 °C for three days, weighing them and then, after threshing, subtracting grain weight. Crop height was assessed at full maturity in trials IC-Crd-18 and IC-Crd-19, measuring five plants per plot with a ruler without stretching them.

The Land Equivalent Ratios (LER) of grain or biomass yields were calculated, when possible, as follows [22]:

where LERpx represents the LER value of a given combination of faba bean and another crop “x” (i.e., pea, wheat or barley). The Y_{ip} and Y_{mp} parameters are the yields (grain or biomass) of faba bean intercropped and monocrop, respectively; Y_{ix} and Y_{mx} are the yields (grain or biomass) of the other crops in intercrop or monocrop, respectively.

Additionally, four trials for cultivar mixtures were performed in 2015–2019 (Table 2). Faba bean cv. Baraca (susceptible to rust) was mixed with resistant cultivar Joya at different proportions (Baraca/Joya: 100/0, 75/25, 50/50, 25/75 and 0/100), in alternate replacement intercropping (Figure 2). Faba bean cv. Joya is similar in morphology and growth cycle to Baraca, differing in the rust resistance, as it is derived from a cross between Baraca with the resistant accessions VF1273. The disease severity (DS) of rust in Baraca was evaluated in the same way as previously described. Grain yields were determined for trials VM-Crd-15 and VM-Crd-19, and plant biomass for trial VM-Crd-19. Plant height was recorded as described before in trials VM-Crd-18 and VM-Crd-19.

Table 2. Field trials carried out for the study of the effect of cultivar mixtures on the system faba bean-*Uromyces viciae-fabae*.

Trial	**VM-Crd-15**	**VM-Crd-17**	**VM-Crd-18**	**VM-Crd-19**
Location	Córdoba	Córdoba	Córdoba	Córdoba
Season	2014/15	2016/17	2017/18	2018/19
Max. T (°C)	33.1	32.2	29.7	35.7
Min. T (°C)	−3.3	−3.4	−3.4	−2.8
Mean T (°C)	11.5	12.1	12.0	12.5
Rain (ml)	155.6	213.6	444.6	110.4

Figure 2. Experimental design for cultivar mixture experiments of two cultivars of faba bean with different degrees of susceptibility to rust (*Uromyces vicia-fabae*): cv. Baraca (susceptible) and cv. Joya (resistant).

2.2. Controlled Condition Experiments

Four experiments on seedlings under controlled conditions were carried out to clarify the mechanisms responsible for the fungal disease reductions in the interaction between the different crops. All experiments were run in completely randomized blocks. The *Uromyces viciae-fabae* isolate CO-07 was used for plant inoculations.

This isolate is part of the faba bean rust isolate collection at IAS-CSIC (Córdoba). Originally, it was isolated from naturally-infected faba bean plants growing in a field in Córdoba. Urediospores were taken from a single pustule and inoculated on a healthy plant under controlled conditions in a growing chamber to produce a monopustular isolate. Growing conditions were: photoperiod of 12 h of visible light (150 µmol m^{-2} s^{-1} photon flux density) at 25 °C and 12 h of darkness at 20 °C. Urediospores were subsequently collected in plastic tubes after aspiring them with the help of a vacuum pump. Then, they were inoculated on faba bean plants to multiply the isolate under the same conditions. Urediospores were again collected and kept in plastic tubes in a freezer at −80 °C.

In the first experiment, the differential behavior of barley and wheat as barriers to rust spore dispersal was assessed. To this end, polystyrene boxes (34 × 55 × 16 cm, width:length:height) were filled with a mixture of sand and peat at a 1:3 rate (v:v) and placed in a growth chamber. Four rows of seeds were sown in each box for three treatments: faba bean monocrop, faba bean/wheat and faba bean/barley intercrops, with five replications (one treatment in each box, fifteen boxes in total). The distance between rows was 7 cm, and the number of seeds per row was 18 for faba bean and 150 for the cereals. The first row was always a cereal in the intercrops, with faba bean in the second and fourth rows (Figure 3). Growing conditions were the same as described above for the multiplication of the isolate. Seventeen days after sowing, faba bean seedlings were inoculated by dusting them with a mixture of rust urediospores and pure talc (1:10), totaling 3 mg of spores per plant. The pathogen spores were homogenously sprayed on the first row of plants perpendicularly to the side of the box. The number of cereal plant leaves was counted at the time of inoculation. Inoculated plants were incubated for 24 h in darkness at 20 °C and 90% relative humidity. Then, growing conditions were reinstated. Fifteen days after inoculation, disease severity was evaluated in faba bean plants in the second and fourth row of each box as follows: three infected leaves per plant were randomly chosen, and the number of pustules in an equally randomly selected area of 1 cm^2 were counted in each of them, then averaged; the number of infected leaves and the total number of leaves per plant were also counted, and their proportion calculated; finally, the average number of pustules per leaf was calculated multiplying the average number of pustules per infected leaf by the proportion of infected leaves in the plant. Cereal plants were subsequently pulled out, dried in an oven at 60 °C for 3 days, and then weighed to determine biomass.

Figure 3. Faba bean cv. Baraca intercropped with barley cv. Henley (**A**) and monocropped (**B**) in the first experiment under controlled conditions for *Uromyces viciae-fabae* management.

In the second and third experiments, the possible interaction of nitrogen nutrition with the intercropping system and rust disease severity was investigated. The experiments were carried out similarly to the previous one, inside a chamber in identical growth conditions. In these experiments, the dimensions of the polystyrene boxes were 27.5 × 34 × 16 cm (width:length:height), so the number of seeds varied: 11 for faba bean and 90 for cereals. For the second experiment, six treatments were included: faba bean monocrop and intercrop of faba bean with barley, each with three levels of nitrogen nutrition. The nitrogen nutrition was performed using irrigation solutions at three different levels: N0, N1 and N2, which were 0, 750 and 1500 mg/l of ammonium nitrate, respectively. Plants were irrigated on demand with one liter of their respective solution per box, twice before inoculation. Treatments were replicated five times. The plant inoculation was conducted as in the first experiment but performed from above the box, perpendicular to the soil, to avoid any barrier effect. Rust severity on faba bean plants was evaluated twelve days after inoculation, calculating the average number of pustules per leaf as in the previous experiment. The objective of the third experiment was to investigate any possible effect of intercropping on the nutrient content of faba bean leaves. It consisted of two treatments, faba bean monocrop and faba bean intercropped with barley, with four replications. No fertilization was added. Twelve days after sowing, the leaves of faba bean plants were detached and frozen until they were taken for analysis of foliar nitrogen content. Nitrogen content was determined by the Kjeldahl method [23].

Finally, the fourth experiment aimed to assess the barrier effect of resistant faba bean cv. Joya on the reduction in rust on susceptible cv. Baraca in the cultivar mixtures field trials. Again, seeds were sown on the smaller polystyrene boxes described above, and four treatments were studied (Figure 4). Growth conditions and inoculation were the same as in the first experiment. Inoculation was performed perpendicularly to the first row, which was sown with the cv. Joya in the three treatments with cultivar mixtures. Disease evaluation was performed as in the previous experiments 12 days after inoculation only for the rows shown in Figure 4. Plant height was determined at the time of inoculation.

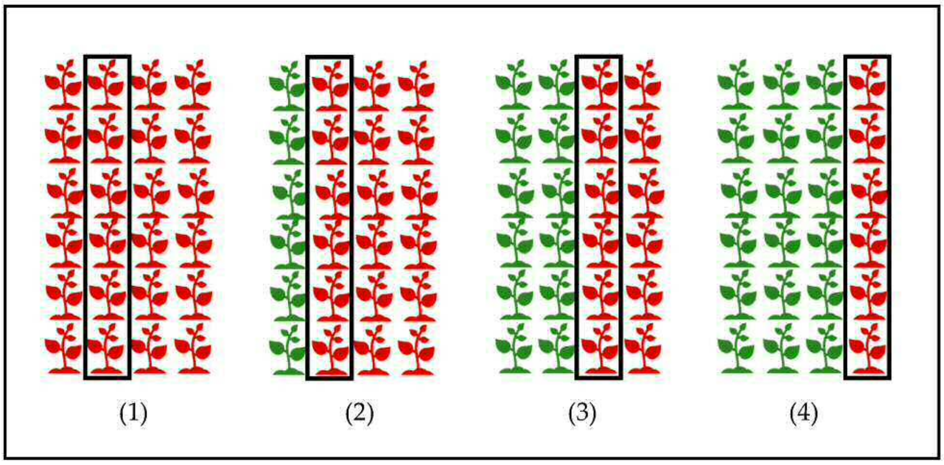

Figure 4. Experimental design for the cultivar mixture experiment in growth chambers under controlled conditions. The susceptible cultivar is Baraca (red), and the resistant one is Joya (green). The four treatments appear numbered: (1) all rows of cv. Baraca, the first one acting as a barrier; (2) one row of cv. Joya acting as a barrier, and three rows of cv. Baraca; (3) two rows of cv. Joya acting as barrier, and two rows of cv. Baraca; (4) three rows of cv. Joya as barrier, and one row of cv. Baraca. The squared rows are those that were evaluated for rust.

2.3. Statistical Analysis

The effect of the various treatments (i.e., different intercropping) on the final disease severity (% leaves covered by pustules) was studied using a Mixed-Effects Model using the treatment as a fixed factor and the environment (defined as the combination of plot-year) as a random factor. The treatment means were compared using Tukey's test at $p = 0.05$. Likewise, a General Linear Model was performed to study the effects of the treatments (intercropping or nitrogen levels) on the rust severity under controlled conditions, followed by Tukey's test. To test whether the LER value of each treatment differed from the hypothesized value ($\mu \neq 1$), the confidential interval (C.I.) for the mean of each treatment was calculated, and a one-sample t-test was performed. Two-way ANOVA was used to study the effects of the crop and cultivation system (intercrop or monocrop) on biomass production. Subsequently, biomass means were compared by Fisher's protected Least Significant

Differences Test (LSD at $p < 0.05$). The effect of the cultivar showing ratio in the crop mixtures was studied by employing the exponential equation of [24]:

$$\partial y/(\partial r) = -by$$

where y represents the severity of symptoms on the susceptible cultivar (SAUDPC), r represents the ratio of the resistant cultivar and b is the rate of decrease in disease per unit increase in the resistant cultivar (i.e., slope). Overall, there was a higher effect with higher disease severity due to the resistant cultivar. The previous equation was linearized as $Lny = a - b \times r$, in which a is a constant. The linearized equation fits the data of the different seasons. It was selected based on the coefficient of determination ($R^2 > 0.450$; $p < 0.001$) and the pattern of residuals and was compared with other exponential models [25].

In the experiments conducted under controlled conditions, disease severity was quantified as the number of rust pustules per leaf. A Generalized Linear Model (GLM) was performed to study the treatment significance. When necessary, this dependent variable was arcsine-transformed for variance homogeneity. After GLM, we compared the means by Fisher's protected LSD at $p < 0.05$. Statistical analyses were performed using SPSS version 23.0 for Windows.

3. Results

3.1. Intercropping in the System Faba Bean/Rust

3.1.1. Field Trials

Disease severity (DS) varied with the environment, being highest on faba bean monocropped at IC-Crd-16 (82%) and the lowest at IC-Crd-19 (6.7%) (Table 3). In most cases, crop combinations brought about a reduction in DS as compared to the monocrop, although with differences between the intercropping types. The analysis across all four trials (Figure 5) showed that the combination of faba bean with barley resulted in the lowest level of rust disease severity (26.5%) as compared to the severity in the faba bean monocrop (34%), which means a significant ($p < 0.05$) average reduction of 22%.

Table 3. Final disease severity (DS, evaluated as the percentage of the whole faba bean plant canopy covered by rust (caused by *Uromyces viciae-fabae*) for each treatment of the different intercropping trials carried out (SE: standard error).

	IC-Crd-16	IC-Crd-18	IC-Alm-18	IC-Crd-19
Faba bean 100%	82.0	13.5	33.7	6.7
Faba bean/barley	68.3	12.1	24.0	1.6
Faba bean/pea	80.6	13.0	24.7	3.4
Faba bean/wheat	74.3	15.9	25.9	2.7
Faba bean 50%	66.5	19.5	39.0	6.6
SE	2.8	2.2	2.8	1.2

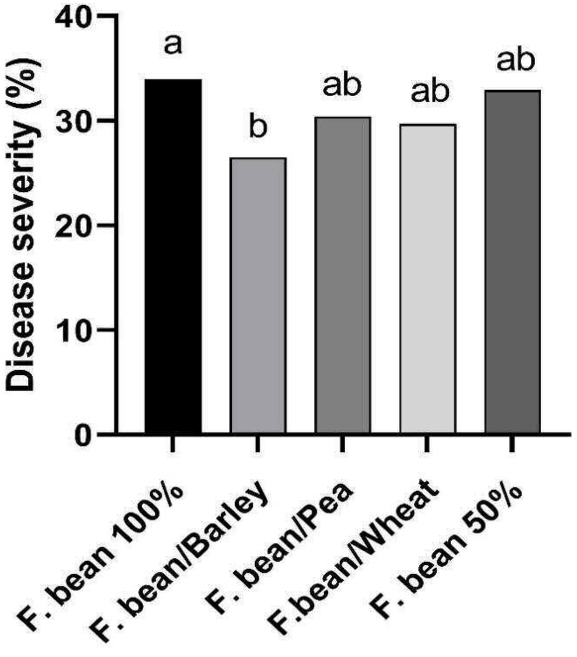

Figure 5. Disease severity of rust (caused by *Uromyces viciae-fabae*) in faba bean in the different treatments evaluated across the four intercropping field trials. Different letters mean significant differences (Friedman's test, $p < 0.05$).

In order to study the effect of intercropping on the grain and biomass yield, we calculated the LER ratios (intercrop yield: monocrop yields). Thus, in the theoretical case of intercropping having no impact on the yields of the crops, LER should equal approximately 1. In our study, LER values for grain and biomass yields did not significantly deviate from 1, i.e., intercropping did not negatively or positively impact faba bean yield or biomass (Table 4).

However, a significant interaction between crop and cultivation system (intercrop or monocrop) was found in the factorial analysis for biomass per row of the companion crops ($p < 0.05$): barley intercropped with faba bean presented the highest biomass per row of all treatments, including barley monocrop; the biomass of wheat intercropped with faba bean was higher than that of wheat monocrop, while pea biomass (intercrop and monocrop) was the lowest of all treatments (Figure 6).

Table 4. LER values for grain yield (trial IC-Crd-19 for the combinations of faba bean with barley or wheat, and trials IC-Crd-16 and IC-Crd-18 for the combination of faba bean with pea) and biomass (trial IC-Crd-19). No significant differences between any of them in each case were detected, and they did not significantly deviate from 1.

	LER Grain Yield	LER Biomass
Faba bean/barley	0.89	1.01
Faba bean/wheat	0.95	1.02
Faba bean/pea	0.85	1.44

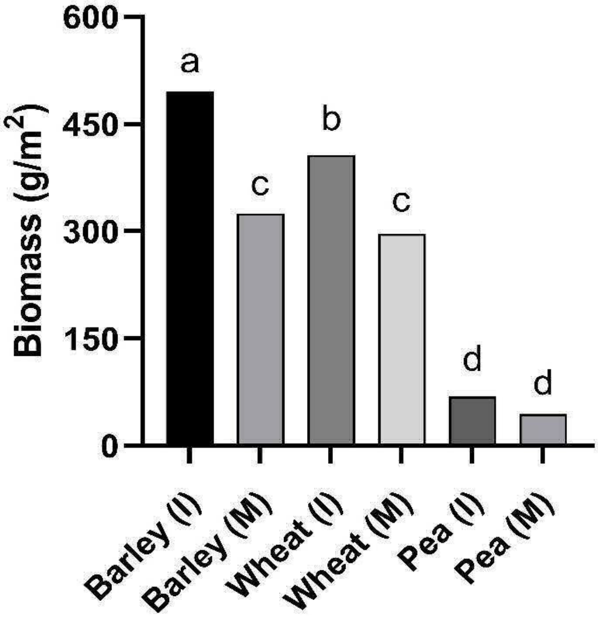

Figure 6. Biomass (g/m^2) for the different companion crops (I: intercrop; M: monocrop) in trial IC-Crd-19. Different letters mean significant differences (LSD test, p <0.05).

The plant height differences between faba bean and their companion crops were determined, resulting in the combination of faba bean with barley presenting the lowest difference, 0.17 cm; in comparison, the differences between faba bean and wheat or pea were similar ($p < 0.05$) and higher than 13 cm.

3.1.2. Controlled Conditions Experiments

In the first experiment, treatments and position row (distance to the point of inoculation) significantly ($p < 0.05$) affected the number of rust pustules per leaf but no interaction between them was detected. The highest infection was quantified on faba bean growing in monocrop (average = 3.15 pustules/leaf). This was reduced on faba bean intercropped with wheat (2.14 pustules/leaf) and further reduced on faba bean intercropped with barley (1.15 pustules/leaf). Leaves of those plants in the row further from the inoculation point presented about half of the pustules as those in the second row (7.8 vs. 3.7 pustules/leaf). The dried biomass and the average number of leaves per plant of barley were higher than those of wheat ($p < 0.05$): 15.5 g and 5.3 leaves/plant vs. 9.02 g and 4.5 leaves/plant.

In the second experiment under controlled conditions, the statistical analysis revealed no significant differences in the number of pustules per leaf for any of the three factors under study, that is, nitrogen level (N0, N1 or N2), cropping system (monocrop or intercrop) or row in the tray (Table 5). Similarly, no significant difference was found in the third experiment for nitrogen content in faba bean leaves either in monocrop (5.78% N content) or intercropped with barley (6.15% N content).

Table 5. Pustules per leaf in faba bean plants grown in monocrop or intercropped with barley at three nitrogen fertilization levels (N0, N1 and N2) and for row position in the tray (2 or 4). No significant differences were found for any of the tested factors.

	N0 (0 mg/L)		**N1 (750 mg/L)**		**N2 (1500 mg/L)**	
	row 2	row 4	row 2	row 4	row 2	row 4
Faba bean	3.6	3.3	3.5	4.8	3.6	3.1
Faba bean/barley	3.3	3.5	3.5	4.6	3.4	2.5

3.2. Cultivar Mixtures in the System Faba Bean/Rust

3.2.1. Field Trials

Rust levels in the different trials covered a span of low to medium infection pressure (Table 6). Disease severity values linearly decreased in susceptible cv. Baraca as the proportion of resistant cv. Joya increased in each experiment, fitting significantly ($p < 0.001$) a linear regression (Figure 7).

S/R in Mixture (%) [a]	VM-Crd-15	VM-Crd-17	VM-Crd-18	VM-Crd-19
100/0	22.4	19.7	16.6	13.2
75/25	19.0	12.1	14.5	10.5
50/50	15.3	13.0	9.2	3.5
25/75	9.4	8.2	7.9	2.5
SE [c]	1.1	2.2	1.0	2.2

Table 6. Final rust severity (DS) for each treatment of the different intercropping trials conducted by mixing the cultivars Baraca (Susceptible) and Joya (Resistant).[a] S/R: proportions of susceptible cultivar and resistant cultivar sown; [b] Disease severity was evaluated in cultivar Baraca. [c] SE: standard error.

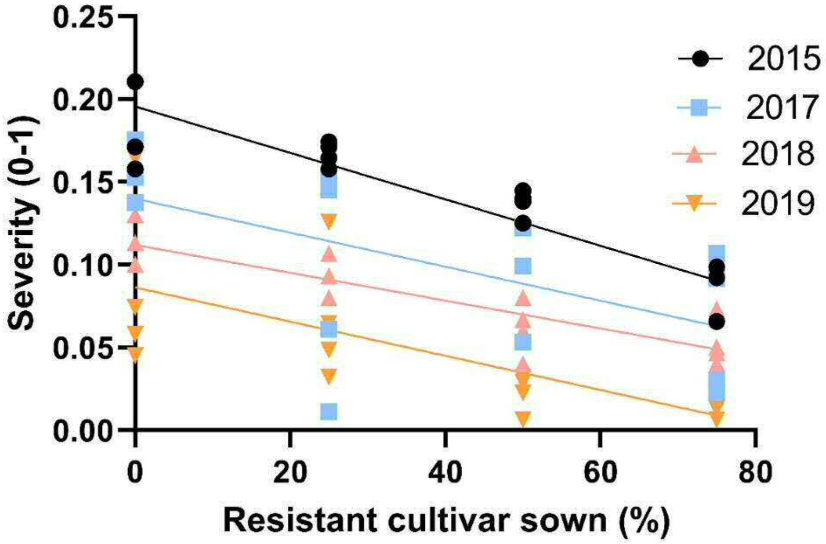

Figure 7. Linear regression between the rust disease severity (linearized as natural logarithm), caused by *Uromyces viciae-fabae* in susceptible faba bean cv. Baraca and the proportions of resistant cv. Joya in four field experiments (in all the cases: $R^2 > 0.450$; $p < 0.001$).

No significant differences were detected in yield for any of the different cultivar combinations. However, results did show that cv. Joya has a significantly higher biomass and is 24 cm taller than cv. Baraca (Table 7).

Table 7. Biomass (VM-Crd-19) and plant height (VM-Crd-18 and VM-Crd-19) for the two cultivars tested in the cultivar mixtures experiments. Different letters mean significant differences (LSD test, $p < 0.05$).

Cultivar	Biomass (g/m^2)	Height (cm)
Joya	524.1 ^{a}	98.2 ^{a}
Baraca	321.0 ^{b}	74.1 ^{b}

3.2.2. Controlled Conditions Experiment

The analysis of the experiment under controlled conditions showed significant differences in the number of pustules per leaf in plants of cv. Baraca depending on whether the previous row consisted of Joya or Baraca: those plants with Joya before them presented less disease incidence (9 vs 12.8 pustules per leaf, $p < 0.05$). On the contrary, the number of rows before the evaluated plants did not appear to influence disease severity. As for plant height, Joya turned out to be nearly 1 cm taller than Baraca (13.1 vs. 12.2 cm, $p < 0.05$).

4. Discussion

In this work, we have assessed the impact of intercropping and cultivar mixtures on the system faba bean-*Uromyces viciae-fabae*, attaining significant reductions in the disease with both systems. Field trials for intercropping found an overall reduction in rust severity of 22% for the combination of faba bean with barley. No effect could be established for any of the other combinations. Recently Shtaya et al. [19] also identified the mixture of faba bean and barley as the most effective in reducing rust (35% in their case) over mixtures with other cereals. Other authors, Guo et al. [20] and Luo et al. [21], only tested faba bean mixed with wheat and obtained rust reductions in the range of 20–50% for the different treatments they tested. On the contrary, Kamalongo and Cannon [26] did not find rust reduction in faba bean combined with wheat. Nevertheless, it is not easy to compare these studies, as the experimental designs differed greatly. We studied rust suppression on faba bean using alternate replacement intercropping, finding that barley appears to be a more effective accompanying crop to suppress rust infection than wheat. Shtaya et al. [19] used mixed intercropping, and Guo et al. [20] and Luo et al. [21] used strip intercropping with a high number of wheat rows separating the rows of faba bean. Kamalongo and Cannon [26] did study alternate replacement for the combination of faba bean and wheat, and their results confirm ours. It can be concluded that faba bean with barley appears to be more reliable in controlling rust than other mixtures.

The combination of faba bean with barley has also been described as effective against *Botrytis fabae*, a causal agent of chocolate spot [15]. Barley also appears as a good partner for other legumes for disease suppression, as it has been reported to help to control *Erysiphe pisi*, the causal agent of powdery mildew, in pea [27]; fungal species (*Ascochyta pisi*, *Mycosphaerella pinodes,* and *Phoma pinodella*) causing ascochyta blight in pea [28]; and *Pleiochaeta setosa* causing brown spot in lupine [29].

Several mechanisms have been suggested to explain the reduction in diseases with intercropping [10]: inoculum dilution effect by the decreased density of the host crop; barrier effect by the added crop to spore dispersion; morphological and physiological changes in the host; changes in the microclimate that make it less favourable for fungal dispersion progression; inhibition of the fungal infection by allelochemicals. In the system faba bean-*U. viciae-fabae*, Shtaya et al. [19] have recently suggested that the reduction might be due to a barrier effect. However, given their experimental design (mixed intercropping), other mechanisms such as inoculum dilution or altered microenvironment may play a greater role. Likewise, Guo et al. [20] and Luo et al. [21] have found variations in the plant microenvironment when intercropped (towards higher humidity and temperature) that might account for at least some part of the decrease, but, considering the experimental design (strip intercropping), it is quite likely that there is a significant barrier effect.

Our results point to a barrier effect, which would be expected given the alternate intercropping system that has been employed. The results of the faba bean at half density support this: faba bean plants are at double the distance than in the normal monocrop, which in principle would render a dilution of inoculum and less humidity due to higher aeration, all of which should lead to a hampering of disease progress and less incidence and severity. However, the disease levels are equal to those of the treatment of monocrop at a normal distance. It is the introduction of a different crop in between the rows of faba bean that hampers infection.

The higher biomass and height of barley as compared to the two other companion crops (wheat and pea) in the field trials might explain why barley performs better as a barrier. In the experiment under controlled conditions, we tested the performances of barley and wheat as barriers, confirming that barley is the most efficient barrier. In this case, the biomass and height of the plantlets of barley were also higher than those of wheat plants. The fact that barley has a more vigorous development than wheat has been previously described [30–32], and our results show that this vigour makes barley a better candidate than wheat as an intercropping partner to control rust in faba bean.

It has been reported that the role of nitrogen in plants' response to fungal infection is diverse, with positive, negative or neutral effects on disease development [33–35]. We studied the impact of nitrogen with two experiments under controlled conditions. In the first of them, no difference was detected in damages on faba bean caused by *U. viciae-fabae* for any of the fertilisation levels tested. This is in contrast with the works of Guo et al. [20] and Luo et al. [21], both reporting that disease increased with N fertilization, which was related to changes in the microenvironment in the first study; the conditions of our experiment, with young plants evaluated over a short period, might account for this difference. It is remarkable, however, that there were no differences in disease levels between monocropped and intercropped faba beans in this experiment, where inoculation had been performed from the top. This appears to confirm the importance of the barrier effect for the control of rust in intercropping. In the second experiment, it was found that intercropping with barley does not alter the nitrogen content in faba bean leaves, which would also disregard a potential effect of intercropping on nitrogen content as a mechanism to reduce rust.

The use of cultivar mixtures to control crop diseases has been very limited and restricted mostly to cereals, mainly wheat [36–40]. For legumes, only a few studies have assessed their effectiveness in controlling anthracnose and rust in common beans [41,42] or powdery mildew in pea [27]. These mixtures appear to avoid the surmounting of pathogens' resistance by reducing the high selection pressure that homogeneous monocultivar crops impose on them [43]. The key issue is to mix cultivars with resistance and susceptibility to the disease in such a proportion that disease severity is kept to acceptable levels, while the resistance is prevented from being broken over. The mechanisms that explain disease reduction are quite similar to those already mentioned for intercropping, although the dilution of inoculum appears, in principle, as the most relevant [11]. Our results show that in the case of the system faba bean/rust, the use of cultivar mixtures may be effective. The fact that the resistant cultivar Joya grew higher and produced more biomass than the susceptible cultivar Baraca raised the question of the importance of the barrier effect in addition to the dilution of inoculum. The differential response of both cultivars when standing in the way of inoculation in the experiment under controlled conditions confirmed that the barrier effect plays an essential role in reducing rust in this particular mixture. This stresses the importance of choosing partners for mixtures based on their contrasting responses to the disease and other factors, such as plant architecture [44], that may provide additional advantages.

In conclusion, in this work, it has been established that the combination of barley and faba bean in alternate intercropping is an excellent tool to help control rust in faba bean and that the barrier effect by the cereal is the main mechanism operating in this situation. Equally, it is the first time that it has been proved that cultivar mixtures in faba bean may be effective in controlling rust. Further work should focus on determining the features that mixed cultivars should possess for optimum performance in a particular environment either for intercropping or cultivar mixtures.

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Chapter III

Use of intercropping to control weeds in faba bean

Introduction

Crop legumes are an essential component of sustainable agriculture, given the ecological services they provide, especially their ability to fix atmospheric nitrogen [1]. However, legume cultivation is hampered by biotic and abiotic stresses, which may seriously impact their yields [2]. One of these is the competition of weeds, which is a serious challenge for managing these crops. Not only may weeds reduce crop yields, but they also may affect the quality of the grain [3], serve as alternative hosts for diseases and pests [4,5], or even hinder harvesting. In general, weed control methods must be implemented for legume crops since these are inefficient competitors [6]. Herbicides are the most widely used weed management, but these present several drawbacks, such as high costs for the farmer, environmental damage, and the eventual appearance of resistance to the active ingredients in weeds. These resistances may be especially problematic, as no herbicides with new modes of action have appeared in recent years [7]. Other strategies are mechanical control and management of the spatial arrangement of crops [8,9].

Faba bean (*Vicia faba* L.) outstands as one of the most cultivated legumes worldwide for its use for both animal feed and human consumption [10]. Weeds are one of the major biological constraints for faba bean, reducing yields by up to 50%, although they appear more competitive with weeds than other legumes [11].

Intercropping is the practice of growing two or more crop species simultaneously in one field. It may help in the control of weeds in many crops [12], complementing herbicides if necessary [13]. In the case of legumes, weed reduction has been reported when combined with diverse crops, such as corn, wheat, oat, barley or rape [14-19]. There are different types of intercropping, depending on the arrangement and sowing densities of the combined crops; mixed intercropping, when plants are completely mixed; and alternate intercropping, when rows of each crop alternate regularly [20]; in addition intercropping all crops are sown at their normal densities, while in replacement intercropping densities are adjusted for the presence of the other(s) crop(s) [21].

Different mechanisms explain the smothering of weeds in intercropping systems. The most common is the competition of crops and weeds for natural resources such as light, water or nitrogen [18]. Another one is allelopathy, which is a characteristic of plants that may be exploited for weed control [22]. Allelopathy is defined as the capability of plants to exert positive or negative influence in the surrounding area by releasing chemicals [23]. It has been known for a long that several crops present allelopathic activity, such as oat, wheat, barley, and sorghum [24].

The main objective of this work was to establish if it is possible to control weeds in faba bean by intercropping, determining the best crop combination if so. To this effect, field trials were performed across different years. Additionally, experiments under controlled conditions were carried out to investigate the possible role of allelopathy in weed control in intercrops of faba bean.

Material and methods

Field trials

Four field trials were carried out from 2014 to 2018 in Cordoba (South of Spain) to assess the effect of different crop combinations on the control of weeds in faba bean (Table 1). The combinations tested were those of faba bean/wheat, faba bean/barley, and faba bean/pea; the monocrops of the four crops were equally tested, as well as a monocrop of faba bean at 50% of sowing density (doubling the distance between rows). The cultivars used were 'Muchamiel' (faba bean), 'Califa' (wheat), 'Henley' (barley), and 'Messire' (pea). Sowing densities were 80 seeds/m^2 for legumes and 200 seeds/m^2 for cereals. Two different intercropping systems were evaluated: alternate with replacement at 50%, where rows of each crop are alternated, giving a final sowing rate of 50/50, i.e., half of the normal density of each crop; and alternate with addition, where rows are also alternated, but to a final rate of 100/100, i.e., the normal density of each crop. Replacement intercropping was evaluated in trials Cordoba-15 and Cordoba-16, and addition intercropping was evaluated in trials Cordoba-16, Cordoba-17, and Cordoba-18. The experimental plots had a length of 3 m and comprised eight rows at 35 cm distance between them in the case of monocrops and replacement intercropping and 16 rows at 17.5 cm in the case of addition intercropping; the monocrop of faba bean at 50% had four rows at 70 cm per plot (Fig. 1). The experiment was designed as a randomized complete block with four replications.

Table 1. Field trials conducted to study the control of weeds in faba bean by intercropping.

Trial	Cordoba-15	Cordoba-16	Cordoba-17	Cordoba-18
Location	Cordoba	Cordoba	Cordoba	Cordoba
Season	2014/15	2015/16	2016/17	2017/18
Intercropping system	Replacement	Replacement & addition	Addition	Addition
Max. T (ºC)	35.3	31.7	32.2	29.7
Min. T (ºC)	−3.3	-2.3	−3.4	-3.4
Mean T (ºC)	12.3	12.6	14.3	12.0
Precipitation (ml)	150	336	143.8	444.6

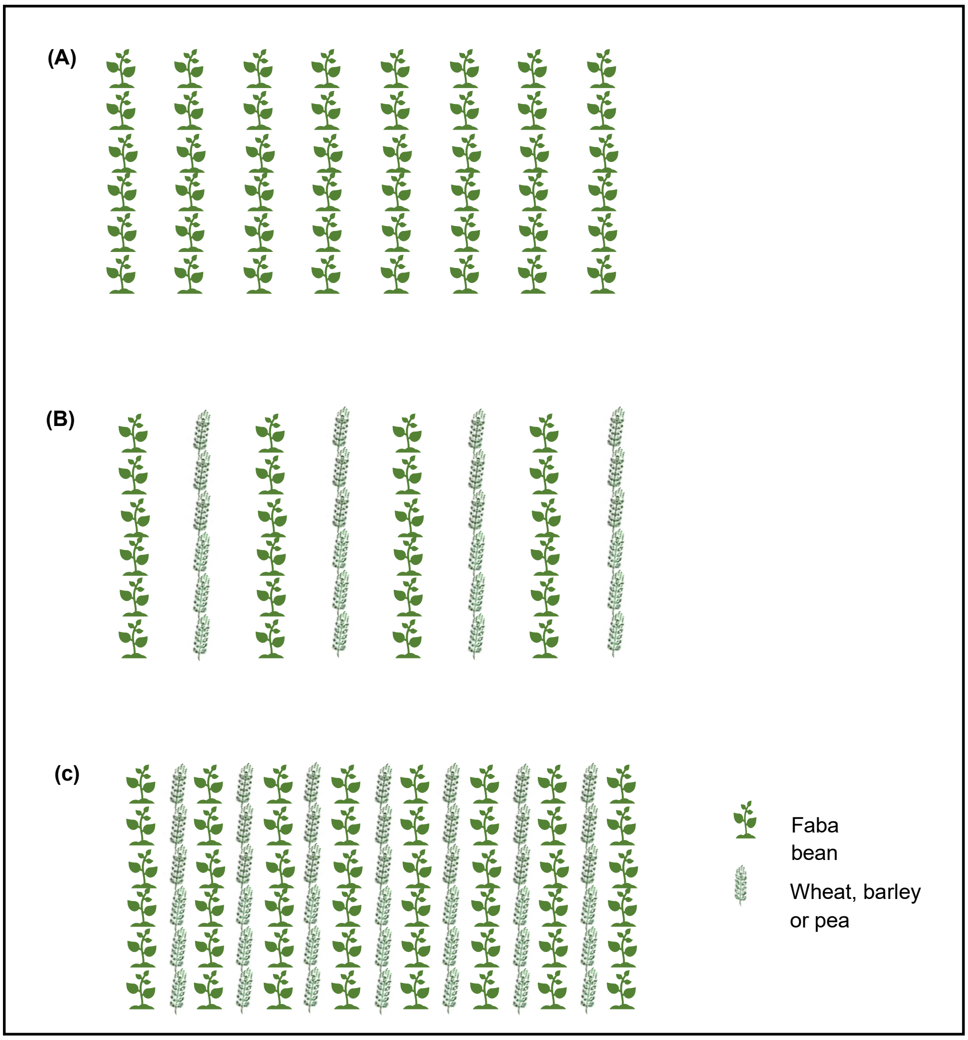

Figure 1. Different cropping systems evaluated in the field trials. A: Faba bean monocrop. B: Alternate replacement intercropping. C: Alternate addition intercropping. Row distances in A and B were 35 cm. and in C 17.5 cm.

Two areas of 0.7 m2 were randomly evaluated in the central part of each plot at the faba bean maturity stage to assess weed infestation. The weeds were counted and classified by botanic families. Two diversification indices were calculated in trials Cordoba-16, Cordoba-17, and Cordoba-18:

Richness (R) and Relative density (D):

R = nFi/nFT

R: Richness; nFi: number of weed families present in a given plot (i); nFT: total number of weed families found in the whole trial

$D_{ji} = (N_{ji}/NT_i)\times100$

Dij: Relative density of weed family j in plot i; Nji: number of plants of weed family j present in plot i; NTi: total number of weed plants in plot i.

Additionally, two evaluators visually estimated the weed coverage as a percentage of the area covered by weeds. At the end of the experiment, in trials Cordoba-16 and Cordoba-17, the weeds in a central area of 2 m2 in each plot were harvested, and their biomass determined by drying them in an oven at 60 ºC for three days and subsequently weighing them.

Controlled-condition experiments

Two experiments were carried out under controlled conditions to investigate a possible allelopathic effect of barley. Thus, four weeds that had grown in the field trials were selected for this: *Polypogon monspeliensis* (L.) Desf., *Matricaria camomilla* L., *Sinapis arvensis* L., and *Medicago truncatula* Gaertm, belonging to the botanical families Poaceae, Asteraceae, Cruciferae and Fabaceae, respectively. In the first one, 10 seeds of barley were sown on plastic pots (5× 5 × 10 cm) filled with a mixture of sand and peat at 1:3 rate (v:v) and grown in a growth chamber with a photoperiod of 12 h of visible light (150 µmol m^{-2} s^{-1} photon flux density) at 25 ºC, and 12 h of darkness at 20 ºC. Two weeks later, the barley plants were removed, and 20 seeds of the same weed species were sown in the same pots maintaining the substrate. Weed plants were counted every two weeks for 42 days. Then, weed plants were removed, dried in an oven at 60ºC for three days, and weighed to obtain their biomass. A treatment control was used, in which weeds were sown in pots where barley has not been previously grown. All this was done in separate pots for each one of the four weeds. The third experiment was carried out in the same way as in the first one, only that in this case the barley plants remained in the pots when weeds were sown, and they grew together till the end of the experiment. The design in the two cases was randomized with seven replications. Each of these experiments was performed twice.

Statistical analysis

For the analysis of weed coverage and biomass in the field trials, we calculated first the contribution of the treatment, year, and treatment×year to the overall explained variance, calculated as the partial omega-squared value (ωp^2) and partial eta-squared values (ηp^2) [25]. Then, considering that the interaction treatment×year explained 79% of the variance and the type of data (counts and percentages), generalized linear mixed models (GLMMs) were applied to compare the treatments for each year. Model fits were evaluated through the residual plots. Treatments were then compared according to the Least Significant Differences at $P \leq 0.05$. As for the diversification indices, they were analysed in each field trial by ANOVA, and means were compared by Tukey test.

In the experiments under controlled conditions, to study how the plant counts depended on the presence or not of barley plants, Poisson regression procedure was permorfed using the maximum likelihood estimation method. For weed biomass, and the proportions comparing methods and weeds, ANOVA analyses were carried out, comparing means by LSD tests.

Data were analyzed using Statistix software (Version 10; Statistix, Tallahassee, FL) and the R statistical software package.

Results

Field experiments

A high pressure of weed infestation was present in all field trials. In the experiments where the replacement intercropping system was used there was no difference in weed coverage between any intercropping treatment and faba bean monocrop (Supplementary Data Table S1). In these trials, faba bean at 50% density presented the highest level of weed infestation, not statistically different from wheat and pea monocrops. That is, intercropping was ineffective in controlling weeds in faba bean when rows of faba bean plants were replaced by rows of other crops (pea, wheat, or barley).

On the contrary, clear differences were found among treatments according to weed infestation values for the addition system. In general, pea and faba bean at 50% density presented the highest levels of weed infestation in all trials, both for weed coverage and weed biomass. The combined analysis of the experiments found the lowest weed coverage for the combination of faba bean with barley, with a reduction of 92.7% about the faba bean monocrop (Fig. 2). As for weed biomass, the combinations of faba bean with barley and faba bean with wheat showed the lowest values, not significantly different from those of barley as a sole crop, getting decreases in comparison with monocropped faba bean of 76.6% and 46.1%, respectively (Fig. 3).

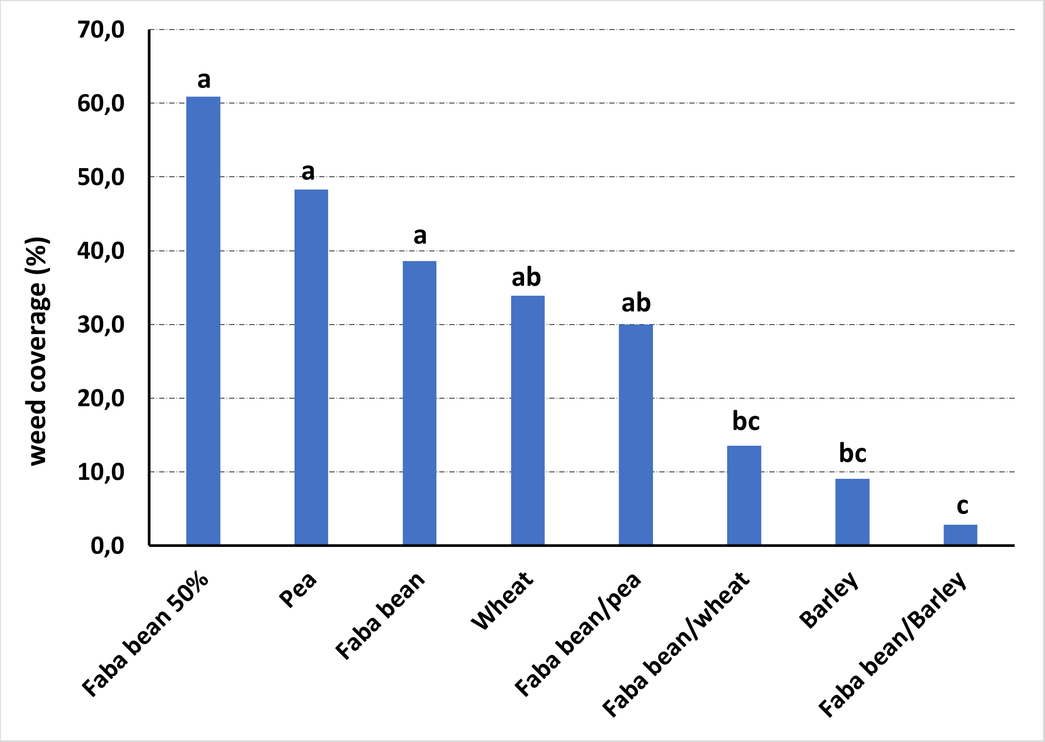

Figure 2. Weed coverage in the different treatments evaluated across the three intercropping field trials where addition alternate intercropping was tested. Different letters mean significant differences (Friedman's test, $p \leq 0.05$).

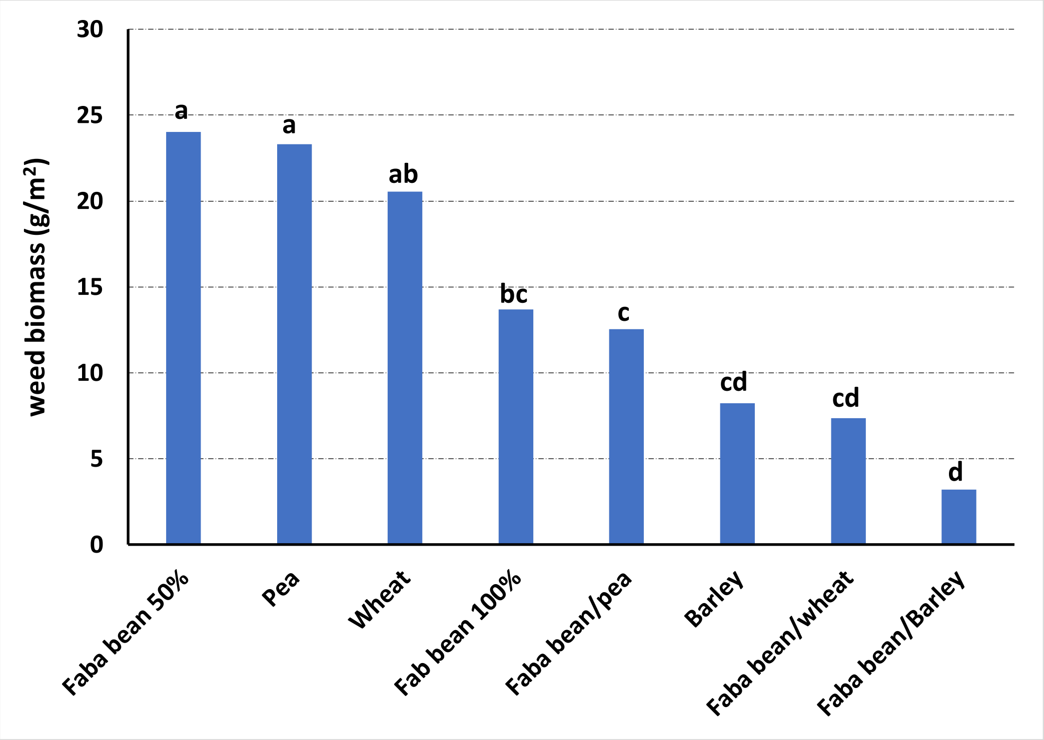

Figure 3. Weed biomass (g/m^2) in the different treatments evaluated across the three intercropping field trials where addition alternate intercropping was tested. Different letters mean significant differences (Friedman's test, $p \leq 0.05$).

The weeds found were classified into 10 different botanical families along the trials Cordoba-16, Cordoba-17, and Cordoba-18 (Table 2). No difference was found for Richness (R) between faba bean monocrop and any of the intercropping mixtures in any of the three trials where it was calculated (Table 3). Equally, no significant differences for Relative density (D) of any of the ten botanical families of weeds was detected for the different treatments tested (Supplementary Data Table S2)

Table 2. Presence of the different botanical families to which the weeds identified belonged in the field trials Cordoba-16, Cordoba-17 and Cordoba-18.

	CORDOBA-16	CORDOBA-17	CORDOBA-18
Amarantaceae		x	x
Asteraceae	x		
Convolvulaceae	x	x	x
Cruciferae	x		x
Cyperaceae		x	
Fabaceae	x	x	x
Papaveraceae		x	x
Poaceae	x		x
Polygonaceae	x	x	x
Quenopodiaceae	x		

Table 3. Weed richness (R) for the different treatments tested in three field trials (Cordoba-16, Cordoba-17, and Cordoba-18). Different letters mean significant differences for each trial (Tukey test, $p < 0.05$). Richness was not measured for barley, wheat and pea monocrops in Cordoba-16.

	Richness (R)		
	Cordoba-16	Cordoba-17	Cordoba-18
Faba bean 100%	35.7 a	78.6 ab	76.2 ab
Faba bean / barley	25.0 a	50.0 ab	42.3 a
Faba bean /wheat	25.0 a	46.4 a	61.9 ab
Faba bean / pea	50.0 a	64.3 ab	85.7 b
Faba bean 50%	46.4 a	75.0 ab	85.7 b
Barley 100%		83.3 b	76.2 ab
Wheat 100%		75.0 ab	95.0 b
Pea 100%		83.3 b	80.9 ab

Controlled-condition experiments

The number of emerged weed plants was significantly lower ($p < 0.05$) in the pots where barley had grown previously (either removed or maintained when weed seeds were sown) than in the pots used as control. The number of plants did not vary as the experiments progressed, so the counts of the three-time points were very similar (data not shown). The results for the final count of plants are shown in Table 4, both for the experiment where barley was removed before sowing the seeds of weeds and for the experiment where barley remained in the pots after the seeds of weeds were sown. The biomass of weeds at the end of the experiments was significantly lower in the pots with barley than in the control ones (Table 5).

Table 4. Number of weed plants per pot for each treatment in the two experiments under controlled conditions: where barley was removed before sowing the weeds and where barley was not removed after sowing the weeds. Different letters mean significant differences for each weed in each type of experiment, i.e., with barley removed or not removed. Differences were significant in all cases (Poisson regression analysis test, p <0.05).

	Barley removed		**Barley not removed**	
	Control	**Barley sown**	**Control**	**Barley sown**
Polypogon monspeliensis	19,9 a	5,0 b	19,7 a	4,6 b
Matricaria chamomilla	18,9 a	5,9 b	18,6 a	5,1 b
Amaranthus retroflexus	11,1 a	3,8 b	11,9 a	2,7 b
Medicago truncatula	5,2 a	1,4 b	7,8 a	1,4 b

Table 5. Biomass of weed plants per pot (mg) for each treatment in the two experiments under controlled conditions: where barley was removed before sowing the weeds and where barley was not removed after sowing the weeds. Different letters mean significant differences for each weed in each type of experiment, i.e., with barley removed or not removed (LSD test, p <0.05).

	Barley removed		**Barley not removed**	
	Control	**Barley sown**	**Control**	**Barley sown**
Polypogon monspeliensis	39.9 a	20.2 b	55.5 a	24.9 b
Matricaria chamomilla	115.4 a	49.0 b	131.6 a	25.6 b
Amaranthus retroflexus	70.6 a	41.7 b	104.6 a	10.1 b
Medicago truncatula	16.3 a	10.6 b	35.0 a	4.8 b

The proportion of reduction of the number of weed plants and weed biomass per pot as compared to the control was calculated in both types of experiments (removing barley before sowing the weeds and not removing it). There were no significant differences in the proportion of the number of plants for the two types of experiments. On the contrary, there were differences in weed biomass per pot for *Matricaria chamomilla* and *Amaranthus retroflexus*: final weed biomass was lower when barley remained in the pots till the end of the experiment (Table 6).

Table 6. Proportion of number of weed plants and biomass per pot as compared to the control for both types of experiments under controlled conditions (removing barley and not removing it just prior to sowing the weeds). Different letters mean significant differences for each weed and parameter: number and biomass of weeds (LSD test, $p < 0.05$).

	Nº of weeds (%)		**Biomass of weeds (%)**	
	Barley removed	**Barley not removed**	**Barley removed**	**Barley not removed**
Polypogon monspeliensis	25.2 a	23.6 a	70.1 a	61.9 a
Matricaria chamomilla	31.1 a	27.3 a	65.5 a	43.7 b
Sinapis arvensis	32.1 a	23.0 a	77.0 a	29.3 b
Medicago truncatula	24.9 a	18.4 a	58.9 a	31.9 a

Additionally, the proportion of reduction of the number of weed plants and weed biomass per pot was compared between the four weed species in the experiment where barley was removed, to investigate if any one of them was more affected by barley than the others, and no significant differences were found between them ($p > 0.05$).

Discussion

In this work, we have evaluated intercropping as a tool to control weeds in faba bean in the South of Spain. The level of control attained has been very high, reaching reductions of 92.7% in weed coverage when combined with barley and 76.6% and 46.1% in weed biomass when mixed with barley and wheat, respectively. The mixture of faba bean and barley, then, might be effective enough to dispense with the application of herbicides in this agroecosystem.

Previously only one work had studied the effect of the combination of faba bean and barley on weeds: Dhima et al. [26] reported that alternate replacement intercropping achieved levels of reduction of corn poppy of around 90%. As for other cereals, the faba bean and wheat mixture has proven to reduce weed biomass by 60% in alternate and mixed replacement intercropping [27]. Equally, Boutagayout et al. [28] found that an alternate intercrop of faba bean with wheat and oat got decreases in weed biomass of around 50%. As far as we know, our work is the first to achieve such a level of weed supression in faba bean, validated across three field experiments. Equally, barley has proved to be successful in reducing broomrape (*Orobanche crenata*) infestation in faba bean [29], and also to be effective in controlling rust disease in faba bean [30]. In these cases, the intercropping systems were different from those assessed in this work, so it would be necessary to integrate them in the best way to maximize the benefits of faba bean/ barley intercropping. Furthermore, it is remarkable that the combination of faba bean and wheat reduced 64% weed biomass compared to the wheat monocrop, making this mixture an exciting option for farmers that can be combined with some herbicide applications.

We did not find differences in weed diversity among all the tested treatments: no weeds belonging to a particular botanic family were more affected by any crop combination or monocrops than others. This is in contrast with what has been found in other intercropping systems [31,32], although there are also situations where weed diversity has not been influenced by intercropping [32]. In our case, the weed community has proved to be relatively stable regardless of the crop or crops present.

Of the two types of intercropping we tested, alternate with replacement and alternate with addition, only the latter was adequate for controlling weeds. The relationship between crops and weeds is based on the competition for available resources such as water, nutrients or light [33,34]. Plant density is one key factor in the improvement of crop competitiveness against weeds [35], and that is precisely the difference between both intercropping systems. In addition, intercropping plant density is doubled in comparison with alternate intercropping. The high weed pressure levels that we observed in the plots with faba bean sown at half density confirm the importance of plant density.

Plant density, however, is not the only mechanism that explains weed suppression in intercropping. If that were the case, we would have gotten similar results with barley, wheat, or pea. Weed pressure levels for them as sole crops illustrate that not all crops have the same competitive ability against weeds: barley presents very low weed infestation compared to the other two, with pea ranking the highest. Other factors influence the performance in the presence of weeds, such as plant architecture, vigor, or allelopathy [36,37].

Barley has been described as one of the most competitive crops against weeds by different authors [38,39]. The rapid biomass accumulation and high growth rates that barley shows at the beginning of its cycle are some of the reasons for this [39]. Another reason is the efficiency of barley in taking nitrogen: it has been reported to be more competitive for nitrogen than pea in intercrops, so depriving weeds of this nutrient. Beyond that, barley is considered a crop with high levels of allelopathy [40], and as many as 44 potential allelochemicals have been identified so far [41]. The two more important are the alkaloids Gramine and Hordeine, which appear in barley plants' leaves, roots, and roots exudates [23,41]. All this has made barley a common partner in crop diversification for weed control [42].

The experiments under controlled conditions aimed at evaluating the role that barley allelopathy might have on our results. Different types of bioassays under controlled conditions may be used to assess the allelopathic ability of a plant species, such as testing extracts from the allelopathic plant [43,44], agar bioassays [38] or pots screenings [45], where plants are grown together in Petri dishes or pots with soil, respectively. We opted for pot screening because it may better reflect the conditions under which allelopathy operates. Besides, our design, in which removing barley plants is compared with the effect of not removing them, allows for discriminating allelopathy from competition effects.

The results from the pots where barley was removed before sowing the weeds show a substantial allelopathic effect against them. All four weed species presented a decrease in plant emergence and biomass that can only be explained by the presence in the soil of chemical compounds previously released by the barley plants. These results also suggest that the main allelopathic effect is related to the first stages of seed germination and seedling development. Remarkably, there were no significant differences between the final number of weed plants in the pots where barley had been removed and in those where it remained till the end. As for weed biomass, however, in the case of two species (*Sinapis arvensis* and *Matricaria camomilla*) the decrease was higher when barley remained than when it was removed. This is probably due to additional allelopathic effects, although it is more difficult to separate them from competition effects in this case. Barley allelopathy had been previously tested on *Sinapis arvensis* [46,47]. Still, as far as we know, this is the first time it has been assessed on the other three weeds.

The fact that there has been no difference in the reduction of emerged plants and biomass between four weeds belonging to such different botanical families points to a global and non-discriminatory effect of barley in our case. These weeds are a sample of the ten families found in our area, and this global effect could explain the lack of differences in the composition of weed communities between the barley intercrops and the monocrops in our field experiments.

In conclusion, this research has established that the combination of faba bean and barley has great potential for the control of weeds in the agroclimatic region of the South of Spain. Further work should focus on developing the optimal intercropping strategy to obtain the best advantages from combining these two crops. It would also be of great interest to identify the allelochemicals behind weed suppression in our experiments and determine their mechanisms of action.

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Supplementary data

Table S1. Global weed coverage (in % of surface) for the different treatments tested in field trials Cordoba-15 (A) and Cordoba-16 (B), with alternate replacement intercropping. Different letters mean significant differences (Tukey test, $p < 0.05$).

A

	Coverage (%)
Faba bean 100%	14,1 a
Faba bean / barley	9,7 a
Faba bean /wheat	9,6 a
Faba bean / pea	11,9 a

B

	Coverage (%)
Faba bean 100%	23.1 abc
Faba bean / barley	18.4 ab
Faba bean /wheat	52.4 abcd
Faba bean / pea	50.4 bcd
Faba bean 50%	70.6 d
Barley 100%	10.4 a
Wheat 100%	52.4 cd
Pea 100%	69.6 d

Table S2. Relative density (D) of the botanical families of weeds for each of the treatments in the three field trials where addition intercropping was carried out: Cordoba-16 (A), Cordoba-17 (B) and Cordoba-18 (C).

A

	Poáceas	***Fabaceae***	***Quenopodiaceae***	***Cruciferae***	***Polygonaceae***	***Convolvulaceae***	***Asteraceae***
F. bean	0.0	0.0	13.1	8.2	13.5	3.8	61.4
F. bean/barley	30.6	0.0	0.0	0.0	0.0	5.6	63.9
F. bean/pea	5.4	5.4	17.7	0.0	5.4	10.3	55.9
F. bean/wheat	11.1	0.0	7.1	0.0	0.0	0.0	81.7
F. beasn 50%	0.0	9.2	16.5	8.2	2.5	0.9	62.8
SD	14,7	6,5	14,6	8,4	13,1	8,1	21,4

B	*Cyperaceae*	*Polygonaceae*	*Amarantaceae*	*Convolvulaceae*	*Fabaceae*	*Papaveraceae*
F. bean	24.0	32.8	17.2	4.9	7.1	13.9
F. bean 50%	32.2	34.5	10.3	4.1	8.8	10.1
F. bean/barley	9.0	52.1	11.8	4.5	4.2	18.5
F. bean/pea	4.4	47.6	8.3	2.6	6.9	30.1
F. bean/wheat	17.6	37.7	11.2	1.9	10.0	21.6
Barley	34.8	34.0	4.9	7.0	15.3	4.0
Pea	25.0	33.0	14.9	2.4	11.1	13.7
Wheat	28.5	40.4	4.8	9.0	7.6	9.7
SD	19.3	23.7	12.4	5.9	11.9	14.6

Conclusions

The following conclusions can be derived from the previous work:

1. It has been established that mixing pea with barley or faba bean in alternate intercropping with replacement may help in the control powdery mildew, with disease reductions reaching 44% and 32% respectively. The mixture with pea, on the contrary, had no effect on disease severity. The higher biomass and height of barley in comparison to the other crops seems to indicate that a barrier effect provided by barley plants plays an important role in the disease decrease.

2. Cultivar mixtures of Messire (susceptible to powdery mildew) and Eritreo (resistant to powdery mildew) reduce disease levels in cv. Messire, being the reduction higher as higher is the proportion of Eritreo in the mixture. The decreases in disease severity ranged 30-70%. This is a good strategy to prevent the resistance in Eritreo to be overcome by the pathogen.

3. The combination of faba bean with barley in alternate intercropping with replacement has achieved a reduction of rust severity in faba bean of 22%, which makes it a useful tool to help control the disease. No disease decreases were obtained when faba bean was mixed with pea or wheat. The barrier effect by barley appears to be a major mechanism behind this effect.

4. The mixture of cv. Joya, which is resistant to rust, with cv. Baraca, susceptible to it, produced reductions in disease severity which were higher as the proportion of Baraca increased. The barrier effect provided by cv. Joya seems to explain an important part of the disease reduction, that may be added to other mechanisms like the effect of dilution of inoculum. Joya produces more biomass and grows higher than Baraca, which stresses the importance in cultivar mixtures of other factors, like plant architecture, beyond disease resistance.

5. Alternate intercropping with replacement has proved unable to control weeds in faba bean when combined with pea, wheat or barley. Alternate intercropping with addition, on the contrary, has obtained reductions in weed pressure when faba bean was combined either with barley or wheat (76.6% and 46.6% decreases in weed biomass, respectively). This stresses the importance of crop densities in the competition with weeds. Diversity of weed communities was not affected by any of the treatments tested.

6. Barley has shown a high level of allelopathy under controlled conditions against four weed species that were present in the field trials: *Polypogon monspeliensis*, *Matricaria camomilla*, *Sinapis arvensis* and *Medicago truncatula*. This allelopathic effect seems to interfere with the first stages of development of the weeds.

7. Further research will be necessary to better identify the mechanisms behind the effects of intercropping on legume diseases. Additionally, it will also be required to develop breeding programs aimed at identifying the genotypes best suited for intercropping, so maximizing the benefits of this agronomic practise.