Spanish Journal of Agricultural Research
13(4), e1009, 13 pages (2015)
eISSN: 2171-9292
http://dx.doi.org/10.5424/sjar/2015134-7905
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)

RESEARCH ARTICLE

OPEN ACCESS

Organic amendments conditions on the control of Fusarium crown and root rot of asparagus caused by three *Fusarium* spp.

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Abstract

Fusarium oxysporum (*Fo*), *F. proliferatum* (*Fp*) and *F. solani* (*Fs*) are causal agents associated with roots of asparagus affected by crown and root rot, a disease inflicting serious losses worldwide. The propagule viability of *Fusarium* spp. was determined on substrate artificially infested with *Fo*5, *Fp*3 or *Fs*2 isolates, amended with either poultry manure (PM), its pellet (PPM), or olive residue compost (ORC) and, thereafter, incubated at 30 or 35°C for different periods. Inoculum viability was significantly affected by these organic amendments (OAs) in combination with temperature and incubation period. The greatest reduction in viability of *Fo*5 and *Fs*2 occurred with PPM and loss of viability achieved was higher at 35°C than at 30ºC, and longer incubation period (45 days). However, the viability of *Fp*3 did not decrease greatly in most of the treatments, as compared to the infested and un-amended control, when incubated at 30ºC. After incubation, seedlings of asparagus `Grande´ were transplanted into pots containing substrates infested with the different species of *Fusarium*. After three months in greenhouse, symptoms severity in roots showed highly significant decreases, but *Fp*3 caused lower severity than *Fo*5 and *Fs*2. Severity reduction was particularly high at 30ºC (by 15 days incubation for *Fs*2 and by 30-45 days for *Fo*5), after PPM treatment, as well as PM-2% for *Fo*5 and *Fs*2 incubated during 30 and 45 days at both temperatures, and with ORC (15-30 days incubation). Moreover, assessment of plants fresh weight showed significantly high increases in *Fo*5 and *Fs2*, with some rates of the three OAs tested, depending on incubation period and temperature.

Additional key words: biofumigation; *Fusarium oxysporum* f. sp. *asparagi*; *Fusarium proliferatum*; *Fusarium solani*; olive residue compost; pelleted poultry manure; poultry manure.

Abbreviations used: CFU (colony-forming units); FCRR (Fusarium crown and root rot); Foa (*Fusarium oxysporum* f. sp. *asparagi*); Fp (*Fusarium proliferatum*); Fs (*Fusarium solani*); OAs (organic amendments); ORC (olive residue compost); PDA (potato dextrose agar); PDB (potato dextrose broth); PM (poultry manure); PPM (pellet of poultry manure).

Citation: Borrego-Benjumea, A. I.; Melero-Vara, J. M.; Basallote Ureba, M. J. (2015). Organic amendments conditions on the control of Fusarium crown and root rot of asparagus caused by three *Fusarium* spp. Spanish Journal of Agricultural Research, Volume 13, Issue 4, e1009, 13 pages. http://dx.doi.org/10.5424/sjar/2015134-7905.

Received: 24 Apr 2015. **Accepted:** 13 Nov 2015

Funding: This research was funded by a JAEPredoc-CSIC grant to AIBB; Ministerio de Agricultura, Alimentación y Medio Ambiente, Spain (project INIA RTA 2006-00045); and Consejería de Economía, Innovación y Ciencia, Junta de Andalucía, Spain (Project P06-AGR-02313).

Competing interests: The authors have declared that no competing interests exist.

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Introduction

Fusarium crown and root rot (FCRR), probably the most important disease of asparagus in the world (Schreuder *et al.*, 1995; Elmer *et al.*, 1996; Blok & Bollen, 1997), has a complex etiology, with several *Fusarium* spp. associated with the roots of asparagus, involved as causal agents of the disease (Blok & Bollen, 1995; Elmer, 2001).

The most important species in Spain are: *Fusarium oxysporum* f. sp. *asparagi* (*Foa*), *F. proliferatum* (*Fp*), *F. solani* (*Fs*) and *F. verticillioides* (syn. *F. moniliforme*) (Tello *et al.,* 1985; Seifert *et al*., 2003; Corpas-Hervias *et al.*, 2006; Wang & Jeffries, 2006). The FCRR of asparagus most frequently affects adult plants, but also devastates seedlings and young plantations. The distribution and importance of these *Fusaria* depend on the geographic area, but the symptoms caused are similar. These include vascular staining and rotting of roots, rhizomes and stems; necrotic lesions on the surface of the root and stem; reduced plant size; yellowing and senescence of stems and crowns and, in the

most severe cases, death of the plant (Johnston *et al.*, 1979; Elmer, 2001). Consequently, crop yield declines slowly, because of the lower production of individual diseased plants and by the decrease in plant density due to the death of the most affected ones.

This disease is difficult to control due to, among other factors, the long-term survival of the pathogen in the soil and its easy propagation by planting material (Elmer, 2001; Corpas-Hervias *et al.*, 2006). Furthermore, chemical treatments are frequently ineffective or provide short-term protection (Elmer, 2001). Moreover, the use of methyl bromide, the most effective fumigant for the suppression of soil pathogens, is no longer allowed, because its destructive effect on the ozone layer in the stratosphere that protects life on Earth from harmful radiations (Gamliel *et al.*, 2000; Katan, 2000; Basallote-Ureba *et al.*, 2010).

Soil solarization is a non-chemical method of soil disinfestation that increases the soil temperature by retaining the energy of solar radiation using transparent polyethylene sheets, under suitable climatic conditions, to reduce pathogen populations and disease incidence. The multiple mechanisms involved determine the thermal inactivation of the pathogen due to increased soil temperature (Katan *et al.*, 1976), or the weakening of pathogen propagules that become more susceptible to competition or antagonistic activity of the indigenous soil microflora (Stapleton, 2000). Soil solarization, either alone or combined with organic amendments, and soil flooding, are effective in controlling many soilborne plant pathogens (Katan, 1981; Blok *et al.*, 2000; Klein *et al.*, 2011; Melero-Vara *et al.*, 2011). In addition, soil solarization frequently enhances plant growth by improving soil structure, releasing nutrients (Chen *et al.*, 1991) and stimulating plant growth promoting rhizobacteria (Gamliel & Stapleton, 1993).

Soil organic amendments (OAs) including animal manures, such as poultry manure (PM), and pellet of PM (PPM), composts (*e.g.*, olive residue compost, ORC) and green manures, may suppress or reduce many soilborne plant pathogens (Gamliel & Stapleton, 1993; Abbasi *et al*., 2002, 2008; Noble & Coventry, 2005; Bonanomi *et al.*, 2007; García-Ruíz *et al.*, 2009; Avilés *et al.,* 2011; Borrego-Benjumea *et al.*, 2014a,b). Organic amendments affect aeration and soil structure, drainage, water holding capacity, nutrient availability and microbial ecology of soil (Davey, 1996). The suppression of pathogens by incorporation of OAs in the soil depends on several mechanisms. A factor involved in the survival of the pathogen is the production of toxic compounds, such as volatile fatty acids (acetic, propionic and isobutyric acids) during the microbial degradation of manure. The mechanism of suppression of microsclerotia and other fungal propagules arisen by nitrogen-rich amendments, such as PM or its pelletized form, is due to the production of ammonia and nitrous acid following degradation of the amendments by microorganisms (Tenuta & Lazarovits, 2002). Another possible mechanism of action of the OAs, such as composts, is the decrease in disease severity due to significant increases of microbial populations present in the amendment (Tsao & Oster, 1981; Conn & Lazarovits, 1999). Thus, the use of compost on asparagus fields enhanced the soil microbial biomass, as well as crop yield and spear number (Ngouajio & Counts, 2012). This fact leads to greater competition for nutrients in the soil and ecological niches, increasing the effect of antagonistic microorganisms, antibiosis, microbial production of lytic enzymes, fatty acid degradation, parasitism, changes in nutrient availability and induction of host resistance (Tsao & Oster, 1981; Hoitink & Boehm, 1999; Borrero *et al.,* 2006; Avilés *et al.*, 2011).

Other studies involving biochar, a C-rich mineral product of biomass pyrolysis, showed reduction of FCRR of asparagus following biochar applications at 10 to 30% (v/v) in *Foa* infested soil (Matsubara *et al*., 2002) and at 1.5 and 3.0% (w/w) in infested soil by *Foa* and *Fp* (Elmer & Pignatello, 2011), associated with root colonization by arbuscular mycorrhizae, though the reduction was more effective with the higher rate of amendment.

A new method of biological soil disinfestation was also proposed to control soilborne pathogens, which combine the addition of OAs to irrigation and air-tight plastic tarping leading to soil anaerobiosis. The amendment of soil with fresh broccoli or grass (3.4 to 4 kg m^2) followed by tarping for 15 weeks was an effective method against a wide range of soilborne pathogens, including *Foa*, where toxic volatile products, including isothiocyanates formed by the hydrolysis of glucosinolates achieved by the enzyme myrosinase, both occurring in cruciferous plants, and confined under the tarp, contributed to the inactivation of fungal propagules, whereas precluding thermal inactivation (Blok *et al.*, 2000). Analogous results were found with *F. oxysporum* f. sp. *lycopersici* (*Fol*) after addition of a number of plant biomass sources such as *Brassica* spp., wheat bran, rice straw, rice bran, or other organic substances such as molasses (1 to 2 kg/m^2 of organic material), followed by irrigation and tarping for three weeks to induce reducing soil conditions (Shinmura, 2004).

This work has been conducted in the framework of organic farming using different by-products from the local agricultural industry (*i.e*., poultry manure, pelleted poultry manure and olive residue compost) as organic amendments, thus promoting more sustainable

agriculture by reusing them, to aim disease control for the pathosystem *Fusarium* pp./asparagus. The objectives were to determine the efficacy of those organic amendments combined with two temperatures and several incubation periods, on populations of *Fo*, *Fp* and *Fs* from Spain as well as the impact of these combined treatments on the FCRR symptoms development and on the fresh weight of asparagus plants. This amplifies the results already obtained with Canadian isolates of *Fo* and *Fs* (Borrego-Benjumea *et al.*, 2014a) with Spanish isolates including one of *F. proliferatum*, the amendment with poultry manure and incubation simulating the conditions in which soil solarization is carried out in southern Spain (periods extended up to 30-45 days, at 30 and 35ºC).

Materials and methods

Fungal isolates

Three monoconidial isolates of *Fusarium* from our collection and maintained in sterile soil at 4ºC, *i.e*. *F. oxysporum* (*Fo*5), *F. proliferatum* (*Fp*3) and *F. solani* (*Fs*2), which had proven pathogenic to asparagus (Corpas-Hervias *et al.*, 2006) were selected. Inocula of these isolates were obtained by incubation of flasks with sterile potato-dextrose broth (PDB) to which four actively growing mycelium disks on potato dextrose agar (PDA) plates per flask were added. Incubation of PDB cultures was for 7 days on an orbital shaker at 150 rpm continuously, at 25ºC and a 12 h photoperiod. Four-layer sterile gauze was used to filter the conidial suspensions recovered from the flasks, and then their concentrations were estimated using a hematocytometer.

Infestation of substrate

Sand:silt mixture (2:1, v/v) was used as substrate in pot experiments conducted in greenhouse. This substrate was autoclaved at 121ºC for 60 min, twice in consecutive days. Afterwards, a volume of each conidial suspension, calculated according to the concentration, was poured in each bag of sterile substrate in order to reach 10^5 microconidia/g, and vigorously shaken under aseptic conditions trying to homogenize the distribution in the substrate. Sterile distilled water was added instead in the un-infested controls. Substrate bags were then incubated at 25ºC for 30 days in the dark, with aseptic aeration every other day in a laminar air flow chamber at the time that the content was homogenized again. Chlamydospore formation was confirmed in all infested bags after the incubation period.

Organic amendments

Three organic amendments obtained as by-products of different agricultural activities, *i.e*., PM, PPM and ORC were tested in this study. Several physicochemical features of those are given in Table 1. Standard techniques were used to measure pH in de-ionized water.

Effect of organic amendments, incubation period and temperature on the viability of propagules of *Fusarium* spp. pathogenic to asparagus

The three isolates of *Fusarium* spp. with two incubation temperatures (30 and 35ºC) during three periods (15, 30 and 45 days), and the three OAs above mentioned, were included in this experiment. After the batches of substrate separately infested with the three isolates were incubated during 30 days, 45 g-aliquots were placed in sterile polypropylene 50-mL test tubes (25 mm diameter) and PM or PPM at rates 1 and 2% (w/w) and ORC at 3 and 6% rates were added separately and homogeneously mixed with the differently infested substrate, then closed with plastic plugs and incubated in the dark at 30 or 35ºC for 15, 30 and 45 days. There were nine replications for every combination of treatments. The rates of OAs used were assessed and selected in a previous study by its effectiveness in controlling *Fusarium oxysporum* f. sp. *dianthi* in carnation (Melero-Vara *et al.,* 2011).

Table 1. Nitrogen, phosphorous and potassium contents, pH, electric conductivity (EC) and C/N ratio of the organic amendments used in this study

Organic amendments	Nitrogen (%)	Phosphorous (%)	Potassium (%)	pH	EC (mS/cm)	C/N ratio
Poultry manure	2.4	3.4	1.9	7.5	2.9	10-11
Pellet of poultry manure	4.0	1.0	2.0	6.3	11.0	6-7.5
Olive residue compost	1.5	0.7	2.0	8.7	3.2-3.5	18-22

For determination of the inocula viability in the substrate, samples (1 g) were taken just after the application of OAs (time 0) as well as after incubation periods of 15, 30 and 45 days, then kept at 4-6ºC until lab processing. Plate dilution method was used with a semi-selective agar medium for *Fusarium* in order to determine inoculum viability (Bouhot & Rouxel, 1971). Original suspensions were obtained by magnetic stirring for 1 min of the substrate samples in flasks with 150 mL sterile water-agar (0.1%), then serial dilutions 10^{-1} and 10^{-2} were made, and three aliquots of 1 mL of each of them were transferred onto Petri plates with that medium (as replications). After incubation at 25ºC for 2 days in the dark followed by three additional days with a 12-h photoperiod, the colony-forming units (CFU) were enumerated in all plates. Inoculum viability was then expressed as log-transformed data of the CFU/g substrate, and percentage reductions of viability were calculated by the quotients between the log-values of CFU for treated and un-treated samples.

Effect of organic amendments, incubation period and temperature on disease development

One bioassay was conducted in a greenhouse using the infested and amended substrate in which seedlings of asparagus `Grande´, moderately susceptible to *Fusarium* spp. were grown (Corpas-Hervias *et al.*, 2006).

Asparagus seeds were surface-disinfested by dipping them for 3 min in aqueous solution of Na-hypochlorite at 20% (50 g/L active chlorine) supplemented with 0.05% Tween 20, then three times washed with sterile distilled water at 5 min interval. Thereafter, disinfested seeds were aseptically placed on Petri dishes (eight seeds per dish) with water-agar 0.6%, and incubated at 28ºC in the dark for 7-8 days. The germinated seeds were planted in flats with autoclaved sand (60 min at 121ºC, twice in two consecutive days), and incubated for 2-3 weeks in a growth chamber (16 h fluorescent light at 23ºC and 8 h dark at 18ºC).

Infested substrate in each tube was added to each of three containers (6 cm × 6 cm × 18 cm) filled with 150 g of infested sand-peat mixture (1:1, v/v). As negative and positive controls, *Fusarium* un-infested and infested substrates lacking organic amendment, were used respectively. One asparagus seedling was transplanted to each container. The flats holding these containers with the plants were incubated in a greenhouse (photoperiod of 16 h and mean temperature ranging 20-24ºC) for 12 weeks, and watered as needed. There were three replications (containers) for each combination of treatments. Independent experiments were performed for every *Fusarium* spp. isolate with a three-factorial design, *i.e*. treatments with OAs (PM, PPM and ORC), temperatures (30 and 35ºC) and periods of incubation (15, 30 and 45 days). Disease symptoms (chlorosis, wilt and necrosis) development was observed weekly until the end of each experiment. Then, asparagus plants were pulled out the containers, their root systems were rinsed under tap water to allow the evaluation of severity of lesions affecting crowns, roots and aboveground plant parts (stems) using a percentage scale 1-100% of tissues showing chlorosis, necrosis or wilt (Molinero-Ruiz *et al.*, 2011). Furthermore, fresh weight of plants from each treatment was recorded. Finally, isolation on PDA dishes was tried with samples of affected tissues, to confirm the infections due to *Fusarium*. Each experiment was repeated.

Data analyses

Homogeneity of variance for the experimental error between replications was shown for the separate analyses of every experiment. The ANOVA for the viability of *Fusarium* were performed after the log-transformation of CFU. The percentages of severity of symptoms were angle-transformed prior to conducting the analysis of variance (ANOVA). Average viabilities, severities and fresh weight of plants were compared by LSD tests ($p \leq 0.05$). Statistix 9.0 (Analytical Software, Tallahassee, FL, USA) was the program used for all the ANOVAs.

Results

Effect of organic amendments, incubation period and temperature on the viability of propagules of *Fusarium* spp. pathogenic to asparagus

Fusarium oxysporum

After 15 days incubation at 30ºC there was a significant reduction of CFU of *Fo5* following the application of PPM-2% (Fig. 1A). When incubation duration was respectively 30 or 45 days the reductions of viability (log values) of this pathogen were 22 and 54% or 26 and 76%, respectively for the amendments with PPM-1 and 2%; in addition to 25% reduction of *Fo*5 viability when substrate amended with PM-2% was incubated for 45 days, but not with PM-1% nor with un-amended control (Fig. 1A).

However, the incubation at 35ºC for 15 days determined reductions in the CFU of *Fo*5 with all organic amendments tested, which were significantly larger for

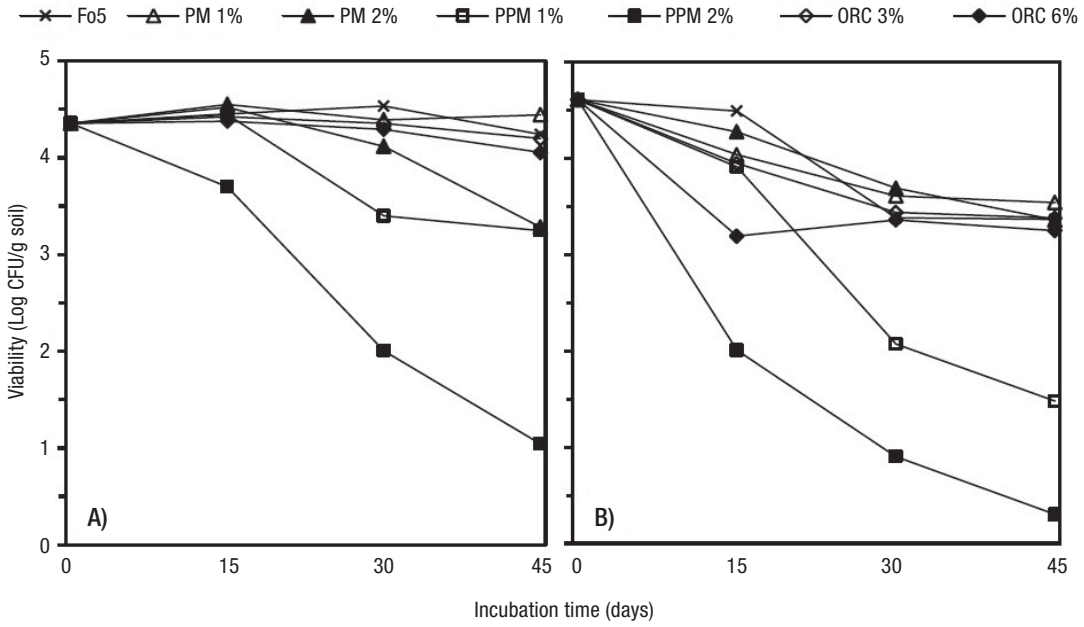

Figure 1. Viability of *Fo*5 isolate of *Fusarium oxysporum* in a substrate incubated at 30ºC (A) and 35ºC (B). After 30 days incubation with *Fo*5, the substrates were amended with poultry manure (PM; 1 and 2%), pellet of PM (PPM; 1 and 2%), and olive residue compost (ORC; 3 and 6%). Values are expressed as the logarithm of colony forming units (CFU)/g soil, and are the average of results from two experiments, each with three replications for every treatment.

longer periods of incubation, especially for PPM-1 and 2% amendments. Thus, viability reduction for the latter rate increased from 57 to 93% as the incubation period increased from 15 to 45 days, but PM-1 and 2% amendments incubated at 35ºC achieved much lower reductions in *Fo5* viability (7-27%) as compared to initial inoculum. The amendment with ORC-6% incubated at 35ºC for only 15 days achieved log CFU reductions of 31%, whereas the un-amended control determined 27% reductions for incubation periods of at least 30 days at this temperature (Fig. 1B).

Fusarium proliferatum

Viability of *Fp*3 propagules in all amended substrates was significantly reduced at both incubation temperatures irrespective of the incubation period, except for the PM-1% amendment after 15 days at 30ºC (Fig. 2). When amended substrates were incubated at this temperature *Fp*3 viability decreased gradually with incubation period, as compared to the un-amended control (Fig. 2). Larger reductions were found in PPM-1 and 2%, respectively reaching 11 and 19%, and 28 and 54%, after 15 and 45 days incubation. Also pathogen populations significantly decreased in 27 and 22%, respectively, after 45 days incubation with ORC-3 and 6%, whereas amendments with PM-1 and 2% determined significant decreases in *Fp*3 viability, respectively reaching 17 and 10% (for log values), after 45 days incubation. In contrast, *Fp*3 populations in un-amended substrate increased slightly over initial inoculum after 15 and 30 days incubation periods (Fig. 2A).

When incubated at 35ºC viability of *Fp*3 propagules in all treatments decreased progressively with time of incubation. The reduction in viability was over 44% (log values) after 15 days incubation for all the amendments except PM-1 and 2% (only 19 and 27%) as well as for the un-amended control. Following incubation for 30 and 45 days, viability was reduced over 35 and 46%, regardless of the treatments (Fig. 2B). Maximal reductions were reached with PPM-1 and 2% amendments, for all incubation periods, although the lowest *Fp3* viability (93% decrease of log value) corresponded to incubation with PPM-2% for 45 days (Fig. 2B).

Fusarium solani

Viability of inoculum of *Fs*2 was significantly reduced both in all amended substrates and their un-amended controls, irrespective of temperature and period of incubation (Fig. 3).

PPM-amended substrates incubated at 30ºC for 15 days significantly reduced *Fs*2 viability log values

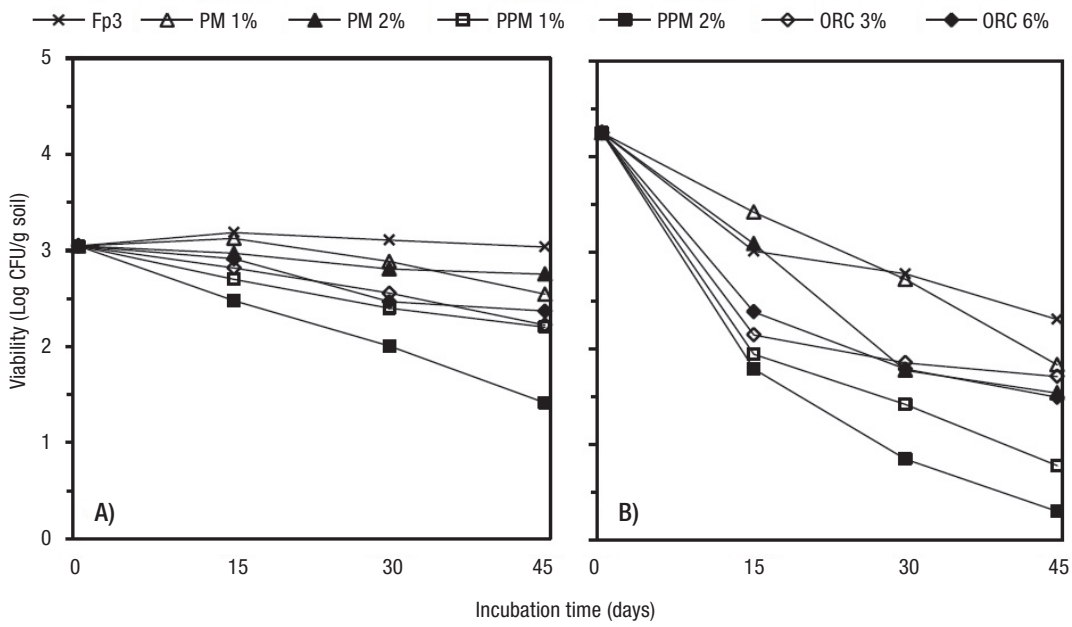

Figure 2. Viability of *Fp*3 isolate of *Fusarium proliferatum* in a substrate incubated at 30ºC (A) and 35ºC (B). After 30 days incubation with *Fp*3, the substrates were amended with poultry manure (PM; 1 and 2%), pellet of PM (PPM; 1 and 2%), and olive residue compost (ORC; 3 and 6%). Values are expressed as the logarithm of colony forming units (CFU)/g soil, and are the average of results from two experiments, each with three replications for every treatment.

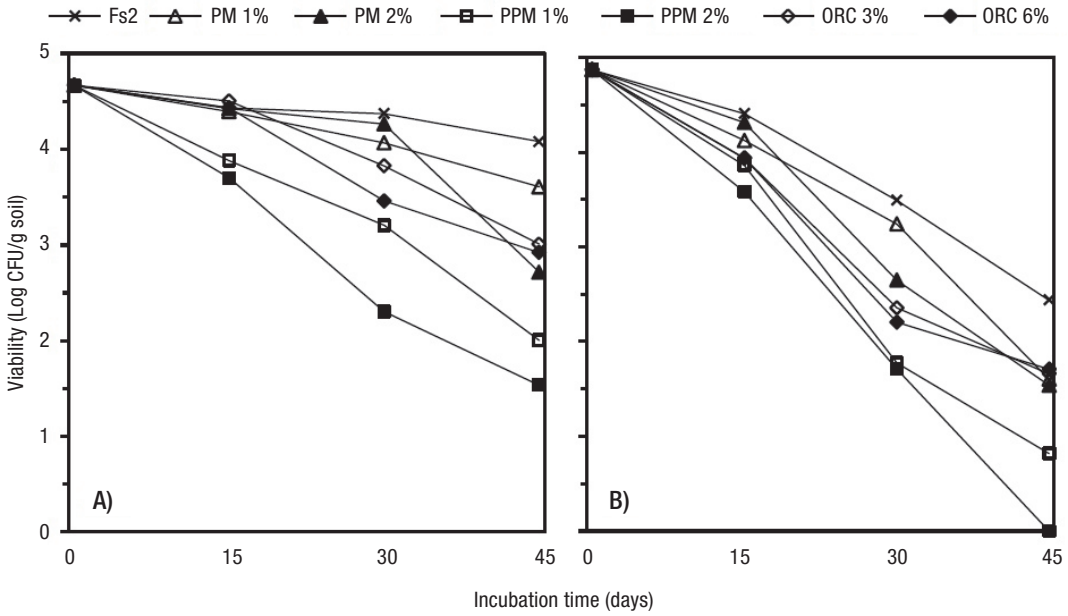

Figure 3. Viability of *Fs*2 isolate of *Fusarium solani* in a substrate incubated at 30ºC (A) and 35ºC (B). After 30 days incubation with *Fs*2, the substrates were amended with poultry manure (PM; 1 and 2%), pellet of PM (PPM; 1 and 2%), and olive residue compost (ORC; 3 and 6%). Values are expressed as the logarithm of colony forming units (CFU)/g soil, and are the average of results from two experiments, each with three replications for every treatment.

(17 and 21%, for rates 1 and 2%, respectively), but the other amendments did not cause significant reductions of *Fs2* viability. After incubating for 30 days, following amendment with PPM-1 and 2%, viability was further decreased (31 and 51%, respectively) whereas ORC amendments achieved 18 and 26% reductions, according to rates. All amendments evaluated reduced log values of inoculum viability by 23-67%, after 45 days incubation, in contrast with a decrease of only 13% for the un-amended control (Fig. 3A).

Viability of *Fs*2 was reduced by 21 and 27% in substrates amended with PPM-1 and 2%, respectively, when incubated at 35ºC for 15 days. These conditions determined 19% reduction of inoculum for ORC-amended substrate at 3 and 6% rates, and only in 15 and 12% for PM-1 and 2% amendments. The latter viability reductions were similar to that achieved in un-amended control (10%). Incubation for 45 days at 35ºC reached log viability reductions over 65% for all amendments, which were maximal (83 and 100%) for PPM-1 and 2%, in contrast with the un-amended control (Fig. 3B).

Effect on the severity of root symptoms

Three months after incubation all the asparagus plants infected by *Fo*5, *Fp*3 and *Fs*2 showed symptoms typically associated with FCRR, such as brown or reddish-brown lesions of roots, necrosis in the insertion of feeding rootlets with storage roots, necrotic flecks, more or less extensive necrosis of the stem base and both types of roots, as well as wet rots of roots. Restricted growth of radical system is eventually observed in the very severe reactions.

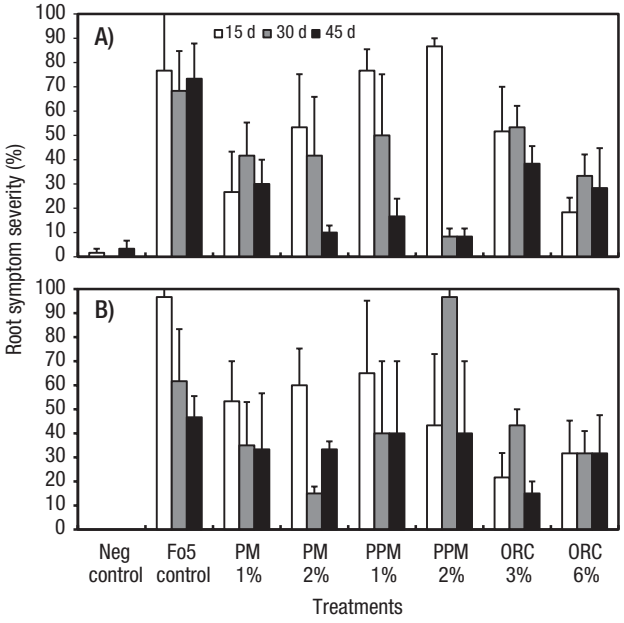

Figure 4. Severity of root symptoms in asparagus 'Grande'. Seedlings were grown in *Fusarium oxysporum* (*Fo*5) infested substrate amended with poultry manure (PM; 1 and 2%), pellet of PM (PPM; 1 and 2%), and olive residue compost (ORC; 3 and 6%) and incubated for 15, 30 and 45 days at 30ºC (A) and 35ºC (B). Values are the average of results from two experiments, each with three replications for every treatment.

Fusarium oxysporum

Addition of OAs to the substrate infested with *Fo*5 showed a significant effect on the reduction of symptoms severity due to FCRR, for most of the treatments evaluated (Fig. 4). After 15 days of substrate incubation at 30ºC, PM-1% and ORC-6% determined severity reductions significantly larger (65 and 76%) as compared to the un-amended control (Fig. 4A). When incubated for 30 days at this temperature, symptoms severity was reduced by 39% for PM-1% and 2% treatments, by 27 and 88% for PPM-1 and 2%, and by 22 and 51% for ORC-3 y 6%, respectively. Severity reductions after 45 days incubation were maximal, when compared to untreated control of *Fo*5-infested substrate, the most effective amendments being PM-2% as well as PPM-1 and 2%, with reductions of 86, 77 and 89%, respectively (Fig. 4A).

Incubation at 35ºC for 15 days resulted in maximal severity reductions (78 and 67%, respectively for ORC-3 and 6% amendments), whereas largest reduction (76%) after incubation for 30 days, corresponded to PM-2% treatment. For substrate incubated for 45 days, reductions in severity were lower than for shorter periods, being 68 and 32% for ORC-3 and 6% respectively, and 29 and 14% for amendments with PM-1 and 2%, and PPM-1 and 2%, respectively (Fig. 4B).

Fusarium proliferatum

Substrate incubation at 30ºC for 15 days, determined 92% reduction of symptoms severity, as compared to the un-amended control, when amended with ORC-3%. The maximal reductions of severity after incubation for 30 days were 89 and 83% respectively for PM-1 and 2%, and 78% for PPM-1%, but also significant for ORC-3 and 6% (72 and 67%, respectively). The period of incubation of 45 days determined 78% reduction in severity for the amendment with PM-2%. All these reductions contrasted with the very high severity of symptoms for PPM-2%, overcoming the values in the untreated, infested substrate (Fig. 5A).

After 15 days substrate incubation at 35ºC, all the amendments evaluated, with the exception of PPM-2%, significantly reduced symptoms severity at both concentrations, those reductions being 80 and 60% for PM-1 and 2%, 67 and 73% for ORC-3% and 6%, respectively, whereas for PPM-1 y 2% they were 53 and 13%. When the incubation period was 30 days, only ORC amendments, at 3 and 6%, reduced severity by 67% (Fig. 5B). After 45 days incubation, the most effective amendments were PPM-1%, PM-2% and ORC-3%, with severity reductions of 82, 72 and 72%, respectively.

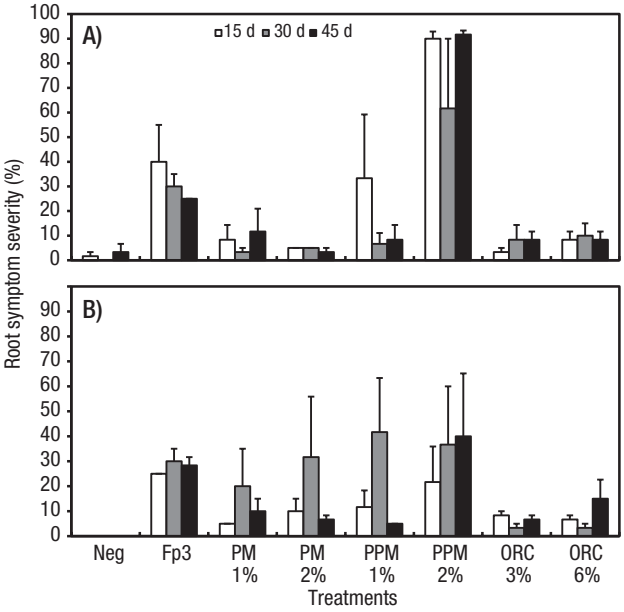

Figure 5. Severity of root symptoms in asparagus 'Grande'. Seedlings were grown in *Fusarium proliferatum* (*Fp*3) infested substrate amended with poultry manure (PM; 1 and 2%), pellet of PM (PPM; 1 and 2%), and olive residue compost (ORC; 3 and 6%) and incubated for 15, 30 and 45 days at 30ºC (A) and 35ºC (B). Values are the average of results from two experiments, each with three replications for every treatment.

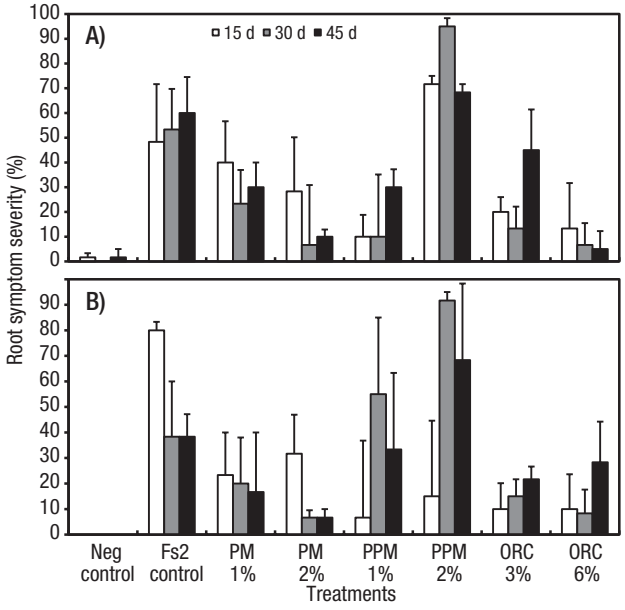

Figure 6. Severity of root symptoms in asparagus 'Grande'. Seedlings were grown in *Fusarium solani* (*Fs*2) infested substrate amended with poultry manure (PM; 1 and 2%), pellet of PM (PPM; 1 and 2%), and olive residue compost (ORC; 3 and 6%) and incubated for 15, 30 and 45 days at 30ºC (A) and 35ºC (B). Values are the average of results from two experiments, each with three replications for every treatment.

Fusarium solani

After 15 and 30 days incubation at 30ºC of the substrate infested with isolate *Fs*2 and amended with PPM-1%, reductions in severity of symptoms on asparagus cv. Grande were *ca.* 79 and 81%, as compared to the un-amended control. Amendments with PM-1 and 2% respectively reduced severity by 17 and 41% after 15 days incubation and by 56 and 88% after 30 days incubation. Severity reductions of 59 and 72%, and 75 and 88% corresponded, respectively, to incubations for 15 and 30 days following amendments with ORC-3 and 6%. After 45 d incubation, PM-1 and 2% determined reductions by 50 and 83%, whereas 92% was found for ORC-6% amendment (Fig. 6A).

Severity reductions for *Fs*2 infested substrate, as compared to the un-amended control, ranged 60-92%, for the different amendments, when incubation was at 35ºC for 15 days. However, after incubation for 30 days at this temperature, severity reductions were as high as 83 and 78% for amendments with PM-2% and ORC-6%. After 45 days incubation the higher decrease (83%) was reached for PM-2% (Fig. 6B). Surprisingly incubation for 30-45 days with PPM-2% largely increased severity over that of the un-amended infested control, at both temperatures, which was also true for the 30 days incubation at 35ºC when amended with PPM-1%.

Effect on fresh weight of plants

Fusarium oxysporum

Fresh weight of asparagus plants grown on *Fo*5-infested substrate and incubated at 30ºC for 15 days, increased by 390 and 216%, over the infested, un-amended control, when amended, respectively, with PM-1 and 2%, and by 173 and 193% for ORC-3 and 6%. Furthermore, incubation for longer determined weight increases of 338 and 314% when PPM-2% was applied. However, when substrate was amended with PPM-1%, significant fresh weight increase (by 140%) only occurred after 45 days incubation (Fig. 7A).

When incubated at 35ºC for 15 days, all organic amendments provided significant increases of the fresh weight of asparagus plants. Incubation at 35ºC for 30 days after PM-2% amendment determined increase by 243% of the fresh weight, but PPM-2% required 45 days incubation to achieve fresh weight increase of 57%. However, maximal fresh weight of plants with this treatment was achieved after only 15 days incubation, similarly to the amendment of the substrate with ORC-6% (Fig. 7B).

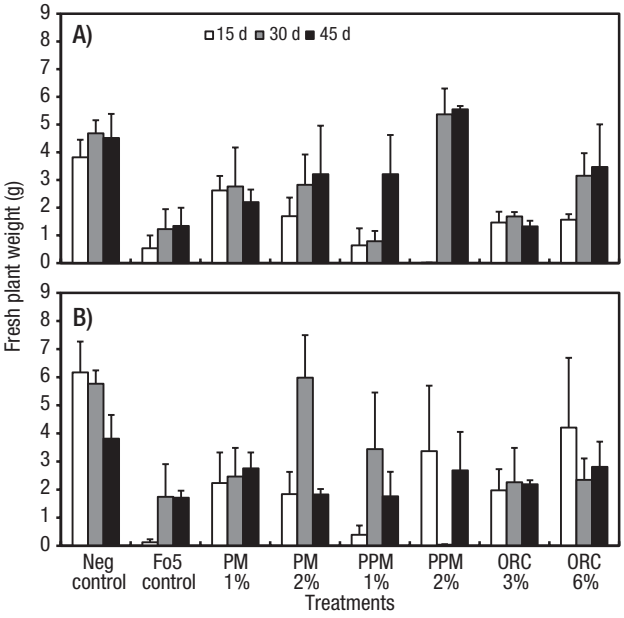

Figure 7. Plant fresh weights of asparagus 'Grande'. Seedlings were grown in substrate infested with *Fusarium oxysporum* (*Fo*5) and amended with poultry manure (PM; 1 and 2%), pellet of PM (PPM; 1 and 2%), and olive residue compost (ORC; 3 and 6%) after 30 days of incubation at 30ºC (A) and 35ºC (B). Values are the average of results from two experiments, each with three replications for every treatment.

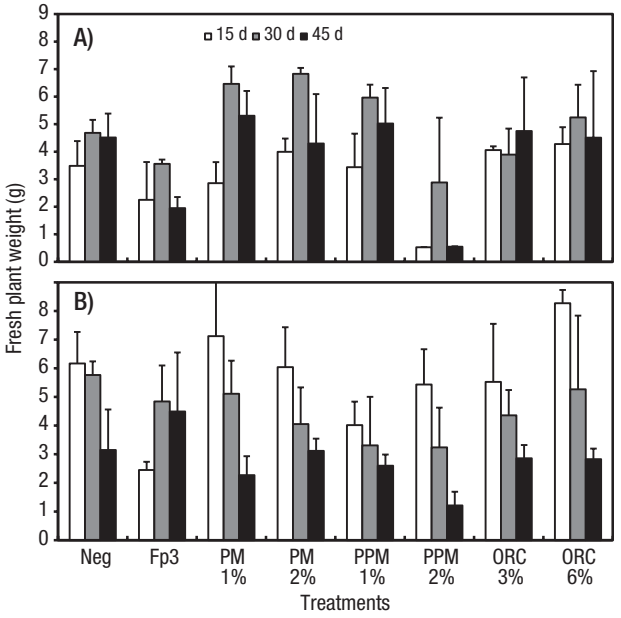

Figure 8. Plant fresh weights of asparagus 'Grande'. Seedlings were grown in substrate infested with *Fusarium proliferatum* (*Fp*3) and amended with poultry manure (PM; 1 and 2%), pellet of PM (PPM; 1 and 2%), and olive residue compost (ORC; 3 and 6%) after 30 days of incubation at 30ºC (A) and 35ºC (B). Values are the average of results from two experiments, each with three replications for every treatment.

Fusarium proliferatum

Fresh weight of plants grown on *Fp*3-infested substrate and incubated at 30ºC for 15 days, increased by 78%, over the infested, un-amended control, with PM-2% amendment, and by 80-85% with ORC-3 and 6%, respectively. As incubation period increased to 30 and 45 days, except for substrate amended with PPM-2%, fresh weight of asparagus plants increased, irrespective of the amendments and rates tested, reaching maximal weight increases of 172, 158% and 144 %, respectively for amendments with PM-1%, PPM-1% and ORC-3% after 45 days incubation (Fig. 8A).

All the organic amendments increased fresh plant weight (by 64-238%) when incubated for 15 days at 35ºC. However, extending this incubation to 30 days determined only slight weight increases for PM-1% and ORC-6% (6 and 9%, respectively), whereas all the other treatments, as well as incubation for 45 days resulted in decreased fresh weight of plants (Fig. 8B).

Fusarium solani

Asparagus plants cv. Grande grown on *Fs*2-infested substrate incubated at 30ºC for 15 days following the amendment with PPM-1% achieved maximal increase of fresh weight (93%) whereas, after 30 days incubation, increases were slightly higher (104%) for PM-2% and PPM-1%, but still higher (118 and 162%) in substrates amended with ORC-3 and 6%, respectively. After 45 days incubation, all amendments evaluated increased the fresh weight of plants, with maximal increases (449%) for ORC-6%, and *ca*. 344% for PM-1 and 2% (Fig. 9A).

When substrate incubation was at 35ºC for 15 days, all the amendments determined asparagus fresh plant weight increases in the range 596-797%. After incubation for 30 days, only ORC-6% achieved significantly higher increase of plant weight (163%). However, incubation for 45 days determined very low weight increases of plants grown in all amended substrates, except for PPM-2% and ORC-3%, which decreased weight (Fig. 9B).

Discussion

The application of organic amendments to the infested substrates combined with temperature and period of incubation considerably reduced the viability of fungal propagules of the three isolates of *Fusarium* (*Fo*5, *Fp*3 and *Fs*2) studied. This agrees with the loss of viability of several soil-borne plant pathogenic fungi, including *Foa*, when amendment of soils with animal

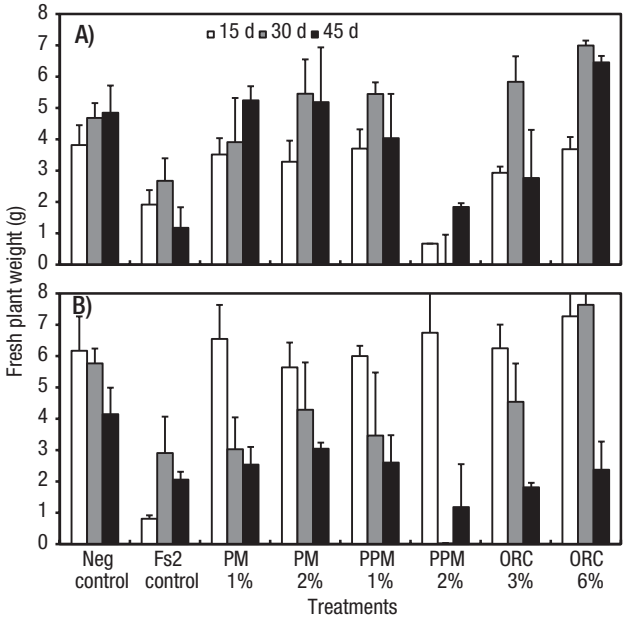

Figure 9. Plant fresh weights of asparagus 'Grande'. Seedlings were grown in substrate infested with *Fusarium solani* (*Fs*2) and amended with poultry manure (PM; 1 and 2%), pellet of PM (PPM; 1 and 2%), and olive residue compost (ORC; 3 and 6%) after 30 days of incubation at 30ºC (A) and 35ºC (B). Values are the average of results from two experiments, each with three replications for every treatment.

and plant residues combined with soil solarization was performed (Gamliel & Stapleton, 1993; Blok *et al*., 2000; Abbasi *et al*., 2002, 2008; Borrego-Benjumea *et al*., 2014b).

The most effective amendment in declining the viability of *Fo5* and *Fs*2 was pelleted poultry manure (PPM-1 and 2%), with a more pronounced effect for the higher application rate, reaching undetectable levels in the case of *Fs*2 after 45 days incubation at 35ºC. This demonstrated a larger effect of temperature for *Fs*2 than for *Fo*5 and seems concomitant with sustained increase of total bacteria populations and overall moderate increases of pH and N content in PPM as compared to the other amendments tested (Borrego-Benjumea *et al.*, 2014b). As a matter of fact, the N-richest amendments, with a low C/N ratio, which release and accumulate higher amounts of volatile toxic compounds such as NH_4 and HNO_2 in the soil during organic matter decomposition, are able to eliminate many soilborne fungi (Tsao & Oster, 1981; Tenuta & Lazarovits, 2002; Borrego-Benjumea *et al.*, 2014a). However, amendments with high C/N ratio may be effective in the control of these pathogens because those compounds can stimulate microbial activity, enhancing depletion of N availability, and, consequently, impairing the pathogen infection process (Snyder *et al.*, 1959).

Decreases in severity of root symptoms in asparagus were much more pronounced in *Fo5*- and *Fs2*-infested substrate amended, respectively, with PM and PPM, and PM and ORC at the higher rates, but the effect varied with incubation temperature. Disease reduction after application of different organic amendments (including poultry manure) on soils infested with *F. oxysporum, F. solani* and *F. equiseti, F. oxysporum* f. sp. *cumini* and *F. oxysporum* f. sp. *spinaciae* were also reported by Escuadra & Amemiya (2008), Israel *et al.* (2011) and Martínez *et al.* (2011). On the other hand, the lower severity of root symptoms caused by isolate *Fp*3, as compared to *Fo5* and *Fs*2 (except for incubation of substrate amended with PPM-2% at 30ºC) is likely due to poor survival of *F. proliferatum* in bare soils, as this species does not produce chlamydospores; therefore the initial inoculum density was lower for this than for the two other species tested (Elmer *et al.*, 1996; Elmer, 2001). This is in agreement with previous results (Reid *et al.*, 2002) that showed a low root severity (20%) using *F. proliferatum* at 1.3×10^4 CFU/g (similar to that in our experiments). For the other treatments, however, we found much lower root severity values than the un-amended control.

The increase of root severity and the negative effect on plant fresh weight observed, in plants established in *Fp3*- and *Fs2*-infested substrates amended with PPM-2%, and incubated for 30-45 days regardless of temperature, suggests a phytotoxic effect of this PPM rate. A similar effect was also observed when high concentrations of PPM were applied to pots with substrate infested with *F. oxysporum* f.sp. *dianthi* and incubated at high temperature before transplanting rooted cuttings of carnation (Nava-Juárez, 2013), but these phytotoxic symptoms were absent from plants growing in greenhouse on soil to which equivalent dosages of PPM were applied (Melero-Vara *et al.,* 2011). The reason is likely that volatile compounds generated by PPM-2%, mainly ammonia, develop more quickly at 30ºC (Lazarovits, 2001; Tenuta & Lazarovits, 2002; Borrego-Benjumea *et al.*, 2014a). To this regard it is interesting the comparison of N content and electrical conductivity between PPM and PM, as those for PPM are, respectively, almost double and 4-times those of PM (Table 1).

However, substrates infested with *Fo5* and then treated with PPM-2%, resulted in the higher plant weight as compared to the un-amended infested control, mainly with 45 days of incubation at 30ºC. At these conditions, all amendments, except PMM-2%, had a positive effect on the vigor of plants growing on *Fp*3- and *Fs2*-infested substrate. This is in agreement with previous results reported on increased plant growth of asparagus `Jersey Giant´ inoculated with one Canadian

isolate of *F. oxysporum* and `Jersey Giant´ and `Grande´ inoculated with another isolate of *F. solani* also from Canada (Borrego-Benjumea *et al.*, 2014b).

For the three pathogens studied, low and high severity values frequently correlated with high and low plant weight values respectively, especially for PM and PPM amendments. Nevertheless this general trend showed variability, according to the type and dosage of organic amendment, in relation to temperature and period of incubation. Therefore these effects were predominant for a mode of action related to toxic compounds release, whereas effects on soil biology and chemistry seem to occur more likely with ORC than with the other amendments tested.

Summarizing, asparagus disease levels decreased and plant weight increased when the integration of organic amendment, temperature and incubation periods used resulted in reducing inoculum viability, which corresponded in most cases with long periods of incubation. However, additional field experiments are required before implementation of our results. From these studies we are able to conclude that different organic amendments are suitable to FCRR management and do not cause phytotoxicity problems (except PPM-2%) to asparagus plants growing in nurseries and fields, such as has been reported for the control of Fusarium wilt of carnation.

Acknowledgements

We are thankful to C. Casanova, A.B. García and N. Lucena for their technical assistance.

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