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## **7. Integrated Management of Fusarium Wilt**

### **Diseases**

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Running title: Fusarium wilts IPM

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**Abstract.** The integrated management concept is one of the fundamental paradigms that have emerged in crop protection in the last 50 yrs and yet a matter for legislation as exemplified by the European Union that recently has established the integrated management as the fundamental procedure for the management of crop diseases, pests and weeds. However, the integrated management is not a panacea for the control of plant diseases. It is an ecology-based approach aiming minimizing damage caused by diseases through ‘*the combined use of all available disease control measures, either simultaneously or in a sequence, through actions taken prior and after establishing the crop*’. In this chapter, we propose and develop a strategy for the integrated management for Fusarium wilts, one of the most devastating and challenging type of diseases impairing agricultural production worldwide, based on the: (i) use of pathogen-free planting material; (ii) site selection to avoid planting into high risk soils; (iii) reduction or elimination of *F. oxysporum* inoculum in soil; (iv) use of biocontrol agents for protection of healthy planting material from infection by resident or incoming inoculum subsequent to planting; (v) use of resistant cultivars regardless the level of resistance; and (vi) choice of cropping practices to avoid conditions favouring infection of the plant. The integrated management of Fusarium wilt diseases is difficult because complexities of target pathosystems are overlaid on the inherent complexities of the management strategy itself. Much research is still needed on population biology and genetic diversity in Fusarium wilt pathogens, disease risk prediction, disease-incidence-yield losses relationships, biological control, biotechnological breeding for disease resistance. On top of difficulties pointed out above, the practice of integrated management requires involvement of well-trained professional plant pathologists able to implement the tenets of the concept at the local level, as well as to incorporate into decision-making framework new knowledge and technologies that may be developed from scientific research. As the demand has increased for knowledgeable practitioners capable of integrating multifaceted controls in rigorous IDM programs, institutional support has declined through declining or even vanishing University education in Plant Pathology and the loss of extension-related activities in commercial agriculture. Erosion at the top of the trickle-down structure responsible for knowledge transfer to the field is one of the most serious threats to IDM.

**Introduction: A concept of Integrated Disease Management (IDM) of diseases caused by soil-borne pathogens**

Phytopathology as a science, and plant pathologists as scientists and practitioners, find themselves under a recurrent challenge for efficiently managing diseases affecting crop plants and forests. While this has remained the main 'raison d'être' of Phytopathology since it was established as a scientific discipline in mid 19<sup>th</sup> century, the means and procedures for achieving that task needed to evolve to satisfy social perceptions and claims about the negative impacts of disease control measures on health and the environment. Those concerns relate mainly to the extensive and intensive use of synthetic chemicals for the control of plant diseases which, with few exceptions such as host resistance-based control strategies, has been the main mean for protecting crop yield from losses caused by diseases. As a result, there have been numerous caveats and changes in legislation to minimize use of chemicals for disease control. For example, the European Commission (EC) has recently completed a Directive 91/414/EEC-based revision of nearly 1,000 phytosanitary active ingredients (a.i.) existing in the market in 1993, of which only 26% including 71 fungicide a.i. and 16 microbial biocontrol agents (but no fumigants) have satisfied the harmonizing process and are authorized for phytosanitary use in the European Union (EU). Following that, further legislation has been promoted on the matter, such as Directive 2009/128/CE that establishes the framework for a sustainable use of pesticide in EU member states, and its subsequent Regulation (EC) 1107/2009 by the European Parliament and the Council relative to marketing of phytosanitary products. With these two legislative packages, the EU has adopted a strategy for the sustainable use of chemicals aimed to a new, productivity and quality-based agriculture as well as to reduce their impact on human health and the environment in a way compatible with the protection of crops yields. That strategy determines more restrictive procedures for the registration and authorization of pest control chemicals, and establishes the Integrated Pest Management (IPM) and use of non-chemical measures as the fundamental procedures for the management of crop diseases, pests and weeds; to be implemented by the member states through specific Actions Plans by year 2014.

The IPM is one of the fundamental paradigms that have emerged in crop protection in the last 50 yrs, for which some 77 definitions have been proposed [41, 117]. This concept derives from 'The Integrated Control Concept' (ICC) established by Stern et al. in 1959 [114]. In this historic article, these authors explained how reducing insecticide dose resolved the

problem of organophosphate-resistant aphids on alfalfa in California by allowing considerable survival of their natural enemies. Although the ICC was addressed to insect pests and based on the integration of chemical and biological controls, it established a new philosophical framework for crop protection and provided a foundation for IPM to develop. Actually, a major contribution of IPM to agriculture has been to demonstrate the need to base all phases of production systems on sound ecological principles, with the ultimate goal of developing agroecosystems economically and ecologically sustainable [66].

The integrated management is not a panacea for the control of plant diseases. It is an ecological approach to maintaining plant health by minimizing damage caused by diseases, through the use of strategies that will vary according to the presence of a variety of factors that modify disease development. Moreover, this approach recognizes that the modifying factors often influence the action of each other [63]. Therefore, the integrated plant disease management (IDM) is not simply the juxtaposition or superimposition of two disease control measures, but the integration of all suitable management measures with the natural regulating and limiting elements in the environments [117]. Under this umbrella, a broad concept of IDM would imply *'the combined use of all available disease control measures, either simultaneously or in a sequence, through actions taken prior and after establishing the crop'* [43]. The basic principles of IDM are to: (i) reach a level of disease control sufficient, though not necessarily total; (ii) assess the pathogen population in order to apply disease control strategies and measures only when necessary; (iii) reduce but not necessarily eliminate completely the use of chemical means of control; (iv) consider the environmental impacts that might result from application of disease control measures; (v) consider all diseases affecting the crop in addition to the specific target of the control measures being applied; and (vi) consider legal and social implications of the actions for disease control measures being implemented.

Diseases caused by soil-borne plant pathogens, if uncontrolled, are amongst the main limiting factors in crop production, particularly when availability of agricultural land and/or demand of food lead to intensive use and continuous cultivation [36, 68]. Soil-borne plant pathogens include many fungi and nematodes as well as some bacteria and parasitic plants. They are characterized by their ability to persist free in the soil for long periods of time either by means of stress-resistant resting structures (e.g., chlamidospores, cysts, egg masses, microsclerotia, seeds, etc.) or through active, saprophytic growth in the soil environment. Plant pathogenic soil-borne fungi were differentiated by Garret [34] into two contrasting types,

namely soil inhabitants and root inhabitants. Garret regarded soil-inhabiting parasitic fungi as primitive, unspecialized microorganisms infecting seedlings and juvenile root tissues, for which parasitism was incidental to an edaphic saprophytic existence and high competitive saprophytic ability. Conversely, root inhabitants were viewed as more highly specialized parasites that exhibit some host-specificity and a delayed destructive effect on the host plant. In the absence of their hosts, root-inhabiting fungi have a transitory existence in soil and low competitive saprophytic ability if any [34]. A large majority of *Fusaria* are soil-borne. While those invading the plant root and foot cortex can be considered close to soil inhabitants, wilting *Fusaria* are a good example of root-inhabiting parasites [34, 79]. Vascular wilts are caused by strains of the highly diverse *Fusarium oxysporum* species complex that display a high degree of pathogenic specificity to host species and cultivars, on which basis they are classified into some 150 *formae speciales* (ff. spp.) [6, 65, 92]. Furthermore, those pathogenic strains are characterized by their ability to invade and colonize the vascular system of the host plant. These diseases are regarded amongst the most devastating and challenging of those that impair agricultural production worldwide. Therefore, this chapter is devoted to the study of strategies of use for the integrated management of *Fusarium* wilt diseases.

### **Principles of disease management**

Integrated Disease Management programs are based on the following principles of disease control: (i) exclusion or (ii) eradication, of the pathogen; (iii) escape from infection; (iv) development and use of genetic resistance against the pathogen; (v) protection of the plant from infection; and (vi) reduction of disease in infected plants [81]. They can be applied by biological, chemicals, cultural, physical, and regulatory methods, depending of the nature of the agents employed. For diseases caused by soil-borne pathogens, such as *Fusarium* wilts, which are mainly monocyclic in nature, the control principles and methods should be targeted to excluding the pathogen, as well as reducing the amount and/or efficiency of the initial inoculum. Therefore, IDM strategies of those diseases within the framework of sustainable agriculture would include: (i) use of pathogen-free planting material; (ii) site selection to avoid planting into high risk soils; (iii) reduction or elimination of *F. oxysporum* inoculum in soil; (iv) use of biocontrol agents for protection of healthy planting material from infection by resident or incoming inoculum subsequent to planting; (v) use of resistant cultivars regardless the level of

resistance; and (vi) choice of cropping practices to avoid conditions favouring infection of the plant [43].

**1. Use of pathogen-free planting material.** Many wilt-inducing *Fusaria* can be transmitted in infected seeds, vegetatively-propagated planting material (e.g., bulbs, cuttings, rootstocks, scions, etc.), or transplants developed from them [9, 92]. Use of infected propagating material can lead to introducing the pathogen or its pathogenic variants (see below) into pathogen-free production areas or pathogen-free soils in areas where the pathogen occurs already. Therefore, the importance of checking the health of that material through certification programs, phytosanitary inspection, and quarantine legislation cannot be too strongly emphasized. Failure in this pursue may lead to the establishment of new pathogens in a country, as it recently happened in Spain with *Fusarium circinatum*, *F. oxysporum* f. sp. *basilici*, *F. oxysporum* f. sp. *radicis-lycopersici*, *Fusarium solani* f. sp. *cucurbitae* race 1, etc. [42, 44]. More importantly, introduced exotic pathogens can potentially be invasive and give rise to devastation in cultivated as well as natural plant communities [e.g., 3, 14, 87]. The European Plant Protection Organization (EPPO), being well aware of such a risk, has placed a warning on quarantine fungal pathogens, of which 21 species are already present in member states and 39 are currently absent from them (<http://www.eppo.org/QUARANTINE/quarantine.htm>).

One of most important difficulties for the detection and identification of *Fusarium* wilt pathogens concerns the similarity in morphology between pathogenic and non-pathogenic strains of *F. oxysporum*. As a result, identification of pathogenic *F. oxysporum* isolates has been based mainly on pathogenicity assays either on various plant species for determination of the *formae specialis* to which it may belong, or on a set of race differential lines, which are laborious and time consuming. Therefore, approaches have been addressed to differentiate *F. oxysporum* ff. spp. as well as for their in planta detection and quantification (e.g., *albedinis*, *basilici*, *ciceris*, *chrysanthemi*, *cucumerinum*, *phaseoli*, *radicis-cucumerinum* and *radicis-lycopersici*) and pathogenic races based on the use of molecular markers identified by genotyping (e.g., RFLP, RAPD, AFLP) or polymerase-chain-reaction (PCR) amplification of transposon insertions [2, 46, 78]. Conventional PCR assays suffer from several technical limitations, which relate to the nature of matrices from which the template DNA is extracted, the components of the reaction mix, cross contaminations, post amplification procedures necessary to detect the amplicons, etc. Most of these limitations can be now overcome by use of recently developed real-time PCR technologies that combine the sensitivity of conventional PCR

with the generation of a specific fluorescent signal. This signal provides real-time analysis of the reaction kinetics and allows quantification of specific DNA targets [99, 110].

**2. Site selection to avoid sowing/planting into high risk soils.** Proper selection of the planting site optimizes the use of *F. oxysporum* ff. spp.-free planting material in non-infested soils. For that purpose, accurate information on the disease history of the field with regard to production of susceptible crops is of utmost importance. Disease risk assessment based on inoculum density (ID)-disease incidence (DI) relationships would be most useful if the inoculum density in soil at planting sites could be estimated to avoid those with high risk for severe disease. Populations of *F. oxysporum* in soil can be assessed by soil dilution plating using selective media. However, this does not allow inferring ID of pathogenic strains because of their morphological similarity with non-pathogenic ones. For example, De Vay et al. [27] assessed the ID of *F. oxysporum* in cotton soils by agar dilution plating but identified colonies belonging to *F. oxysporum* f. sp. *vasinfectum* by further pathogenicity assay to cotton seedlings. That allowed estimating the number of *F. oxysporum* f. sp. *vasinfectum* colony forming units (CFU) g<sup>-1</sup> soil and relating a range of 1,100 to 2,608 CFU g<sup>-1</sup> soil to increase of Fusarium wilt incidence over physiological time in degree days and effects on crop growth and yield. Ben Yephet et al. [11] used a similar approach for Fusarium wilt in carnations and found that 6, 25, 120, 770, and 3,500 CFU g<sup>-1</sup> soil of *F. oxysporum* f. sp. *dianthi* determined a final DI of 2, 5, 13, 34, and 57 %, respectively; the flower yield being related inversely to ID of the pathogen. Conversely, disease risk can be made by directly bioassaying the planting soil with susceptible and resistant host cultivars. Kraft et al. [67] developed a soil-sample bioassay to determine predominance of *F. oxysporum* f. sp. *pisi* in pea soils, whereby soil sampled from planting sites were tested for disease developed on race differential lines under controlled environment. This allowed selecting the appropriate resistant cultivar for the planting site assayed.

The procedures referred above are too laborious and time consuming to be of practical use for implementing IDM strategies on a commercial scale. These difficulties would be overcome by molecular protocols for the specific detection and quantification of the pathogen in soil. Recently, Zambounis et al. [118] sequenced a portion of the ribosomal intergenic spacer (IGS) region of *F. oxysporum* f. sp. *vasinfectum* and used the sequence data to develop two specific quantitative polymerase chain reaction (qPCR)-based protocols for the quantification of pathogen

genomic DNA obtained from soil substrates. Use of these protocols with sterile soil artificially infested with a range of conidial inoculum allowed detection of fungal DNA as low as  $5 \text{ pg } \mu\text{l}^{-1}$  of target DNA, with detection sensitivity lower than  $10^4$  conidia  $\text{g}^{-1}$  soil. However, the practical use of those protocols was not validated with naturally infested soils. In a similar study using primers internal in the sequence of a *F. oxysporum* f. sp. *ciceris*-specific SCAR marker [47], Jiménez-Fernández et al. [46] developed a real-time qPCR protocol that allowed quantifying the pathogen DNA up to 1 pg in soil. Moreover, validation of this protocol using field soil infested with several races of the pathogen allowed quantifying as low as 45 CFU of *F. oxysporum* f. sp. *ciceris*  $\text{g}^{-1}$  dry soil. Additional studies on the relationship of concentration of pathogen DNA in soil with inoculum potential would be of much importance for predicting Fusarium wilt risk in chickpea crops based on the use of quantitative models between disease development, inoculum density and other factors in this pathosystem [90].

**3. Reduction or elimination of *F. oxysporum* inoculum in soil.** Soils infested with *F. oxysporum* ff. spp. can be recovered for agricultural production by reducing the amount of initial inoculum and/or its potential for disease to levels below the threshold for severe disease. The initial inoculum of *F. oxysporum* ff. spp. can be reduced by means of chemical, biological or physical disease control methods [9]. Achieving that aim by cultural practices such as crop rotation is of lesser efficacy because of the ability of chlamidospores of pathogens for long survival in soil. However, use of crop rotations in the integrated management of Fusarium wilt diseases should not be disregarded to avoid recurrent increase of inoculum in soil. The use of cultural practices for reducing disease potential or mitigating disease effects on crop yield is discussed latter.

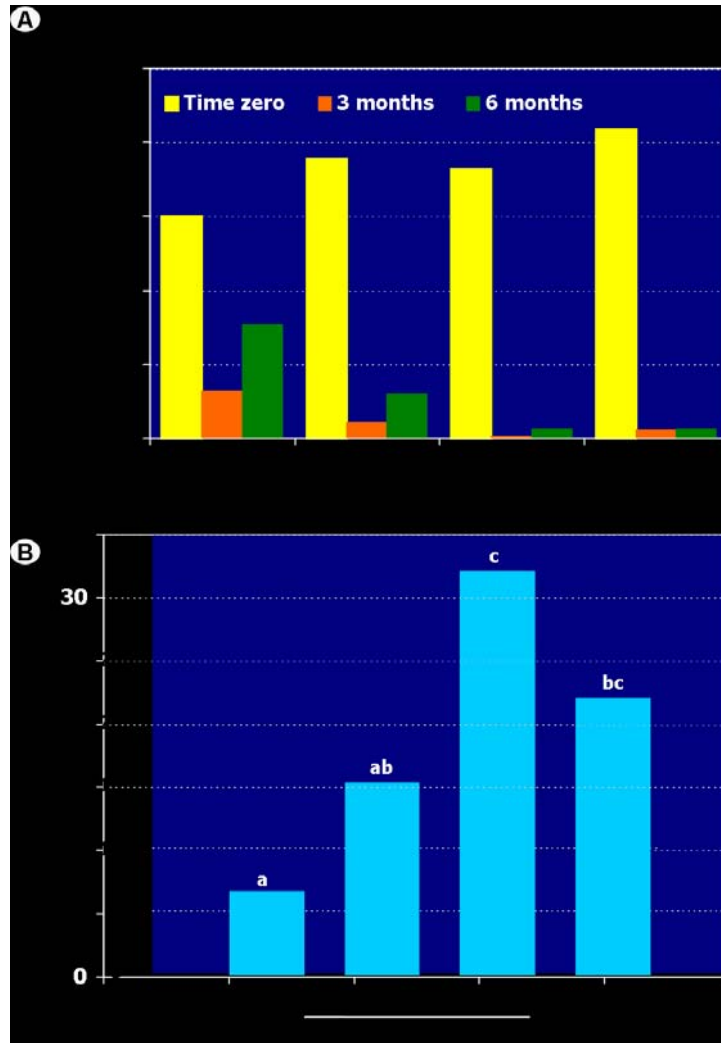
**3.1. Chemical methods.** Soil treatment with broad-spectrum fumigants such as methyl bromide, chloropicrin, or methyl isothiocyanate both alone or in mixtures successfully controlled Fusarium wilt of tomato and increased crop yield [9]. However, the efficiency of soil fumigation is curtailed by either survival of pathogens in soil layers below the depth of effective fumigation, or reintroduction of them through infected planting material or by conidia carried in the air or irrigation water [60, 101]. Also, methyl isothiocyanate is prone to enhanced biodegradation in soil by adaptation of microbial populations to use the compound as an energy source. This adaptation may be induced by repeated or even single applications of methyl isothiocyanate-generating formulations to a field,



thus seriously compromising its efficacy [28, 84, 113]. Methyl bromide is scheduled for worldwide withdrawal from routine use as a fumigant in 2015 under the directive of the Montreal Protocol on ozone-depleting substances. Moreover, none of other fumigants tested against Fusarium wilt diseases satisfied the established harmonizing criteria in the recent revision of marketed active ingredients completed by the EC within the framework of EEC Directive 91/414; therefore the use of those fumigants will not be allowed in the EU in the near future.

**3.2. Physical methods.** Soil solarization and flooding have been successfully used for the control of Fusarium wilt diseases.

**Soil solarization.** This is a hydrothermal process that occurs when moist soil is covered with thin (25 to 50  $\mu\text{m}$ ), transparent plastic polyethylene or polyvinyl sheets during a period of high temperature and intense solar radiation. Soil solarization has become a widely and extensively used technology for the management of soil-borne plant pathogens after the pioneering work of Katan et al. [61]. In this landmark publication, these authors showed that soil solarization for 2 weeks during summer time in Israel reduced the populations of buried inoculum of *F. oxysporum* f. sp. *vasinfectum* and *Verticillium dahliae* by 94 to 100% at 5 cm, 67 to 100% at 15 cm, and 54 to 74% at 25 cm. Average maximum temperatures of the solarized soil were 50.7°C at 5 cm and 40.8°C at 15 cm, while the average maximum temperatures of the non-solarized soil were 37.6°C and 32.4°C, respectively. Following that paper, a large number of papers and several reviews have been published on the fundamental aspects of soil solarization and its effectiveness for controlling many different pathogens and pests under a wide range of climates and different cropping systems [56, 57, 59, 60, 62]. Soil solarization compares favourably with chemical fumigation and thus it can be conceived as an alternative to it. In a study done in plastic houses at Almería, south-eastern Spain, in a soil artificially infested with the highly virulent race 2 of *F. oxysporum* f. sp. *niveum*, *F. oxysporum* populations in the upper 15 cm were reduced by 94, 99, and 97 % following solarization for 1 and 2 months or fumigation with metham-Na at a rate of 480 L ha<sup>-1</sup>, and remained at a low level for 6 months thereafter. Solarization for 2 months completely controlled Fusarium wilt in watermelon and gave a fruit yield almost five times that of plants in untreated soil (Fig. 1). Solarization for 1 month and fumigation with metham-Na doubled and tripled fruit yield, respectively and slowed or retarded disease development [35].



**Figure 1.** Effects of soil solarization and fumigation with metham-Na on: **A**, numbers of propagules of *Fusarium oxysporum* f. sp. *niveum* in soil before treatment, or 3 and 6 months after treatment; **B**, yield of a subsequent watermelon crop in a plastic house at Almería, southern Spain (González-Torres et al., 1993).

The success of soil solarization is based mostly on thermal sensitivity of most plant pathogens, which rarely can grow at temperatures above 35°C. Rather, most pathogen propagules are unable to withstand temperatures of 50°C for more than a few hours, and longer periods at lower temperatures may also be lethal. The temperatures in the solarized soil are 5 to 15°C higher than those in comparable non-solarized ones; and effective disease control was obtained with maximum temperatures within the range of 45 to 50°C and 38 to 45°C at depths of 10 and 20 cm, respectively. The thermal decline of soil-borne organisms during solarization depends on both the soil temperature and exposure time, which are inversely related. Since the upper layer of soil is heated more intensively than the lower ones, the solarization period should last at least 4 to 5 weeks to achieve control at all desired depths; however, the longer the solarization, the greater the depth of effective activity, and the higher the pathogen-killing rates. Nevertheless, sublethal heating may have a 'weakening' effect in surviving propagules and reduce their inoculum potential. Freeman and Katan [33] found that sublethal heating of conidia and chlamydo spores of *F. oxysporum* f. sp. *niveum* caused a delay in germination, reduction in growth of conidial and chlamydo spores germ tubes, and enhanced decline in viability of propagules beyond a low initial killing. Similarly, Arora et al. [5] reported that sublethal heating of *Fusarium oxysporum* f. sp. *ciceris* chlamydo spores resulted in C exudation in soil and reduced aggressiveness on chickpeas compared with that of unheated chlamydo spores.

In addition to the physical effect of heat, soil solarization may contribute to disease control by microbial and chemical changes in soil associated with solar heating. Thus populations of microbial flora antagonistic to pathogens may increase and attack the pathogen propagules, especially if they are weakened by sublethal heating [5, 108, 109]. A synergistic interaction similar to that between microbial antagonists and weakened inoculum may be responsible of the increased effects of combining soil solarization and fumigation with metham-Na on killing of propagules of *F. oxysporum* f. sp. *vasinfectum* and control of Fusarium wilt of cotton, compared with either individual treatment [10].

**Flooding.** Flooding was a well established pre-planting practice in ancient agriculture in the near and far East [107]. It can be regarded as a soil disinfestation method harming soil-borne pathogens by reduction of O<sub>2</sub>, increase of CO<sub>2</sub>, or a diversity of microbial interactions that result in toxic substances to pathogens upon anaerobic processes [15]. In modern agriculture, a number of reports since 1948 indicated efficient control of

several bacterial, fungal, and nematode diseases, including Fusarium wilts [107]. A classical example of large-scale use of flooding for the control of Fusarium wilts is that of Panama disease of bananas. Studies by Stover [105, 106] indicated that flooding for 3 to 4 months with a minimum of 30 cm of water significantly reduced populations of *F. oxysporum* f. sp. *cubense* in soil and controlled Fusarium wilt. Flooding can be used as a cultural practice for management of Fusarium wilts only in countries where large resources of water are available and level land is suitable for the construction of water retention. Nevertheless, occurrence of natural flooding by intense, prolonged rains and crop rotations that involve flooding of soil may provide opportunities to take advantage of the underlying mechanisms against soil-borne pathogens. Katan [58] refers a noticeable benefits on crop health of vegetables grown in soil flooded by rain for several weeks in winter and spring in the Gaza region; and rotation of cotton with paddy rice effectively reduced populations of *V. dahliae* and *F. oxysporum* f. sp. *vasinfectum* and controlled Verticillium and Fusarium wilt diseases of cotton in California and China, respectively [20, 97].

**Sanitation.** This disease control method includes practices that remove and destroy sources of inoculum from affected plants or infested debris. The systemic infection of the host plant that characterizes Fusarium wilt gives rise to formation of abundant chlamydospores in above-ground organs that can become incorporated into soil after harvest of affected crops and contribute to build up of soil-borne inoculum. Burning or flaming residues of affected crops to achieve thermal killing of pathogen resting structures would reduce that effect thereby reducing disease risk in subsequent host crops. *Cephalosporium graminearum* invades the vascular system of wheat plants extending to the heads so that considerable amount of inoculum remains in straw after harvest. Burning the straw of *Cephalosporium* stripe-affected wheat crops destroys much potential inoculum and markedly reduces disease in subsequent wheat crops [13, 15]. Burning is contrary to longstanding conservation policy and considered a destructive practice, but similar thermosanitation with lesser environmental impact can be achieved by flaming crop debris with propane or oil fuelled flamers that allow more controlled heating. This technique has successfully been applied to potato crops before harvest to greatly reduce the amount of *V. dahliae* microsclerotia added to the soil by infected potato stems and possibly would be of similar use against Fusarium wilt-affected annual crops. Burying infested debris is an ecologically sound practice and helps destroying inocula of fungal

pathogens lacking specialized resting structures but it is unlikely to contribute destroying the latter.

**3.3. Organic amendments.** This term is used here for all organic matter incorporated in the soil. Organic amendments have been used to promote crop growth and yields since ancient in agriculture [107], but their deliberate application for the purpose of plant disease management is a more recent approach. Organic amendments cover a range of inputs, including animal (cattle, poultry, swine) and green manures, composts, high N-containing products (blood, bone, and meat meal, fish meal, soy meal), etc.

Although there are sufficient data to indicate that organic materials reduce disease incidence caused by a wide range of plant pathogens, our level of understanding of the mechanisms involved is still limited [7]. Elegant research by Lazarovits and co-workers has convincingly shown that production of ammonia ( $\text{NH}_3$ ) and nitrous acid ( $\text{NO}_2\text{H}$ ) upon microbial degradation of N-containing products eradicate soil-borne propagules of several plant pathogens, including *F. oxysporum* ff. spp. and *V. dahliae*, but the efficacy of these materials is related to soil properties [75]. Ammonia forms in soil from accumulation of non-toxic ammonium ( $\text{NH}_4^+$ ), reaching equilibrium accumulates at high pH (8.5 to 9.5). However, this accumulation and production of  $\text{NH}_3$  are impaired by nitrification ( $\text{NH}_4^+$  to  $\text{NO}_2^-$  and  $\text{NO}_3^-$ ) in soils with high organic C. Thus,  $\text{NH}_3$  formation is enhanced by inhibiting nitrification in organic soils or diluting the organic matter content to below 2% by addition of sand. Use of low rates (0.25 to 0.50%) of high-N products showed a delayed eradication of pathogens compared with that caused by higher rates (2%), which was related to the formation of  $\text{NO}_2\text{H}$  from  $\text{NO}_2^-$  at pH below 5.5.  $\text{NO}_2\text{H}$  is 300 to 500 times more toxic than  $\text{NH}_3$  to *V. dahliae* microsclerotia. Amendment of soil with liquid swine manure ( $55 \text{ hL ha}^{-1}$ ) is also effective in eradicating the same pathogens of above, but again soil pH is critical to the activity of the amendment. At pH lower than 5, the eradicating activity against *V. dahliae* microsclerotia is related to formation of  $\text{NO}_2\text{H}$  and presence of the non-ionized, acidic form of volatile fatty acids, with acetic acid representing 60% of the active ingredients, and butyric, caproic, isobutyric, isovaleric, propionic, and valeric acids the remainder. However, at pH >8.5 the killing of those propagules is due to formation of  $\text{NH}_3$ .

Toxic compounds released from microbial degradation of green manure are also efficient in the eradication of soil-borne fungi, including *F. oxysporum* ff. spp. A specific term, 'biofumigation', was originally coined to describe the suppression of pathogens by biocidal hydrolysis

products, notably isothiocyanates released by glucosinolate-containing plants in soil [64]. However, the term has since been popularized to now include a range of other organic amendments. Biofumigation can involve incorporating fresh plant material as green manure, or utilizing processed plant products high in glucosinolates such as seed meal. Apparently, biofumigation is not yet sufficiently powerful or practical in implementation as a management strategy of soil-borne pathogens since its efficacy is influenced by the many factors involved in the technique. For instance, fungal pathogens vary in sensitivity to different types of isothiocyanates (i.e., aromatics, sulphur-substituted aliphatics), and pathogen propagules may differ in sensitivity to a given isothiocyanate [103]. Also, caution should be taken to conceive isothiocyanates as the sole mechanism associated with biofumigation involved in the control of soil-borne pathogens. Firstly, the microbiological decomposition of plant tissues also releases other products that are biologically active, such as volatile fatty acids and ammonia [75]. Secondly, the incorporation of organic amendments themselves increases the total microbial populations in soil by 10- to 1000-fold, many of which can be antagonists to plant pathogens. Thus, the toxicants generated by microbial decomposition do not kill all microorganisms, but rather exert a selective influence on the microbial population possibly because some can detoxify or utilize the degradation products for growth. Finally, the inactivation of pathogens after additions of large quantities of organic matter may be due to anaerobic and strongly reducing soil conditions that develop mainly if the soil is covered with an airtight plastic sheet as shown by Block et al. [12]. These authors found that inocula of *F. oxysporum* f. sp. *asparagi*, *Rhizoctonia solani*, and *V. dahliae* were reduced if soil amended with 3.4 to 4.0 kg fresh weight m<sup>-2</sup> of fresh broccoli or grass was covered with plastic sheeting, but the pathogens were not or hardly inactivated in amended, non-covered soil or non-amended, covered soil.

**4. Use of biocontrol agents for protection of healthy planting material from infection by resident or incoming inoculum subsequent to planting.** The efficiency of soil disinfestation and use of pathogen-free planting material in the management of Fusarium wilt diseases would be enhanced if this material is protected further from pathogen inoculum residual in soil or incoming from outside sources in infested debris or water by introducing biocontrol agents into soil or the rhizosphere [21]. Actually, a considerable number of Fusarium wilt diseases were reportedly controlled by treatment with a range of microbial agents, including fungi (e.g., non-pathogenic strains of *F. oxysporum*, *Penicillium oxalicum*, and

*Trichoderma* spp.) and rhizobacteria (e.g., *Bacillus* spp., *Paenibacillus* spp. and fluorescent *Pseudomonads*) isolated from Fusarium wilt-suppressive soils or compost, healthy plants, or epiphytic microflora [1, 25, 29, 32, 38-40, 69, 72, 98]. For example, seed treatment with a non-pathogenic *F. oxysporum* isolate suppressed Fusarium wilt in 'ICCV 4' chickpeas by 30 to 45% or 78% after challenge inoculation with highly virulent *F. oxysporum* f. sp. *ciceris* race 5 by root dipping or sowing in infested soil under optimal conditions for the disease, respectively [38, 39]. Similarly, rhizosphere colonization of 'PV 61' chickpea seedlings by the same nonpathogenic *F. oxysporum* isolate, *Bacillus subtilis* or *Trichoderma harzianum* prior to transplanting into soil infested with race 5 significantly reduced the final disease intensity and the area under disease progress curve (AUDPC) between 14 and 33% and 16 and 42%, respectively, under conditions that resulted in 100% disease in the untreated controls [40]. Also, in a series of elegant studies Melgarejo and co-workers showed that infesting melon, tomato and watermelon seed beds or transplanting substrates with  $10^6$  to  $10^7$  conidia of *P. oxalicum* g<sup>-1</sup> of substrate suppresses Fusarium wilt of melon, tomato and watermelon by 30, 28 to 72, and 54%, respectively [25, 72].

**4.1. Factors influencing performance of biocontrol agents.** Of some 41 products currently registered or marketed (mainly in the USA and Israel) for control of soil- and seed-borne plant pathogens, eight bacterial and five fungal formulations include *Fusarium* spp. or *F. oxysporum* as the target pathogen [115]. Similarly, some 16 microbial products are included in CEE 91/414 Directive, Annex I for control of fungal diseases [86]. However, biological control of Fusarium wilt diseases has not reached yet application in commercial agriculture because of inconsistent performance common to most biological control agents [8, 31]. That inconsistency is due mainly to the influence of abiotic and biotic factors, including soil temperature and type, the nature and mechanism of action of the biocontrol agent, host plant genotype, inoculum density of the target pathogen etc., on biocontrol activity.

**Nature and mechanisms of action of biocontrol agents.** Most reports on biocontrol using *Trichoderma* spp. referred the biocontrol agent as *Trichoderma harzianum*. However, *T. harzianum* is conceived currently as a species complex that includes *T. harzianum sensu stricto*, *T. asperellum*, *T. atroviride*, and *T. longibrachiatum* [37]. This genetic complexity may harbour diversity in the mechanisms of action involved in biocontrol and may eventually give rise to variability in biocontrol efficiency. Studies on *Trichoderma* spp. antagonistic mechanisms have

shown the involvement of antibiosis, competition for nutrients or infection loci, mycoparasitism, and induction of systemic resistance in the plant. Most efficient biocontrol strains display more than one of those mechanisms, either simultaneously or sequentially, but only one of them may be mainly involved in some strains. For example, the strain *T. harzianum* T35 was shown efficient in biocontrol of Fusarium wilt of cotton and melon. This activity was attributed to competition for nutrients with the pathogen, which resulted in reduction of germination rate of chlamydospores of *F. oxysporum* f. sp. *melonis* and *F. oxysporum* f. sp. *vasinfectum* in the rhizosphere of cotton and melon plants in the presence of *T. harzianum* T35 [102].

Non-pathogenic isolates of *F. oxysporum* suppress Fusarium wilts by saprophytic competition for nutrients (mainly C sources) in the soil and rhizosphere, as well as for infection sites on and in the root, and through induction of systemic resistance in the infected host plant. Although the different mechanisms of action described above do not exclude each other, one of them may predominantly be expressed in biocontrol by a given non-pathogenic strain and that may bear implications for its mode of application [1, 32]. For example, Larkin and Fravel [73] demonstrated that at glucose concentrations  $\geq 0.2 \text{ mg g}^{-1}$  of soil non-pathogenic *F. oxysporum* Fo47 significantly inhibited chlamydospore germination of pathogenic *F. oxysporum* ff. spp. and reduced subsequent germ tube growth in soil. Conversely, the biocontrol isolate *F. oxysporum* CS-20 had no effect on germination or germ tube development of the pathogen, but both Fo47 and CS-20 strains were able to induce systemic resistance in some plant species. When competition is the main mechanism of action, typically the population of the biocontrol fungus must be at least as large, if not larger, than that of the pathogen in order to achieve control. However, if induction of resistance is the main mechanism of action, control can often be achieved when the pathogen population is much greater than that of the biocontrol fungus. Thus, strain CS-20 significantly reduced Fusarium wilt incidence in tomato at an inoculum density  $10^3$  times lower than that of the pathogen, but strain Fo47, which functions mainly through competition, was only effective when it was introduced at concentrations 10 to  $10^2$  times higher than the pathogen concentration [73]. Comparatively, *P. oxalicum*, whose main mechanism of biocontrol of Fusarium wilts is also induction of resistance, must be applied at an inoculum density of similar or 10 times higher than that of the pathogen within a threshold of  $10^6$  to  $10^7$  *P. oxalicum* conidia  $\text{g}^{-1}$  of substrate [72].

**Inoculum density of the target pathogen.** The inoculum level of the target pathogen may determine success or failure of biocontrol conferred



by a microbial antagonist regardless its nature and mechanism of action. Treatment of 'ICCV 4' and 'PV 61' chickpea seeds with non-pathogenic *F. oxysporum* Fo90105 ( $3 \times 10^6$  conidia seed<sup>-1</sup>) reduced Fusarium wilt incidence and AUDPC by 78 and 53%, and 97 and 71%, respectively, at an inoculum density of 500 chlamydospores g<sup>-1</sup> of soil of *F. oxysporum* f. sp. *ciceris* race 5. However, this protection from disease was annulled with an inoculum density of 1,000 chlamydospores g<sup>-1</sup> of soil [39]. Also, prior colonization of 'PV 61' chickpea rhizosphere by *Bacillus subtilis* GB03 ( $1 \times 10^6$  CFU g<sup>-1</sup> of soil) reduced Fusarium wilt incidence and AUDPC by 22 and 40% at an inoculum density of 1,300 chlamydospores g<sup>-1</sup> of soil of *F. oxysporum* f. sp. *ciceris* race 5, but this disease suppression was annulled with an inoculum density of 6,700 chlamydospores g<sup>-1</sup> of soil of the pathogen [40].

Similarly, studies under glasshouse conditions by De Cal et al. [25] indicated that rhizosphere populations of *P. oxalicum* of  $1.75 \times 10^7$  conidia g<sup>-1</sup> of root failed to suppress Fusarium wilt of melon with an inoculum density of  $0.6 \times 10^6$  CFU g<sup>-1</sup> of peat substrate of *F. oxysporum* f. sp. *melonis* that determined 100% disease in the untreated control. Comparatively, *P. oxalicum* at a dose of  $2.32 \times 10^7$  conidia g<sup>-1</sup> of root suppressed by 58% Fusarium wilt of watermelon with *F. oxysporum* f. sp. *niveum* inoculum density of  $3.1 \times 10^4$  CFU g<sup>-1</sup> of peat substrate that determined 59% disease in the untreated control. However, 100 and 30% suppression of melon wilt by *P. oxalicum* were achieved in microplot experiments where  $4.3 \times 10^3$  CFU g<sup>-1</sup> of soil of *F. oxysporum* f. sp. *melonis* resulted in 39 and 13% Fusarium wilt in the untreated control in two consecutive years.

Several authors have emphasized that for efficient plant disease biocontrol there must be established a dose-response relationship between pathogenic and biocontrol strains, which can be influenced by the pathogen virulence and mechanism of action of the microbial antagonist [51, 73]. Montesinos and Bonaterra [85] used mathematical relationships between the inoculum densities of the pathogen and biocontrol agent that allowed relating the pathogen virulence with efficiency of biocontrol. Using those models on a range of pathosystems and hosts (although none were Fusarium wilt diseases) allowed demonstrating an inversely proportional relationship between efficiency of a biocontrol agent and ED<sub>50</sub> of the pathogen, and that biocontrol efficiency decreases as virulence of the pathogen increases [30].

**Soil temperature.** Temperature can have an effect on the efficiency of biocontrol of Fusarium wilt diseases that may vary according to other interacting factors, such as the inoculum density of the pathogen and

nature of biocontrol strain. For example, Landa et al. [69] reported that *P. fluorescens* RGAF 19 and RG 26 strains non-inhibitory of in-vitro growth of *Fusarium oxysporum* f. sp. *ciceris* suppressed Fusarium wilt in 'PV 61' chickpeas only at a soil temperature of 20 or 30°C and at inoculum densities below 250 chlamydo-spores g<sup>-1</sup> of soil. These temperatures were suboptimal for disease compared with 25°C, at which temperature increasing inoculum densities of the pathogen did not influence Fusarium wilt in chickpea. Comparatively, at 20 and 30°C disease development increased as inoculum density did from 250 to 1,000 chlamydo-spores g<sup>-1</sup> of soil compared with 25 to 100 chlamydo-spores g<sup>-1</sup> of soil. Similarly, Larkin and Fravel [74] investigated the influence of temperatures within the range of 22 to 32°C, and other environmental factors, on the suppression of Fusarium wilt of tomato by non-pathogenic strains of *F. oxysporum* CS-20 and CS-24. While strain CS-20 significantly reduced disease incidence at all temperatures within that range by 59 to 100% relative to the untreated control, strain CS-24 reduced disease at high temperatures but was less effective at 27°C, which was the optimum temperature for disease development.

**Host plant genotype.** Whereas the influence of abiotic and microbial factors on inconsistency of biocontrol has been studied in a number of cases, the putative effect of the host genotype on such inconsistency has been considered rather recently. Hervás et al. [36] found that prior seedling inoculation with non-pathogenic *F. oxysporum* isolates Fo90105 protected chickpea cv. ICCV 4 from Fusarium wilt after challenge inoculation with *F. oxysporum* f. sp. *ciceris* race 5, but the former isolate failed to induce resistance in cv. JG 62 despite this cultivar was more susceptible to that race than cv. ICCV 4. In a subsequent study, seed treatment with Fo90105 significantly suppressed disease in 'ICCV 4' chickpeas in soil infested with 500 chlamydo-spores g<sup>-1</sup> of soil of *F. oxysporum* f. sp. *ciceris* race 5, but this effect was higher and more consistent in cv. PV 61, which showed to be as susceptible as cv. ICCV 4 in the untreated controls [39]. Moreover, an increase of pathogen inoculum density from 500 to 1,000 chlamydo-spores g<sup>-1</sup> of soil annulled protection from disease in 'PV 61' but significantly increased the amount of Fusarium wilt in 'ICCV 4' treated with isolate Fo90105 compared with that in the untreated control. Interestingly, Forsyth et al. [29] recently reported a similar phenomenon, whereby one endophytic *F. oxysporum* isolate reduced Fusarium wilt in banana cvs. Cavendish and Lady Finger whereas another non-pathogenic isolate increased Fusarium wilt severity on 'Cavendish' and had no disease suppression effect on 'Lady Finger'.

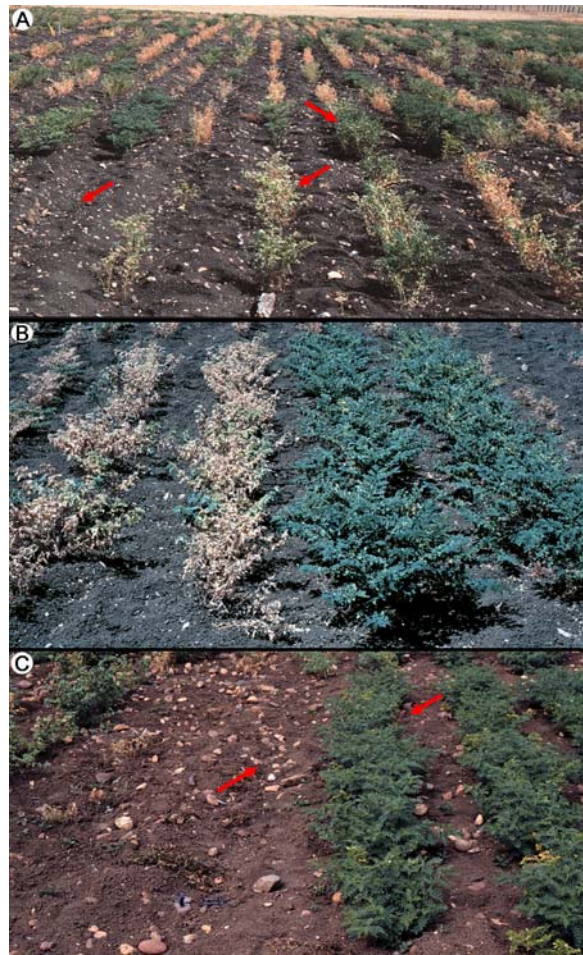
The role of the genetics of the host plant in Fusarium wilt suppression indicated by results of above does not necessarily concern to non-pathogenic *F. oxysporum* only, as shown by Hervás et al. [40]. Thus, the difference in the extent of Fusarium wilt suppression conferred by non-pathogenic *F. oxysporum* Fo90105 in 'ICCV 4' and 'PV 61' chickpeas was later demonstrated to occur also in the suppression conferred by *B. subtilis* isolate GB03 and *T. harzianum* KRL-AG2, which again was higher and more consistent in 'PV 61' than in 'ICCV 4'. The effect of the host plant genetics on the inconsistency of disease suppression by biocontrol agents may not necessarily involve the reaction of the host plant to the pathogen; but rather, it may involve effects on traits of the microbial antagonist essential for biocontrol. Research by Weller and co-workers has convincingly shown that strains of *P. fluorescens* producing the antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) play a key role in the suppressiveness of some soils against soil-borne pathogens, including *formae speciales* of *F. oxysporum* [114]. Moreover, a positive relationship often exists between rhizosphere colonization and antagonistic activity by 2,4-DAPG-producing *P. fluorescens* strains and the level of disease suppression, and both are modulated by the host crop genotype [26].

**4.2. Strategies to counteract lack of consistency of biocontrol performance.** Consistency and efficiency of biocontrol can be improved by repeated applications of a biocontrol agent, use of a mixture of biocontrol species or strains, and use of biocontrol agents individually or in mixture combined with organic amendments and composts, among other strategies. However, these strategies have not been investigated in depth until recently. For example, a single application of *P. oxalicum* to tomato seedlings before transplanting can suppress Fusarium wilt in tomatoes to a degree, but this suppression lasted for some 60 days only. Supplementing that single application with one to three additional ones after transplanting prolonged the duration and improved the efficiency of control of Fusarium wilt beyond that conferred by the sole initial application, especially when disease incidence was high [24, 72]. These authors attributed the biocontrol improvement to better spatial coverage and improved contact by *P. oxalicum* with plant roots that resulted in persistence of the induced resistance mechanism by the biocontrol agent that relates to renewed or prolonged cambial activity in the plant and formation of additional secondary xylem.

It would seem logical that combining the use of a number of biocontrol agents as a mixture, especially of different species, might result in treatments that could improve consistency of biocontrol of soil-borne

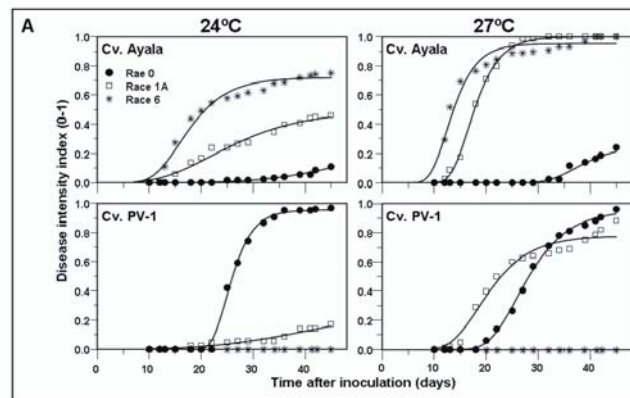
diseases through longer persistence of the microbial agent in the rhizosphere, provision of a wider array of biocontrol mechanisms, and/or functioning under a broader range of environmental conditions. However, this strategy has received little attention until the last decade [21]. Early studies by Lemanceau and co-workers demonstrated that the combinations of different *Pseudomonas* spp. and non-pathogenic *F. oxysporum* were more effective in controlling Fusarium wilts than when these antagonists were used individually [76, 77]. However, that increased effectiveness may not be necessarily straightforward since it can be influenced by compatibility among the combined microbial agents. Hervás et al. [40] found that *B. subtilis* isolate GB03, non-pathogenic *F. oxysporum* Fo90105, and *T. harzianum* KRL-AG2 effectively colonized the chickpea rhizosphere alone or in combination and significantly suppressed Fusarium wilt, but the combination of these microbial antagonists was not more effective than each of them alone. Although the combination of *B. subtilis* + *T. harzianum* effectively reduced Fusarium wilt, this combination treatment was not better than *T. harzianum* alone or *B. subtilis* alone. In contrast, the combination of *B. subtilis* + non-pathogenic *F. oxysporum* Fo90105 was less effective in reducing disease development than either antagonist alone.

The use of microbial antagonists for amending conducive or moderately suppressive organic amendments and compost is also a viable strategy for improving consistency/and or efficiency of biocontrol. Sant et al. [98] found that adding a conidia suspension of *T. asperellum* strain 34 to a carnation growth substrate based on grape marc compost (compost: peat 1:1, v/v) effectively restored natural suppressiveness of this compost against Fusarium wilt of carnation, which was lost while adapting the physical properties of the compost to carnation growth conditions. Similarly, Wu et al. [116] reported that amendment of enzymatically-hydrolyzed oil rapeseed cakes with spore suspensions of *Paenibacillus polymixa* and *T. harzianum* reduced the incidence of Fusarium wilt of watermelon by 85 to 75% under greenhouse conditions. These authors attributed this effect to induction and enhancement of systemic resistance in the plant by those microorganisms.



**Figure 2.** Resistance to Fusarium wilt in chickpeas. **A**, Screening of chickpea breeding germplasm for resistance to the disease in a field plot naturally infested with races 0, 1A, 5, and 6 of *Fusarium oxysporum* f. sp. *ciceris* (arrows indicate lines with complete or late-wilting resistance; early wilting genotypes died soon after plant emergence and have disappeared already); **B**, chickpea breeding line with complete resistance (two rows, right) to *Fusarium oxysporum* f. sp. *ciceris* race 0; left two rows are of susceptible line; **C**, chickpea breeding line CA-336-14-3-3 showing complete resistance (two rows, right) to *Fusarium oxysporum* f. sp. *ciceris* race 5 (susceptible line PV13, left, showed early wilting reaction and has disappeared already, arrow).

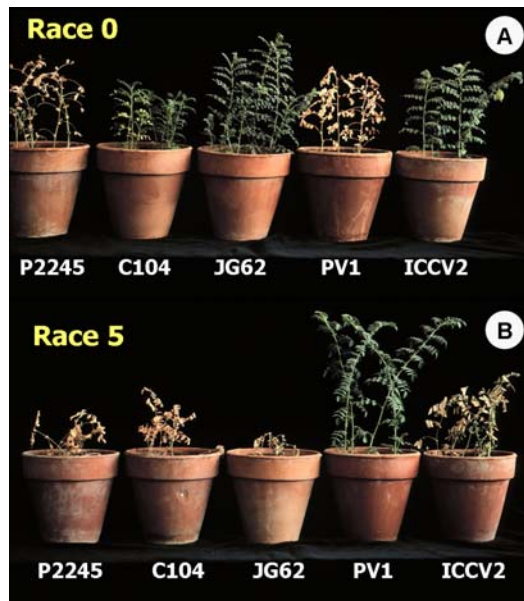
**5. Use of resistant cultivars.** The use of resistant cultivars is the most practical, cost-efficient, and environmentally safe control method for the management of Fusarium wilt diseases (Fig. 2). However, several factors that impinge upon resistance to disease can seriously limit its use and effectiveness, including genetic and pathogenic variability, and the evolutionary pattern of the pathogen, availability of resistance sources, co-infection of the plant by other pathogens, genetics and penetrance of resistance (i.e., reduced expression as a result of interaction between host genotype and inoculum load, temperature, seedling age), etc. For example, partial and complete resistance of ‘Ayala’ and ‘PV1’ chickpeas to *F. oxysporum* f. sp. *ciceris* race 1A, respectively, are fully expressed at 24°C but both cultivars become highly susceptible at 27°C (Fig. 3) [71].



**Figure 3.** Effect of incubation temperature (23°C vs 27°C) on disease reaction of chickpea cvs. Ayala and PV-1 to races 0, 1A and 6 of *Fusarium oxysporum* f. sp. *ciceris* (Landa et al., 2006)

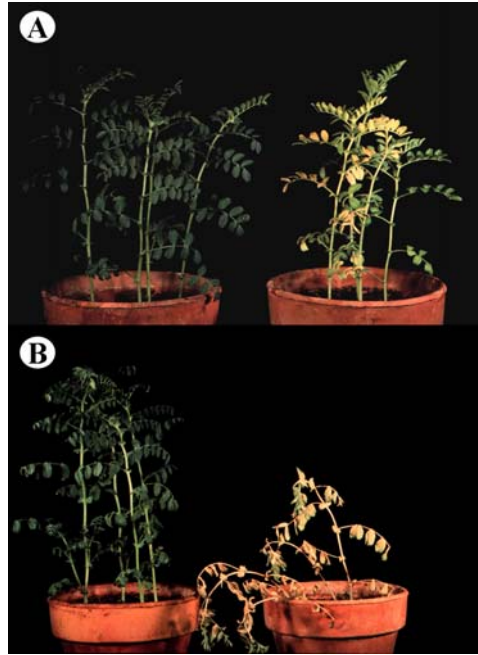
**5.1. Evolutionary patterns of *F. oxysporum* ff. spp.** Fusarium wilt pathogens are currently conceived as host-adapted populations of mitotic clonal lineages within the *F. oxysporum* species complex (i.e., *formae speciales*), in which host-specific pathogenicity evolved just once from a parasitic but non-pathogenic ancestor (i.e., monophyletic *formae speciales*), or is an evolutionary convergent trait that evolved multiple

times independently (i.e., polyphyletic *formae speciales*) [94, 96]. Most *F. oxysporum* ff. spp. are polyphyletic (e.g., ff. spp. *asparagi*, *cubense*, *cucumerinum*, *dianthi*, *lactucae*, *lini*, *lycopersici*, *melonis*, *phaseoli*, *vasinfectum*, etc.) [6, 94] and a few of them are monophyletic (e.g., ff. spp. *albedinis*, *ciceris*, *conglutinans*) [6, 48, 65]. That a given *formae speciales* is polyphyletic has important implications for the development and deployment of resistant genotypes of the host plant. Thus, each clonal lineage might be expected to have unique pathogenic properties and the underlying genes for resistance in the host and virulence in the pathogen may differ in different areas [96]. Actually, that a pathogen lineage may have different evolutionary histories in different geographic areas make uncertain that resistance developed against local populations in an area remains effective when used in areas evolutionary different.



**Figure 4.** Reaction of five chickpea race (Leith to right: PV 13, C 104, JG 62, PV 1, and ICCV 2) to inoculation with races 0 (**A**) and 5 (**B**) of *Fusarium oxysporum* f. sp. *ciceris*. Note the yellowing syndrome caused by race 0 on 'PV 13' and 'PV 1', and complete resistance in 'JG 62' (**A**); and flaccidity and plant death caused by race 5 on all lines, except 'PV-1' that shows complete resistance (**B**).

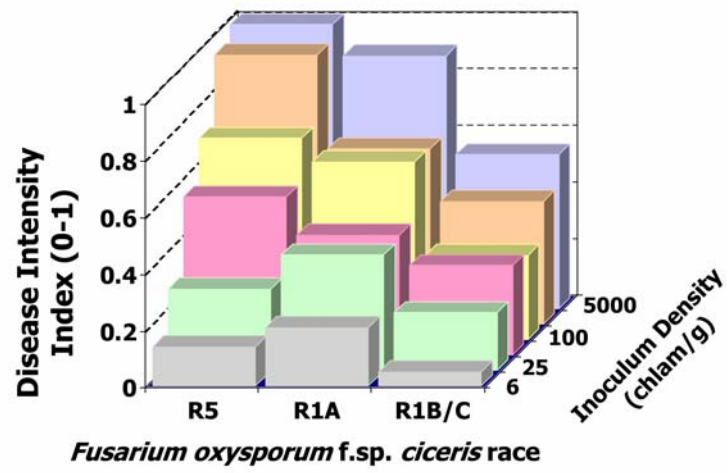
**5.2. Genetic and pathogenic variability.** Whether monophyletic or polyphyletic, *F. oxysporum* ff. spp. also exhibit a wide pathogenic variation within their populations, which are referred to as pathogenic races and pathotypes or symptoms types. For example, three races have been described in *F. oxysporum* f. sp. *lycopersici*, four in ff. spp. *cubense*, *melonis* and *pisi*, five in f. sp. *fabae*, seven in f. sp. *phaseoli*, eight in ff. spp. *vasinfectum* and *ciceris*, etc. Pathogenic races are identified by the disease reactions on a set of differential host cultivars, or even species (e.g., ff. spp. *cubense* and *vasinfectum*) (Fig. 4). Race differentiation may be an imperfect measure of pathogenic diversity within populations of polyphyletic *formae speciales*, and both its use and designation has been questioned in some cases [22, 23]; however, it still provides crucial information for disease management [96].



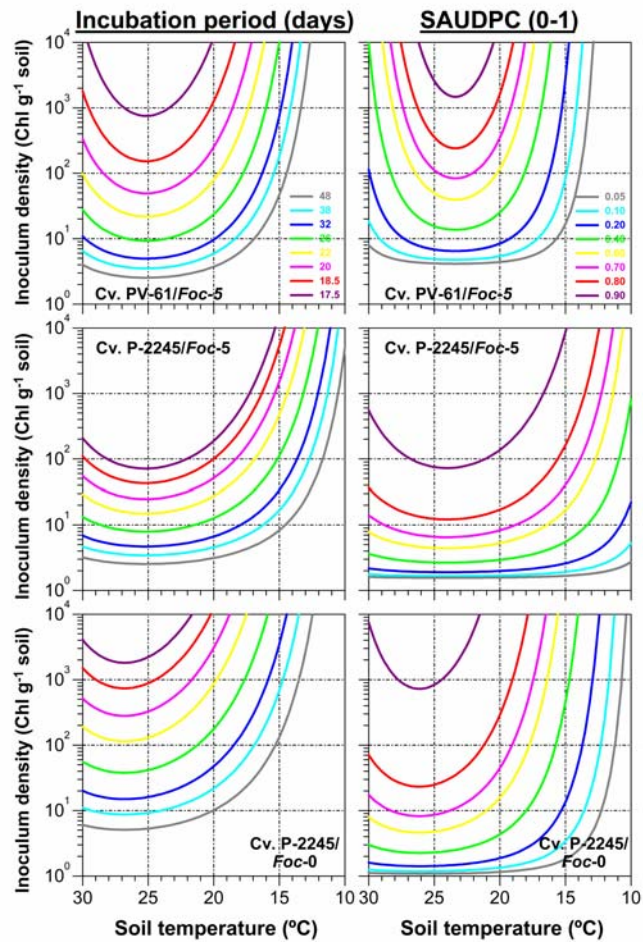
**Figure 5.** Disease syndromes caused by *Fusarium oxysporum* f. sp. *ciceris* pathotypes in chickpeas grown in artificially-infested soil. **A.** Yellowing caused by race 0 (yellowing pathotype) on ‘P-2245’ plants after 24 days incubation at 25°C; **B.** Wilting caused by race 5 (wilting pathotype) on PV-60’ plants after 24 days incubation at 25°C.



In *F. oxysporum* f. sp. *ciceris*, two pathotypes have been distinguished besides the eight pathogenic races (races 0, 1A, 1B/C, and 2 through 6), based on the distinct yellowing (slow, progressive and acropetal foliar yellowing) or wilting (fast, severe chlorosis and flaccidity) syndromes with brown vascular discoloration that pathotype isolates cause in susceptible chickpeas (Fig. 5). The eight *F. oxysporum* f. sp. *ciceris* races differ in their geographic distribution, pathotype, virulence (herein defined as a quantitative measure of pathogenicity of a strain indicated by the intensity of symptoms caused on individual hosts or host genotypes) and temperature range, all of which have an influence on management of Fusarium wilt in chickpeas. Races 0 and 1B/C belong to the yellowing pathotype whereas races 1A through 6 belong to the wilting pathotype. Races 2, 3, and 4 have only been reported in India, whereas races 0, 1B/C, 5, and 6 are found mainly in the Mediterranean region and the USA (California) [50]. Unlike the other races, race 1A is more widespread and has been reported in India, California, and the Mediterranean region. Regarding differences in virulence, 5,000 chlamyospores  $g^{-1}$  of soil of race 1B/C (yellowing pathotype) cause same amount of disease in 'PV 61' chickpeas that 1,000 chlamyospores  $g^{-1}$  of soil of race 1A (wilting pathotype), and the amount of disease developed with 5,000 chlamyospores  $g^{-1}$  of soil of race 1A is equal to that developed with 1,000 chlamyospores  $g^{-1}$  soil of race 5 (wilting pathotype) (Fig. 6). Thus, it appears that collectively, the yellowing *F. oxysporum* f. sp. *ciceris* pathotype is less virulent than the wilting one, but differences in virulence to a chickpea cultivar may also occur between races within a *F. oxysporum* f. sp. *ciceris* pathotype. Quantitative nonlinear models of infection by races 0 and 5 in chickpea cultivars differing in susceptibility indicated a temperature x race virulence (or cultivar susceptibility) interaction in chickpea wilt. Moreover, the models estimated 22 to 26°C as the most favorable soil temperature for infection of cvs. P-2245 (most susceptible) and PV-61 (less susceptible) by race 5, and 24 to 28°C for infection of 'P-2245' by race 0. At 10°C, no disease developed except in the most compatible interaction 'P-2245'/race 5. At optimum soil temperature, maximum disease in 'P-2245' developed with 6 and 50 chlamyospores  $g^{-1}$  soil of races 5 and 0, respectively; and in 'PV-61' with 1,000 chlamyospores  $g^{-1}$  soil of race 5. Risk threshold charts indicated that limitation in disease by a deficient factor is compensated by another factor (Fig. 7). These charts can be applied to predict the potential threat of Fusarium wilt in a geographic area based on soil temperature, the race and inoculum density in soil, and susceptibility of cultivars [90].



**Figure 6.** *Fusarium* wilt disease intensity in chickpea cv. PV-61 grown for 48 days at 25°C in soil infested with different inoculum densities of *Fusarium oxysporum* f. sp. *ciceris* races 1A, 1B/C, and 5.



**Figure 7.** Charts for risk prediction for Fusarium wilt disease progress curve elements (reciprocal of incubation period, and standardized area under disease intensity index progress curve [SAUDPC]) in chickpea cvs. P-2245 and PV-61 grown in soil artificially infested with *Fusarium oxysporum* f. sp. *ciceris* races 0 (*Foc-0*) and 5 (*Foc-5*) based on inoculum density and soil temperature (Navas-Cortés et al., 2007).

**5.3. Evolution of new pathogen strains and the breakdown of resistance.** Development of new, more virulent strains of the pathogen can give rise to resistance-breakdown and thus threatens the efficient use of resistant cultivars in the management of Fusarium wilts as exemplified by *F. oxysporum* f. sp. *lycopersici* on tomatoes and *F. oxysporum* f. sp. *pisi* on peas [9, 67]. Spontaneous mutations appear to occur frequently in *F. oxysporum* and successive accumulation of those that result in increased virulence can be favoured by repeated interaction with susceptible or resistant cultivars. For example, *F. oxysporum* f. sp. *vasinfectum* in Australia appears to have evolved from a local lineage of native *F. oxysporum* associated with wild cotton (*Gossypium* spp.) and mildly virulent to *Gossypium hirsutum* [22] since continuous interactions of wild cotton-associated *F. oxysporum* with the cultivated species led to a gradual increase of virulence to susceptible *G. hirsutum* [112]. Conversely, in northwest WA and southwest British Columbia, growing *F. oxysporum* f. sp. *pisi* race 1-resistant peas in monoculture in the presence of races 1 and 2 led to great economy losses and the development of races 5 and 6 [67]. New races of *F. oxysporum* ff. spp. can also develop and become established in infested soils cropped to susceptible cultivars of the host. In southern Spain, repeated cropping of susceptible chickpeas in a field plot infested with low virulent *F. oxysporum* f. sp. *ciceris* race 0 resulted in the development of more virulent races 1A, 5, and 6; and a similar development of races 0 and 6 occurred in another plot without history of chickpeas that had been artificially infested with race 5 and subsequently grown in monoculture to susceptible chickpeas [Jiménez-Díaz and Jiménez-Gasco, unpublished].

New pathogenic races of *F. oxysporum* ff. spp. can evolve from local non-pathogenic *F. oxysporum* isolates, such as it appeared to have occurred with race 1 of *F. oxysporum* f. sp. *melonis* in Maryland [4], as well as from previous existing races [e.g., race 3 of *F. oxysporum* f. sp. *lycopersici* from race 2 in California, 16]. In *F. oxysporum* f. sp. *ciceris*, an intraspecific phylogeny of races was inferred whereby each of the eight races forms a monophyletic lineage. Also, a simple stepwise pattern of evolution of the races was demonstrated whereby virulence has been acquired according to two simplest scenarios of few parallel gains or losses [49]. The scenario based on the gains, but not loss of virulence is consistent with the yellowing race 0 being ancestral to wilting races and race 1B/C being its closest race in evolutionary terms. This inferred scenario would be consistent with race 0: (i) being pathogenic on the fewest race-differentials of all races; (ii) being the most widespread race in the Mediterranean region, although it has not been reported from the

Indian subcontinent, and (iii) showing the highest molecular diversity of all races. A second scenario of race evolution proposes race 1A as the common ancestor of all races, which would be consistent with this race being the most widespread geographically. The stepwise evolution of races in *F. oxysporum* f. sp. *ciceris* suggests that it would take longer for the pathogen to overcome two resistance genes and provides support for pyramiding resistance genes as a strategy for resistance breeding, being thus of importance for the development and use of resistant cultivar in the management of Fusarium wilt of chickpea.

The potential for development of new strains of the pathogen may not give rise to resistance-breaking necessarily. In spite of the race-specific nature of complete resistance to *F. oxysporum* f. sp. *ciceris* in chickpea, there is no evidence to date of resistance breakdown suggesting that there may be little or no selection for resistance-breaking races in Fusarium wilt of chickpea. For example, a high diversity of *F. oxysporum* f. sp. *ciceris* races exists in the Mediterranean Basin where resistant cultivars generally have not yet been used. Conversely, widespread use of race 1A-resistant cultivars in India has not led yet to reports of race 6, which specifically overcomes that resistance and derives from race 1A. Rather, races 2, 3, and 4, which are virulent to race 1A-resistant cultivars, were reported in India long before the release of these cultivars.

**5.4. Interaction with plant-parasitic nematodes.** Co-infection of the host plant by some plant-parasitic nematodes can seriously limit valuable race-specific resistance to the interacting fungus and increase Fusarium wilt severity in susceptible cultivars [18, 19, 27, 80]. Although Fusarium wilt alone is a serious disease of cotton, disease incidence and severity is often greater in the presence of the root-knot nematode *Meloidogyne incognita* and the nature of the interaction between the fungus and the nematode is influenced by the population levels of the two pathogens [23, 27]. The leading role of *M. incognita* in the disease complex is indicated by the fact that nematode resistance was more effective than wilt resistance in suppressing wilt symptoms when either resistance was present alone, and nematode resistance combined with intermediate wilt resistance was highly effective in protecting plants from root-knot nematodes and *F. oxysporum* f. sp. *vasinfectum* race 1 [27, 111].

Infection of chickpea by root-knot nematodes *Meloidogyne artiellia*, *M. incognita* or *Meloidogyne javanica* can breakdown resistance to *F. oxysporum* f. sp. *ciceris*, but such effect may be strongly influenced by factors in the pathosystems [19]. For instance, co-infection of the plant by *F. oxysporum* f. sp. *ciceris* race 5 and the cereal and legume root-knot

nematode *M. artiellia* significantly increased wilt severity in chickpea genotypes with partial resistance to Fusarium wilt regardless of the ID density of the fungus and the geographic origin of the nematode populations, except for cv. CPS 1 in which the increase in Fusarium wilt severity occurred only with the highest ID of *F. oxysporum* f. sp. *ciceris* race 5 [18]. Also, and more important, co-infection by the two pathogens overcame complete resistance to race 5 in some chickpea genotypes (e.g., lines CA 334.20.4, CA 336.14.3.0, and UC 27), but not in others (line ICC 14216 K). Such resistance breakdown occurred irrespective of the initial ID of the fungus and the geographic origin of the nematode (e.g., lines CA 334.20.4, and CA 336.14.3.0), or required a high ID of the fungal pathogen (e.g., cv. UC 27) [18]. Interestingly, this genotype-specific resistance breakdown by *M. artiellia* infection did not occur for other pathogenic races of *F. oxysporum* f. sp. *ciceris*, such as races 0, 1A, and 2. Thus, infection by the nematode does not compromise complete resistance of 'UC 27' and 'ICC 14216 K' chickpeas to *F. oxysporum* f. sp. *ciceris* race 0 and of 'ICC 14216 K' chickpeas to races 1A and 2 [91]. Contrary to above, co-infection of chickpeas by *Pratylenchus thornei* and *F. oxysporum* f. sp. *ciceris* race 5 did not modify Fusarium wilt disease reaction in chickpea genotypes susceptible or resistant to the fungus. However, root infection by the nematode significantly increased the numbers of *F. oxysporum* f. sp. *ciceris* propagules in roots of wilt-susceptible 'CPS 1' and wilt-resistant 'UC 27' at low and high ID of the nematode, respectively [17].

**5.5. Genetics of resistance.** Resistance to pathogenic races of *F. oxysporum* ff. spp. can be monogenic or oligogenic and polygenic, and of complete or partial (intermediate) phenotype. In Fusarium wilt of tomato, monogenic resistance derived from accessions of *Lycopersicon pimpinellifolium* is of complete phenotype against *F. oxysporum* f. sp. *lycopersici* races 1 and 2, and intermediate for race 3 (although it appears that the accession source can have an influence on the level of resistance conferred by *I* gene against race 1) [9]. A comparable situation seems to occur with the resistance of 'WR-315' chickpea against race 3 of *F. oxysporum* f. sp. *ciceris*, which varied from complete to partial depending upon the accession source [45].

Success in the development of resistance can be limited by the underlying genetic system and/or resistance sources. For instance, the use of host resistance to manage Fusarium wilt of cotton has been moderately successful. Resistance in Egyptian cottons (*G. barbadense*) is controlled by two dominant genes and seems to be more complete than that in

Upland cottons (*G. hirsutum*) where it is quantitatively inherited and controlled by several major genes with minor modifying genes. Although commercial Upland cotton cultivars with moderate to high levels of wilt resistance have been produced, none of them carry complete resistance and their yield and fiber quality are lower than those of wilt-susceptible cultivars [23]. Comparatively, good progress has been made in the identification of sources of resistance and development of Fusarium wilt-resistant, high-yielding chickpea cultivars. Resistance to *F. oxysporum* f. sp. *ciceris* races was identified mainly in 'desi' germplasm (small, angular, colored seeds, grown mainly in the Indian subcontinent) as well as in wild *Cicer* spp. (Fig. 2A). Resistance against races 0 and 5 was identified in entries of *C. bijugum*, *C. cuneatum*, *C. judaicum*, but their use as sources of resistance is curtailed by crossability with cultivated *C. arietinum*. Valuable resistance has also been identified in 'kabuli' germplasm (rams-head-shaped and beige- to white-colored seeds, grown mainly in the Mediterranean Basin) of medium to large seed size. In Spain, 'kabuli' germplasm lines were identified with resistance against specific races (e.g., lines CA-334.20.4, CA-336.14.3.0, and ICC-14216K resistant to race 5). At ICRISAT, race 1A-resistant 'kabuli' chickpeas 'ICCV-2' through 'ICCV-6' were developed from complex crosses involving different resistant 'desi' parents. In Mexico, Fusarium wilt resistance from 'desi' line L 1186 was introgressed into 'kabuli' cvs. Macarena and Breve Blanco to develop resistant 'Surutato-77' and 'Sonora-80' chickpeas; later, cvs. UC 15 and UC 27 were developed at UC Davis using Sonora-80 as resistant parent. Further testing at Spain showed that cvs. UC 15 and UC 27 are resistant to races 0, 1A, and 3 through 6 [45].

The genetics of resistance to individual *F. oxysporum* f. sp. *ciceris* races has not been fully clarified yet. Whereas resistance against most of the pathogen races is either monogenic or oligogenic and of complete phenotype, the genetics of resistance to races 1B/C and 6 is yet to be determined. Resistance to races 0, 1A, and 2 was reported to be either digenic or trigenic, but recent studies using 'WR 315' chickpeas suggest that single genes might govern resistance to races 1A, 2, 3, 4 and 5. In addition to that, a late wilting phenotype characterized by a delay in the onset of disease symptoms in partially resistant genotypes was found to be a monogenic trait and controlled by three independent genes, each of which delays that onset. Furthermore, a combination of any of the two late-wilting genes is required for complete resistance to race 1. The complete-resistant phenotype, such as that of 'WR-315' to races 0 and 5 and 'JG-62' to race 0, characterizes by confinement of the pathogen

within the cortical tissues of roots and lower stem without development of localized cell and inability to colonize the vascular tissues. While genotypes with complete resistance show no disease under natural infections in the field, late-wilting genotypes may develop disease few weeks before harvesting but show low incidence or no disease at flowering time (Fig 2B,C) [45].

**6. Choice of cropping practices to avoid conditions favouring infection of the plant.** Environmental factors such as temperature, nutrients and soil pH can significantly influence development of Fusarium wilt diseases and proper choice of cultural practices to take advantage from such influence can contribute to management of Fusarium wilts.

**6.1. Choice of sowing date.** For most Fusarium wilts, except Fusarium wilt of melon, optimum disease development occurs at temperature within the range of 25 to 28°C [55, 69, 90, 92, 101]. On the contrary Fusarium wilt of musk melon develops most severely at 18 to 22°C [82]. Therefore, adjusting the sowing date to escape from the optimum temperature range during crop growth should contribute to reduce disease development. Delaying lettuce planting in south-western Arizona (USA) from September to December reduced the incidence of Fusarium wilt from 92.3 to 2.0% in one year and from 74.2 to 0.7% in another [83]. That delay was associated with a reduction in the mean soil temperature at the 10-cm depth from 26 to 14°C in September and December for both years, respectively.

In Mediterranean environments, advancing the chickpea sowing date from early spring to early winter contributes to control of Fusarium wilt by significantly delaying epidemic onset, slowing down epidemic development, and reducing the final amount of disease [70, 88]. These effects result from a lowering of soil and air temperatures during the early growth stages of the crop. Moreover, the delay in epidemic onset decreases yield loss in a linear relationship while yield loss due to the disease is increased exponentially with the rate of disease progression favoured by the delay of sowing [89]. However, these benefits can be overridden if the cultivar sown is highly susceptible to wilt, a highly virulent race of *F. oxysporum* f. sp. *ciceris* prevails in soil, or both. This emphasizes the usefulness of chickpea cultivars with a late-wilting disease reaction. Efficiency in the combined use of late-wilting chickpeas and choice of sowing date for the management of Fusarium wilt in chickpeas can be reinforced if integrated with seed and soil treatment with biological control strains, such as *B. subtilis* GB03 and *P. fluorescens* RG 26, applied either



alone or each in combination with non-pathogenic *F. oxysporum* Fo90105 [70].

**6.2. Altering soil nutrients and pH.** Limiting the availability of micronutrients for which *F. oxysporum* f. sp. *lycopersici* has relatively high requirement for growth and sporulation (such as Cu, Fe, Mn, Mo, and Zn), by liming to pH 7.0 to 7.5, consistently reduced Fusarium wilt in tomato grown in low-pH soil [55]. This effect was due to increased pH and not to increase of Ca content in plants since soil amendment with  $\text{SO}_4\text{Ca}$  neither increased soil pH nor reduced the amount of disease compared with liming, although the two amendments gave rise to similar content of Ca in plant tissues. Suppression of the disease was also due to reduced availability of P and Mg in high-pH soil since high-superphosphate amendments resulted in increased diseases at soil pH 6 but not at pH 7 [55]. Raising soil pH by use of nitrate N or hydrated lime ( $\text{CaOH}_2$ ) also reduced Fusarium wilt of chrysanthemum caused by *F. oxysporum* f. sp. *chrysanthemi* and *F. oxysporum* f. sp. *tracheiphilum*, and use of both together have an additive effect in suppressing the disease [92].

## Conclusions and future prospects

The integrated management of Fusarium wilt diseases is a difficult task because complexities of target pathosystems are overlaid on the inherent complexities of the management strategy itself. Much research is still needed on population biology and genetic diversity in Fusarium wilt pathogens, as well as on disease risk prediction, disease-incidence-yield losses relationships, biological control, biotechnological breeding for disease resistance, etc., which should be carried out under a system approach. Actually, plant pathologists have not yet reached a level of efficiency on the integrated management of plant diseases comparable to that achieved by agricultural entomologists in the integrated management of agricultural pests. Zadoks [117] attributed that lack of success both to particular complexities of agricultural pathosystems as well as to an insufficient scientific and technological knowledge about them and the measures for their control. Recently, Pinstrup-Andersen [95] emphasized that widespread application of IPM *sensu lato* is still curtailed by insufficient knowledge on target diseases and pests and that, consequently, recommendations for its application based on generalizations should be considered with caution.

The integrated management is a skilful solution for the plant diseases problems faced by current agricultural production. However, on top of difficulties pointed out above, putting it into practice requires involvement of well-trained professional plant pathologists able to implement the tenets of the integrated management concept at the local level, as well as to incorporate into decision-making framework new knowledge and technologies that may be developed from scientific research. As the demand has increased for knowledgeable practitioners capable of integrating multifaceted controls in rigorous IDM programs, institutional support has declined through declining or even disappearing University education in Plant Pathology and the loss of extension-related activities in commercial agriculture. Erosion at the top of the trickle-down structure responsible for knowledge transfer to the field is one of the most serious threats to IDM. Lack of appropriate and specific training in Plant Pathology: (i) seriously limits proper communications among those that at different levels may be involved in strategic actions concerning IDM programmes; (ii) make more difficult transferring new knowledge and technologies derived from research; and more importantly (iii) limits an adequate social perception of the true nature and magnitude of plant diseases as a threat to food production.

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