The effect of combining two biological control microbes on seed and root colonization

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The effect of combining two biological control microbes on seed and root colonization

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Abstract:

Clonostachys rosea strain IK726 and *Pseudomonas chlororaphis* strain MA342 are two promising biocontrol microbes which both has various biocontrol mechanisms to control many seed and soil-borne plant diseases. Because a potential additive effect may exist between these two biocontrol microbes, like an expanded antagonistic spectrum and enhanced persistence of biocontrol effect, we expected to get a more competitive biocontrol with a mixture of IK726 and MA342. Strong colonization and high persistence ability on seed and root is one important trait of a potential biocontrol agent. This study was aimed to estimate the colonization ability on seed and root under the circumstances of joint and alone applications of IK726 and MA342 as seed-dressing on spring wheat cultivar Dacke. By comparing the dilution plating results of joint and alone inoculated treatments, we observed that IK726 slightly reduced the initial seed inoculum of MA342. But in the treatment with or without IK726, MA342 persisted a stable population density in root that is distinct from previous research. We got an unexpected result from dilution plating that IK726 has not been detected on the root of spring wheat which has been confirmed by PCR results. Mycelial growth and sporulation lagged behind root-tip elongation, and nutrient deficiency might be the causes of the poor performance of IK726 on spring wheat root.

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1. INTRODUCTION

1.1 The General Concept of Biological Control of Plant Diseases:

Biological control of plant diseases is the utilization of one or more living organisms which have antagonistic effect against various plant pathogens to achieve the suppression of plant diseases (Baker *et al.*, 1982b; Baker, 1987; Pal *et al.*, 2006). The real value of biological control of plant diseases is reflected in the less usage of chemical pesticides (Monte, 2001). According to the objective of biocontrol of plant diseases which was given by Baker and Cook (1982b), any living organism which is able to decrease the inoculum density of plant pathogen or weaken its pathogenicity or reduce the infection of a host plant can theoretically become a potential biological control agent (BCA).

The global screening of potential plant disease BCAs have been going on for years. Among these BCAs, there are listed various living microorganisms including bacteria, fungi, and also including virus, etc. But the most common BCAs are either bacterial or fungal antagonists isolated from the rhizosphere and used to control seed or soil-borne pathogens, like *Rhizoctonia* spp., *Gaeumannomyces* spp., *Sclerotina* spp., *Phytophthora* spp., *Pythium* spp., and *Fusarium* spp., etc (Baker, 1968) (Table 1.).

Table 1. Biocontrol of various seed and soil-borne diseases by selected antagonistic micro-organisms.

	Genera	Different isolates	References
Bacterial antagonistic micro-organisms	*Pseudomonas*	*P. fluorescens* 2-79	(Thomashow *et al.*, 1988)
		P. fluorescens Pf-5	(Howell *et al.*, 1979)
		P. fluorescens CHA0	(Keel *et al.*, 1992)
		P. putida WCS358	(Geels *et al.*, 1983a)
		P. chlororaphis MA342	(Hökeberg *et al.*, 1997)
	Bacillus	*B. subtilis* BD170	(Broggini *et al.*, 2005)
		B. pumilus CL45	(Leifert *et al.*, 1995)
		B. cereus UW85	(Handelsman *et al.*, 1990)
	Serratia	*S. marcescens* B2	(Someya *et al.*, 2000)
		S. plymuthica HRO-C48	(Kurze *et al.*, 2001)
	Agrobacterium	*A. radiobacter* 84	(Moore *et al.*, 1979)
	Streptomyces	*S.griseoviridis* K61	(Tahvonen, 1982)
Fungal antagonistic micro-organisms	*Trichoderma*	*T. harzianum* 1295-22	(Sivan *et al.*, 1991)
		T. harzianum T-39	(De Meyer *et al.*, 1998)
	Clonostachys	*C. rosea* J1446	(McQuilken *et al.*, 2001)
		C. rosea IK726	(Knudsen *et al.*, 1995)
	Coniothyrium	*Coniothyrium minitans*	(Campbell, 1947)
	Chaetomium	*Chaetomium globosum*	(Hubbard *et al.*, 1982)

The seed and soil-borne diseases cause heavy yield losses annually and are hard to control. Contrary to the ineffectiveness of conventional control methods such as application of chemical pesticides, crop rotation and selection of resistant cultivars, biological control method undergo extensive testing (Schroth *et al.*, 1982; Haas *et al.*, 2005). Many milestone BCAs such as *Agrobacterium radiobacter* K84 and *Trichoderma harzianum* T-22 has withstood the practical field test (Cooksey *et al.*, 1982; Harman, 2000). Any one of the

BCAs performed with multiple biocontrol mechanisms and it is not easy to determine which specific mechanism plays a key role. We chose *Pseudomonas* spp. as examples of bacterial antagonists, *Trichoderma* spp. and *Clonostachys* spp. as examples of fungal antagonists to discuss some well-known types of biocontrol mechanisms in the next section.

1.2 Biological Control Mechanisms

Strong colonization ability has always been regarded as a key assessment criteria of an eligible biocontrol microorganism (Harman, 2000). Colonization ability improves the survival and competitiveness of biocontrol microbe for nutrients and habitats. More information about colonization ability was discussed in the section 1.3.

Many bacterial and fungal antagonists are antibiotic producers. Many fluorescent pseudomonads (Table.1.) produce a broad-spectrum antibiotic, 2,4-diacetylphloroglucinol (2,4-DAPG or Phl), which plays a key role in wheat take-all decline (Bonsall *et al.*, 1997). Phenazines (Phz) and its derivatives (phenazine-1-carboxylic acid, PCA; phenazine-1-carboxamide, PCN) can broadly inhibits the growth of fungi and bacteria by the accumulation of toxic superoxide and hydrogen peroxide (Haas *et al.*, 2003; Mavrodi *et al.*, 2006).

Many antibiotic toxins are produced by *Trichoderma* spp., including trichodermin, gliotoxin, trichobrachin, trichovirin, viridin and peptide antibiotics, etc. These toxins can damage fungal pathogen cell wall and cause mycelium crack and cytoplasm leakage (Hou *et al.*, 1972).

Siderophores inhibits the pathogen growth in the form of iron chelate. *P. putida* vigorously deprived iron from native soil-borne pathogens by producing extracellular siderophore, pseudobactin (Kloepper et al., 1980).

Lyases produced by BCA (chitinase, β-1,3-glucanase, cellulase and proteinase) is regarded as an important factor to inhibit plant pathogen mycelium growth and spore germination. Naik isolated one antifungal *Pseudomonas* strain from rice root which was found to produce chitinase and proteinase (Naik *et al.*, 2006). Exoenzymes of fungal antagonists are essential in the process of mycoparasitism, especially in *Trichoderma* spp. and *Clonostachys* spp.. Four steps are usually involved in the mycoparasitism: i). target location; ii). recognition; iii) contact and penetration; iv). nutrient acquisition (Durrell, 1968; Pachenari *et al.*, 1980).

Many *Pseudomonas* and *Trichoderma* strains can elicit plant defense through the activation of the SA or JA/ET signaling pathways. Various biocontrol

microbe expressed effectors (or called microbe-associated molecular patterns, MAMPs) like siderophores, lipo-polysaccharides and cellulases, etc which have been confirmed prime plant defence or induce resistance in host plants (Peer *et al.*, 1991; Yedidia *et al.*, 1999).

Promotion of plant growth through enhancing the Fe and P uptake of plants (Yedidia *et al.*, 2001) and regulating phytohormones (Contreras-Cornejo *et al.*, 2009) have been observed in many *Trichoderma* or PGPRs mediated plant-microbe interactions.

1.3 Colonization Ability of Biocontrol Agent

A positive relationship between colonization ability and disease suppression has been observed in numerous BCA studies (Weller, 1984; Bahmen *et al.*, 1987; Weller, 1988). It has been widely accepted that strong colonization ability is a prerequisite for BCA to suppress soil-borne pathogens at the potential infection sites (Bahmen *et al.*, 1987; Handelsman *et al.*, 1996). So it is extremely essential to accumulate knowledge of the spatio-temporal dynamics and microbial ecological processes of seed and root colonization by an antagonist. Because the most common inoculation method for antagonistic microorganism is seed dressing, we summarized the general process of introduced antagonist

spreads from the inoculated seed or seed piece to the root system. This process could be divided into two phases.

Phase Ⅰ starts from sowing and ends before seed germination. The main events are activation of the antagonist inoculum and establishment of an antagonist population in the spermosphere. Because of the seed imbibition, at the beginning of Phase Ⅰ, the water potential drives the water flow from soil to seed. Although the antagonist-treated seed started with a high dosage of inoculum, it was not easy for the bacterial antagonists to massively diffuse to the surrounding soil by countering the water flow (Scher *et al.*, 1985; Haas *et al.*, 2005). In contrast, fungal antagonist could expand their habitat into the soil mass by active mycelium growth and sporulation (Lübeck *et al.*, 2002). The introduced antagonist and the native soil microbes which respond to seed exudates with chemotaxis all contribute to the microbial balance in the spermosphere (Weller, 2007).

Phase Ⅱ is the process of the introduced antagonist and native root-associated microbes to establish a population density and persist in the rhizoplane, rhizosphere or inside the root. Some unpredictable factors were excluded, like sudden flooding which can disturbs the natural distribution of the antagonist. The geotropic growth of young root started when the seed was germinated.

During the root-tip was pushing forward, the border cells of the root cap were programmed separated from root-tip. Some fresh separated cells mixed with the cell contents after cytolysis formed a sheath-like mucigel that worked as lubricant (Hawes *et al.*, 1998). This mucigel contained abundant ions which resulted in potential gradient across interface of the root-tip and its adhesive soil. Certain root-associated microbes can sense this gradient and move to the root (Baker *et al.*, 1982a). This mucigel directly carried the introduced antagonist from seed to deeper root system. Most of the fungal antagonist spread to the deeper soil layer without multiplication, but bacterial antagonit with short life-cycle maybe can reproduce (Weller, 1988). During the root-tip moving downwards, root cap mucigel carried antagonists or the native root-associated microbes were all gradually diluted (Baker *et al.*, 1982a). Part of the microbes continuously attached to the root surface as inoculum, colonized on the maturing root later and fed on the nutrient from the exudates of root hairs (Weller, 1984; Haas *et al.*, 2005). The population density of fungal inoculum usually declined faster on root than bacterial inoculum during root elongation (Baker *et al.*, 1982a). One reason was the longer life cycle of fungus as we said before, the root tip mucigel carried fungal inoculum was hardly reproduced during the root-tip elongation. Another reason was, compared to bacteria, the bigger fungal spores confronted more resistance during the interaction between root tip and soil mass. For these two reasons, we can

conclude that the rate of root colonization of fungal inoculum was greatly falling behind of root tip elongation. So the initial population establishment of fungal inoculum on root relies on the rate of spore germination and mycelium growth. But whether fungal or bacterial antagonist, they are all dependent on the root exudates for living. About 20% plant dry weight was released into the rhizosphere, the whole released materials composed of root cap cells, mucigel, nutrient exudates of root hair and lysates (Kluepfel, 1993). The root exudates contained organic acids, amino acids, large proteins, carbohydrates, vitamins and other secondary metabolites (Rovira, 1969; Bais *et al.*, 2006). The root exudates is the initial driving force of the rhizosphere effect: drastic population increase of different root-associated microbes (Rouatt *et al.*, 1960) and alter the composition of the microflora in the rhizosphere under different physiological or pathological stages (Katznelson, 1946; Rovira, 1956). Part of the mucigel-carried microbes was lost during root-tip pushing soil mass. Aimed to quickly colonise the root, certain microbial characteristics were observed. The flagella and pili were contributed to a short movement of rhizobacteria that penetrated the mucigel layer to get closer to root surface (Hamdi, 1971; De Weger *et al.*, 1987; Vesper, 1987). Lipopolysaccharides and agglutinin has been confirmed that involved in the microbe-plant cell to cell recognition and the microbe attachment to the root surface (Douglas *et al.*, 1982; Martinez-Gil *et al.*, 2010; Nian *et al.*, 2010), and biofilm formation can enhance this

attachment to epidermal cell and root hair (Danhorn *et al.*, 2007). Some endophytic bacteria can aggregated and even lived inside the epidermal and cortical cells (Chin-A-Woeng *et al.*, 1997; Troxler *et al.*, 1997; Bloemberg *et al.*, 2000; Prieto *et al.*, 2011). Hydrophobins have been proved to enhance *Trichoderma* attachment to the root surface (Viterbo *et al.*, 2006) and plant cell wall degrading enzymes was relevant to the population establishment of *Trichoderma* spp. (Ahmad *et al.*, 1987). Due to my research mainly related to the living antagonistic fungus (*C. rosea* IK726) and bacteria (*P. chlororaphis* MA342), so these two strains discussed into details hereinafter.

1.3.1 Colonization Pattern of MA342

P. chlororaphis MA342 was originally isolated from Swedish soil and selected for its biocontrol activity in green house bioassay (Hökeberg *et al.*, 1997). It has been exploited as a commercial BCA for seed dressing (Hökeberg, 1998). It has been proved that MA342 control seed-borne pathogens partly via antibiosis, 2,3-deepoxy-2,3-didehydrorhizoxin (Hökeberg, 1998). Promotion of plant growth, induction of host plant resistance and competitive colonization all contribute to its biocontrol ability (Hökeberg, unpublished). According to the previous research, a competitive colonizer was performed in the spermosphere, but contrary in the rhizosphere (Johnsson *et al.*, 1998). Tombolini et al. (1999)

selected a green fluorescent protein coded transformant MA342G2 and studied its colonization pattern on barely seed after sowing. The initial distribution of MA342G2 on barely seed 1-2 days after sowing has been determined and the isolate was shown to be an excellent spermosphere colonizer. The attachment sites included the external cracks and the parenchymatous layer of the glume. More aggregation of MA342G2 that close to the barely seed embryo was observed when seed began to germinate that actually formed a physical barrier to protect young roots . But MA342G2 did not occur inside the embryo that might limit the biocontrol effect to certain seed-borne pathogens (Hökeberg, 1998).

1.3.2 Colonization Pattern of IK726

Clonostachys rosea is a saprotrophic soil fungus (Schroers *et al.*, 1999). *C. rosea* strain IK726 was isolated from barely roots (Knudsen *et al.*, 1995) and evaluated over a hierarchic screening process as a potential BCA against seed and soil-borne diseases in Denmark (Knudsen *et al.*, 1997). In the field experiment of barley and wheat seed treated with IK726 against *Fusarium culmorum*, the seed-inoculum dosages of fresh or stored IK726 conidia were all highly correlated with the biocontrol efficacy (Jensen *et al.*, 2000). A web of hyphe of *gfp* transformant IK726d11 with sporulation was detected on carrot

seed during biopriming which confirmed IK726 was a competitive colonizer (Jensen *et al.*, 2004). After the germination of inoculum spores, new IK726d11 hyphe colonized on intact tissue of carrot roots with sporulation, and then actively moved to deeper root by constantly hyphal growth and sporulation (Lübeck *et al.*, 2002). As we said in section 1.3, the colonization rate of fungal antagonist and the root tip elongation is asynchronous. Fungal antagonist posses merits e.g. quick spores germination and fast mycelium growth rate is always expected by researchers. The active nutrient up-taking ability is essential for fungal growth that can assist antagonist to take the leading-position in root colonization. In the soil condition with limited organic matter which is the carbon source of IK726, IK726 mainly present as spores with limited germination and hyphal growth (Lübeck *et al.*, 2002). When extra organic matter added into soil, the population density and the growth rate of IK726 increased, but other soil microoganisms decreased (Ravnskov *et al.*, 2006). On the contrary, without extra organic matter IK726 stimulated biomass increase of some bacteria species.

2. Objective

IK726 and MA342 have been proved to function as biocontrol microbes in the field. It has been introduced in section 1.3.2 that IK726 can increase the

biomass of other soil microbes (Ravnskov *et al.*, 2006) including *Pseudomonas* spp. (Johansen *et al.*, 2005). An additive effect among IK726 and MA342 was expected that control seed and soil-borne pathogens with broader antagonistic spectrum and longer duration. The aim of the current study is to test if these two antagonists are compatible for cereal seed dressing. Conventional dilution plate counting and PCR detection methods were used in monitoring the colonization abilities of two antagonists on the seed and root of spring wheat.

3. Materials and Methods

3.1 Experimental design

We designed four seed dressing treatments to investigate the colonization ability of the two antagonists on spring wheat: two treatments were only inoculated with MA342 and IK726 respectively; the third treatment was dual inoculation of MA342 and IK726; the fourth treatment was inoculation of tapwater as inoculum control. We detected the colonization of the two antagonists on seed by dilution plating on the same day we inoculated the seeds. Root samples was collected on 7 and 10 days after sowing and also analyzed by dilution plating. PCR detection primers of MA342 and IK726 has been prepared to check the two antagonist colonization on molecular level.

3.2 *P. chlororaphis* and *C. rosea* inocula

Stock culture of MA342 was offered by Annika Gustafsson, BioAgri AB. MA342 was recultured on vegetable peptone agar (VPA, Thermo Scientific Oxoid) and incubated in 28℃ growth chamber for 2 days until single colonies formed. Two Erlenmeyer flasks (250ml) of vegetable peptone broth (VPB, Thermo Scientific Oxoid) were prepared and both inoculated with single colony of MA342 in shake culture, 120 rpm in room temperature for 48 hours. One flask was for the DNA extraction, the other one was for the seed dressing. We calculated the concentration of VPB inoculated MA342 after shaking culture by series dilution plate counting which was about 9×10^{9} -1×10^{10} cfu/ml, Table 2.

Stock culture of IK726 was retained in -80℃ in our own lab. IK726 was reculture on potato dextrose agar (PDA, BD Difco) plates and incubated in 25℃ growth chamber (in dark) for 14 days. Then we transferred the PDA plates to room temperature in day light condition to induce sporulation. Sporulated PDA plates were washed with 5 ml sterile water. We filtrated the mycelium mixed spore solution through glass fibre. The spore concentration was determined with a hemacytometer and adjusted to 2×10^{9} cfu/ml, Table 2.

3.3 Seed dressing

For each treatment, 2 ml inoculum was added to 100 g spring wheat seeds, Table 2. For the MA342 and IK726 inoculated alone treatments, inoculum were obtained from half dilution of the MA342 and IK726 stock suspensions in section 3.2 respectively. Disease-free spring wheat cultivar Dacke was offered by BioAgri AB. Seed dressing was performed in a spinning disc lab (Rosengren).

Table 2: Initial concentrations and dosages of two antagonists in four seed dressing treatments

	Concentration of inoculum	Dosage/ 100 g seeds	cfu/seed
MA342	4.5×10^9 cfu/ml	2 ml	$3.2\text{-}4.5 \times 10^6$
IK726	1×10^9 cfu/ml	2 ml	$7.1 \times 10^5 – 1.0 \times 10^6$
MA342+ IK726	MA342:9×10^9cfu/ml IK726: 2×10^9cfu/ml	1 ml 1 ml	$3.2\text{-}4.5 \times 10^6$ $7.1 \times 10^5 – 1.0 \times 10^6$
Tapwater control	----	2 ml	----

3.4 Growth condition

We split the plastic centrifuge tubes (12cm length, 4cm diameter) longitudinally and bonded the two pieces by adhesive tape. Two seeds were sowed in each tube. We put six tubes in one container as six replicates for each seed dressing treatment, Fig 1. All tubes and containers were filled with quartz sands. Tapwater was added at the beginning. No extra fertilizer solution was added. The potting experiment was operated in the growth chamber, 12℃, under long-day light condition (dark:light=8h:16h).

Fig 1. Climate chamber experiment set-up for testing the colonization ability of two BCAs

3.5 Seed and root sampling and colonization test

10 seeds were randomly selected from each seed dressing treatment before sowing. The seeds were washed by shaking with 10 ml 10mM $MgSO_4$ and 20

glass beads for 15 minutes, Fig 2. Series 10 fold dilutions from 10^{-2} to 10^{-5} were made.

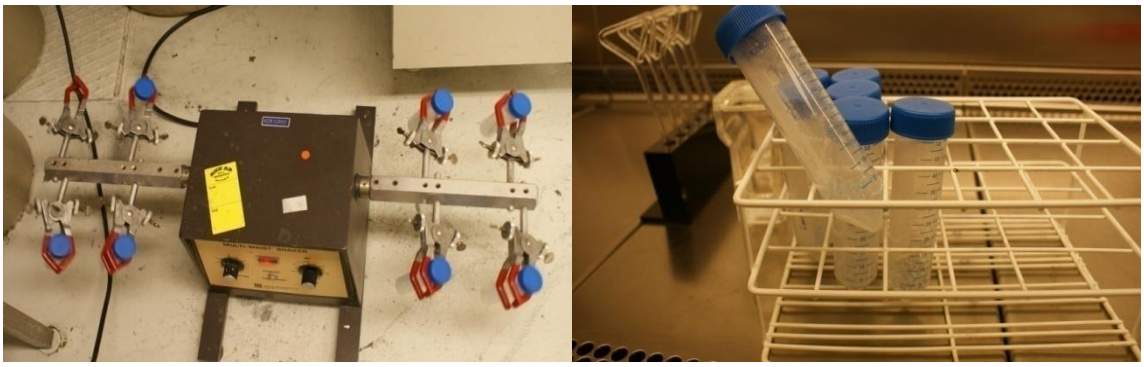

Fig 2. Shaking machine and series 10 fold dilution

We took 0.1 ml from each dilution and spread it on plate counting agar where PDA and PF (fluoresent pseudomonds medium, BD Difco) were used for IK726 and MA342 respectively. All plates were incubated in 28 ℃ growth chamber (no light). Visible colonies on each plate were counted after 48 hours incubation for MA342 and 96 hours incubation for IK726 respectively.

For the root colonization test, plants were sampled on day 7 and 10 after sowing. Wheat seedlings were gently pulled out of the tubes, Fig 3. On the first sampling (day 7), only 1 cm root tips were collected from each plant, two root tips from two plants in each tube were washed by shaking with 2 ml 10 mM $MgSO_4$. Another sampling site was added on the second sampling time (day 10), 1 cm root segment was cut off 1 cm beneath the seed, Fig 3 (right). The abovementioned dilution plating method was used.

Fig 3. Root sampling sites for testing the root colonization ability of two BCAs
(Left: root sampling on day 7; Right: root sampling on day 10. Red bracket: sampling sites)

3.6 PCR detection primers of two antagonists

CRnc A/B, a detection primer pair for IK726, was offered by Magnus Karlsson (Department of Forest Mycology and Plant Pathology, SLU), Table 2.

The detection primer pair for MA342 was designed in this study. The genomic DNA (prepared in section 3.2) of MA342 was isolated by following a modified CTAB protocol (Rogers *et al.*, 1985; Griffiths *et al.*, 2000). A primer pair targeting a 345 bp the *Pseudomonas* spp. gene for ribosomal protein S11 (S11A/B; Tm: 56 ℃ ; forward: CAAAACCTGCTGCTCGTCCTC; reverse: TGCGGGATTGGCGTCAC) was designed using the PrimerSelect (DNASTAR) software. The primers were designed to conserved regions based on an

alignment between the *S11* gene from *P. fluorescence*, *P. fulva*, *P. mendocina* and *P. putida*. The *S11* sequences were retrieved from the genome sequences of the included species, which are available at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). The S11A/B primer pair was used in PCR to amplify the partial *S11* gene from MA342. PCR reaction was performed using the following conditions: one cycle of 98°C for 30 sec, 30 cycles of 98°C for 10 sec, 55°C for 20 sec and 72°C for 2 min. The purified PCR product was sequenced by Macrogen Inc (Seoul, Korea) in both forward and reverse directions. The sequence result was evaluated by using the BioEdit package version 7.1.3 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and confirmed by BlastX searches. A new primer pair (PC For/Rev, Table 2) was designed using the PrimerSelect (DNASTAR) software, based on the amplified MA342 *S11* gene, for detection of MA342. There was no cross amplification among these two detection primers, PC For/Rev and CRnc A/B.

Table 2. Detection primer pairs for MA342 and IK726

	Forward primer 5'-3'	Reverse primer 3'-5'	Amplicon(bp)	Tm
PC For/Rev	GCGGACTGCGGATTCACGAC	TAACGCTCTTTCCTGGGCTACCTC	178	60
CRnc A/B	TTTCTCGGCCTTTGTCCACTAACG	CGCCCCGCCCCCATTCTA	124	60

3.7 PCR detection of seed and root colonization of two antagonist

A subset of the seed and root samples were selected from the four treatments for PCR detection. The reactions were performed using the following conditions: one cycle of 98°C for 30 sec, 30 cycles of 98°C for 10 sec, 60°C for 20 sec and 72°C for 2 min. The last cycle was followed by 10 min at 72°C and then 4°C. The PCR products were analyzed by electrophoresis on 2 % agarose gel. The PCR results were compared with the results of dilution plating, section 3.5.

4. Results

4.1 Seed colonization of two antagonists on spring wheat

In the two antagonist alone inoculated treatments, the population densities of MA342 and IK726 were log 5.68 cfu/seed and log 4.80 cfu/seed respectively, Fig. 4. In the dual-inoculated treatment, the population of MA342 was 10-fold decreased (log 4.43 cfu/seed) and the population of IK726 was stable (log 4.73 cfu/seed).

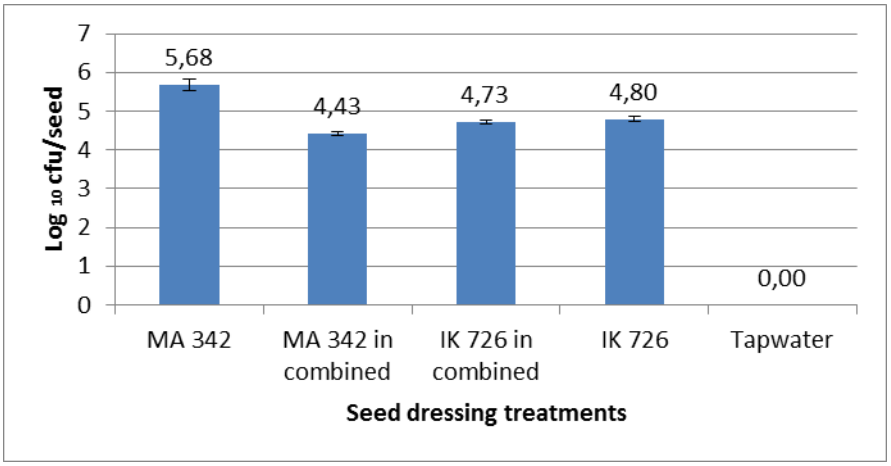

Fig. 4. Population densities of MA342 and IK726 in all four treatments on seed. Error bar represent standard errors of the means of three replicates.

4.2 Root colonization of two antagonists on spring wheat

On the 7 days after sowing, only MA342 was found on root tips with density above log 3 cfu/cm root, Fig. 5. In both IK726 alone and dual-inoculated treatments, plating results indicated that IK726 population of original dilution were all under the lower limit of countable range (30-300 colonies).

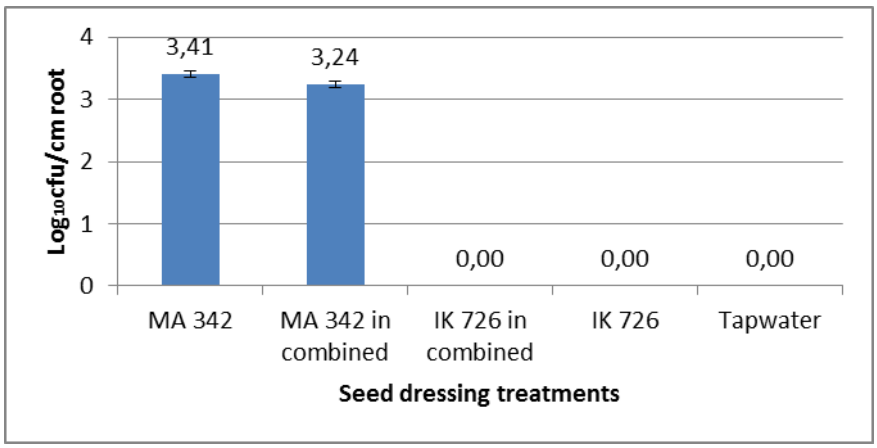

Fig. 5. Population densities of MA342 and IK726 in all four treatments on root (sampling on 7 days after sowing). Error bar represent standard errors of the means of three replicates.

On the 10 days after sowing, the population density of MA342 was about log 3 cfu/cm root in all the close to seed samples, Fig. 6.1. But in all root tips samples, population density of MA342 had declined to about log 2 cfu/cm root, Fig. 6.2. The same plating results were obtained in all treatments with IK726, where plating results were all under the lower limit of countable range (30-300

colonies).

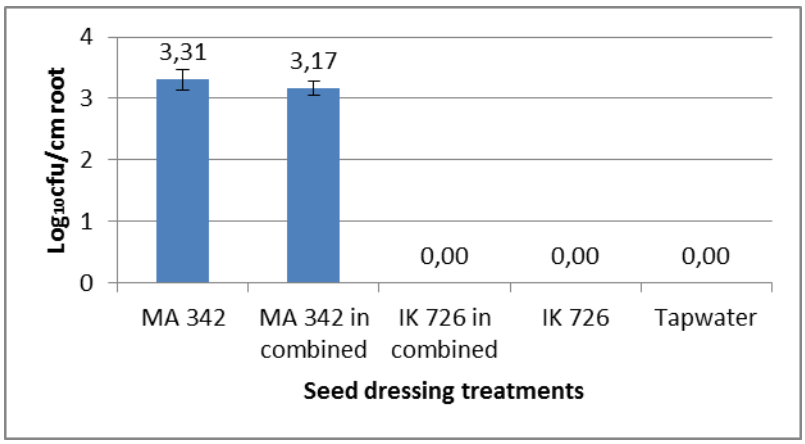

Fig. 6.1
1 cm root segment beneath the seed

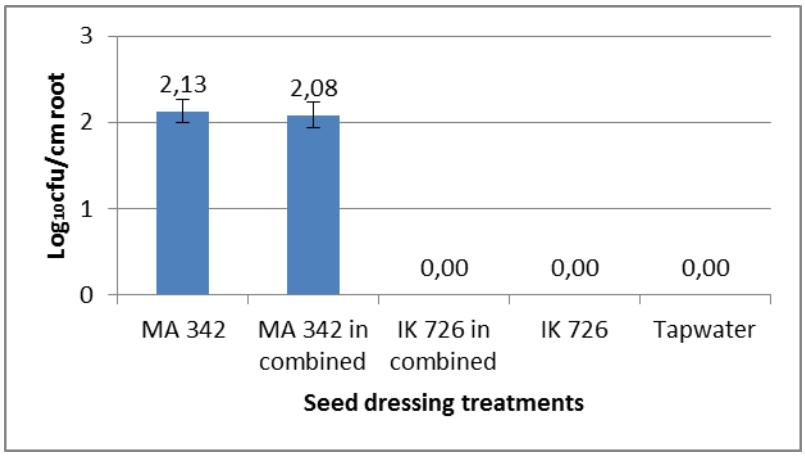

Fig. 6.2
1 cm segment of root tip

Fig. 6 Population densities of MA342 and IK726 in all four treatments on root (Sampling on 10 days after sowing). Error bar represent standard errors of the means of three replicates.

4.3 PCR detection of seed and root colonization of two antagonists

The electrophoresis identified a total of 4 bands of among 6 samples, Fig.7. Two amplification bands belongs to MA342 and IK726 alone inoculated seed samples respectively. Two other bands all belongs to MA342 alone and dual inoculated root samples (day 7). No amplification band was detected in IK726

alone and dual inoculated root samples (day 7).

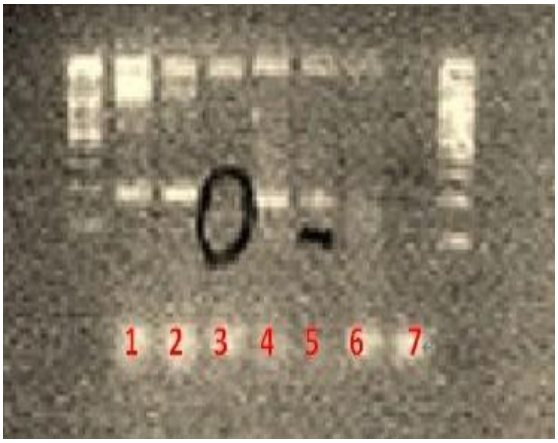

Fig.7. Loading PCR samples from the left to the right:

1. MA342 (root, alone treatment)
2. MA342 (root, combined treatment)
3. IK726 (root, combined treatment)
4. MA342 (seed, alone treatment)
5. IK726 (seed, alone treatment)
6. Tap water (seed)
7. Negative control

Specific detection primers: CRnc for IK726; PC For/Rev for MA342

5. Discussion

5.1 Seed colonization of two antagonists on spring wheat

Theoretically, the initial inoculum population density of the antagonist affects its subsequent persistence in the rhizosphere. In the dual inoculated treatment, the population density of MA342 was 10 fold decreased compared to that in the MA342 alone inoculated treatment. One reasonable explanation is that seed has a limited carrying ability for microorganisms. The inoculated antagonists had suffered through a competition for the attachment sites on spring wheat seed. IK726 may has a stronger ability to seize the limited attachment sites on spring wheat seed.

5.2 Root colonization of two antagonists on spring wheat

C. rosea is a worldwide isolated soil saprophytic fungus. We expected IK726 performs as an active root colonizer. But our root sampling results suggested a fact that there was no trace of IK726 in the rhizosphere and the dilution plate counting results were confirmed by PCR detection results. Most possible reason is nutrient deficiency, especially lack of organic matter in the potting system. Since only tap-water was added without extra fertilizer, the root exudates became the only nutrient source. About the importance of organic matter in IK726 life cycle we already discussed in section 1.3.2. Clearly without sufficient carbon source supply, only small amount root exudates of two wheat seeds in each tube clearly was not enough to support a high population density of IK726 in the rhizosphere. The other important reason is, unlike the root colonization of most bacteria that can be directly carried and distributed by root tip mucigel, the much larger-size IK726 spores have to actively explored the nutrient substrates in the form of hyphal growth. Besides only a limited number of IK726 spores germinate in the absence of organic nutrients. So obviously, the slow hyphal growth was not able to keep pace with root tip elongation. And hypha lack of sporulation can be easily break down into pieces during sampling and shaking. So the real population of IK726 was greatly underestimated by technical error. That may be the reason that we did not detect IK726 in any root

tip sample by dilution plating. To solve this problem, a transformant encoding a fluorescent reporter protein, like GFP or RFP, is highly recommended in the micro-ecology assay.

Different pseudomonads isolates demonstrated variant root colonization patterns due to different genotypes and different preferences to host plants and soil types (Geels *et al.*, 1983b; Kluepfel, 1993; Lugtenberg *et al.*, 1999). As we discussed in the section 1.3, antagonistic microbes were always aggregated and multiplied on the root hair zone where the root exudates is rich. In contrast, the population on the root tips is much lower than that on the root hair zone. That is why the population density of MA342 was 10 fold declined from the mature root tissue to the root tip (10 days after sowing). In our study, only 1 cm segment of tiny young root was harvested (Fig. 3) and initial concentration was about 10^6 cfu/seed. These parameters were far below the common sampling amount (1 g of root tissue) and inoculum density (10^{8-9} cfu/seed). So, according to our results, Fig. 6, MA342 was a powerful root colonizer which is distinct from the previous study of MA342 (Johnsson *et al.*, 1998). MA342 retained at the level of log 3 cfu/cm root from day 7 to day 10. Also, the root colonization of MA342 was as good in the alone inoculated treatment as in the dual inoculated treatment at both sampling times. These results also indicated MA342 has a good persistence in the rhizosphere of spring wheat.

6. Conclusion

IK726 may have a stronger ability than MA342 to compete for the limited attachment sites on spring wheat (cultivar Dacke) seed. MA342 is a powerful root colonizer that retained a stable population density at the level of log 3 cfu/cm root in the rhizosphere from day 7 to day 10. The poor root colonization performance of IK726 on spring wheat may be caused by nutrient deficiency, slow hyphal growth and technical error. We suggest in the future researches of IK726, organic matter should be considered as an important restriction factor of growth of IK726. Proper level of organic matter should be introduced into potting system to support the population of IK726 in the rhizosphere. And transformants encoded different fluorescent reporter proteins should be considered to introduce in the study of root colonization of two BCAs.

7. **References**

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