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Enhancement of control by nutrient formulations of selected biocontrol agents against fusarium crown and root rot of tomato

(Peningkatan kawalan oleh agen kawalan biologi dengan formulasi nutrien terhadap penyakit reput pangkal dan akar fusarium pada tomato)

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Key words: nutrient enhancement, fusarium crown and root rot, *Bacillus megaterium* c96, *Pseudomonas cepacia* c91, tomato

Abstrak

Dua asingan bakteria yang dikenal pasti sebagai *Bacillus megaterium* c96 dan *Pseudomonas cepacia* c91 telah digunakan untuk mengkaji kesan penambahan nutrien di dalam formulasi kawalan biologi. Kedua-dua asingan diperoleh daripada kerja-kerja saringan yang dijalankan untuk program penyelidikan bagi kawalan penyakit reput pangkal dan akar fusarium pada tomato. Penggunaan medium cecair YPG (yis, pepton, glukosa) pada kepekatan 1% atau 10% di dalam formulasi kawalan biologi tidak meningkatkan aktiviti kawalan biologi kedua-dua asingan tersebut. Bagaimanapun, apabila punca nutrien disediakan secara berasingan, yis didapati berkesan (p = 0.01) meningkatkan aktiviti kawalan biologi *P. cepacia* c91 tetapi kurang berkesan terhadap *B. megaterium* c96. Keputusan yang sama diperoleh apabila formulasi tersebut diuji di dalam percubaan jangka panjang. Kesan Fe, Mg dan Zn telah diuji dan keputusannya menunjukkan yang bakteria sebagai agen kawalan biologi memberikan tindak balas positif terhadap penambahan Mg dan Fe dengan penurunan peratus penyakit secara berkesan, tetapi Zn memberikan kesan kerosakan kepada kedua-dua asingan bakteria yang diuji.

Abstract

Two bacterial isolates identified as *Bacillus megaterium* c96 and *Pseudomonas cepacia* c91 were used in this study to investigate the effect of nutrient amendment in the biocontrol formulation. Both isolates were obtained from screening works conducted in the research programme for the control of Fusarium crown and root disease of tomato. The use of 1% or 10% concentrations of YPG (yeast, peptone, glucose) liquid medium in the biocontrol formulation did not enhance the biocontrol activity of both isolates. However, when these nutrient sources were prepared individually, yeast was found significantly (p = 0.01) enhancing the biocontrol activity of *P. cepacia* c91 but was less effective with *B. megaterium* c96. Similar results were obtained when the formulation was tested under long-term experiment. The effects of Fe, Mg and Zn were examined and the results indicated that the bacterial biocontrol agents responded positively to the addition of Mg and Fe with significant reduction in the percentage of disease infection but Zn shows some deleterious effect on both isolates tested.

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Introduction

The tomato crop is commercially important throughout the world both for fresh fruit and the processed food industries. Tomato has been bred for fruit production under a wide range of environmental conditions, from temperate climates to the warm humid tropics and to the hot arid deserts (Watterson 1986). Cultivated tomato is highly susceptible to many pathogens. There are about 200 diseases known to infect tomatoes and losses of yield due to diseases could be as high as 70–95% (Lukyanenko 1991).

Recently, fusarium crown and root rot (FCRR) of tomato caused by *Fusarium oxysporum* f.sp. *radicis-lycopersici* (Forl) has been identified as one of the most prevalent soil-borne diseases limiting greenhouse tomato production in some of the tomato major growing areas such as, Japan (Sato and Araki 1974), Canada (Jarvis et al. 1975), France (Couteaudier et al. 1985), United Kingdom (Hartman and Fletcher 1991) and Southern Florida (Datnoff et al. 1995). Following the unsatisfactory performance of chemical control and the frequent outbreak of the disease in sterilized soils (Jarvis 1977), biological control is considered an attractive alternative to the use of chemicals and fumigation.

Several work have been reported on the successful use of biocontrol on FCRR using microbial antagonists. A mixture of six fungi, which included *Gliocladium* spp. and *Fusarium* spp., considerably reduced disease severity (Reyes 1978). Other microorganisms such as, *Penicillium funiculosum*, *Trichoderma harzianum* and *Aspergillus ochraceus* have reduced the activity of the pathogen in fumigated soil (Morois and Mitchell 1981). More recently Rattink (1993) reported some success in suppression of the pathogen in a recirculation substrate using *T. harzianum*.

In general, antagonistic microorganisms are often inconsistent in their efficacy compared to chemical pesticides. In order for a biocontrol agent to persist and control the disease effectively, it needs to establish a high population level, survivality and adaptability to the environment where it is introduced. The biocontrol activity of antagonists may be improved by the addition of appropriate nutrients (Papavizas and Lewis 1981; Nelson et al. 1988) for instance; *Trichoderma* spp. and *Gliocladium virens* were activated by addition of wheat bran (Lewis et al. 1991) while $CaCl_2$, KCl or $CaCO_3$ was used successfully to improve the efficiency of *Candida* spp. in controlling Botrytis fruit rot on apple (McQuilken et al. 1994). Despite the substantial progress in the field of biological control, there is no doubt that with the addition of nutrients to the biocontrol preparation, its application could be greatly improved in the control of FCRR in tomato.

This paper presents the results of a nutrient amendment and the formulation study on two biocontrol agents namely *Bacillus megaterium* c96 and *Pseudomonas cepacia* c91 for the control of fusarium crown and root rot disease of tomato.

Materials and methods

Bacterial strains and fungal isolate

Two bacterial strains *B. megaterium* c96 and *P. cepacia* c91 were obtained from a series of screening work against *F. oxysporum* f.sp. *radicis-lycopersici* as previously reported (Omar 1999). These strains were isolated from Cyclamen roots and able to give a significantly higher percentage of disease reduction when compared with other isolates tested. Bacterial strains were maintained on either nutrient agar (NA) slants at 4 °C or in the form of frozen culture containing 15% glycerol at –20 °C.

Fungal isolates of *F. oxysporum* f.sp. *radicis-lycopersici* (Forl) known to be highly virulent on tomato were obtained from University of Nottingham. The isolates were originally from ADAS, Cambridge. The stock was maintained on slants of potato dextrose agar (PDA) at 4 °C.

Bacterial cell and fungal conidia suspension preparation

Bacterial cell suspensions were prepared by streaked inoculated on NA slant and incubated for 24 hours at 30 °C. The slants were then flooded with 10 mL sterile distilled water (SDW) and the bacteria were scraped from the agar surface with a sterile plastic loop. The suspensions were made homogeneous by agitation using Fison 'Whirlmixer'. Flasks (250 mL) containing 50 mL of the appropriate medium were inoculated with 100 µL of the bacterial suspension. All flasks were incubated in a Nobel MKV orbital shaker at 30 °C and 250 rpm.

Microconidia of Forl were obtained by flooding 10 mL of SDW onto 14-day-old colonies grown on PDA at 20 °C. Conidia were dislodged by scraping the agar surface with a sterilized glass rod. The resulting slurry was strained through 4 layers of sterilized muslin cloth to remove mycelial debris. The filtrate was collected and the spores were washed twice with SDW and separated by centrifugation at 3 000 rpm for 5 minutes. The concentration of microconidia in the suspension was adjusted to 5 x 10^5 conidia/mL by dilution with SDW.

Seedling bioassay

Tomato seeds cv. Ailsa Craig were sown in plastic plant plugs (1.5 cm) filled with Levington Professional F2 peat-based potting compost. After 9 days, the seedlings were drenched with 1 mL of tested microbial suspension at the concentration of approximately 10^8 cfu/mL using Gilson pipette. After 24 hours the seedlings were drenched with 1 mL of Forl conidia suspension at 5 x 10^5 conidia/mL. The seedlings were grown for another 10 days before transplanted into larger plant plug (9 cm) filled with loam-based (John Innes No. 2) compost. Seedlings were raised in growth room with temperature regime maintained at 20 °C day, 15 °C night, with 16 hours photoperiod. The plants were watered everyday and wilting or dead seedlings were recorded whenever seen and on day 14, the plants were cut at the stem-based and the incidence of stem vascular staining was examined and presented as percentage of infected plants.

Effect of concentration of yeast peptone glucose (YPG) in biocontrol formulation

To test the effect of nutrient addition in the biocontrol formulation, yeast, peptone and glucose were used. The bacterial isolates c96 and c91 were grown in YPG liquid medium for 72 h as previously described. When the incubation was completed the cultures were harvested by centrifuge at 16 000 g for 10 min and the supernatant decanted. Cells collected at the bottoms of the tubes were resuspended in either 1% or 10% YPG solution or $^1/_4$ strength Ringer's solution and the volumes were made up to 50 mL. The suspensions were applied to the roots of the seedlings as described earlier. The experiments were conducted in Completely Randomized Design (CRD) with 3 replications. Disease assessment was made at 14 days after transplanting (DAT) and presented as previously described.

Effect of different nutrient sources in formulation of biocontrol

The aim of the experiment was to determine whether any nutrient source in YPG, which represent the source of carbon, nitrogen and trace elements, would be beneficial in the enhancement of the activity of the biocontrol agents. The experiment employed the procedure as previously described. The nutrient sources, yeast extract, bacteriological peptone and D-glucose were prepared individually as a 10% solution of their concentration in YPG medium. The bacterial suspension was prepared and inoculated onto the seedlings root as described in the seedling bioassay. The experiment was conducted in CRD with 3 replications. Infected seedlings were recorded at 14 DAT and the data were presented as percentage of infected plants.

Effect of yeast in formulation of biocontrol agents for long-term control

The yeast biocontrol formulation was prepared as described in the previous experiment. Seedlings were germinated and grown as described in the seedlings bioassay. The yeast formulation and the control treatment where the bacterial were resuspended in the $^1/_4$ strength Ringer's solution was applied to the seedling as described in the previous experiment. Ten days after inoculation of Forl the seedlings were transplanted into 13 mm pots filled with John Innes No. 2 loam-based compost. The treated plants were maintained in the glasshouse and the pots were arranged in CRD with 3 replicates consisting of 6 plants in each treatment. The plants were regularly assessed for disease symptom development. Disease severity was determined by using a subjective foliar disease rating scale *(Table 1)*. At 35 days after transplanting, a final score of foliar symptom was made and the plants were uprooted and the lower stem and tap root was longitudinally sectioned for examination and measurement of the vascular tissue discolouration length.

Effect of trace element in formulation of biocontrol agents

To establish the effect of trace elements on biocontrol activity, the procedure described in previous experiment was employed. Washed cells of both bacterial isolates from 50 mL YPG culture were prepared and resuspended in each of the solutions containing 0.1 g/litre of $FeCl_3$, $MgSO_4$ and $ZnSO_4$. The control treatment was resuspended in $^1/_4$ strength Ringer's solution. The biocontrol formulation and the Forl conidia suspension were applied to the seedlings as previously described. The experiment was conducted using CRD with 3 replicates and each treatment consisted of 24 seedlings. The seedlings were assessed for disease infection at 14 DAT. The experiments were conducted from September 1997 until April 1998.

Statistical analysis

Analyses were performed using the 'StatMost' Statistical Analysis and Graphic, version 2.50. Mean treatments were compared by using Duncan's Multiple Range Test (DMRT) at $p = 0.05$. Errors bars represent the standard errors of the means.

Results

Effect of concentration of YPG in biocontrol formulation

Figure 1 shows that the effect of nutrient amendment on the percentage of infection was not significantly different, except *B. megaterium* c96 in 1% YPG treatment, when the mean treatments were compared using DMRT ($p = 0.05$). Even though *B. megaterium* c96 in 1% YPG was less active in controlling the disease, generally both isolates were able to bring down the percentage of infection regardless of nutrient amendment. These results have established that both YPG concentrations of 1% and 10% did not give a significant effect on the biocontrol activity of either *B. megaterium* c96 or *P. cepacia* c91.

Effect of different nutrient sources in formulation of biocontrol

It was observed that the addition of certain nutrient sources to the formulation of the biocontrol agents influenced the ability to reduce disease *(Figure 2)*. Yeast as a source of trace elements significantly ($p = 0.01$) reduced the percentage of infection with both biocontrol isolates. The reduction of the infected plant was about 20% when compared with plants treated with nutrient unamended formulation. Other nutrients did not significantly influence the percentage of infection, and gave similar results to those obtained using $^1/_4$ strength Ringer's solution. However, overall results showed that both isolates were able to reduce disease below 40% which was a highly significant ($p = 0.01$) reduction when compared to the control. Yeast extract is suggested as a promising nutrient in the formulation of the biocontrol agents.

Table 1. Foliar disease rating for estimating disease severity (based on Hartman and Fletcher 1991)

Score	Disease level (%)	Symptom
0	0	plant foliage green, healthy
1	25	lower leaves yellow
2	50	lower leaves brown and dead
3	75	top leaves wilting, lower leaves dead
4	100	whole plant wilt or dead

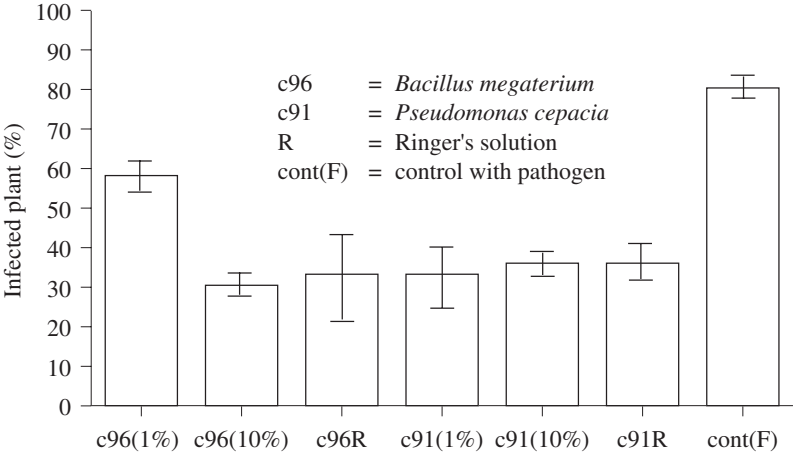

Figure 1. Effect of 2 concentrations of yeast peptone glucose broth in biocontrol agent's formulation on the percentage of infected plant. Figures in parentheses represent the concentration of YPG in bacterial cell suspension. Error bars represent the standard error of 3 replicates mean

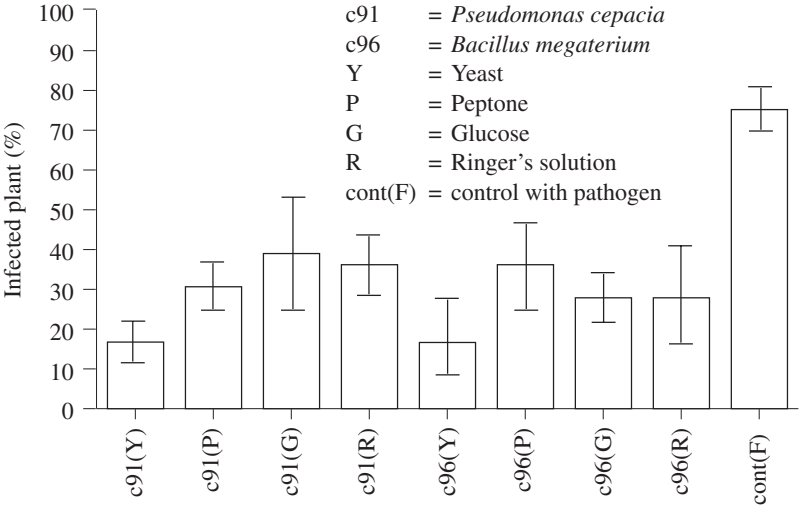

Figure 2. Effect of formulation of biocontrol agents with different nutrients sources on the percentage of infected plants. Error bars represent the standard error of 3 replicates mean

Effect of yeast in formulation of biocontrol agents for long-term control

Following the encouraging results obtained in the previous experiment, when yeast extract was used in the formulation, attempts were made to further evaluate the consistency of control achieved by yeast addition in a long-term study. The addition of yeast to the formulation did not significantly enhance the efficacy of control by c96, where about 50% disease severity was recorded using this treatment *(Figure 3a)*. However, isolate c91 amended with yeast gave a considerable reduction in the percentage of disease severity. When the stem of the fruited plants were examined, stem vascular staining was observed in all treatments. The level of protection was low when the plants were exposed to a longer growing period.

To further assess the level of protection with the formulation, stem vascular staining length was measured and the results are presented in *Figure 3b*. These results demonstrated that the length of vascular staining was significantly ($p = 0.01$) lower in isolate c96 amended with yeast, but yeast did not enhance isolate c91 in reducing the severity of the vascular infection of the plants. The presence of yeast in the formulation may provide an initial boost to the bacterial biocontrol agents, but may also be utilized by the pathogen and lead to poor long-term protection.

Effect of trace elements in formulation of biocontrol agents

It was clearly shown in the previous investigation that yeast was able to enhance the efficacy of control given by both bacterial isolates at least in the early stage of infection. Further investigation on the trace elements have shown some convincing results. Both bacterial isolates responded positively to the addition of Mg and Fe in their biocontrol activity against Forl *(Figure 4)*. Addition of both nutrients reduced significantly ($p = 0.01$) the percentage of infection when compared with the control without BCAs. However, there were non-significant difference in the percentage of infection when compared with the control of biocontrol agents in Ringer's solution. Addition of Zn to the formulation may have a deleterious effect on both isolates and resulted in low biocontrol activity. The positive effects of Mg and Fe in the formulation of the BCAs may be due to the stimulatory effect on the production of antibiotics or the growth of the bacterial isolates in the soil.

Discussion

The introduction of bacterial biocontrol agents or antagonists for managing soilborne pathogens is often inconsistent in performance from one locality to another, and this has been a primary obstacle to its commercial development (Weller and Thomashow 1994). One of the ways to make it more conducive for their growth and survival, is to regulate an existing nutritional environment, as competition for substrates in soils and rhizospheres is intense (Colbert et al. 1993). There is a need to establish a high population level of the antagonists in the root system for effective disease control. Investigation into biocontrol activity with yeast peptone glucose nutrient (YPG) solution in a biocontrol nutrient formulation revealed that such activity was not enhanced either at 1% or 10% concentration. The use of a complete nutrient source like YPG, which contains carbon, nitrogen and trace elements which are needed by microorganisms for growth, has proved to be a better choice in promoting biocontrol activity. The complete nutrient improves growth and development of biocontrol agents as well as pathogens. Thus nutrient amendment may affect growth and survival of some plant pathogens, yet stimulate the growth and survival of other microbial populations, including antibiotic-producing bacteria, actinomycetes and fungi (Huang and Huang 1993). The results of this study indicated that either the activity of the biocontrol agents was not enhanced or at the

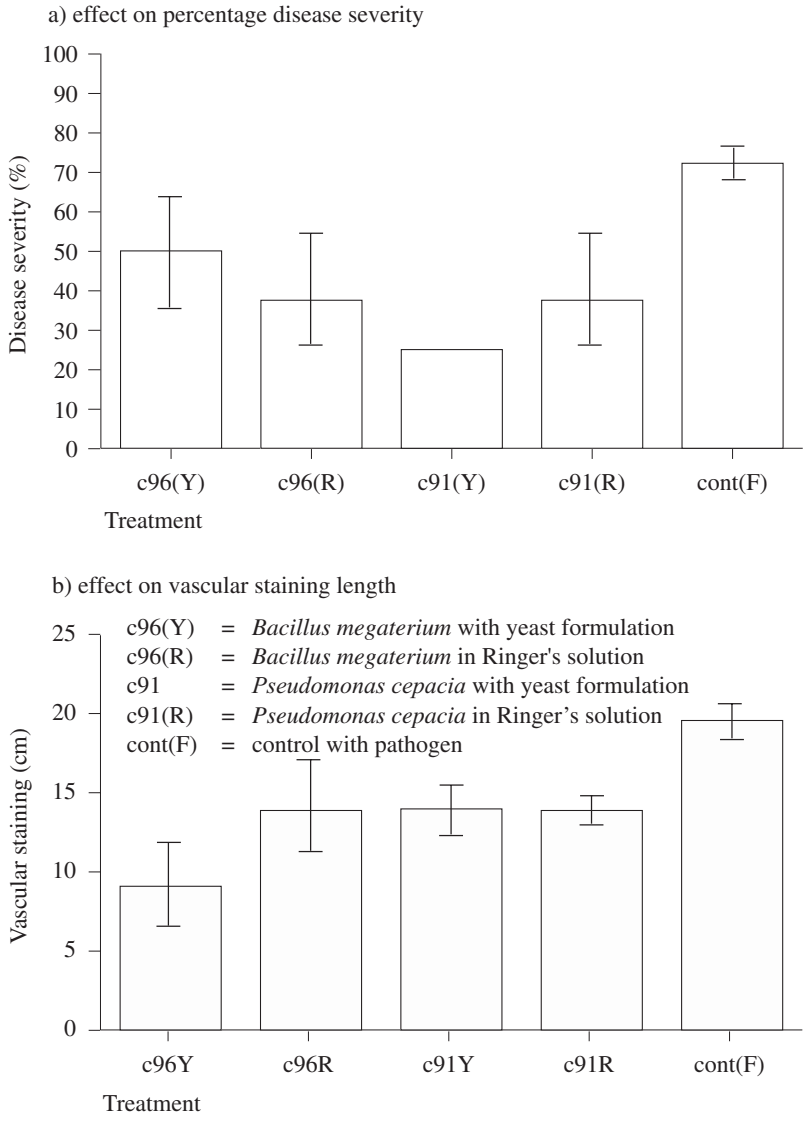

Figure 3. Effect of yeast in formulation of biocontrol agents in long-term experiment. Error bars represent the standard error of 3 replicates mean

same time the pathogen was stimulated by the nutrients to be more virulent and caused more severe infection on the tomato plants.

One way to make it more effective would be to provide a substrate that could be selectively utilized by the bacterial isolates used. Examination of the effectiveness of individual nutrient sources of yeast, peptone and glucose showed that yeast extract used in the formulation significantly enhanced the biocontrol activity of both bacterial isolates. Yeast extract, which is rich in trace minerals, is believed to have stimulated the growth and antibiotic production and increased the competitive effects of the introduced bacterial biocontrol agents in the soil. This idea is based on the fact that certain diseases can be managed with mineral fertilization regimes. There are a few cases, in which minerals appear to reduce disease, for example fusarium crown and root rot of

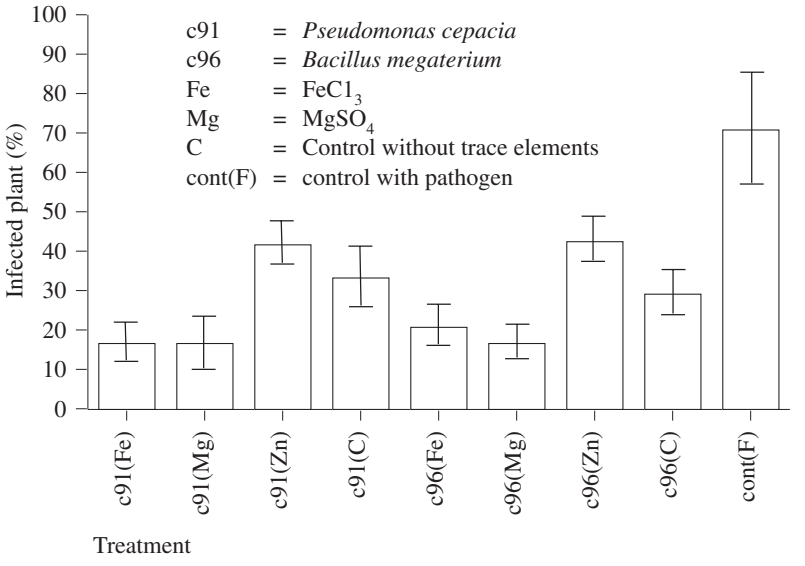

Figure 4. Effect of trace elements in formulation of bacterial isolates on percentage of infected plants. Error bars represent the standard error of 3 replicates mean

asparagus and take-all disease of wheat, by exerting an indirect beneficial effect on indigenous and introduced antagonistic microorganisms (Huber 1989; Elmer 1995).

The effect of yeast in the formulation of biocontrol agents was further examined in a long-term control experiment. The results from this experiment indicated that yeast consistently enhanced the biocontrol activity of *P. cepacia* c91 but was less effective with *B. megaterium* c96. A one time addition of yeast in the formulation could be enough for the enhancement of the early growth phase of the bacteria, but when the yeast had been utilized, it may become a limiting nutritional factor and affect the competitiveness of the BCAs against pathogen in long-term control.

Yeast extract was identified as a source of trace minerals which seemed to be important in the enhancement of the biocontrol agents activity selected for this work. Identification of mineral amendments that favour biocontrol may also provide clues to soil factors or components of nutrient solutions in hydroponics culture that will improve the level and reliability of biocontrol (Duffy and Defago 1997).

The effects of Fe, Mg and Zn were examined and the results indicated that the bacterial biocontrol agents responded positively to the addition of Mg and Fe but Zn shows some deleterious effect on the bacterial isolates tested. These results suggested that it may be possible to enhance the level of biocontrol through manipulation of certain selective mineral sources used for production of the antagonists. These results concur with Fiddaman (1994) who reported that the addition of iron (III) promoted in vitro antagonism of *Rhizoctonia solani* AG4 and *Pythium ultimum* K874A by *Bacillus subtilis*.

Similarly, Slininger and Jackson (1992) found that growth and antibiotic production by *Pseudomonas fluorescens* strain 2–79 increased with additions of $MgSO_4$, H_3BO_4 and $FeSO_4$ and in contrast with the results obtained in this study, $ZnSO_4$ interacted with iron to maximize phenazine-1-carboxylic acid (PCA) accumulation. However, it was reported that the requirement of an iron-zinc combination for optimal bacterial secondary metabolism is unusual (Weinberg 1977, 1986). Nevertheless, zinc soil content has been found to be positively correlated with

the biocontrol activity of introduced *P. fluorescens* 2-79 (Weller and Thomashow 1994). Duffy and Defago (1997) reported that zinc had no direct effect on fusarium crown and root rot in soilless tomato culture when used alone, but did reduce disease by 25% in the presence of the *Pseudomonas* strain CHAO, indicating that it indirectly reduced disease through some influence on the interaction between the biocontrol agent and the pathogen.

The tomato crown and root rot pathogen produced fusaric acid with nonspecific phytotoxic activity that contributes to wilt and rot of various crops caused by *Fusarium oxysporum* (Remotti and Loffler 1996). However, in in vitro studies with the pathogen, zinc at concentrations as low as 10 mg/mL disrupted production of the phytotoxin fusaric acid (Duffy and Defago 1997) as these trace elements are essential in improving the biocontrol activity, a formulation that efficiently supply minerals to the antagonists may further improve their availability and efficacy on the control of plant disease. However, this approach should be looked into in further detail. Additional research is also needed to determine the role of the minerals in gene expression that influence both antibiosis and colonization which are important to antagonism in the rhizosphere.

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