



Comparison of AMF and PGPR inoculants for the suppression of *Verticillium* wilt of strawberry (*Fragaria* × *ananassa* cv. Selva)

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Abstract

Verticillium wilt, caused by *Verticillium dahliae*, is widespread in intensive strawberry (*Fragaria* × *ananassa*) growing areas. It is effectively controlled in site preparation by soil fumigation with methyl bromide but with the ban on ozone-depleting chemicals, which applies to methyl bromide, there has been an international effort to find alternatives, which will protect the crop from planting to fruit harvest and in runner production. These include chemical replacements and alternative disease control strategies. In this study, biological control, based on single and dual inoculation with a commercial arbuscular mycorrhizal inoculant containing *Glomus* spp. and a commercial plant growth promoting rhizobacterial inoculant containing a *Bacillus* sp., has been evaluated in the field. Control plants inoculated with *V. dahliae* had wilt symptoms, and marketable fruit yield was significantly reduced by approximately 60% whereas the yield of plants from runners inoculated with the AMF or PGPR inoculants did not differ significantly from that of the non-inoculated controls. Generally, marketable fruit size and plant and root fresh weight were not significantly different in the inoculated plants compared with the non-inoculated controls. There were no significant differences in the protection afforded by the AMF and PGPR inoculants and dual inoculation did not give greater protection than single inoculation.

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Keywords: *Bacillus subtilis*; Biological control; *Glomus mossae*; *Glomus intraradices*

Abbreviations: AMF, arbuscular mycorrhizal fungi; MALDI-TOF, matrix-assisted laser desorption ionization-time-of-flight mass spectrometry; PGPR, plant growth promoting rhizobacteria; PPF, photosynthetic photon flux

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1. Introduction

Worldwide, strawberry production in 2002 according to the Food and Agriculture Organization of the United Nations (FAO, 2003) was estimated at over 3 million metric tonnes with 1.07 million metric tonnes

produced in the European Union and Accession States and 0.91 million metric tonnes produced in the North American Free Trade Area. These figures, however, mask the regional concentration of the industry where 0.76 million tonnes are produced in the USA (70% in California and 30% in Florida), and 0.33 and 0.2 million tonnes produced in Spain and Poland, respectively. The production of strawberry planting material (runners) and fruit is heavily dependent on the use of methyl bromide as a soil fumigant in site preparation but there is no fungicide for control of the disease in the growing crop (Maas, 1998; Hancock, 1999). Methyl bromide is being phased out under the Montreal Protocol on ozone-depleting chemicals (Ristaino and Thomas, 1997). There have been intensive efforts to find alternatives to methyl bromide in strawberry production with much of the research focusing on chemical alternatives (Duniway, 2002). The replacement chemicals, however, have tended to be less efficacious and to be influenced by soil type (Csinos et al., 2002). Many have not been approved for use on strawberry or are themselves under investigation for safety (Saethre et al., 1999). While plant breeding, including genetic engineering, may provide resistant cultivars in the future, these approaches are longer term, and in the case of genetic engineering, are currently facing consumer resistance in some markets.

Organic production of strawberry has been successful in California where the reduction in yield is compensated for by the price premium in the fresh strawberry market. While organic production is an expanding market, it may be limited to the niche sector for fresh strawberries and not applicable for the mass market (ca. 70% of production) or the processing sectors (ca. 30% of production). Nevertheless, its expansion suggests that disease in strawberry may be at least partially controlled by a combination of solarization (Hancock, 1999), crop rotation and promotion of antagonistic soil microorganisms (Ristaino and Thomas, 1997; LaMondia et al., 2002). Solarization is not universally applicable, e.g. to the high altitude areas where strawberry runner production takes place, and has been reported to be ineffective in controlling *Verticillium* wilt in strawberry in successive years (Hartz et al., 1993). Crop rotation poses problems related to the availability of equivalent high value crops for the rotation. There is also a problem in the transition from the use of methyl

bromide to the establishment of a biologically buffered, pathogen suppressive soil. It has been estimated that crop losses may be up to 35% in the first year following the withdrawal of methyl bromide (USDA, 2000).

Verticillium wilt of strawberry, caused by *Verticillium dahliae*, is widespread in strawberry growing regions and is particularly severe in areas such as Greece, where the plants can be stressed by sudden changes in temperatures in spring (Maas, 1998). Crop rotation, solarization, planting of certified *Verticillium*-free runners and the use of partially resistant varieties (Tahmatsidou et al., 2002) contribute to disease control. In this study, commercial AMF and PGPR inoculants to control *Verticillium* wilt in the growing crop were compared in support of the latter strategies. The hypothesis that additive effects may be obtained by dual inoculation was investigated also.

2. Materials and methods

2.1. Plant material

Conventional runners were obtained from certified plants of *Fragaria × ananassa* Duch. ‘Selva’ grown outdoors in the National Agricultural Research Foundation (NAGREF) facilities in Thessaloniki, Greece. ‘Selva’ is the main commercial strawberry variety grown in the trials area but is susceptible to *Verticillium* disease.

2.2. *Bacillus* inoculation

Commercial *Bacillus* inoculum was obtained from FZB Biotechnik GmbH (Berlin, Germany) as a dry mixture of corn starch, skimmed milk powder and glycerol with freeze dried spores of the non-pathogenic microorganism *Bacillus subtilis* FZB24[®]. To confirm spore viability and counts, 0.01 g of *B. subtilis* FZB24[®]-WG was suspended in 100 ml of sterile distiller water. Three dilutions of 1:100 each were made. One millilitre of each dilution was placed in sterile petri dishes and mixed with 15 ml of cooled, molten (approximately 40 °C) potato dextrose agar (PDA; Oxoid, Basingstoke, UK) medium, which was allowed to solidify (two plates per dilution per medium). The plates were incubated at

23 °C for 24 h and bacterial colonies were then counted. Single colonies were stained for Gram and catalase reactions and the presence of endospores and further characterized by MALDI-TOF (see below).

For inoculation, runners were dipped in 0.1% (w/v) *B. subtilis* FZB24[®]-WG suspension in warm (approximately 40 °C) SDW for 10 min. Plants were watered with an additional 0.02% (w/v) *B. subtilis* FZB24[®]-WG suspension in warm (approximately 40 °C) SDW 7 days after planting in raised beds; 50 ml of *B. subtilis* suspension was applied per plant.

2.3. Preparation of *Bacillus* material for MALDI-TOF analysis

Mass spectral analysis of membrane proteins of whole bacteria were obtained using a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (Kratos Analytical, Manchester, UK) in the linear mode at an accelerating voltage of 20 kV using a 3 µs delay time. *Bacillus* spp. were grown on TSA plates for 24 h prior to analysis. Cultures stored at -40 °C were passaged twice on TSA to obtain isolated colonies. Colonies were then removed from the plates using sterile disposable plastic loops and suspended in 1 µl of the matrix (α -cyano-4-hydroxycinnamic acid (HCCA), in acetonitrile–0.1% trifluoroacetic acid 70:30, v/v). The 20 well sample slide was prepared by flushing each well with the matrix. Then, 0.5 µl of the bacteria/matrix suspension was placed in the well and allowed to air dry. Insulin chain B oxidized (3494 Da), insulin (5730 Da) and apomyoglobin (16,951 Da) (Sigma Chemical Co., Dublin, Ireland) were used as molecular weight standards. Each sample was analyzed using linear, positive ion mode with a pulsed extraction of 7000. The laser power was set to just above the threshold of ionization, which allowed peaks to be detected over the mass range of interest (500–10,000 Da). Each spectrum consisted of the ions from 200 laser shots. Spectra were compared with a reference *Bacillus* database.

2.4. Mycorrhizal inoculation

Commercial mycorrhizal inoculum 'Vaminoc-G[®]' containing spores of *Glomus mossae* and *Glomus intraradices* (John Hooker, personal communication)

was obtained from MicroBio Ltd. (Harpenden, Herts, UK). For inoculation of the runners, 6 g of Vaminoc[®] were added to the planting hole in the raised beds, ensuring that good contact was achieved with runner roots. Root samples for mycorrhizal staining were taken once during plant culture and at the end of the culture period. Sampled roots were cut to approximately 2 cm lengths and stained with trypan blue solution according to the method of Phillips and Hayman (1970) for hyphae, arbuscule and vesicle observation using a binocular microscope with 400× magnification.

2.5. Dual inoculation

Roots of runners were dipped in 0.1% (w/v) *B. subtilis* FZB24[®]-WG suspension in warm (approximately 40 °C) SDW for 10 min. Six grams of Vaminoc-G[®] were added into the planting hole in the raised beds ensuring that good contact was achieved with the plant roots. Plants were watered with an additional 50 ml per plant of 0.02% (w/v) *B. subtilis* FZB24[®]-WG suspension in warm (approximately 40 °C) SDW, 7 days after planting in raised beds.

2.6. Pathogen inoculum

The isolate of *V. dahliae* used was a local isolate obtained from the Benakio Phytopathological Institute, Athens, Greece and was grown in the dark at 23 °C on potato dextrose agar (Sigma Chemical Co., Dublin, Ireland) plates. Stock cultures were grown in the dark on potato dextrose agar and transferred to fresh medium every 10 weeks. For inoculum production, mycelial blocks (0.5 cm × 0.5 cm) were taken from 10-day old cultures and transferred to 100 ml of Sinha and Wood (1968) medium and incubated on an orbital shaker at 23 °C at 120 rpm for 7 days. The liquid cultures were homogenized and diluted to 10⁷ conidia/ml. Liquid inoculum (200 ml) was added to a mixture of sand and corn flour (95:5, v/v) (Paplomatas, 1991) that had been autoclaved at 121 °C, 105 kPa for 1 h in 750 ml aluminum containers. The inoculum was incubated at 23 °C for 10 days. In the disease challenge, 10 ml of solid inoculum was used to inoculate each plant in the raised bed field trials. *V. dahliae* inoculum was added to a hole made in

the substrate near the stem, ensuring that good contact was achieved with the plant roots. Based on preliminary studies, inoculation was carried out 2 weeks after planting. Plants were regularly examined for disease symptoms (Maas, 1998; Hancock, 1999) and stem sections of infected and control plants were cultured on PDA medium in pathogen re-isolation studies. Symptoms were scored on the basis of a phenotypic resistance scale from 1 to 5 where 1 was no symptoms, 2 was 25% decrease of the foliage, 3 was 50% decrease of the foliage, 4 was 75% decrease of the foliage and 5 was plant death.

2.7. Field trials

The field trials took place in raised beds at the National Agricultural Research Foundation in Thessaloniki, Greece. The soil was disinfected with SOBROM™ (active ingredients: methyl bromide 98% and cloropicrin 2%) to eliminate in native inoculum and two row beds were formed. Plastic drip irrigation was laid down the middle of the bed, which was covered with black plastic mulch. Bayleton® (active ingredient: triadimefon) was applied to control grey mould. For the raised bed trials, 10 plants were used for each treatment in a complete randomized design. A spacing of 30 cm between rows and between plants was used.

2.8. Data collection and analysis

Fruits were harvested when ripe. Mean marketable fruit yield per plant (MFYP) and mean marketable fruit weight (MFW) were determined. Marketable fruits were fruits weighing at least 8 g. After 8 months in cultivation, plants were uprooted and plant fresh weight (PFW) and root fresh weight (RFW) were

estimated. Data were analyzed using the Kruskal–Wallis test ($P = 0.05$).

3. Results

3.1. Inoculation with FZB24®-WG

Marketable fruit yield of runner-derived plants of ‘Selva’ was significantly reduced by inoculation with *V. dahliae*, this was associated with *Verticillium* wilt symptoms in the plants and by re-isolation of the pathogen. The yields of AMF-inoculated (FZB24®-WG inoculated) and AMF-inoculated (FZB24®-WG inoculated) *V. dahliae*-challenged plants was increased compared to the non-inoculated controls but the difference was not significant (Table 1). Marketable fruit weight did not vary significantly between the control and treatments. Plant fresh weight and root fresh weight were reduced by approximately 40% in the *V. dahliae*-challenged plants which showed disease symptoms and increased by from 36 to 91% in the other treatments which were symptomless, these differences were not significant.

3.2. Inoculation with Vaminoc-G®

Marketable fruit yield was significantly reduced in control plants derived from ‘Selva’ runners by challenge inoculation with *V. dahliae* (Table 2). There was no difference in the mean weight of the marketable fruit (Table 2). Marketable fruit yield, while increased by approximately 20% in the AMF-inoculated, and AMF-inoculated + *V. dahliae*-challenged treatments, the increase was not significantly different from the non-inoculated controls.

Table 1

Evaluation of *Bacillus subtilis* FZB24®-WG, a commercial PGPR inoculant, for the suppression of *Verticillium* wilt in plants derived from runners of *F. × ananassa* ‘Selva’ in a raised bed trial

| Treatments | Plants derived from runners | | | |
|--|-----------------------------|---------|---------|---------|
| | MFYP (g) | MFW (g) | PFW (g) | RFW (g) |
| Control | 285.2a | 16.6a | 104.7ab | 10.0ab |
| + <i>Bacillus subtilis</i> FZB24®-WG | 367.9a | 17.2a | 200.0a | 20.4a |
| + <i>Bacillus subtilis</i> FZB24®-WG + <i>V. dahliae</i> | 300.7a | 17.6a | 117.9b | 16.3a |
| <i>V. dahliae</i> | 94.7b | 16.6a | 73.2b | 6.8b |

Treatments sharing a common letter in the same column are not significantly different ($P = 0.05$), using the Kruskal–Wallis test.

Table 2

Evaluation of Vaminoc-G[®], a commercial AMF inoculant, for the suppression of *Verticillium* wilt in plants derived from conventional runners of *F. × ananassa* ‘Selva’ in a raised bed trial

| Treatments | Plants derived from runners | | | |
|---|-----------------------------|---------|---------|---------|
| | MFYP (g) | MFW (g) | PFW (g) | RFW (g) |
| Control (no inoculation) | 285.2a | 16.6a | 104.7ab | 10.0ab |
| +Vaminoc-G [®] | 389.5a | 19.7a | 183.1a | 15.0a |
| +Vaminoc-G [®] + <i>V. dahliae</i> | 345.0a | 19.4a | 139.2a | 17.3a |
| + <i>V. dahliae</i> | 94.7b | 16.6a | 73.2b | 6.8b |

MFYP: median marketable fruit yield per plant; MFW: median marketable fruit weight; PFW: plant fresh weight of runners; RFW: root fresh weight of runners. Treatments sharing a common letter in the same column are not significantly different ($P = 0.05$), using the Kruskal–Wallis test.

Plant and root fresh weights were significant higher in the inoculated and inoculated *Verticillium*-challenged plants compared with the *Verticillium*-inoculated plants, which showed disease symptoms. Plant and root fresh weights decreased by approximately 40% in the *V. dahliae*-challenged plants and increased by 27–48% in the AMF-inoculated and AMF-inoculated + *V. dahliae*-challenged treatments but these changes were not significant (Table 2). The presence of the pathogen was confirmed in inoculated plants by re-isolation from infected tissues. *V. dahliae* was not detected in non-inoculated plants. The AMF-inoculated *V. dahliae*-challenged plants showed no disease symptoms.

3.3. Dual inoculation with Vaminoc-G[®] and FZB24[®]-WG

Marketable fruit yield in the dual-inoculation trial was significantly reduced by inoculation with *V. dahliae* associated with *Verticillium* wilt symptoms in the plants and by re-isolation of the pathogen (Table 3). Fruit yield was increased by approximately

25% in the AMF and PGPR dual-inoculated plants and in the dual-inoculated *V. dahliae*-challenged plants (Table 3). The latter showed no symptoms of *Verticillium* wilt. Fruit weight and plant fresh weight did not significantly differ from the uninoculated controls in any of the treatments (Table 3).

Plant weight was reduced by 40% in the *V. dahliae*-inoculated plants and increased in the dual-inoculated and dual-inoculated pathogen challenges but not significantly (Table 3). Root fresh weight was reduced in the *V. dahliae*-inoculated plants and increased in the dual-inoculated plants, significantly so in the dual inoculated, *V. dahliae*-challenged plants (Table 3).

3.4. Confirmation of the persistence of Vaminoc-G[®] and FZB24[®]-WG

The confirmation of the persistence of the AMF inoculant was based on examination of the cleared stained roots for the present of vesicle and/or arbuscules. The latter were present in the AMF treatments, single and dual inoculations and in the AMF-inoculated *V. dahliae*-challenged treatments but

Table 3

Evaluation of dual inoculation with AMF and PGPR inoculants for the suppression of *Verticillium* wilt in plants derived from conventional runners of *F. × ananassa* ‘Selva’ in a raised bed trial

| Treatments | Plants derived from runners | | | |
|--|-----------------------------|---------|---------|---------|
| | MFYP (g) | MFW (g) | PFW (g) | RFW (g) |
| Control | 285.2a | 16.6a | 104.7ab | 10.0ab |
| Vaminoc-G [®] + <i>Bacillus subtilis</i> FZB24 [®] -WG | 352.0a | 18.2a | 176.6a | 17.3a |
| Vaminoc-G [®] + <i>Bacillus subtilis</i> FZB24 [®] -WG + <i>V. dahliae</i> | 358.4a | 19.7a | 225.1a | 33.9b |
| <i>V. dahliae</i> | 94.7b | 16.6a | 73.2b | 6.8b |

Treatments sharing a common letter in the same column are not significantly different ($P = 0.05$), using the Kruskal–Wallis test.

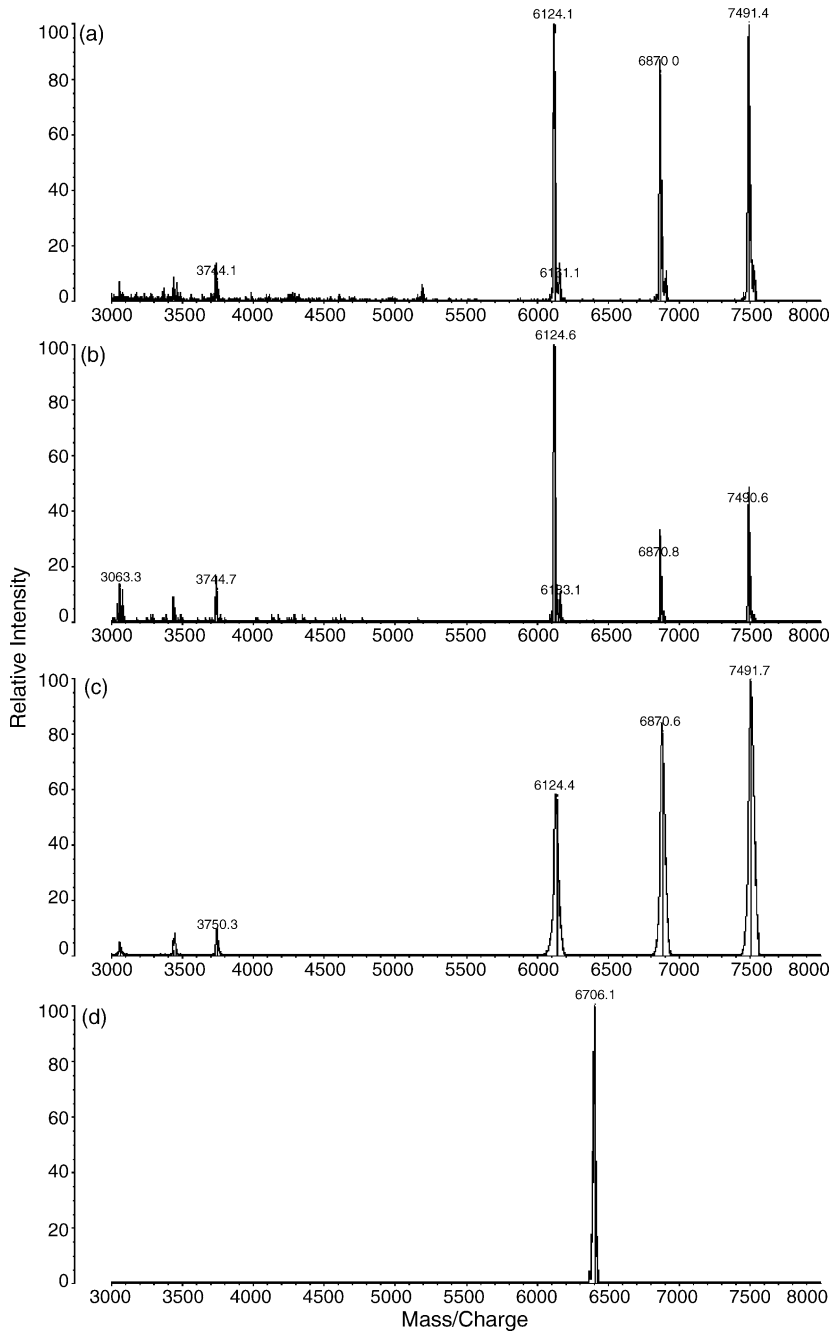


Fig. 1. MALDI-TOF mass spectra of whole *Bacillus* isolates. Representative spectra: (a) *Bacillus subtilis* FZB24[®]-WG control; (b) *Bacillus* from *Bacillus subtilis* FZB24[®]-WG inoculated; (c) *Bacillus* from micro-runner *Bacillus subtilis* FZB24[®]-WG-inoculated, *V. dahliae*-challenged plants; (d) *Bacillus* from non-*Bacillus subtilis* FZB24[®]-WG-inoculated control runners. Note the characteristic peaks of *Bacillus subtilis* FZB24[®]-WG at 6124, 6870 and 7491 Da.

absent from the non-AMF-inoculated controls indicating the absence of infectious soil mycorrhizal propagules.

Bacillus species were isolated following heat treatment of the root extracts. The isolates were Gram positive and the intact bacteria analyzed by MALDI-TOF MS. The FZB24[®]-WG inoculant, in addition to the characteristic *B. subtilis* markers (Hathout et al., 1999), had three characteristic peaks at 6124, 6870 and 7491 (Fig. 1). The inoculant FZB24[®]-WG was detected in all the PGPR singly or dual-inoculated plants and in the inoculated *V. dahliae*-treated plants (Table 4). Other *Bacillus* spp. were detected in the non-PGPR-inoculated controls and in all the treatments (Table 4).

4. Discussion

Methyl bromide is a very potent fumigant (Tomlin, 1997). It has contributed to the successful expansion of the strawberry industry in the USA and Europe and is equally important in other intensive strawberry production areas. In spite of extensive and heavily financed research into methyl bromide alternatives, no universal replacement has emerged (Noling, 2002) and exemptions from the 2005 deadline for the banning of methyl bromide have been sought for 15 crops in the USA which include strawberry. Any alternative to methyl bromide fumigation is likely to be more expensive but the consumer appears willing to pay a higher price where the human health and environmental benefits are evident (Anderson et al., 1996).

Here biological control of *Verticillium* wilt of strawberry has been investigated using commercial inoculants on the premise that pathogen inoculum pressure may increase following withdrawal of methyl bromide. The mycorrhizal inoculant, Vaminoc-G[®], has previously been shown to be beneficial to strawberry in protecting against red core (syn. Red stele) disease of strawberry caused by *Phytophthora fragaria* (Rafferty et al., 2003). The beneficial effects of FZB24[®]-WG on strawberry have not been reported previously but this inoculant has been reported to be beneficial against diseases of other crops, e.g. against *Rhizoctonia solani* in potato (Schmiedeknecht et al., 1998). Coincidentally, *Rhizoctonia* spp. are also an important pathogens of strawberry (Maas, 1998).

V. dahliae resulted in losses of approximately 60% here with a reduction in plant and root fresh weight also of approximately 40% for plants from runners. These results reflect the development of the disease where *Verticillium* wilt is expressed in mid-season, early fruit set and plant growth is not affected. Similar results were obtained ‘Elvira’ and for plants established from microplants and micro-runners (runners from microplants) for both ‘Selva’ and ‘Elvira’ (data not presented).

Inoculation of plants (with root inoculants) in vivo always poses the risk that environmental isolates may compete, even out-compete, the inoculants. This is usually addressed by confirming the persistence of the inoculant by re-isolation and characterization. That the AMF inoculant persisted was easily confirmed in this case as the soil, as confirmed by examination of the roots of uninoculated controls, did not contain infectious AMF inoculum while the roots of mature

Table 4
Plate counts for pasteurised ‘Selva’ root samples at a dilution factor of 10⁻⁴

| Treatment | Inoculated with <i>Bacillus subtilis</i> FZB24 [®] -WG | <i>Bacillus</i> CFUs per 100 µl at 10 ⁻⁴ * | CFUs positive for <i>Bacillus subtilis</i> FZB24 [®] -WG |
|-----------|---|--|---|
| R-A | – | 4 | 0 |
| R-F | + | 5 | 2 |
| R-A-F | + | 4 | 2 |
| R-A-V | – | 7 | 0 |
| R-F-V | + | 6 | 3 |
| RA-F-V | + | 8 | 2 |

CFU were based on the average of three replicate plates. Treatment codes are: the plant material (R: runner); the inoculant (A: Vaminoc; F: FZB24[®]-WG) and V is *V. dahliae* challenge.

AMF-inoculated plants contained vesicles. The persistence of the bacterial inoculant was shown by isolation of *Bacillus* species from the rhizosphere and characterization by MALDI-TOF MS fingerprinting (Elhanany et al., 2001).

Both Vaminoc-G[®] and FZB24[®]-WG had similar positive, but not statistically significant effects in increasing yield in inoculated plants. This growth promotive effect also was observed in pathogen-challenged plants and may have been caused by the inoculant formulations. Generally, the effects on fruit size were not significant but the trend was towards an increase in fruit size in both inoculated, non-challenged and pathogen-challenged trials. The effects on plant and root fresh weight were also positive in non-challenged and challenged plants. Both inoculants performed equally well. Dual inoculation did not result in better protection or higher yields than single inoculation with either inoculant.

While there may be some common elements in the mechanisms involved in biological control by AMF and PGPR, there are also differences. Both may sensitize the pathogen defenses of the host plant such that these are rapidly up regulated on pathogen attack (Lusso and Kuc, 1999). Both may promote plant growth but by different mechanisms; in the case of the AMF by facilitating mineral and water uptake (Mukerji et al., 2000); in the case of PGPR by release of plant growth regulators (Koch et al., 1998). Both may also suppress disease by blocking root entry points and influence pathogen inoculum in the soil by antibiosis. The hypothesis that dual inoculation with AMF and PGPR may have additive beneficial effects was not confirmed here. In other studies, dual or multiple inoculations have been neutral, positive or negative depending on the inoculants used (Cordier et al., 2000). Nevertheless, this approach deserves further investigation as, so long as there is no associated yield penalty to the plant, biological control that depends on more than one mechanism may be more durable in the field (De Boer et al., 2003).

The results, which have been repeated, using the cv. Elvira, while encouraging, should only be regarded as preliminary given that methyl bromide-fumigated soil was used. However, as with all attempts at biological control, reproducibility soils needs to be confirmed, especially in non-fumigated soils. In the latter,

variability in the native soil flora and fauna, and competition with/antagonism to the inoculants, may mitigate against success and may reveal differences in the efficacy of AMF versus PGPR inoculants or the greater durability of treatments involving inoculants with different mechanisms of action. In conclusion, biological inoculants may have the potential to contribute to sustainable alternative strategies, i.e. not involving hazardous soil sterilants, for the control of *Verticillium* wilt of strawberry.

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