Final report of the project "Evaluation of biocontrol agents for control of root diseases in hydroponic systems" project nr: 0456018

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Objectives

The performed project focused on the antagonistic potential of the bacterial strain *Pseudomonas fluorescens* 5.014 together with other commercially available biocontrol agents (bca) against root pathogens commonly found in closed hydroponic greenhouse systems. The results were expected to provide more knowledge about the efficiency of using this strain as a bca in closed hydroponic systems. Our partial aims were to evaluate: (i) the bacterial strain *P. fluorescens* 5.014 together with a mixture of biological control agents with different biological control mechanism against four root pathogen *Pythium aphanidermatum*, *Pythium ultimum*, *Phytophthora cryptogea* and *Fusarium oxysporum* on tomato and sweet pepper grown under controlled conditions in climate chamber (phytotrone) as well as in the greenhouse and (ii) the bca against root pathogens in two different growing media.

Conclusions

1. The results from the conducted project reveal on variations in the efficiency of the used biocontrol agents depending on the type of pathogen present in the system, the number of biocontrol agents acting together, type of the cultivation system, liquid or aggregate hydroponics, as well as on the type of growing media.
2. In Liquid hydroponics,
    a. Mycostop was the most effective biocontrol agent followed by Binab T and Gliomix. The effect of these agents decreased by combination
    b. The bacterial strain *P. fluorescens* 5.014 showed a biocontrol effect towards *Pythium aphanidermatum* and *P. ultimum* when applied individually. The biocontrol effect of this strain towards *P. ultimum* increased by combination with other agents.
    c. No effect towards the root pathogens *Phytophthora cryptogea* and *Fusarium oxysporum* could be noticed by *P. fluorescens* 5.014.
3. In substrate based cultivation system only commercial products showed antagonistic effect towards the tested pathogen.
4. In the greenhouse, the efficiency of these agents decreased with time.

Background

Due to low microbial biodiversity in the nutrient solution and roots, closed hydroponic systems are considered to be sensitive from a biological point of view (Paulitz 1997). The re-use of excess nutrient solution in these systems enhanced the risk of dispersal of plant pathogen. The oomycetes *Pythium* and *Phytophthora* spp. are important root pathogens in hydroponic systems with or without growing medium (Stanghellini & Rasmussen, 1994). The presence of the infective motile zoospore stage makes these pathogens potentially the most damaging in soilless cultivation systems particularly in early stages of plant growth. Restrictions on the use of chemical protection agents has encouraged the use of other methods of disease control, such as filtration, heat, UV irradiation (Ehret et al. 2001) and biological control using beneficial rhizobacteria (McCullagh *et al.*1996; Paulitz 1997). Several studies have shown the ability of these bacteria to protect against root diseases (Paulitz 1997), to have a plant growth promoting effect or to break down plant growth inhibitory compounds (Caspersen et al. 2000).

In general, the rhizobacterial may act by diverse antagonistic mechanisms against root pathogens. These mechanisms depend on the species and isolates of the antagonist and the

pathogen, the crop and whether the disease occurs in the field or in the greenhouse (Thrane et al. 2000). In addition to external root colonization, the rhizobacteria may compete with the indigenous microorganisms on space and nutrient in the rhizosphere and spermosphere (Lynch 1990). Rhizobacteria can also protect against root pathogens by producing primary and secondary metabolites such as antimicrobial agents, siderophores and hydrogen cyanide (O´Sullivan & O´Gara 1992; Kumari & Srivastava 1999). Moreover, rhizosphere organisms may produce lytic enzymes with antifungal properties. This can be mediated by the production of extracellular hydrolytic enzymes by *Trichoderma* spp. The role of these enzymes in biocontrol is supposed to be connected either to their saprophytic lifestyle or to their direct action against plant pathogens (Thrane et al. 2000). Furthermore, strains of the actinomycete *Streptomyces* have shown to antagonize several plant pathogenic fungi *in vitro*. Mechanisms of fungal inhibition were elucidated by tracing secondary metabolites and fungal cell wall degrading activity (Berg et al. 2001).

Outcome

The following publications, presentations and manuscripts were achieved in this project:

* *Publications in national journals within the horticultural branch*

1. Khalil S. & Alsanius B.W. 2007. Know How- en viktig förutsättning för fungerande biologisk bekämpning i slutna odlingssystem. Gurk- och Tomatbaldet Nr 3, November 2007
2. Khalil S. & Alsanius B.W. 2006. Fungerar bio-bekämpning i slutna odlingssystem mot rotsjukdomar? Viola 111(4):20-21.

* *Publications in international journals*

1. Khalil S. & Alsanius B. 2006. Biochemical characterization of biocontrol agents used for control of root pathogens. Communications in Agricultural and Applied Biological Sciences 71 (3 Pt B):979-84 17390847 (P,S,E,B) **Biochemical characterization of biocontrol agents used for control of root pathogens.**

* *Presentation at the grower meeting on 7/mars-2007- Hässleholm*

1. Alsanius B.W. & Khalil S. 2007. Möjligheter att integrera biologisk och kemisk bekämpning i ett slutet system med rening

* *Manuscripts*

1. Evaluation of biocontrol agents against different root pathogens on hydroponically grown tomato (to be submitted to Biological control).
2. Utilization of carbon sources by *Pythium aphanidermatum*, *Phytophthora cryptogea*, *Phytophthora capsici*, *Fusarium oxysporum* f.sp. *radicis-lycopersici* and *Fusarium solani* (to be submitted to *Antonie van Leeuwenhoek*).
3. Growing media- as a key factor to explain interactions between biocontrol agents, root pathogens and resident microflora in closed hydroponic system with tomato (to be submitted to Canadian Journal of Plant Pathology).
4. Combining biological control agents to enhance suppression of root pathogens in closed hydroponic systems (to be submitted to Crop protection).

* *Homepage*

The project is represented at växtskydd Alnarp (http://www.vaxtskyddalnarp.se)

Material and Methods

Biocontrol agents

1. *P.* 5.014: The bacterial strain *P. fluorescens* 5.014 isolated from a closed hydroponic tomato culture (Waechter- Kristensen et al. 1994) has showed both plant growth promoting effects and some antagonistic effect against the root pathogen *Pythium ultimum* on tomato seedlings as well as on older plants (Hultberg et al. 2000; Khalil 2001).

2. Binab T (Binab Bio- innovation AB, Sweden) is marketed in Sweden as a biocontrol agent against fungal diseases on potato and vegetables in both greenhouses and fields. It is microbial active ingredients are *Trichoderma polysporum* and *T. harzianum*.
3. Gliomix (Kemira Agro Oy, Finland) is a beneficial microbial product based on *Gliocaldium cantenulatum* and used in seedling production of vegetables, herbs and ornamentals. It enhances the emergence and growth of seedlings and is used in control of damping-off, wilts and root diseases in greenhouse.
4. Mycostop (Kemira Agro Oy, Finland), based on *Streptomyces griseoviridis*, is a biological fungicide for greenhouse vegetables, ornamentals and herbs. It controls a wide range of fungal pathogens which cause seed- and soil-borne damping-off and root diseases.

The bacterial strain *Pseudomonas fluorescens* 5.014 was available at our laboratory. The commercially developed biocontrol agents were obtained from Econova Predator Helsingborg, Sweden.

Pathogens

The pathogens *Pythium aphanidermatum* (PA), *Phytophthora cryptogea* (PC) and *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FO) were obtained from Centraalbureau voor Schimmelcultures, The Netherlands. *Pythium ultimum* (PU) was kindly provides by Prof. W. Wohanka, Geisenheim, Germany. All pathogens were maintained in the dark on potato dextrose agar (PDA) at 23^{o}C during laboratory storage.

Experimental set- up

Tomato (*Lycopersicon esculentum* cv. Ingar) was used as a model plant. The project was performed in three different phases: (i) a bioassay to evaluate the application time, rate and *In vitro* antagonism of the biocontrol agents, (ii) climate chambers experiments in the phytotrone and (iii) a greenhouse experiment. In the experiments in climate chamber, the biocontrol agents were evaluated in two different hydroponic systems, liquid- and growing media- based hydroponics. In liquid-based hydroponic system, the biocontrol agents were tested individually or in combination with each others. In the growing media-based system, the agents were evaluated individually in two growing media, peat and pumice. In both cultivation systems, the pathogens were tested separately and each pathogen was investigated for a period of four weeks. In total 32 experiments including repetitions were performed (Fig. 1). In the greenhouse the four bca were evaluated against two pathogens, PA and PC, in hydroponic system with pumice for four months. Sample collection and evaluation of the biocontrol activity of the tested strains were performed three times during the duration of the experiment.

Analyses

All experiments were analysed with respect to growth measurements, microbiological and plant pathological analysis. Growth measurements included measurements of the plant length, fresh and dry weight of the roots, shoots and leaves. In the greenhouse, the number of flowers and fruit size was determined. In addition to the assessment of the pathogens on selective media, disease incidence was determined by measuring the percent infected root as affected by the different treatments.

The impact of the pathogen and the biocontrol agents on the resident microflora was studied by viable count, sole carbon source utilization patterns and the molecular method PCR-DGGE (Fig. 1). Viable count was used for the enumeration of different microbial groups, included the tested pathogens and bca, on selective media.

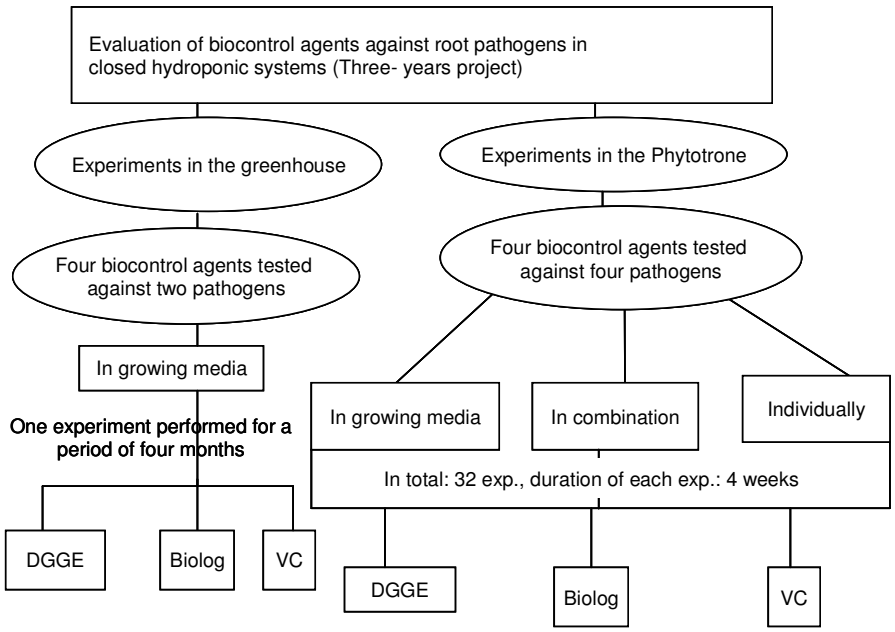

Figure 1. Schematic picture of the experiments performed within the project

Analyses using sole carbon source utilization patterns (SCSU) were performed on the indigenous microflora as well as on the pure cultures of the bca and the pathogens in order to distinguish them from those of the resident microflora. Furthermore, ecological assessment of the bca, pathogens and resident microflora was performed using the molecular technique PCR-DGGE (polymerase chain reaction of 16S or 18S DNA sequences, followed by denaturing gradient gel electrophoresis). However, both viable count and SCSU were performed on all samples collected in the experiments conducted in the climate chamber and the greenhouse. The molecular method PCR- DGGE, on the other hand, was developed and optimized during the duration of the project. Due to the lacke of time, analysis using this technique was performed only on samples collected from liquid hydroponics.

Application of the biocontrol agents and the pathogens

The bacterial strain *P. fluorescens* 5.014 (stored at -80^{0}C in 50% glycerol (v/v) in King´s medium B) applied to the plants by seed inoculation as described by Hultberg & Alsanius (1998). The commercial bca were applied at recommended use rates. Binab T and Gliomix were applied as 1% mixture, respectively. Mycostop was applied as 0.1% mixture.

Zoospore suspensions of PU, PA and PC and conidial suspensions of FO were used as infection mean. Zoospore production of PA was performed according to Postma et al. (2005). The fungus was cultured in 100 ml Erlenmeyer flasks containing 20 ml of V8 liquid medium for 7-10 days at 25^{o}C in the dark. The mycelium was then washed four times with 50 ml of sterilized tap water and placed in Petri dishes with 20 ml sterilized tap water. After about 5 hours the zoospores were released. The fungal inoculum of PU was prepared as described by Hultberg et al. (2000). The isolates were grown for 3 weeks at 22 ºC in 20 ml of Schmitthenner's medium. The mycelium was briefly homogenized in a blender and diluted with NaCl.

Zoospore suspension of PC was prepared according to Pegg & Jordan (1990) with slight modification. A 4 day-old culture grown in V8 liquid medium for 5 days at 20^{o}C in darkness without shaking. Thereafter, the mycelium was washed four times with sterile distilled water and re-incubated in 10 ml sterile distilled water at 20^{o}C for further 5 days. To stimulate

zoospore release, the mycelium was then washed in sterile distilled water, chilled at 4^{o}C for 40 min and re-incubated at 20^{o}C for 1h. For FO, conidia and mycelium from 10-days old colonies were harvested according to Boari & Vurro (2004) by adding distilled water to the colony and gently scraping the surface with spatula.

Cultivation systems

The treatments included in the experiments are presented in the Table 1. In each experiment, four replicates of each treatment were used. In liquid hydroponics, the tomato seedlings were put into black polyethene foam mats (PEN 334, Åkesson & Örbo, Mönsterår, Sweden) and placed in 1L black plastic containers at the density of two plants in each container. The containers were placed in a climate chamber with a light intensity of 250 µmol $m^{-2}s^{-1}$ at 24^{0}C and 75% relative humidity. In the experiment with *P. ultimum*, the temperature in the chamber was 19^{0}C.

Table 1*. Treatments included in the experiments*

Treatments	**Cultivation systems**		
	Liquid hydroponic		*Growing media- based hydroponic*
	BCA applied individually	*BCA applied in combination*	*BCA applied individually in climate chamber and the Greenhouse experiment*
A	Control	Control	Control
B	Exposure to the pathogen	Exposure to the pathogen	Exposure to the pathogen
C	Exposure to the pathogen + P. fluorescens 5.014	Exposure to the pathogen + Binab T	Exposure to the pathogen + Mycostop
D	Exposure to the pathogen + Binab T	Exposure to the pathogen + Mycostop	Exposure to the pathogen + Binab T
E	Exposure to the pathogen + Gliomix	Exposure to the pathogen + BinabT + Mycostop	Exposure to the pathogen + Gliomix
F	Exposure to the pathogen + Mycostop	Exposure to the pathogen + BinabT + Mycostop+ P. fluorescens 5.014	Exposure to the pathogen + P. fluorescens 5.014
G		Exposure to the pathogen + BinabT + Mycostop+ P. fluorescens 5.014+ Gliomix	

In growing media- based hydroponics, tomato seeds (*Lycopersicon esculentum* cvs. Ingar) were sown in 3 L buckets filled with either peat or pumice as growing media. Two seeds per bucket were used. Aliquots of 10ml of Binab T, Gliomix or Mycostop were added to each plant. Plants of the controls treatment and treatment with only the pathogen received 10 ml of sterile distilled water and propagule suspensions respectively. A week later, each plant in the treatments with the biocontrol agents was drenched with 10 ml of the fungal zoospore or conidial suspensions. The addition of the biocontrol agents and the pathogen was performed once more during the cultivation period. The buckets were placed in a climate chamber with a

light intensity of 250 µmol $m^{-2}s^{-1}$ at 24^0C and 75% relative humidity. Ebb and flow irrigation system using nutrient solution containing in (mM): 12.5 NO_3-N, 1.2 P, 4.7 K, 1.1 Mg, 0.6 S and 4 Ca and in (µM): 10 Mn, 25 B, 0.75 Cu, 18.3 Fe 5 Zn and 0.5 Mo was used. The electrical conductivity (EC) and the pH of the nutrient solution were monitored daily during the duration of the experiments. The EC was kept at 2.9 mS cm^{-1}, whereas pH was kept between 5.6 and 6 through the addition of 1M of HCl or NaOH. The growing media were moistening with nutrients twice a week. A complete randomized block design with four replicates of each treatment was used.

Results and discussion

Application time and *In vitro* antagonism

For successful biocontrol in closed hydroponic systems, application of the the biocontrol agents has to be performed prior to the addition of the pathogen. This was indicated for both the bacterial strain *P. fluorescens* 5.014 as well as for the commercial products (Khalil & Alsanisu 2006a). Moreover, both seed inoculation by the bacterial strain and application of mixtures of the commercial products have shown to be suitable means for the application of bca in closed hydroponic systems. For all pathogens used in this study, the test of *In vitro* interactions showed that *Trichoderma* strains in the biocontrol product Binab T rapidly colonized the PDA medium and grew over the mycelium of the pathogens (see lägesrappot 2006). A noticeable inhibition of the pathogen growth was also found for *S. griseoviridis* in the Mycostop product. A very slight inhibition of the pathogens by *Gliocladium* spp. in the Gliomix product was noticed after one week of growth on PDA plates. No inhibition of the pathogens was observed for the bacterial strain *Pseudomonas fluorescens* 5.014.

For the strains used in this study, the actual mechanisms responsible for disease inhibition were not investigated. However, previous survey showed that the production of extracellular hydrolytic enzymes by *Trichoderma* spp. has been shown to be an important factor for disease reduction by these fungi (Thrane et al. 2000). Actinomycetous bacteria have been recognized as sources for several secondary metabolites and antibiotics (Tanaka & Ōmura 1993). The presence of an inhibition zone occurring between the pathogens and *Streptomyces griseoviridis* in Mycostop *In vitro* studies (see lägesrapport 2006) might suggest that production production of antibiotic compounds are involved in the antagonism observed *In vivo*. The mode of action for *Gliocladium cantenulatum* has been reported to be due to mycoparasitism and to the production of cell-wall-degrading enzymes (McQuilken *et al.* 2001). For the bacterial strain 5.014, investigations performed by Hultberg et al. (2000) have shown that inhibition of the growth of *P. ultimum* was due to the production of siderophore.

Moreover, the beneficial relationship between the plants and the introduced strains is an indication for a successful use of different strains for microbial control of root diseases in hydroponic systems. In all investigations performed, a plant growth promotion effect could be achieved by the bacterial strain as well as by the commercial bca.

Application of the biocontrol agents individually

The results indicated variations in the efficiency against the tested pathogens due to the type of the pathogen presents in the system. All the strains in the commercial products were effective against the root pathogens PU, PA and FO. Binab T. and Mycostop were more effective than Gliomix (Fig. 2). Previous studies conducted by Rose et al. (2003) showed that *Gliocaldium cantenulatum* was the most effective biocontrol agent against FO and PA, respectively. However, these studies were performed in substrate-based hydroponic systems with rockwool while liquid hydroponics were used in the present study. This might suggest that the efficiency of the agents could also be affected by the type of the hydroponic system used. Effectiveness against the root pathogen PC was only shown by the biocontrol strains in

Binab T and Mycostop. However, these evaluation experiments in the climate chamber and test of *In vitro* antagonism showed no inhibition of the root pathogen PC by neither Gliomix nor the bacterial strain.

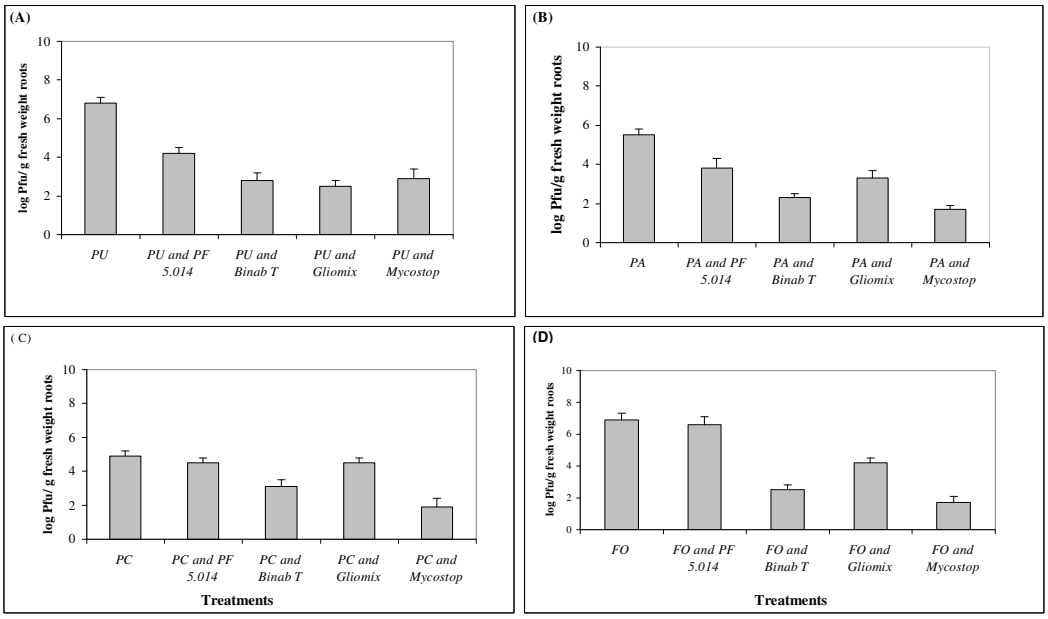

Figure 2. The efficiency of BinabT, Gliomix and Mycostop and the bacterial strain *P. fluorescens 5.014* against root diseases of tomato caused by (A) *Pythium ultimum* (B) *P. aphanidermatum* (C) *Phytophthora cryptogea* and (D) *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Each bar represents the mean ± SE, n=4.

Application of the biocontrol agents in combination

A disease reduction estimated by the amount of pathogen could be achieved when the bca´s were used in combination. However, this reduction was noticed to decrease depending on the number of the bca present in the system (Fig. 3). In the case of PU, the amount of pathogen decreases when the bacterial strain *P. fluorescens* 5.014 was combined with Binab T and Mycostop.

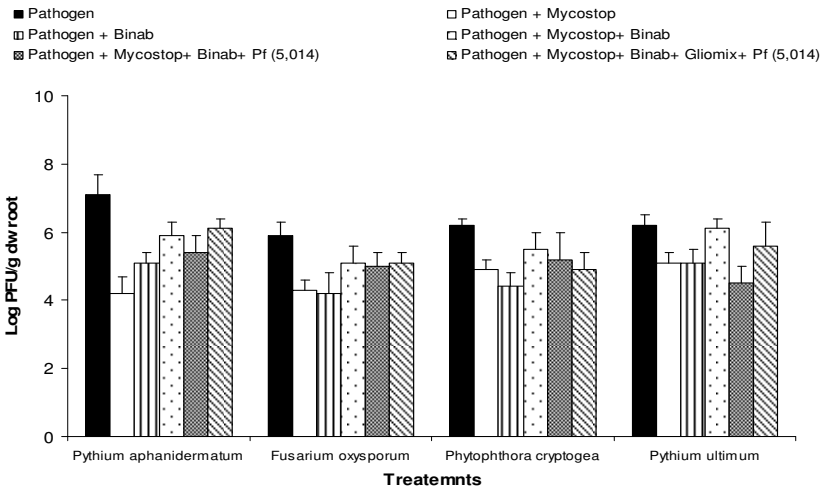

Figure 3. The effect of the bca used in combination on the amount of root pathogens *Pythium ultimum*, *P. aphanidermatum*, *Phytophthora cryptogea* or *Fusarium oxysporum* f. sp. *radicis-lycopersici* in liquid hydroponic system. Each bar represents the mean ± SE, n=4.

The use of combined bca intends to achieve better results based on the fact that each biocontrol agent may use different mechanisms to fight the pathogen. Different studies indicated the synergism effects achieved by combining bca (de Boer et al. 1999). This could, however, not be indicated in this study. This might raise questions about the application rate, mode of action, colonization ability of the biocontrol strains. However, more investigation concerning the mode of action, application rate and impact of biotic and abiotic factors under such conditions is essential.

Application of the biocontrol agents in two growing media

The results from these investigations pointed out variations in the biocontrol activity of bca depending on the type of pathogen present in the system and the type of the growing media used (Khalil & Alsanius 2007). Binab T and Gliomix were more effective peat than Mycostop. However, this is not surprising since these strains have been reported to have an enhanced biocontrol effect in food based substrate and substrates with high organic matter (Papvizas 1985). The bacterial strain 5.014 showed no effect against the tested pathogens neither in peat nor in pumice. In the greenhouse experiments, the bacterial strain showed no biocontrol effect against the tested pathogens (Fig. 4). However, in previous investigations an antagonistic effect against PU in liquid hydroponics could be indicated (Khalil 2001). This also indicates the variations in the efficiency of this strain due to type of hydroponic system. All the commercial bca showed lower biocontrol activity at the end of the cultivation period.

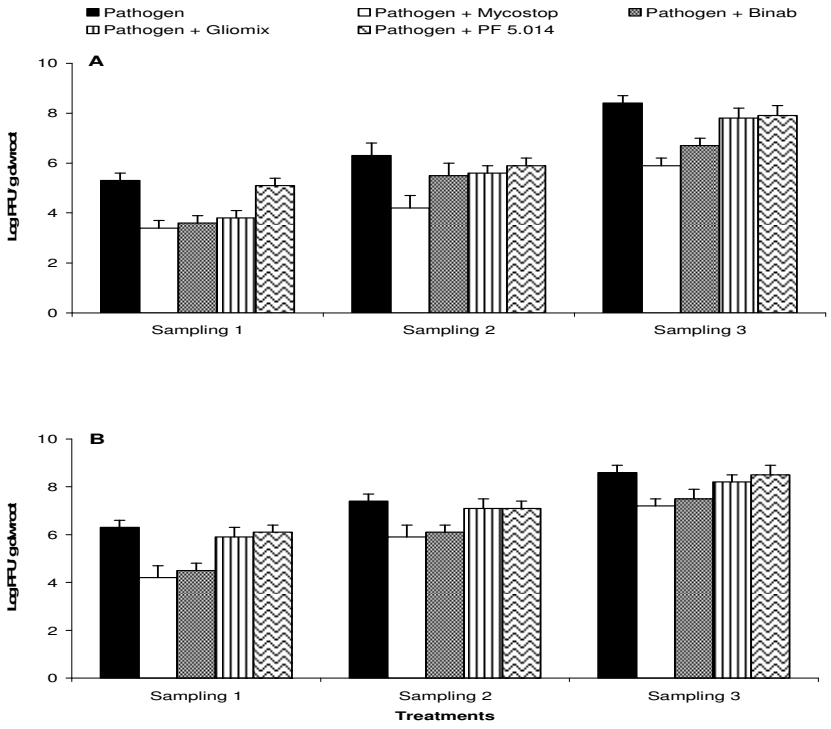

Figure 4. The biocontrol activity of BinabT, Gliomix and Mycostop against root diseases of tomato caused by (A) *Pythium aphanidermatum* and (B) *Phytophthora cryptogea* in pumice. Each bar represents the mean ± SE, n=4.

Microbiological analyses on the resident microflora, as judged by viable count and SCSU patterns, indicated differences in the composition and function of the microflora due to the introduction of the biocontrol and the pathogen on the resident microflora. The molecular fingerprinting PCR-DGGE showed also shifts in the genetic diversity of the resident microflora due to the treatments.

Analysis of the pure cultures of the biocontrol strains using SCSU patterns indicated differences in the utilization patterns of the biocontrol agents and the pathogens. SCSU started earlier and a broader spectrum of compounds was utilized when the active ingredient in Mycostop was inoculated as compared to the two other products. This could indicate the more efficient use of carbon sources by Mycostop than Binab T and Gliomix. For the pure cultures of the pathogens, the utilization pattern of the root pathogen PC differed from those of PA and FO which were similar in their utilization profiles.

Future research

Levels of disease suppression expressed in experiments during this project indicates the variable effect of the biocontrol agents in hydroponic recirculated system concerning both the bacterial strain *Pseudomonas fluorescens* 5.014 and the commercial biontrol products, Binab T, Gliomix and Mycostop. In order to achieve effective biocontrol strategies in these systems more knowledge about the biocontrol mechanisms used by these strains is of particular importance. Interactions between the introduced biocontrol strains and biotic and abiotic factors in the system are also of concern. Future research within this area should also be focused on the development of molecular technique in detecting both the pathogen and the biocontrol agent in the cultivation system as well as on stress factors that affect the plants health and the performance of the biocontrol agent and the pathogen. Interactions between *Pseudomonas* and *Pythium* spp. are also of interest.

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