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Interactive effects of arbuscular mycorrhiza fungi *Glomus intraradices* and *Trichoderma harzianum* against Fusarium wilt of tomato

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Abstract

Biological control agents (BCA) are important as some establish symbiosis with plants hence controlling plant diseases, improving plant nutrients uptake and water absorption. Use of BCA in soil borne disease management is not fully harnessed and is also faced with inconsistencies in developing their formulations. We therefore investigated the use of arbuscular mycorrhiza fungus (AMF), *Glomus intraradices*, and *Trichoderma harzianum* (T-22) against soilborne pathogen *Fusarium oxysporum* f. sp. *Lycopersici* (Fol) in tomato. *G. intraradices* isolate 510 held on expanded clay as carrier material was incorporated into the substrate during germination of tomato seeds and at the transplanting stage. T-22 inoculum was also initiated from potato dextrose agar and inoculated at each transplanting stage, while Fol was applied through drenching. To test the possible synergistic effects, AMF and T-22 were applied in combination under varying niches. Results showed that application of AMF and T-22 together had significant reduction (30.5% $p<0.005$) in Fol. Tests under varying phosphorous (P) regimes revealed significant reduction in wilting symptoms by 40.3% ($p<0.005$) following Fol infection. Plants grown under high levels of P showed typical Fol symptoms characterized by yellowing and gradual wilting, while plants with low levels of P wilted directly without undergoing the yellowing stages. The results show the significant role of AMF and T-22 as BCA against the soil-borne pathogen Fol and contributes to development of safe and sustainable disease management strategy.

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Introduction

Fusarium oxysporum is an ubiquitous soilborne pathogen that includes various pathogenic strains which lead to economic losses in tomatoes (*Solananum lycopersicum*) (Gordon and Martyn, 1997; Fravel *et al.*, 2003; Di Pietro *et al.*, 2003). Chemicals applied to control Fol have been unsuccessful as they barely target their ecological niches but also pose dangers to the environment (Lucas, 2006). Abuscular mycorrhiza fungi is shown to present bio-protection effects against fungal phytopathogens including *Phytophthora*, *Fusarium*, *Pythium* and *Rhizoctonia* (Pozo *et al.*, 1996; Cordier *et al.*, 1998; Akköprü and Demir, 2005). Its ubiquitous nature is a fundamental component for development, exploitation and adaptation to interactions with the other rhizosphere microfloral of which knowledge of mode of interactions can be harnessed for development of an effective biological control strategy (Linderman, 1994; Fillion *et al.*, 1999; Vazquez *et al.*, 2000). Trichoderma is a soilborne fungus commonly used as an antagonist against fungal pathogens and as a bio-pesticide, which is exploited for its biological control potential (Harman, 2000; Harman *et al.*, 2004). Moreover, control may be achieved indirectly by inducing host plant resistance where not only microbial organisms have been used but also compounds that mimic their cellular components to induce resistance (Kiirika *et al.*, 2014).

The efficacy of these biological control agents and consistence performance in controlling soil borne diseases are the hurdles that must be overcome if their application is to be widely used even for commercial purposes. Attempts to combine alternative treatments to improve performance have been made but their application is faced with similar constraints as the level of protection is influenced by factors such as the pathogen strain, environment, soil type and nutrients (Guetsky *et al.*, 2001; Larkin and Fravel, 2002; Spadaro and Gullino, 2005). Optimal plant nutrition is essential for a successful disease management where manipulation of mineral nutrients such as nitrogen, phosphorous, sulphur and potassium have been shown to contribute to control of various plant diseases. Phosphorus (taken up by plants as $H_2PO_4^-$ and HPO_4^{2-}) is shown to have multiple roles not only as an essential plant nutrient but also mitigates disease development in plants (Sweeney *et al.*, 2000). High levels of phosphorous in the soil however shown to reduce host's ability to develop AMF symbiosis (Balzergue *et al.*, 2013). In the current study, we investigated the interactions of AMF and T-22 under varying phosphorous regimes aimed at controlling Fol. Tests to determine control effects of AMF and T-22 against Fol, tests were carried out under different substrates containing different P regimes. We hypothesized that application of AMF and T-22 inhibits Fol infection and the effects will be synergistic for combined applications.

Materials and methods

Plant materials and culture conditions

Tomato seeds of cultivar Cuor Di Bue were raised in trays containing quartz sand and transplanted after 10 days into 9 cm diameter plastic pots (volume 230 ml). Six weeks later, seedlings were transplanted into bigger pots of 17cm diameter (volume 2 l) and kept under greenhouse conditions (20 ^{o}C day/night, 12 h light per day/30 K Lux and 70% RH) and watered regularly to the soil field capacity.

Microbial culture and inoculation

AMF and T-22 isolates were taken from the collection of the Institute of Horticultural Production Systems, section Phytomedicine. The inoculum of *G. intraradices* isolate 510 on expanded clay as carrier material (Dehne and Backhaus, 1986) was incorporated into the substrate during germination and at each transplanting stage at 5 % (v/v). T-22 inoculum was initiated from potato dextrose agar, cultured into liquid conical flasks with sterile media consisting of perlite pre-soaked with 2 % malt extract solution (Kraftnahrung rein, from Villa Natura), at 2 % (v/v) into the culture substrate at each transplanting stage. Fol inoculation was done through drenching by pouring direct into the pot at 30 ml spore suspension (1.5 x 10^8 spore/ml) at the second transplanting stage.

Substrate and fertilization

Pure quartz sand with and without 30 % (v/v) fine ground white peat from Lithuania (calcium carbonate at 500 g/100 l added to increase pH) were used as substrates. Fertilization was carried out in two nutrient regimes; Hewitt (Hewitt, 1966) low phosphorous (LP), by 12.85 g/l $NAH_2PO_7{\cdot}H_2O$) and Hewitt high phosphorous (HP) (128.5 g/l $NAH_2PO_7{\cdot}H_2O$).

Experimental setup

Interactions between AMF and T-22 and their effect when applied together on Fol were tested using tomato cultivar Cuor Di Bue. Experiments were conducted under greenhouse conditions, 16 h light per day with additional light using Phillips lamps SRG 102/400 (195 µmol sec^{-1} m^{-2}) during winter. Disease progress was monitored on tomato plants growing with temperature maintained at 22 °C (day) and 18 °C (night) (Fig. 1). The experiment consisted of treatments arranged in factorial design with Fol infected or not infected (control) and subjected to the two P-levels and substrates: (i) without T-22 or AMF (control), (ii) with AMF alone, (iii) with both T-22 and AMF, (iv) with T-22 alone. Two conditions were compared, during summer and winter periods which enabled identification of differences due to changes in different environmental conditions of the two seasons. Furthermore, we investigated the influence of P on Fol disease symptom expression. To observe whether plants would start showing yellowing symptoms of Fusarium wilt when supplied with more P, plants with LP for AMF and control but only in the substrate sand were included in the setup with the aim of increasing the level of P in this extra set of plants from LP to HP after the initial wilt symptoms were observed.

Assessments and data collection

To assess the effects of different treatments, parameters including plant growth, disease development, severity and incidence were considered. AMF and T-22 establishment and intensity were observed at different plant growth stages to monitor their development and possible interacting effects. Analysis of P content in the plant tissue was carried based on the phosphorous acid colorimetric determination method with ammonium molybdate vanadate by Gericke and Kumries (1952) to decipher differences in its content based on the P nutrition level. In order to monitor disease development and severity, visual symptoms were assessed. Symptoms of Fol infection were observed including wilting and yellowing and each scored separately using a scale of 0-4 according to the following classes: 0=no symptoms, 1=< 25 % leaves with symptoms, 2=26-50 % leaves with symptoms, 3=51-75% leaves with symptoms and 4= 76-100 % leaves with symptoms.

Confirmation of disease incidence and development in the plants were carried out according to Grunewaldt-Stöcker (1994): Fol was re-isolated from the stem cross sections at different heights (stem base, 25 cm and 50 cm). The outer surface of the stem was sterilised with 70 % ethanol and thin cross sections cultured on Komada Agar (Komada, 1975). The growth of the mycelia from the vascular bundles was then assessed with 1/3, 2/3, 3/3 reflecting the number of infected bundles. Re-isolation of Fol from the substrate was done using Komada agar following the basic dilution-plating technique: 15g of the substrate was diluted in 150 ml sterile water, stirred for 10min (750 rpm), a defined volume of a 10^{-3} dilution was then plated out on Komada agar in 3 replicates, and then incubated for 5-6 days at 23 °C after which colonies formed per plate were counted. This was only done for the AMF/T-22 sand HP treatment for the first experiment as majority of plants in the treatment showed no symptom development and was therefore necessary to confirm the success of inoculation by determining the pathogen presence in the rhizosphere.

Trichoderma infection and intensity estimation

T-22 was estimated from the rhizosphere dilution-plating at the second transplanting and harvest stages. Briefly, quantitative isolation of T-22 was done using selective culture medium (Steinmetz, 1994 - unpublished): 15g of the substrate diluted in 150 ml sterile water, stirred for 10 minutes (750 rpm) and a

defined volume of a 10^{-3} dilution was then plated out on TSA agar (Lewis and Papavizas, 1984) in triplicates. The culture was then incubated for 5-6 days at 23 °C and colonies formed per plate counted as colony forming units (CFU)/g substrate using the following formula:

$$\text{CFU} = \text{Number of colonies/ml} \times \frac{1}{\text{oven dry weight of 1g fresh soil} \times \text{dilution factor}}$$

AMF colonisation assay

AMF colonisation was quantified also at the second transplanting and at harvest stages. Samples for the assay were collected from washed root systems by cutting a transverse band of about 1cm width at around 5cm below the root crown. Staining was done following Vierheilig *et al.* (1998): roots were soaked in 3 % KOH overnight, washed several times with tap water, soaked overnight in ink-vinegar-solution (1 % ink and 5 % household vinegar in a dest.). Estimation of AMF colonisation was then done under a light microscope (Axiolab, Zeiss) using a scale from Backhaus (1984) as follows: 0 = no infection, 1-up to 30 % of the root section colonized, 2-up to 60 % of the root section colonized and 3-colonisation of the whole root.

Statistical analysis

The statistical analysis was done using the R software, version 2.9.0. To determine the interactions with regard to the effect of phosphorous, substrate, and biological control agents, a fit of an ordered logistic regression model was used.

The model worked at estimating the probability of an individual to get a score of 0, < 1, < 2, or < 3 for the AMF establishment. Parameter estimates were the log difference of odds, comparing the factors substrate, phosphorous and the treatments. The confidence intervals (CI) at 95 % were then calculated for the parameters and a significant difference was shown if the value 1 was not included in the interval. A Chi-square test of independence with multiplicity adjustment by Bonferroni was then done at a *p*-value ≤ 0.05. The model was also used to plot a model demonstrating the differences in symptom development in terms of wilting and yellowing as influenced by phosphorous level at the probabilities of getting the different scores of 1, 2, 3 or 4 and is shown in the results segment at the probability of getting a score of 3. For the analysis of disease severity and *Trichoderma* establishment, the treatment effects were tested using analysis of variance (ANOVA). The significant differences were then tested by Tukey multiple range tests (*p*-value ≤0.05). For disease severity, the area under disease progress curve (AUDPC) was calculated using the formula below and data subjected to ANOVA.

$$AUDPC = \sum_{i=1}^{n-1} [(x_i + x_{i-1})/2](t_i - t_{i-1})$$

Where, x_i and x_{i-1} are the wilt index, and t_i and t_{i-1} are consecutive evaluation dates (with t_i-t_{i-1} is equal to 1 day).

Results

Establishment of AMF and T-22 under different substrates and P-Levels

Analysis of interacting effects of AMF and T-22 as well as the influence of different substrates (sand and sand-peat mixture) and P-levels (low P and high P) revealed that P level had a significant influence on mycorrhiza colonisation with the logistic model showing differences regardless of substrate, with or without T-22.

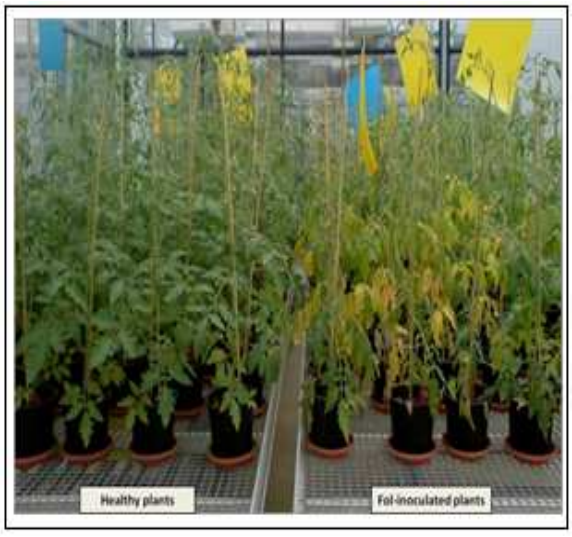

Fig. 1. Tomato plants under greenhouse conditions showing Fol wilting symptoms. Plants inoculated with Fol (right) healthy plants without Fol inoculation (left).

This effect of P on mycorrhiza colonization was evident both at the initial establishment (after 6 weeks) with a confidence interval (CI) of 3.38-16.62 and at harvest (after 13 weeks) CI of 10.02 - 23.28. A Chi-square test of independence revealed high significant differences in mycorrhiza colonization for treatments under LP compared to treatments under HP (Fig. 2A and B).

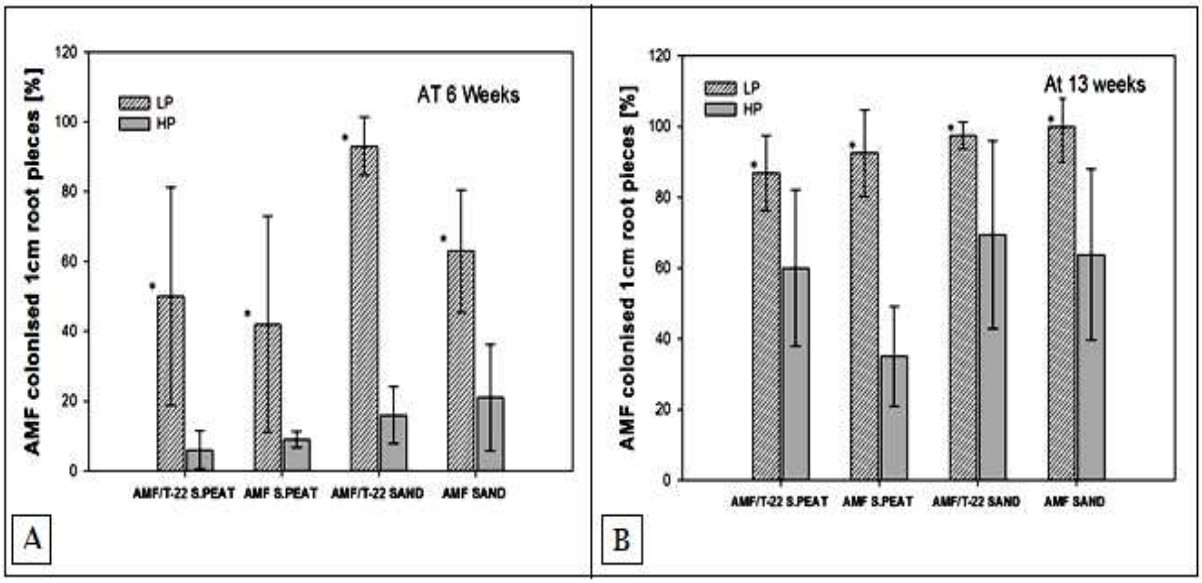

Fig. 2. AMF colonisation is influenced by different P levels (low and high levels). The graph shows data of mean frequency values ± SD; n= 8; after inoculation with AMF alone and in combination with *Trichoderma* (AMF/T-22) in the different substrates sand (SAND) and sand peat (S.PEAT) after (A) six weeks and (B) 13 weeks. Significant differences between LP and HP in each treatment are indicated by (*) according to Chi-square test of independence (*p*-value ≤0.05).

c) The substrate type also had significant effects on AMF colonization with plants grown in sand showing higher colonisation compared to sand peat at 6 weeks (Fig. 3A and B) CI (1.18 -6.41) and after 13 weeks (Fig. 3C and D) CI (1.83-4.43). At 6 weeks, these differences were clearly seen in the AMF/T-22 in low P having significantly higher mycorrhiza colonisation under sand substrate compared to sand peat (Fig. 3A). At 13 weeks, high influence was under the AMF treatment in HP having significantly higher colonisation in sand compared to sand peat (Fig. 3D).

AMF colonisation appeared not to be affected by the addition of T-22 which is evidenced by almost similar AMF-colonisation pattern levels at both the combined treatment AMF/T-22 and individual AMF treatment (Fig. 4A-D). The only significant difference was detected at 6 weeks (Fig. 4B) whereby the combined application of AMF/T-22 shows higher colonisation in the substrate sand at LP-level.

Substrate was observed to have significant effect ($p \leq 0.05$) on T-22 establishment (Fig. 5). Treatments under sand-peat had significantly higher levels of T-22 compared to treatments under sand regardless of P levels both at 6 weeks (Fig. 5A and B) and at harvest (Fig. 5C and D). P level and AMF did not show significant effect on T-22 establishment.

Effects of AMF and T-22 on Fusarium wilt development under different substrates and P-levels

To determine the biological control effects of AMF and T-22 on Fol when either applied alone or in combination (AMF/T-22), a mixed model ANOVA analysis of the AUDPCs under all substrate and P levels was done. Results by ANOVA revealed significant effect of substrate with treatments under sand-peat which had significantly higher disease severity compared to treatments under sand regardless of P-level or biological control treatment (Fig. 6A and B).

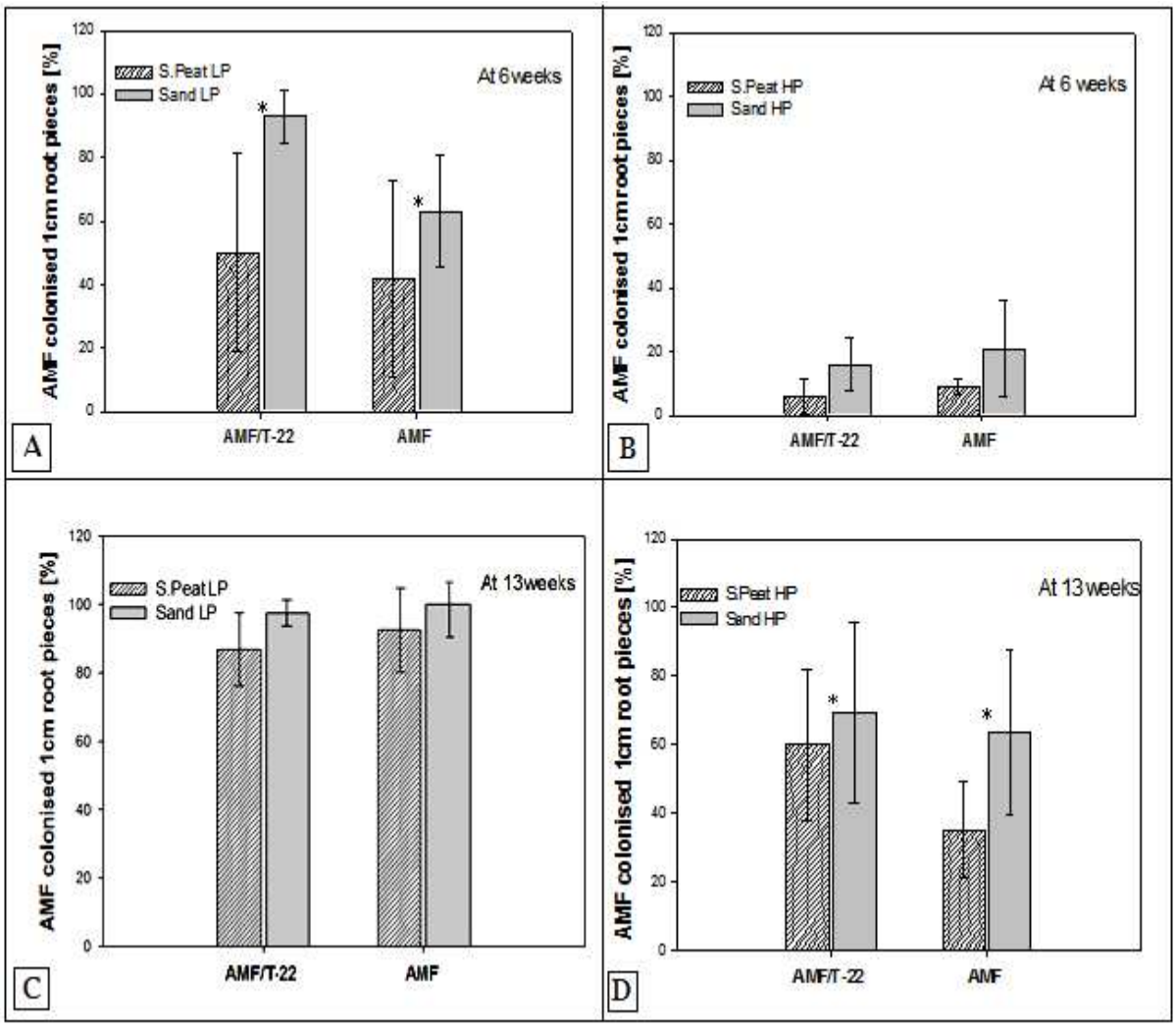

Fig. 3. AMF colonisation is influenced by substrate sand peat and sand within the same P levels. The graphs shows data of mean frequency values ± SD; n= 5 and 8 at (A, B) 6 weeks and (C, D) 13 weeks respectively; after inoculation with AMF alone and in combination with T-22 (AMF/T-22) at equal levels of phosphorous. Significant difference between the substrates Sand peat (S.Peat) and Sand in each treatment are indicated by (*) according to Chi-square test of independence (*p*-value ≤0.05).

Results are also presented as disease progress curves for the treatments with T-22 and AMF at LP or HP both during summer and winter periods (Fig. 8) where the developmental trend was similar for all the treatments.

A Tukey's multiple range tests was then done to compare treatments within the same substrate and under the same P-levels (Fig. 8A-H) While no significant differences were detected in the substrate sand peat at either HP or LP, the disease progress curves show the combined treatment of AMF/T-22 having the lowest severity level compared to the control (Supplementary fig. 1A and B) The most pronounced and significant effect was observed in the substrate sand with HP where the combined treatment AMF/T-22 showed a significant lower disease severity compared to all the other treatments (Fig. 1C) Re-isolation of the pathogen from the substrate of this particular treatment confirmed pathogen presence in the treatment (data not shown). In the sand substrate at LP, no significant differences were detected between the treatments with no clear trend in the disease progress curves, but in the AMF treatment, the trend was lower compared to others (Fig.8D). The re-isolation of the pathogen incidence from the stems from all the treatments inoculated with Fol was consistent with the visual wilt

development scores (data not shown) with the plants showing symptoms visually also showing infection in the selective agar media, while those that did not have any symptoms visually not showing any infection.

The second experiment was done in winter and allowed us to observe the differences due to different environmental conditions as compared to those of summer in the first experiment. The greenhouse temperature during summer period ranged from 28-32 °C while in winter, temperature was maintained between 22-25 °C. Establishment of AMF and T-22 was again determined at initial stages and at harvest but this was only done to confirm their establishment. For T-22, this was done in both treatments containing T-22 and in the control to determine whether there was any indigenous *Trichoderma* spp. in the substrates used. The presence of T-22 in the rhizosphere of treatments inoculated with T-22 was confirmed, but no indigenous T-22 was detected in the controls. The colonisation of AMF on the roots treated with AMF was also confirmed.

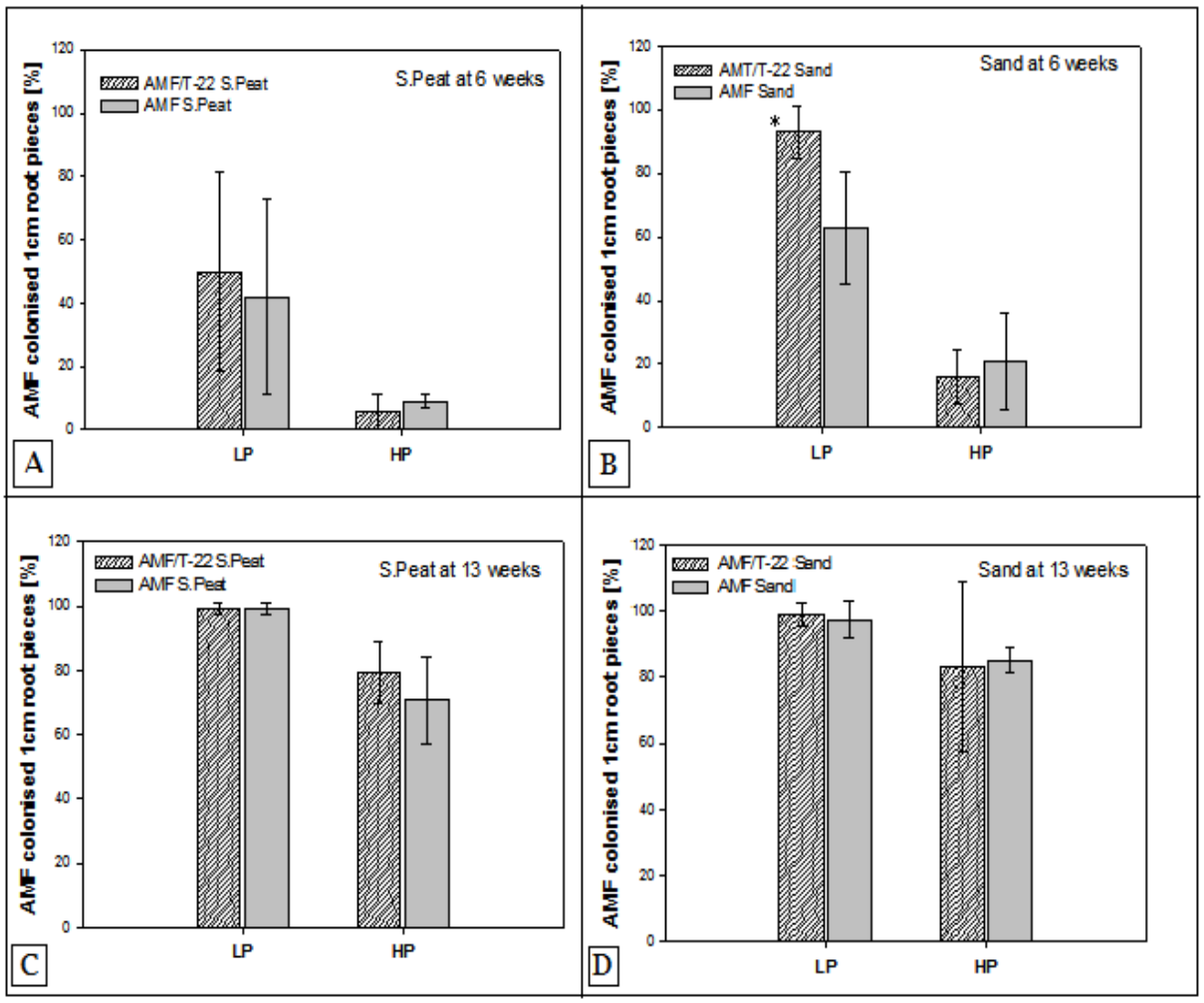

Fig. 4. AMF colonisation is influenced by addition of T-22. The graphs shows data of mean frequency values ± SD; n= 5 and 8 at (A, B) 6 weeks and (C, D) 13 weeks respectively; after inoculation with arbuscular mycorrhiza fungi (AMF) alone and in combination with *Trichoderma* (AMF/T-22) at the same phosphorous levels within the same substrate, Sand peat and Sand. Significant difference between AMF and AMF/T-22 at the same Phosphorous level (LP or HP) is indicated by (*) according to Chi-square test of independence (p-value ≤ 0.05).

In general, the disease incidence and severity levels in winter were lower than in summer and so was the progress of disease development which took four weeks in winter as compared to only two weeks in summer. Due to the fact that the incidence and severity levels were very low in winter (Fig. 8E-H)

with only half of the plants within each treatment showing symptoms, no statistical analysis was possible and therefore only the trends of the disease progress curves are compared. The disease progress curves indicate that the biological control agents AMF and T-22 alone or combined also reduced the severity levels of the disease in winter as their progress curves are lower in comparison to the controls. The other trends on the effect of P and substrate on disease development was the same as in the first experiment.

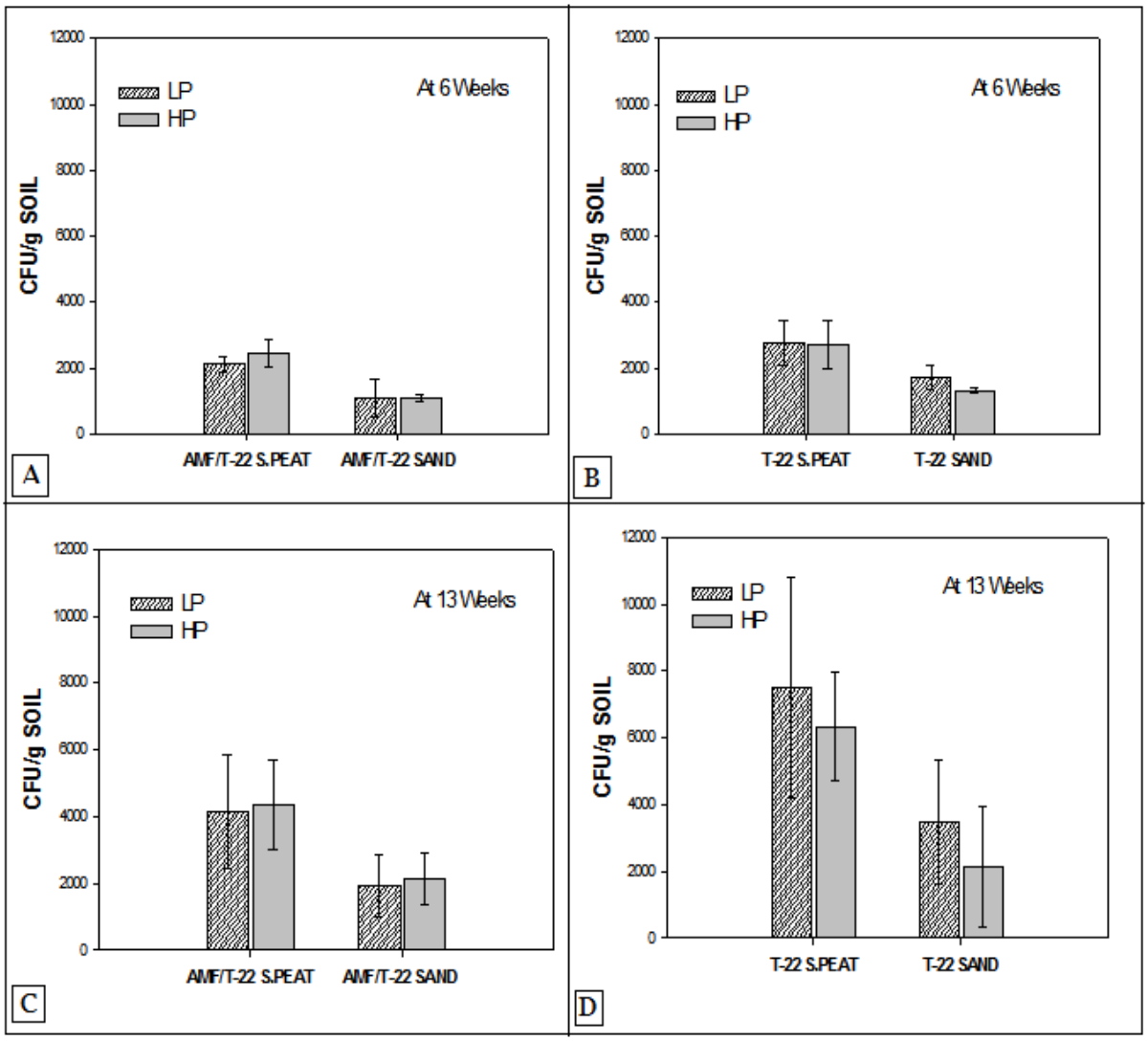

Fig. 5. Colony forming units (CFU) of T-22 as influenced by substrates, P and AMF. Included are the ± standard deviation; n= 5 and 8 at (A, B) 6 weeks and (C, D) 13 weeks respectively, after inoculation with T-22 alone or in combination with AMF (AMF/T-22) under the same substrate Sand or Sand peat and P levels (LP or HP) at 6 weeks and at 13 weeks.

Effect of P on Fusarium wilt development

From our investigations, we observed an unexpected influence of P on fusarium wilt symptom development. It was interesting to note that plants grown under low P did not develop the typical expected symptoms associated with Fusarium wilt. A prominent and distinctive symptom development was observed between plants under HP and LP. Plants under HP developed typical Fol symptoms of first yellowing then gradually wilting while those under low P directly wilted without yellowing.

This difference was clear across the whole data set regardless of the treatment or substrate. To demonstrate this, the scores of both the wilting and yellowing symptoms were fit into a logistic regression model showing the probability to get a score of 1, 2, 3 or 4 within the period of disease development.

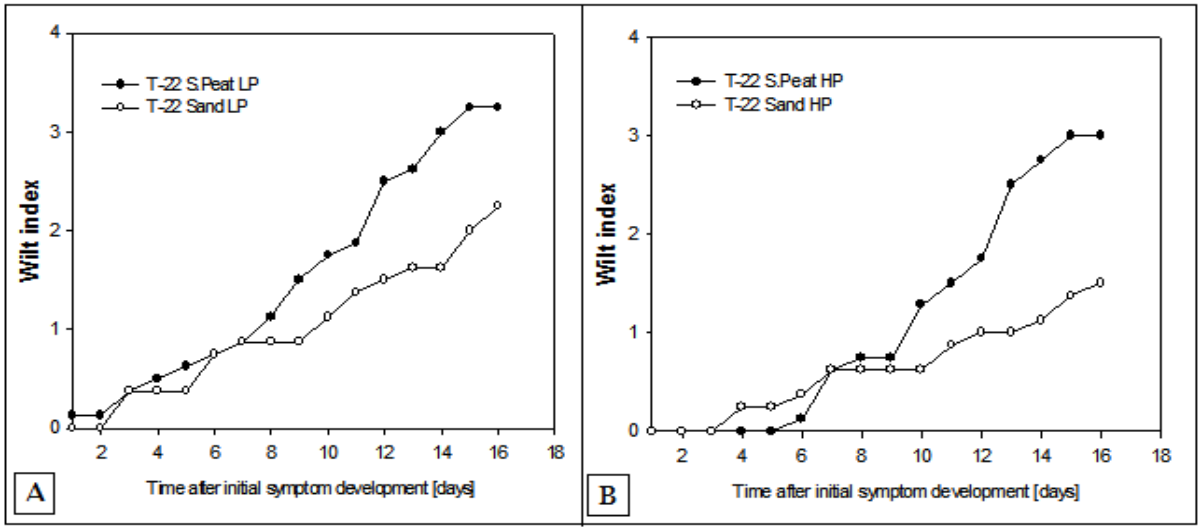

Fig. 6. Disease severity is influenced by substrate within the same P levels. The figure shows disease progress curves within 18 days for treatments with T-22, at (A) low phosphorous level ; (B) high phosphorous level.

The model was then able to show the disease progress curves of both wilting and yellowing and how this was influenced by the P level across the whole data set. This is best shown and illustrated here at the probability of getting a score of 3 (Fig. 9) and was clearly observed on plants (Fig. 10).

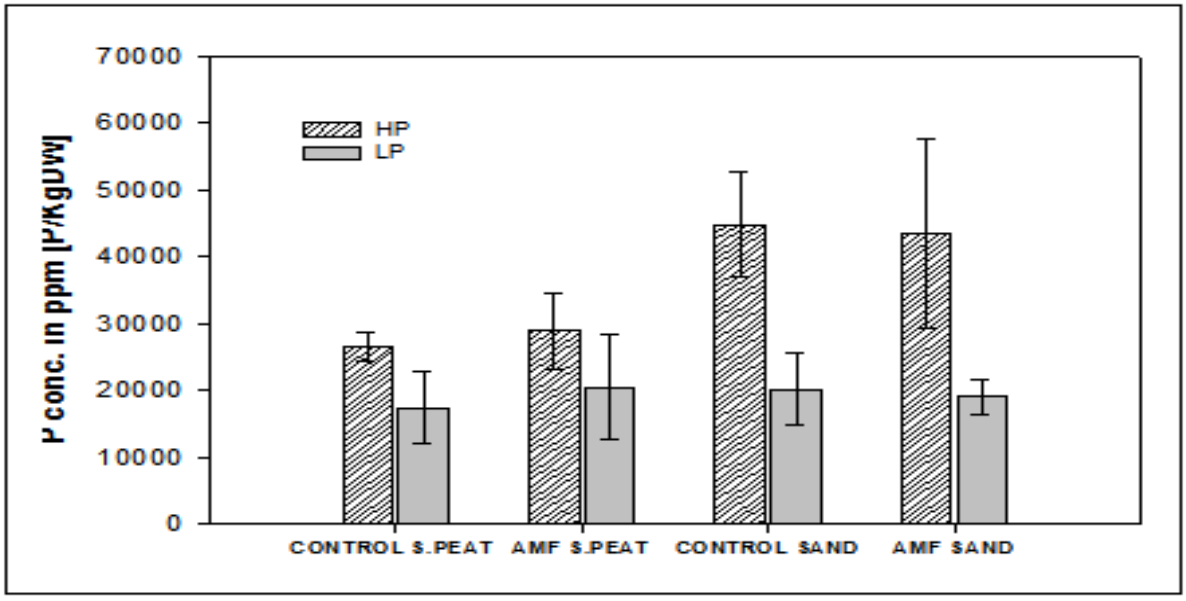

Fig. 7. The P concentration in plant tissue in treatments with high HP and LP level. Included are the ± standard deviation; n= 10 in treatments with AMF and without any biological control (control) under the same substrate sand or sand peat (S.Peat). Significant differences in HP and LP within the same treatment are indicated by (*) according to Tukey multiple range test (p-value ≤ 0.05).

Plant tissue analysis of control and AMF treatments was done to determine actual differences in P content for the treatments with HP and LP. The ANOVA showed interaction effects at P and substrate with no interactions at the treatment levels. This is shown in (Fig. 7) where HP has significant higher concentration levels than LP. The substrate interaction effect was brought out by a significant difference between the treatments in sand having higher concentrations than those in the sand peat at HP.

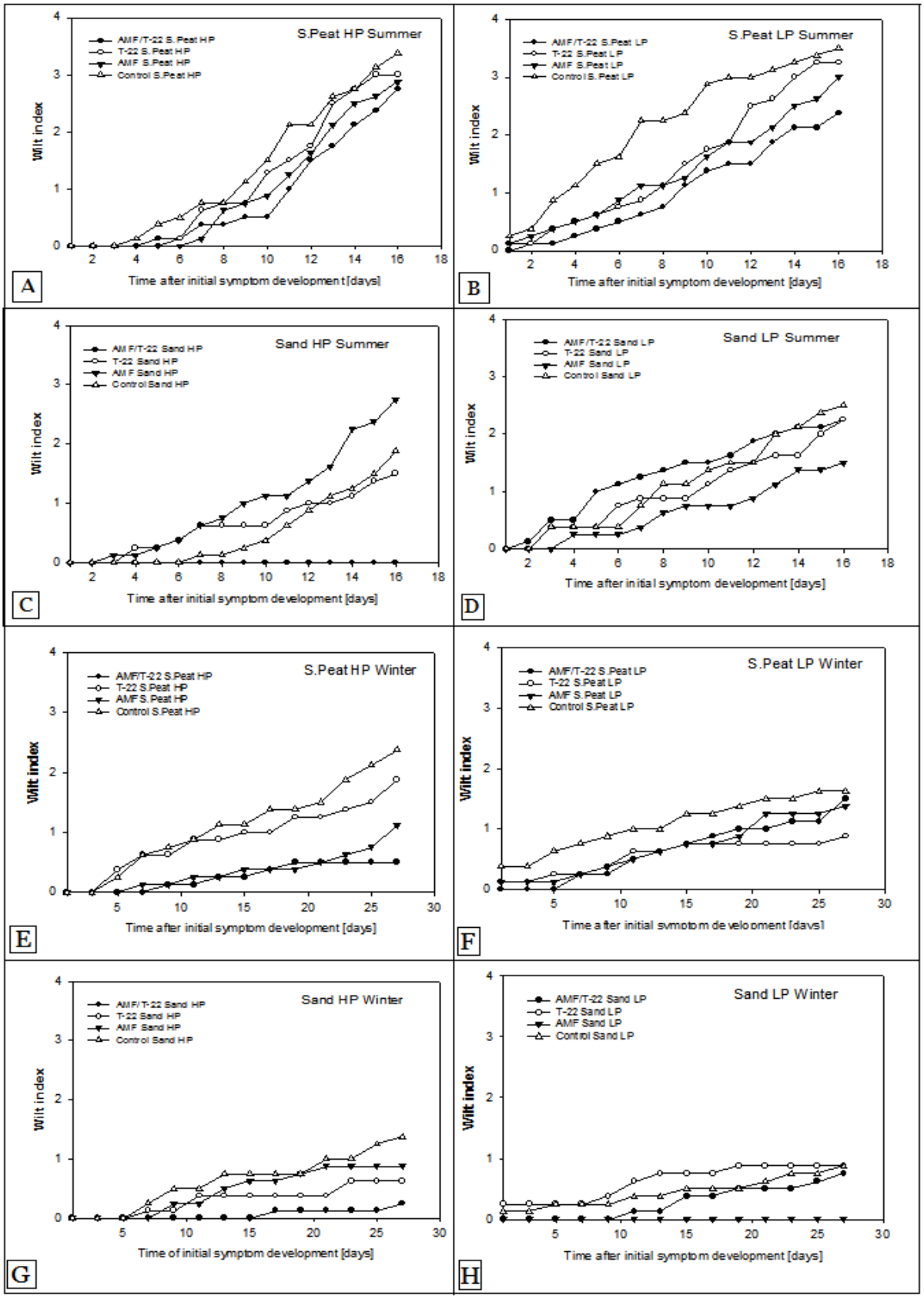

Fig. 8. The effect of AMF and T-22 inoculations on disease severity in summer and winter periods. The figure shows disease progress curves for treatments with AMF, T-22, their combination AMF/T-22 and without AMF or T-22 (control) as they occurred in summer (A, B, C, D) and winter (E, F, G, H) in treatments within the same substrate and P-levels.

To further ascertain the effect of P on Fusarium wilt symptom development and expression, a sub-experiment was set-up. A sub-set of treatments (AMF and control under substrate sand) were first grown under LP levels, inoculated with Fol and upon symptom development, the P level was then increased to the HP level. The idea was to increase the level of P to that of HP after the initial wilt symptoms were detected and observe whether these plants would start showing the yellowing symptoms with the P increase. The disease development period was not long enough i.e. 18 days from initial symptom development to maximum severity level to clearly show the effect of increasing the P level. Within that period, fertilization with the increased level of P had been done only two times. Nevertheless, the observations made towards the end of the experiment showed that, after increasing the level of P, plants began to wilt and started showing yellowing symptoms too.

Discussion

Synergistic effects of AMF and T-22 by dual inoculation against Fusarium wilt of tomato, with the aim to contribute to development of effective biological control strategies against soil-borne pathogens. Preliminary investigations on the influence of the two biological control agents on each other based on establishment were conducted and no antagonistic effects were observed. Establishment levels of T-22 and AMF were similar in the dual inoculation in comparison to their individual inoculation implying that both organisms have a non-competitive growth.

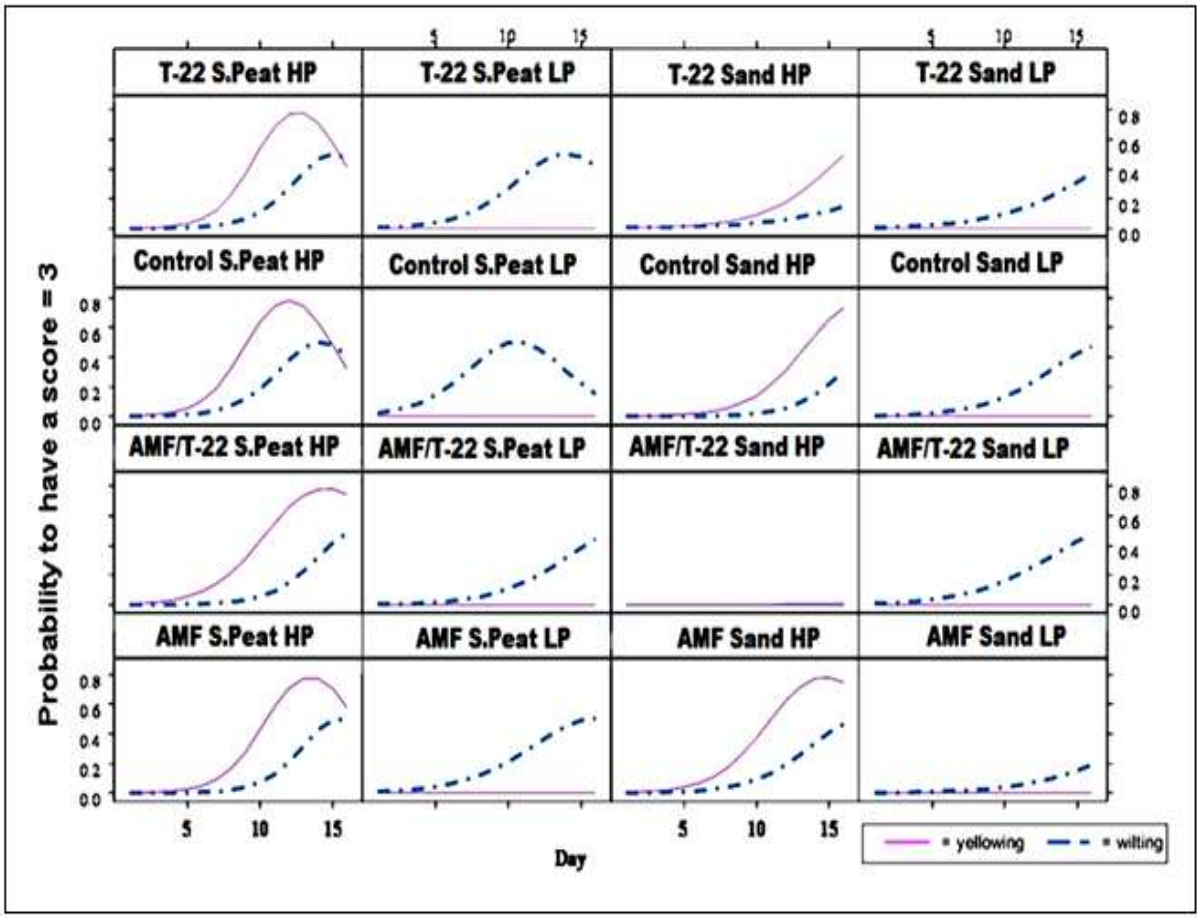

Fig. 7. A logistic regression model showing the distinct disease symptom expression (wilting and yellowing) as influenced by P levels. Different treatments starting from top to bottom which include; (a) T-22, (b) Control, (c) AMF/T-22 and (d) AMF. The columns indicates the P level and substrate starting from the left with; Sand Peat HP, Sand Peat LP, Sand HP and Sand LP. The straight line and the dotted lines represent the yellowing and wilting curves, respectively.

The trend of wilt symptom development shows that the combined treatments had lower severity levels than the individual treatments as well as the control.

This may be attributed to synergistic or additive effects between AMF and T-22. Synergy between mycorrhiza and *Trichoderma spp*. on enhancement of growth and biological control effects were reported earlier (Camprubi *et al*., 1995; Datnoff *et al*., 1995; Fillion *et al*., 1999).

The most significant and conspicuous observation was observed with sand the sand substrate with HP whereby the combined treatment of AMF/T-22 showed significantly minimal disease infection compared to the single inoculated treatments or the control. Re-isolation of the pathogen from the substrate of this particular treatment confirmed its presence, while the analysis of the pathogen incidence from the stems gained no growth on the selective agar media. This indicates mechanisms other than competition and mycoparasitism. Thus, a possibility of synergistic induction of resistance in the dual application could have played a key role which remains to be investigated. Cross-talk between AMF and *Trichoderma* during interactions with the host plant affects disease resistance through their influence on phytohormone synthensis or transport. Martinez-Medina *et al*. (2011) reported that the co-inoculation of AMF and *Trichoderma* in *Cucumis melo* increased the hormonal profile of SA and JA that plays a key role during plant resistance responses as compared when applied singly. Thus, from our observations, the AMF colonisation took place congruent to pathogen attack which combined with the presence of T-22 resulted in the minimal disease infection observed in the AMF/T-22 sand HP treatment. However, the results give no evidence that mycorrhiza was essentially responsible for that effect, as there was no difference in AMF colonisation compared to sand peat treatment. Also, the presence of T-22 in lower density in sand than in the sand peat also gives no real explanation for the increased disease suppression. Consequently, it would only suffice to conclude that other components and/or factors of that rhizosphere condition may have enhanced or complimented the defence mechanisms, the speculations that remain to be investigated.

The enhancement of the resistance levels caused by both AMF and T-22 towards attainment of high resistance has been investigated using *Trichoderma*, where transformants with over-expression of chitinase genes were shown to confer higher defence (Howell, 2003). Improved biological control of Fol by AMF has also been achieved by the combination of AMF with hormonal elicitors like salicylic acid and jasmonic acid (El-Khallal, 2007). On the other hand, research on the effect of soluble substances released by extra-radical mycelium of AMF (Fillion *et al*., 1999) showed evidence that these substances have some stimulatory effect on the growth of *Trichoderma*. So, the question would be whether there is also a possibility that AMF may enhance the production of lytic enzymes in *Trichoderma* which can be studied via molecular techniques. Furthermore, the speculative influence of T-22 and AMF on each other require also further investigations either by: a) comparing the dual application of AMF/T-22 to that of a *Trichoderma* transformant e.g. without chitinase with the assumption that it`s production would be the prominent mechanism of action leading to the highly induced mechanism in the dual application or, b) Comparing the resistance conferred by application of AMF/T-22 to that of AMF/JA-SA or to that of a transgenic resistant variety by monitoring the gene expression.

Our results showed that application of P influences Fol disease development as manifested by reduced symptom expression. This could not only be attributed to the function of P as an essential mineral element required by plants but also its indirect role in mitigating disease development. Thus, healthy and vigorous root system will better compensate for infections especially by roots pathogens. Plants under low P application exhibited higher disease severity and wilted directly without the typical Fol symptom development process involving yellowing of leaves as observed under high P. P treatment was shown to

influence the population of Fusarium spp. by affecting the structural population density, virulence and disease incidence levels (Woltz and Jones, 1973; Yergeau *et al.*, 2006). Yergeau *et al.* (2006) reported the effects of varying P regimes on Fusarium crown and root rot of *Asparagus officinalis*, where disease incidence was significantly low for plants under low P. Direct wilting of plant under low P regime further shows the essential role of P during the plant's metabolic processes. As an essential mineral element that functions as plant structural compound, P is shown also to act as a catalyst in various biochemical reactions in plants (Marschner, 1995; Raghothama, 1999; Raghothama, 2000; Walters and Bingham, 2007). Under optimal supply, P is taken up in the form of orthophosphate (Pi) and translocated into the cytoplasm (Jain *et al.*, 2005). However, under low P supply in the soil, the intracellular Pi can be reduced significantly in the cell affecting the supply of key energy carrier molecules such as ATP/ADP that are essential for plant cellular biochemical processes such as photosynthesis (Marschner, 1995; Rao, 1997; Raghothama, 1999; Jain *et al.*, 2005). Low Pi concentration in the cytoplasm affects photosynthetic process by suppressing the expression of light-regulated *psbO* and *psbP* genes that encode oxygen-evolving proteins of PSII complex (Jain *et al.*, 2005). Thus, due to the suppressive effects of P deficiency in the cells, physiological adaptive changes that culminate to hampered photosynthesis occurred leading to direct wilting without yellowing.

Fol infection was shown to limit the photosynthetic rates of tomato leaves by decreasing the light-saturated rate of CO_2 assimilation accompanied by decreased maximum carboxylation velocity and in turn reducing the maximum quantum efficiency of PS II (Nogues *et al.*, 2002).

Results on plant tissue analysis showed that the concentration of P was significantly reduced in plants grown under low P as compared to high P infected with Fol. Pi is critical for energy metabolism in the host plant which implies that processes such as photosynthetic activity were interfered with.

Yellowing of leaves is usually associated with chlorophyll breakdown, which is an elaborate and highly energy consuming process (Matile *et al.*, 1996). Since plants with low P are expected to have a lower metabolic energy due to the significantly reduced photosynthesis, this process would cause a physiological blocking of the chlorophyll breakdown in a bid to save energy, resulting in expression of Fusarium symptoms only by wilting.

The role of P was further shown by replenishing the LP plants with additional P in order to attain the HP status but after the onset of initial symptoms. Interestingly, a similar process of direct wilting as for the plants under HP was observed where plants first showed the yellowing symptoms (data not shown). Thus, P application delayed wilting symptoms as observed also with AMF treatment. Previous studies showed that the effects of low Pi on photosynthesis can be reversed by increasing the levels of P to the optimum (Rao, 1997; Jain *et al.*, 2005). Moreover, Jain *et al.*, (2005) observed that increased P content lead to the up-regulation of the genes associated with photosynthesis that had initially been down regulated due to P deficiency. Precise mechanisms underlying the effects of P on Fusarium symptom expression due to P deficiency is not clearly understood. Molecular and proteomic tools could possibly be used in future studies to understand the cellular metabolic activities ensued upon Fusarium infection, where potential genes or protein components that most likely support the possible physiological adaptive mechanisms could be characterized.

Conclusion

Results of our investigations shows that AMF and T-22 can co-exist and interact with each other in the rhizosphere without having any detrimental or antagonistic effects on their establishment. Furthermore, we report also the synergistic effects of AMF and T-22 against the soil-borne pathogen Fol which contribute to development of soil borne disease management strategies. Application of AMF in combination with other biological control organisms can further be studied to reduce their variability in

efficacy and determine the appropriate application rates.

This has also been shown in several other studies with AMF, whose effects are shown to be enhanced either by combining with biological control organisms or elicitors like salicylic acid and jasmonic acid. However, interacting factors that affect and manipulate their efficacy remain to be elucidated in order to exploit the full potential of the biological control organisms with the keen interest of conserving the environment by reducing pesticide use in cropping systems. Our study shows that substrates, P nutrition as well as environmental conditions influences the establishment, growth and development of the biological control agents used as well as that of the pathogen.

These findings can be used in predicting how these biological control agents would develop under certain conditions that could be used to understand the functioning of different biological systems, hence facilitating development of highly specific and reliable biological control strategies.

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References

Akköprü A, Demir S. 2005. Biological control of Fusarium wilt in tomato caused by *Fusarium oxysporum* f.sp. *lycopersici* by AMF *G. intraradices* and some *Rhizobacteria*. Journal of Phytopathology **153,** 544-550.

Azcon-Aguilar C, Bago B. 1994. Physiological characteristics of host plant promoting an undisturbed functioning of the mycorrhizal symbiosis. In impact of arbuscular mycorrhizas on sustainable agriculture and natural ecosystems. S. Gianinazzi and H. Schüepp (eds) p 61-67. Birkhäuser Verlag Basel/Switzerland.

Azcon-Aguilar C, Barea JM. 1996. Arbuscular mycorrhizas and biological control of soil-borne plant pathogens –an overview of the mechanisms involved. Mycorrhiza **6,** 457-464.

Backhaus GF. 1984. Untersuchungen zur Nutzung der endotrophen (VA) Mykorrhiza in der gärtnerischen Pflanzenproduktion. PhD Thesis, Leibniz University Hannover.

Bulluck III, LR, Ristaino JB. 2002. Effect of synthetic and organic soil fertility amendments on southern blight, soil microbial communities and yield of processing tomatoes. Phytopathology **92,** 181-189.

Camprubi A, Calvet C, Estaun V. 1995. Growth enhancement of *Citrus reshni* after inoculation with *G. intraradices* and *Trichoderma aureoviride* and associated effects on microbial populations and enzyme activity in potting mixes. Plant and Soil **173,** 233-238.

Cordier C, Pozo MJ, Barea JM, Gianinazzi S, Gianinazzi-Pearson V. 1998. Cell defence responses associated with localized and systemic resistance to *Phytophthora parasitica* induced in tomato by an arbuscular mycorrhizal fungus. Molecular Plant Microbe Interactions **11(10),** 1017-1028.

Datnoff LE, Nemec S, Pernezny K. 1995. Biological control of Fusarium crown and root rot of tomato in Florida using *Trichorderma harziunum* and *G. intraradices*. Biological Control **5,** 427-431.

Dehn HW, Backhaus GF. 1986. The use of vesicular-arbuscular mycorrhizal fungi in plant production. I. Inoculum production. Journal of Plant Diseases and Protection **93,** 415–424.

DI Pietro A, Madrid MP, Caracuel Z, Delgado-Jarana J, Roncero MIG. 2003. *Fusarium oxysporum*: exploring the molecular arsenal of a vascular wilt fungus. Molecular Plant Pathology **4(5),** 315-325.

EL-Khallal SM. 2007. Induction and modulation of resistance in tomato plants against Fusarium wilt disease by bioagent fungi (arbuscular mycorrhiza) and/or hormonal elicitors (Jasmonic Acid & Salicylic Acid): 2-changes in the antioxidant enzymes, phenolic compounds and pathogen related- proteins. Australian Journal of Basic and Applied Sciences, **1(4),** 717-732.

Fillion M, St-Arnaud M, Fortin JA. 1999. Direct interaction between the arbuscular mycorrhizal fungus *Glomus intradices* and different rhizosphere microorganisms. New Phytology **141,** 525-533.

Fravel D, Olivain C, Alabouvette C. 2003. *Fusarium oxysporum* and its biological control. New Phytologist **157,** 493-502.

Garcia C, Pascual JA, Mena E, Hernandez T. 2004. Influence of the stabilization of organic matter on their biopesticide effect in soils: Bio-resource Technology **95,** 215-221.

Garcia-Garrido JM, Ocampo JA. 2002. Regulation of plant defence response in arbuscular mycorrhizal symbiosis. Journal of Experimental Botany **53,** 1377-1386.

Gericke S, Kumries B. 1952. Die Kolorimetische phosphosaurebestimmung mit ammonium-Vandat-Molybdat und ihre Anwenduny in der Pflanzensnslyze. Z. Phanzenehrung Dungung Bodenkude **59,** 235-247.

Godeas A, Fracchia S, Mujica MT, Ocampo JA. 1999. Influence of soil impoverishment on the interaction between *Glomus mosseae* and saprobe fungi. Mycorrhiza **9,** 185-189.

Gordon TR, Martyn RD. 1997. The evolutionary biology of *Fusarium oxysporum*. Annual Review Phytopathology **35,** 111-128.

Grunewaldt-Stöcker G. 1994. Zum Einfluß von *Acremonium ochraceum* auf die Xylementwicklung in Tomatenpflanzen. Mitt. a. d. Biol. Bundesanst. **301,** 135.

Guetsky R, Dinoor A, Shtienberg D. 1998. Micro-organisms combinations for the biological control of gray mold (*Botrytis cinerea*) in strawberries. Phytoparasitica **26,** 174.

Guetsky R, Shtienberg D, Elad Y, Dinoor A. (2001). Combining biological control agents to reduce variability of biological control. Phytopathology **91,** 621-627.

Handelsman JO, Stabb EV. 1996. Biological control of soilborne plant pathogens. The Plant Cell **8,** 1855-1869.

Harman GE. 2000. Myths and dogmas of biological control: changes in perceptions derived from research on T. harzianum T-22. Plant Disease **84,** 377-393.

Harman GE, Howell CR, VIterbo A, Chet I, Lorito M. 2004. *Trichoderma* species-opportunistic, avirulent plant symbionts. Nature Review Microbiology **2,** 43-56.

Hewitt EF. 1966. Sand and Water Culture Methods Used in the Study of Plant Nutrition, Commonw. Agric. Bur. Tech. Comm. 22, 2nd Ed., England.

Hoitink HAJ, Boehm M.J. 1999. Biological control within the context of soil microbial communities: A substrate-dependent phenomenon. Annual Rev. Phytopathology **37,** 427-46.

Hoitink HAJ, Madden LV, Dorrance AE. 2006. Systemic resistance induced by *Trichoderma* spp.: interactions between the host, the pathogen, the biological control agent and soil organic matter quality. Phytopathology **96,** 186-189.

Howell CR. 2003. Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: The history and evolution of current concepts. Plant Diseases **87,** 4-10.

Jain A, Cao A, Karthikeyan AS, Baldwin JC, Raghothama KG. 2005. Phosphate deficiency suppresses expression of light-regulated psbO and psbP genes encoding extrinsic proteins of oxygen-evolving complex of PSII. Current Science **9,** 1592-96.

Kiirika LM, Stahl F, Wydra K. 2013. Phenotypic and molecular characterization of resistance induction by single and combined application of chitosan and silicon in tomato against *Ralstonia solanacearum*. Physiolological and Molecular Plant Pathology **81**, 1-12. http://dx.doi.org/10.1016/j.pmpp.2012.11.002.

Kiirika LM, Bergmann HF, Schikowsky C, Wimmer D, Korte J, Schmitz U, Niehaus K, Colditz F. 2012. Silencing of the Rac1 GTPase MtROP9 in M. truncatula stimulates early mycorrhizal and oomycete root colonizations but negatively affects rhizobial infection. Plant Physiology **159,** 501-516. http://dx.doi.org/10.1104/pp.112.193706

Kok CJ, Hageman PWT, Postma MJ, Roozen NJM, Van Vuurde JWL. 1996. Processed manure as carrier to introduce *Trichoderma harzianum*: Population dynamics and biological control effect on *Rhizoctonia solani*. Biological control Science and Technology **6,** 147-161.

Komada H. 1975. Development of a selective medium for quantitative isolation of *Fusarium oxysporum* from natural soil. Review Plant Protection Research **8,** 114-124.

Larkin RP. Fravel DR. 1998. Efficacy of various fungal and bacterial biological control organisms for control of Fusarium wilt of tomato. Plant Diseases **82,** 1022-1028.

Larkin RP, Fravel DR. 2002. Effects of varying environmental conditions on biological control of Fusarium wilt of tomato by non-pathogenic *Fusarium* spp. Phytopathology **92,** 1160-1166.

Lewis JA, Papavizas GC. 1984. A New approach to stimulate population proliferation of *Trichoderma* species and other potential biological control fungi introduced into natural soils. Phytopathology **74,** 1240-1244.

Linderman RG. 1994. Role of VAM fungi in biological control. In F.L. Pfleger and R.G. Linderman (eds.), Mycorrhiza and plant health. p 1-26. St Paul, Minnesota, USA, APS Press.

Lucas P. 2006. Diseases caused by soil-borne pathogens. In B. M. Cooke, D. Gareth Jones and B. Kaye (eds.), The Epidemiology of Plant Diseases, 2nd edition, p 373–386. Springer publishers, Printed in the Netherlands.

Marschner H. 1995. Mineral Nutrition of Higher Plants. London, UK: Academic Press.

Marinez-Medina A, Roldan A, Albacete A, Pascual JA. 2011. The interaction with arbuscular mycorrhizal fungi or T. harzianum alters the shoot hormonal profile in melon plants. Phytochemistry **72 (2-3),** 223-229. http://dx.doi.org/10.1016/j.phytochem.2010.11.008

Matile P, Hortensteiner S, Thomas H, Krautler B. 1996. Chlorophyll breakdown in senescent leaves. Plant Physiology **11(2),** 1403-1409.

Noble R, Coventry E. 2005. Suppression of soil-borne plant diseases with composts: a review. Biological control Science and Technology **15,** 3-20.

Nogues S, Cotxarrera L, Leonor A, Trillas MA. 2002. Limitations to photosynthesis in tomato leaves induced by Fusarium wilt. New Phytologist **154,** 461-470.

Ozbay M, Newman SE. 2004. Fusarium crown and root rot and control methods. Plant Pathology Journal **3(1),** 9-18.

Pal KK, Gardener BM. 2006. Biological control of

plant pathogens. The Plant Health Instructor http://dx.doi.org/10.1094/PHI-A-2006-1117-02.

Parniske M. 2008. Arbuscular mycorrhiza: the mother of plant root endosymbioses. Nature reviews microbiology **6,** 763-775. http://dx.doi.org/10.1038/nrmicro1987.

Pozo MJ, Dumas-Gaudot E, Slezack S, Cordier C, Asselin A, Gianinazzi S, Gianinazzi-Pearson V, Azcon-Aguilar C, Barea JM. 1996. Induction of new chitinase isoforms in tomato roots during interactions with *Glomus mosseae* and/or *Phytophthora nicotianae* var *parasitica*. Agronomie **16,** 689-697.

Pozo MJ, Azcon-Aguilar C. 2007. Unravelling mycorrhiza-induced resistance. Current Opinion in Plant Biology **10,** 393-398.

Rust KM. 2010. The changing role of insecticides in structural pest control. Haye's Handbook of pesticide toxicology (3rd edition) p 257-270. http://dx.doi.org/10.1016/B978-0-12-374367-1.00005-7

Pozo MJ, Verhage A, García-Andrade J, García JM, Azcón-Aguilar C. 2009. Priming plant defence against pathogens by arbuscular mycorrhizal fungi. C. Azcón-Aguilar *et al.* (eds.), Mycorrhizas - Functional Processes and Ecological Impact p 123-135 © Springer-Verlag Berlin Heidelberg.

Raghothama KG. 1999. Phosphate acquisition. Annual Review Plant Physiology and Plant Molecular Biology **50,** 665-93. http://dx.doi.org/10.1146/annurev.arplant.50.1.665

Raghothama KG. 2000. Phosphate transport and signaling. Current Opinion in Plant Biology **3**:162-l87.

Rao M. 1997. The role of phosphorus in photosynthesis. In Handbook of Photosynthesis (ed. Pessarakli, M.), p 173-194. Marcel Dekker, New York.

Spadaro D, Gullino ML. 2005. Improving the efficacy of biological control agents against soilborne pathogens. Crop Protection **24,** 601-613.

Sweeney DW, Granade GV, Eversmeyer MG, Whitney DA. 2000. Phosphorus, potassium, chloride, and fungicide effects on wheat yield and leaf rust severity. Journal of Plant Nutrition **23,** 1267-1281.

Termorshuizen AJ, Jeger MJ. 2008. Strategies of soilborne plant pathogenic fungi in relation to disease suppression. Fungal Ecology **1,** 108-114.

Vazquez MM, Cesar S, Azcon R, Barea JM. 2000. Interactions between arbuscular mycorrhizal fungi and other microbial inoculants (*Azospirillum, Pseudomonas*, Trichorderma) and their effects on microbial populations and enzyme activities in the rhizosphere of maize plants. Applied Soil Ecology **15,** 261-272.

Vierheilig H, Coughlan AP, Wyss U. 1998. Ink and vinegar, a simple staining technique for arbuscular-mycorrhizal fungi. Applied Enviroonmental Microbiology **64,** 5004-5007.

Vierheilig H, Steinkellner S, KHaosaad T, Garcia-Garrido JM. 2008. The biological control effect of mycorrhization on soilborne fungal pathogens and the autoregulation of the AM symbiosis: One mechanism, two effects? A. Varma (ed) Mycorrhiza p 307-320 Springer-Verlag Berlin Heidelberg.

Walters DR, Bingham IJ. 2007. Influence of nutrition on disease development caused by fungal pathogens: implications for plant disease control. Annals of Applied Biology **151,** 307-324.

Whipps JM. 2001. Microbial interactions and biological control in the rhizophere. Journal of Experimental Botany **52,** 487-511.

Woltz SS, Jones JP. 1973. Tomato Fusarium wilt

control by adjustments in soil fertility. Proceedings Florida State Horticultural Society **86,** 157-159.

Yergeau E, Sommerville DW, Maheux E, Vujanovic V, Hamel C, Whalen JK, St-Arnaud M. 2006. Relationship between Fusarium population structure, soil nutrient status and disease incidence in field-grown asparagus. FEMS Microbiology Ecology **58(3),** 394-403. http://dx.doi.org/10.1111/j.1574-6941.2006.00161.x.

Yigit F, Dikilitas M. 2007. Control of Fusarium wilt of tomato be combination of fluorescent Pseudomonas, non-pathogen Fusarium and *Trichoderma harziunum* T-22 in greenhouse conditions. Plant Pathology **6,** 159-163.

Balzergue C, Chabaud M, Barker DG, Bécard G, Rochange SF. 2013. High phosphate reduces host ability to develop arbuscular mycorrhizal symbiosis without affecting root calcium spiking responses to the fungus. Front in Plant Science**4,** 426. http://dx.doi.org/10.3389/fpls.2013.00426.