Induced systemic resistance in cucumber and Arabidopsis thaliana by the combination of Trichoderma harzianum Tr6 and Pseudomonas sp. Ps14

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HIGHLIGHTS
- Trichoderma and Pseudomonas strains were isolated from cucumber rhizosphere.
- Strains were tested for eliciting systemic resistance against Fusarium in cucumber.
- Combining Trichoderma and Pseudomonas leads to enhanced induced resistance.
- This enhanced effectiveness is observed in cucumber but not in Arabidopsis.
- In cucumber the enhanced effectiveness is paralleled by enhanced priming of defense genes.

ARTICLE INFO

Article history:
Received 27 September 2012
Accepted 28 January 2013
Available online 5 February 2013

Keywords:
Arabidopsis thaliana
Botrytis cinerea
Cucumber
Defense genes
Fusarium oxysporum f. sp. radicis cucumerinum
Induced systemic resistance
Pseudomonas
Trichoderma

ABSTRACT

Trichoderma species and fluorescent Pseudomonas spp. have been reported to induce systemic resistance in plants. In this study the effectiveness of a combination of these biological control agents on the efficacy of induced resistance was investigated in cucumber and the model plant Arabidopsis thaliana. Trichoderma harzianum Tr6, and Pseudomonas sp. Ps14, both isolated from the rhizosphere of cucumber, were tested as a single application and in combination for their abilities to elicit induced resistance in cucumber against Fusarium oxysporum f. sp. radicis cucumerinum and in A. thaliana against Botrytis cinerea. The combination of Tr6 and Ps14 induced a significantly higher level of resistance in cucumber, which was associated with the primed expression of a set of defense-related genes upon challenge with Fusarium. In Arabidopsis both Ps14 and Tr6 triggered ISR against B. cinerea but their combination did not show enhanced effects. In the induced systemic resistance-defective Arabidopsis mutant myb72, none of the treatments protected against B. cinerea, whereas in the SA-impaired mutant sid2 all treatments were effective. Taken together, these results indicate that in Arabidopsis Ps14 and Tr6 activate the same signaling pathway and thus have no enhanced effect in combination. The enhanced protection in cucumber by the combination is most likely due to activation of different signaling pathways by the two biocontrol agents.

1. Introduction

The rhizosphere is a nutrient-rich habitat and harbors a large variety of bacteria and fungi that each can have neutral, beneficial or deleterious effects on the plant (Berendsen et al., 2012). Some of these organisms can improve plant growth by different mechanisms (Lugtenberg and Kamilova, 2009; Van der Ent et al., 2009b). Fluorescent Pseudomonas and Trichoderma species are important groups of plant growth-promoting microorganism reported to protect plants against pathogens by mechanisms such as antagonism, competition, and induced systemic resistance...
Rhizobacterial determinants that can elicit ISR include flagella, phenolic compounds and quorum sensing molecules (Bakker et al., 2007; De Vleesschaauwer and Höfte, 2009; Van der Ent et al., 2009b).

Specific recognition between plants and ISR inducing organism appears to be required for the onset of ISR (Conrath et al., 2002). Rhizobacterial determinants that can elicit ISR include flagella, lipopolysaccharides, iron regulated metabolites, antibiotics, volatiles, phenolic compounds and quorum sensing molecules (Bakker et al., 2007; De Vleesschaauwer and Höfte, 2009; Van der Ent et al., 2009).

Following perception of the inducer, plants activate a signal-transduction pathway in which phytohormones such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA) are key signaling molecules (De Vleesschaauwer and Höfte, 2009; Pieterse et al., 2009). Studies with Arabidopsis thaliana (Arabidopsis) mutants deficient in hormone-regulated signaling pathways and Pseudomonas fluorescens strain WCS417r demonstrated that in this combination ISR is SA-independent, but requires an intact response to ET and JA (Pieterse et al., 1998, 1996; Van Wees et al., 1997). The transcription factor MYB72 is an important factor in induced resistance by both P. fluorescens WCS417 and Trichoderma asperellum T34 and thus appears to be a common node in ISR by PGPR and PGPF (Segarra et al., 2009; Van der Ent et al., 2008).

Trichoderma spp. are plant symbionts that live free in the rhizosphere (Harman et al., 2004a). Presence of fungal prey and root derived nutrients are major attractants for Trichoderma spp. (Druzhinina et al., 2011). Trichoderma spp. can produce several plant defense eliciting MAMPs such as xylanases, peptidols, swolenin, and cerato-platanins (Druzhinina et al., 2011; Harman et al., 2004a; Shoresh et al., 2010). An early report of induced resistance by Trichoderma spp. is on Trichoderma harzianum T39, which reduced gray mold in bean (De Meyer et al., 1998). Since then T. harzianum T22 (Harman et al., 2004b), T. asperellum T203 (Shoresh et al., 2005), T. harzianum T39 (De Meyer et al., 1998), Trichoderma hamatum 382 (Khan et al., 2004) and Trichoderma koningiiopsis Th003 (Moreno et al., 2009) were reported to induce resistance in different plant species against a variety of diseases. The interaction between some Trichoderma strains and plants seems to be associated with ET and JA signaling (Bae et al., 2011; Korolev et al., 2007; Segarra et al., 2009; Shoresh et al., 2005). However, inoculation of roots with Trichoderma did not affect levels of JA and SA (Segarra et al., 2006; Shoresh et al., 2005).

Combining biological control agents can be more effective in the suppression of plant disease, providing enhanced efficacy and reliability from field to field relative to single biocontrol strains (Lutz et al., 2004; Stockwell et al., 2011). Most biocontrol products available on the market are based on combinations of microbial agents. Compatibility and effectiveness of combinations of Trichoderma with other biocontrol organisms is an important issue (Lorio et al., 2010). Enhanced efficacy of mixtures of PGPR and PGPF strains against plant diseases have been reported (Harish et al., 2009; Jetiyannon and Klopper, 2002; Leeman et al., 1996; Shanmugam et al., 2001), including combinations of Trichoderma and Pseudomonas (Latha et al., 2011; Lutz et al., 2004). However, effectiveness of combined application of Trichoderma and Pseudomonas strains that both elicit induced resistance has yet not been studied.

In the present study Trichoderma and Pseudomonas isolates from the cucumber rhizosphere were screened for their ability to elicit ISR against Fusarium oxysporum f. sp. radicis cucumerinum (Forc) on cucumber. Possible additive effects of combinations of induced resistance eliciting strains were tested against Forc on cucumber and Botrytis cinerea on wild-type and mutant Arabidopsis.

2. Materials and methods

2.1. Trichoderma and Pseudomonas isolates

Roots of cucumber plants with adhering rhizosphere soil were sampled from different farms in Iran. Isolation of Trichoderma and Pseudomonas from the root material was carried out by grinding 1 g of the roots in 10 ml of 10 mM MgSO4 and plating serial dilutions on selective media. Trichoderma was isolated from roots and rhizosphere by serial dilution plating on Trichoderma selective medium as described by Davet and Rouxel (Davet and Rouxel, 2000). Selective medium S1 (Gould et al., 1985), was used for isolation of fluorescent Pseudomonas spp. from the rhizosphere of cucumber. The plates were incubated at 28 ºC. After 2 (Pseudomonas) and 4 days (Trichoderma) colonies were selected and for both the bacteria and the fungi pure cultures of 20 strains were obtained. Trichoderma isolate Tr6 and Pseudomonas Ps14 were studied in the greatest detail. For Ps14 a rifampicin resistant derivative was isolated, according to the method described by Glandorf et al. (1992) to facilitate colonization studies. In experiments with A. thaliana, T. asperellum T34 and P. fluorescens WCS417 (Segarra et al., 2009), were included. Fusarium oxysporum f.sp. radics cucumerinum (Forc) strain F42 was obtained from the agriculture research center of Jiroft, Jiroft, Iran. The Trichoderma strains and Forc F42 were grown on potato dextrose agar (PDA, Difco) and the Pseudomonas strains on Kings medium B (KB, King et al., 1954).

2.2. Identification of Pseudomonas and Trichoderma

Pseudomonas isolate Ps14 was identified by sequencing region V6-V8 of the 16S rRNA gene with primers 968F_GC and 1401R (Nobel et al., 1996). Colony PCR was performed as previously described (Sheu et al., 2000), briefly; a reaction master mix was prepared contain 12 µl MQ, 2 µl Taq buffer containing MgCl2 (Invitrogen®), 2 µl of 2 mM dNTPs (Invitrogen®), 2 µl forward and reverse primer (Bioloegio10 pmol), and one µl Phusion® High-Fidelity DNA Polymerase (Finnzymes). One bacterial colony was added to 19 µl of the master mix. PCR was performed using a thermo cycler (Hybaid, Ashford, UK). PCR conditions were 2 min at 95 ºC followed by 35 cycles of 30 s at 95 ºC, 30 s at 55 ºC, and 1 min at 72 ºC, followed by 5 min at 72 ºC. Trichoderma isolate Tr6 was identified by sequencing of ITS1, 5.8S, and ITS2 rRNA with primer pair ITS1 and ITS4 (5'-TCCGTAAGTGACCTGGCGG-3' and 5'-TCCGTCGTTATGATATGC-3') (Chaverri et al., 2003; Samuels, 2006; White et al., 1999). DNA extraction was carried out by DNasy Qiagen kit according to manufacturer instruction. PCR reaction and PCR condition was used as described for 16s rRNA gene. The PCR product was checked after electrophoresis on a 1.5% agarose gel in 1X TAE buffer (40 mM Tris–acetate/1 mM EDTA, pH 8). Subsequently the PCR product was purified using Illustra GFX PCR DNA and Gel Band Purification kit (GE, Diegem, Belgium) according to the manufacturer’s instructions. The purified PCR products were send to the Macrogen (Amsterdam, the Netherlands) for sequencing. For each amplicon both strands were sequenced using the primer used to generate it.

2.3. Plant material

Cucumber seeds (Cucumis sativus L., cv. F1 Barez SF) were obtained from Hamon Agriculture Co. (Tehran, Iran). The seeds were
surface sterilized for 30 s in 1% sodium hypochlorite and rinsed three times with sterile distilled water (Khan et al., 2004). Treated seeds were incubated at 28 °C for 24 h between layers of sterile wet filter paper. After 24 h, germinated seeds were planted in 9-cm pots containing a mixture of sand and potting soil that had been autoclaved twice for 20 min with a 24 h interval. Plants were cultivated in a growth chamber with a 16 h day (24 °C) and 8 h night (20 °C) cycle at 70% relative humidity. The seedlings were watered two times a week, alternating half strength Hoagland and tap water (Hoagland and Arnon, 1950). For split root experiment the germinated seeds were planted in 60-ml pots filled with two times sterilized vermiculite. Seeds of wild-type A. thaliana Col-0 and mutants ein2–1 (Guzmán and Ecker, 1990), myb72–1 (Van der Ent et al., 2008), and sid2–1 (Nawrath and Métraux, 1999), were sown in autoclaved river sand, and incubated at 100% relative humidity and 21 °C, and 8 h photoperiod. Two-week-old seedlings were transplanted into 60-ml pots containing a potting soil-sand mixture (12:5 V/V) that had been autoclaved. A. thaliana two weeks old seedlings were transplanted into 60-ml pots containing a density of 10^7 spores/ml. For soil drench, spore suspensions of Forc were prepared in half strength potato dextrose broth (PDB) at a density of 10^5 spores/ml. Forc was cultured on half strength PDA for 2 weeks at 21°C (10^5 spores/g of soil) or Trichoderma and Pseudomonas isolates were screened for activity against Forc in this split root system. Three days after transplanting, one part of the root system was inoculated with the biocontrol agent and after another three days the other part was inoculated with the pathogen. The plants were kept at 24 °C, 70% relative humidity and a 16 h photoperiod and they were watered twice a week with sterilized tap water. After four weeks disease symptoms were scored. Disease was scored in 5 classes (0–4) with 0 = healthy, 1 = mild stem rot, 2 = spreading stem rot (less than 2 cm), 3 = severe spreading, and 4 = dead. The disease incidence index was calculated by transforming the classes to % as 0 = 0%, 1 = 25%, 2 = 50%, 3 = 75%, and 4 = 100%.

2.6. Screening for induced resistance in cucumber

Two weeks old seedlings, grown on sterilized vermiculite, were carefully removed from the substrate and the root system was gently divided into two equal parts. Subsequently these plants were transplanted into two pots (filled with an autoclaved mix of potting soil and sand) such that the two separate parts of the root system were in separate pots (Khan et al., 2004; Liu et al., 1995; Zang et al., 1996). Trichoderma and Pseudomonas isolates were screened for activity against Forc in this split root system. Three days after transplanting, one part of the root system was inoculated with the biocontrol agent and after another three days the other part was inoculated with the pathogen. The plants were kept at 24 °C, 70% relative humidity and a 16 h photoperiod and they were watered twice a week with sterilized tap water. After four weeks disease symptoms were scored. Disease was scored in 5 classes (0–4) with 0 = healthy, 1 = mild stem rot, 2 = spreading stem rot (less than 2 cm), 3 = severe spreading, and 4 = dead. The disease incidence index was calculated by transforming the classes to % as 0 = 0%, 1 = 25%, 2 = 50%, 3 = 75%, and 4 = 100%.

2.7. Control of Fusarium in cucumber by the combination of Trichoderma and Pseudomonas

T. harzianum Tr6 and Pseudomonas sp. Ps14 were mixed through sterilized soil to a final inoculum density of 10^5 spores or cfu per g of soil. Four treatments were compared, control, T. harzianum Tr6, Pseudomonas sp. Ps14, and the combination of Tr6 and Ps14, with 15 replicates for each treatment. Three weeks old seedlings were inoculated with Forc using stem inoculation (see inoculation of plants). After 2–4 weeks disease incidence was scored. Spatial separation of the pathogen and the antagonists was tested at the end of the bioassay by culturing stem and root samples on selective media.

2.8. A. thaliana – Botrytis cinerea bioassays

P. fluorescens WCS417r and T. asperellum T34, both well studied for eliciting induced resistance in A. thaliana (Pieterse et al., 1996; Segarra et al., 2009), were studied for their combined effects against B. cinerea. The involvement of MYB72 was investigated using A. thaliana mutant myb72-1 (Van der Ent et al., 2008). The abilities of strains Tr6, Ps14 and their combination to elicit ISR against B. cinerea were studied on A. thaliana Col-0, and mutants myb72-1, sid2-1 (Nawrath and Métraux, 1999) and ein2-1 (Guzmán and Ecker, 1990) to study the involvement of MYB72, SA, and ET signaling, respectively. The percentage of spreading lesions was scored three days after inoculation with the pathogen.

2.9. Colonization of cucumber and Arabidopsis rhizosphere by Tr6 and Ps14

In bioassays with cucumber and Arabidopsis, population densities of the introduced beneficial microorganisms were studied both when they were singly inoculated and for the combined inoculation. Rhizosphere samples were obtained from plants at four weeks after inoculation of the roots. 0.5 g of the sample was suspended in 5 ml of sterile 0.01 M MgSO4 and shaken with 0.5 g glass beads (0.6–0.8 mm diameter) for 1 min on a vortex at maximum speed. Numbers of colony forming units (cfu) of Pseudomonas sp. Ps14 were determined by dilution plating on KB agar supplemented with 40 mg/l ampicillin, 13 mg/l chloramphenicol, 100 mg/l nalidixic acid (Delvocid, DSM, Delft, NL) and 150 mg/l rifampicin.
(Doornbos et al., 2010). Population densities of Tr6 were determined by dilution plating on Trichoderma selective medium as described by Davet and Rouxel (Davet and Rouxel, 2000). In the control treatments of the different experiments no counts were observed for Trichoderma showing that indigenous populations were below detection.

2.10. Gene expression in cucumber and Arabidopsis

To evaluate expression of defense related genes in the different experiments samples of cucumber stems and Arabidopsis leaves were flash frozen in liquid nitrogen and stored at −80 °C until required. RNA was extracted individually from the samples of three replicates for each treatment. Samples were ground with mortar and pestle in liquid nitrogen and total RNA was extracted with RNeasy Qiagen kit according to the instructions of the manufacturer. Extracted RNA was treated with DNase (Ambion). Before cDNA synthesis RNA samples were screened for genomic contamination by PCR with primer pair (5′-CTGTCGCTTCCCATTTATG-3′ and 5′-TTGGGATTGAAGTTAGCC-3′) to amplify an intron sequence of a gene encoding the HSP70 gene for cucumber samples (Wan et al., 2010), and with primer pair EIl2 (5′-ATTATCACCCCTTGGCCACT-3′ and 5′-CCGTATGCGAAAGAACAAC-3′) for EIN2-like gene for Arabidopsis samples (Pozo et al., 2008).

Subsequently, DNA-free total RNA was converted into cDNA using oligo-dT primers (Invitrogen, Breda, the Netherlands), 10 mM dNTPs, and Revert Aid H minus Reverse Transcriptase (Fermentas) according to the manufacturer’s instructions.

2.11. qPCR

The following primers were used: EF 1-α, forward 5′-CTGTGCTGCTTCCCATTTATG-3′ and reverse 5′-AGGTTGGAAGAGGAAGAC-3′; CHIT1, forward 5′-GCTGCTGCAACCCCTGACA-3′ and reverse 5′-AGTGGCTGCTAACCACCATC-3′; CACS, forward 5′-TGGGAAGATTCTTATGAAGTGC-3′ and reverse 5′-TGGTGGGATTGAAGTTAGCC-3′ for 16S rRNA gene of Ps14; β-Actin2, forward 5′-CAATTTACTTCAACCAAACT-3′ and reverse 5′-ACCUGGCTCTCGGATACAACAAC-3′; PAL1, forward 5′-ATGGACGCACTCCTCCAAGGA-3′ and reverse 5′-CCATGGATCCTCAGCACCT-3′; PR1, forward 5′-GTCCCAACAATTGCACCAC-3′ and reverse 5′-TCATCCACCCACAACTGAAC-3′; b-Actin2, forward 5′-TGGTCACTGCAACCCTGACA-3′ and reverse 5′-GTCAAAATACTGGGAAGATC-3′; EF 1-α; PAL1, encoding TGA, and PR1, encoding β-1,3-Glucanase, respectively. Expression levels of these related genes in cucumber, we studied expression of CHIT1, β-1,3-Glucanase, and PAL1, encoding Chitinase, glucanase, and phenylalanine ammonia-lyase, respectively. Expression levels of these genes were determined in a 0 to 96 h time series after challenge inoculation with Fusarium. In control treated plants, Forc inoculation resulted in a gradual increase in the expression of β-1,3-Glucanase. In plants pretreated with either Ps14 or Tr6, this augmented expression was significantly increased, indicating that β-1,3-Glucanase expression is primed by the treatment with the

2.12. Statistics

Statistical analysis was performed with SPSS 16.0. Results from the bioassays for control of Fusarium stem and root rot in cucumber were analyzed after Box–Cox transformation, using ANOVA followed by Duncan post hoc test. Gene expression levels were analyzed by one way ANOVA followed by the Duncan post hoc test. The colonization data were log transformed and analyzed by ANOVA followed by Duncan post hoc. The A. thaliana – B. cinerea bioassays were analyzed using non parametric logistic regression analysis.

3. Results

3.1. Isolation of Trichoderma and Pseudomonas and screening for induced resistance

Twenty isolates of Trichoderma and twenty isolates of Pseudomonas were isolated from cucumber rhizosphere on selective media. To select isolates that effectively elicit induced resistance in cucumber against Forc F42, they were screened in a split root system. Pseudomonas isolates Ps9, Ps14, Ps12, and Ps6 and Trichoderma isolates Tr6 and Tr9 significantly reduced disease severity in these experiments (Fig. 1A and B). Both in vitro (growth on plates) and in vivo (colonization of the cucumber rhizosphere) no significant antagonistic interactions between the Pseudomonas and Trichoderma isolates were detected (data not shown). Based on their performance in the ISR bioassay, Ps14 and Tr6 were selected for further experiments. A homology search in GenBank DNA sequence database, using BLASTn, the V6-V8 region sequence of the 16S rRNA gene of Ps14 revealed 99% and 98% homology to Pseudomonas sp. (accession number AB714640 and AY365082). Sequencing of ITS1, 5.8S, ITS2 of Trichoderma isolate Tr6 and a homology search on TrichOKEY 2 (http://isth.info/tools/molkey/index.php) and BLASTn showed 99% homology with T. harzianum (accession numbers JN942884 and HQ608137). Sequences were deposited in Genbank with accession numbers JX411632 for Ps14 and JX411633 for Tr6.

3.2. Combined effect of Trichoderma and Pseudomonas in control of Fusarium stem and root rot of cucumber

To investigate the effect of combined treatment with Ps14 and Tr6 on the level of ISR, cucumber plants were inoculated with different densities of both strains and the level of resistance against Forc was evaluated. Starting densities of 103 cfu per g of soil for both Tr6 and Ps14 controlled disease more effectively than higher densities of 105 and 107 cfu per g (data not shown). As shown in Fig. 2, both Tr6 and Ps14 induced resistance against Forc F42, but the combination of Tr6 and Ps14 suppressed the disease significantly better compared to the individual treatments.

3.3. Expression of defense-related genes in cucumber before and after challenge inoculation with Fusarium

To investigate effects of Tr6 and Ps14 on expression of defense related genes in cucumber, we studied expression of CHIT1, β-1,3-Glucanase, and PAL1, encoding Chitinase, glucanase, and phenylalanine ammonia-lyase, respectively. Expression levels of these genes were determined in a 0 to 96 h time series after challenge inoculation with Fusarium. In control treated plants, Forc inoculation resulted in a gradual increase in the expression of β-1,3-Glucanase. In plants pretreated with either Ps14 or Tr6, this augmented expression was significantly increased, indicating that β-1,3-Glucanase expression is primed by the treatment with the
biological control strains. Interestingly, the primed expression pattern of β-1,3-Glucanase was significantly more pronounced in the combination treatment (Fig. 3), which parallels the enhanced level of protection observed in these plants (Fig. 2). For the relative expression of CHIT1 at the different time points after inoculation with Forc a similar result was observed (Fig. 3). Also for PAL1 primed expression was observed in the Ps14, Tr6 and the combination treatments, but the combination treatment did not result in enhanced up-regulation (Fig. 3). Whereas expression of PR-1 was not affected by the Ps14 treatment, it was primed by Tr6, and in the combination treatment this was significantly more pronounced (Fig. 3). Finally expression of LOX1 was primed by all treatments but there was no enhanced effect of the combination treatment (Fig. 3). Thus suppression of Fusarium in cucumber by the biocontrol agents is accompanied by primed expression of several defense related genes and augmented disease suppression by the combination of Ps14 and Tr6 is accompanied by enhanced priming for some of these genes.

3.4. Does combined application of Pseudomonas and Trichoderma lead to enhanced induced resistance in Arabidopsis?

To study if the combined application of Trichoderma and Pseudomonas also leads to more effective induced resistance in Arabidopsis we studied two combinations for their abilities to elicit ISR against B. cinerea. First of all the combination of Ps14 and Tr6 was studied. P. fluorescens WCS417 and T. asperellum T34 were used as a control as it was previously demonstrated that they elicit ISR in Arabidopsis and that for both micro-organisms this is dependent on the A. thaliana transcription factor MYB72 (Segarra et al., 2009; Van der Ent et al., 2008). The combination of WCS417 and
T34 did not lead to improved control of *B. cinerea* as compared to the single treatments (Fig. 4) and the biocontrol agents and their combination were not effective in the *myb72* mutant (Fig. 4), confirming earlier observations (Segarra et al., 2009). *Trichoderma* Tr6, *Pseudomonas* Ps14, and their combination significantly reduced the percentage disease caused by *B. cinerea* in Arabidopsis (Fig. 5). However, in contrast to their effects in cucumber, the combination of the two biocontrol agents did not result in improved disease control. ISR elicited by Tr6 and Ps14 was not dependent on salicylic acid as both micro-organisms also reduced disease in the *sid2* mutant (Fig. 5). In the *myb72* mutant, none of the treatments reduced disease (Fig. 5) suggesting that also for these micro-organisms ISR is dependent on MYB72. We studied the expression of PDF1.2 and PR1 in Arabidopsis both before and 48 h after infection with *B. cinerea*. At 48 h after infection expression of PDF1.2 was primed in the treatments with Ps14 and Tr6, and no enhanced effect was observed for the combination treatment (Fig. 6). Expression levels of PR1 were not affected by the treatments with *Trichoderma*, *Pseudomonas* and their combination (Fig. 6).

**Fig. 3.** Relative expression of: β-1,3-Glucanase, CHIT1, PAL1, PR1 and LOX1 genes in stems of cucumber grown in soil treated with *Pseudomonas* sp. Ps14 (PS), *T. harzianum* Tr6 (TR) and their combination (PSTR) at different time points after stem inoculation with *Fusarium*. For each time point bars with different letters are significantly different (Duncan post hoc test, *P* < 0.05).

**Fig. 4.** Effects of root colonization by *T. asperellum* T34, *P. fluorescens* WCS417r and their combination on disease development by *B. cinerea* inoculated on the leaves of *A. thaliana* Col-0 and its mutant *myb72*. Numbers of spreading lesions were counted three days after inoculation with the pathogen and data were analyzed by logistic regression. Bars with different letters are significantly different (*P* < 0.05).
3.5. Compatibility between Trichoderma Tr6 and Pseudomonas Ps14 in the rhizosphere

Root colonization by Trichoderma Tr6 and Pseudomonas Ps14 was studied in both cucumber and Arabidopsis. When introduced alone, the Pseudomonas strain reached population densities between $10^6$ and $10^7$ cfu per gram of root on both plant species. In the combined treatment population densities of Ps14 were not influenced by the presence of Trichoderma (Fig. 7A and C). For Trichoderma Tr6 similar results were obtained. The fungal strain reached population densities around $10^4$ cfu per gram of root on both plant species, and these densities were not influenced by the presence of Pseudomonas Ps14 in the combination treatment (Fig. 7B and D). Thus it appears that these two biological control agents are compatible in the rhizospheres of cucumber and Arabidopsis.

4. Discussion

Combinations of biocontrol agents can result in more effective and robust control of plant diseases (De Boer et al., 2003; Leeman et al., 1996). We studied possible enhanced effectiveness of combinations of Trichoderma and Pseudomonas in ISR in cucumber and Arabidopsis. Using a split root system in cucumber it was demonstrated that Trichoderma isolates Tr6 and Tr9 and Pseudomonas isolates Ps6, Ps9, Ps12, and Ps14, all isolated from cucumber rhizosphere, can elicit ISR in cucumber against Forc F42. Isolates Tr6 and Ps14 most effectively reduced disease, and these strains were used in the rest of this study. The strains were identified as T. harzianum (Tr6) and Pseudomonas sp. (Ps14), based respectively on ITS and 16S sequences.

Enhanced efficacy of biocontrol of plant diseases by combinations of Trichoderma and Pseudomonas have been reported (Latha et al., 2011; Lutz et al., 2004), but combined effects of strains that induce resistance have as such not been studied. ISR by the combination of Ps14 and Tr6 provided significantly better protection of cucumber against Forc F42 than individual application of the biocontrol agents. Trichoderma and Pseudomonas could not be detected on the non-inoculated plant parts, this is indicative of persistent spatial separation of the biocontrol agents and the pathogen in these bioassays. In addition, Tr6 and Ps14 did not show in vitro antagonistic activity against Forc F42 (data not shown). Thus the control of disease by Tr6, Ps14 and their combination...
must be plant mediated. In *A. thaliana* simultaneous activation of salicylate and jasmonate dependent defense pathways lead to enhanced induced resistance (Van Wees et al., 2000). Thus the enhanced effectiveness of Tr6 and Ps14 in cucumber suggests that *Trichoderma* and *Pseudomonas* trigger different signal transduction pathways. Induced resistance for many strains of fluorescent *Pseudomonas* spp. is SA-independent and JA and ET signaling dependent (Bakker et al., 2007; Pieterse et al., 2003; Van Loon et al., 1998).

Some studies reported that ISR by *Trichoderma* agents involves JA and ET signaling (Bae et al., 2011; Djonovic et al., 2007; Korolev et al., 2007; Moreno et al., 2009; Segarra et al., 2009; Shoresh et al., 2005; Viterbo et al., 2007), whereas in other cases it seemed SA-dependent (Alfano et al., 2007; Shoresh and Harman, 2008). Moreover activation of both the SA and JA pathway by some strains of *Trichoderma* has been reported (Harman, 2011; Salas-Marina et al., 2011; Segarra et al., 2007; Yoshioka et al., 2012).

Colonization of the rhizosphere by PGPRs and PGPFs leads to primed defense reactions such as up regulation of genes encoding PR proteins and genes which are involved in defense signaling pathways after pathogen challenge (Conrath et al., 2006; Conrath et al., 2002; De Vleesschauwer and Höfte, 2009; Pozo et al., 2008; Shoresh et al., 2005; Van der Ent et al., 2009a; Van Wees et al., 1999). After challenge inoculation with Forc F42, primed expression of the *CHIT1*, β-1,3-Glucanase, *PAL1*, *PR1* and *LOX1* in stems of cucumber plants treated with Ps14, Tr6, or their combination was observed. These results are consistent with Shoresh et al. (2005) who reported primed gene expression in cucumber by *T. asperellum* T203 against *Pseudomonas syringae* pv. *Lachrymans* and with reports on priming of defense-related genes by *P. fluorescens* WCS417 in *A. thaliana* (Hase et al., 2003; Pieterse et al., 2000; Van Wees et al., 1999).

*Arabidopsis* is a model plant for induced resistance and different mutants in induced resistance dependent defense pathways are available. We used *A. thaliana* mutants sid2 and myb72 to study respectively the involvement of SA signaling and of the transcription factor MYB72, in ISR by *Trichoderma*, *Pseudomonas* and their combination. Transcription factor MYB72 is required in the roots during early signaling steps of *P. fluorescens* WCS417r and *T. asperellum* T34-mediated ISR (Segarra et al., 2009; Van der Ent et al., 2008). Indeed bioassays withWCS417r, T34 and their combination confirmed that MYB72 is essential for the onset of ISR, in this case against *B. cinerea*. As expected the combination of these two well-studied biocontrol agents did not result in enhanced disease control, since they appear to activate the same signal transduction pathway. Real time qPCR showed up regulation of PDF1.2. Based on the observation that the combination of Ps14 and Tr6 shows enhanced effectiveness in a cucumber ISR bioassay we expected similar effects in *Arabidopsis*. However, whereas both biocontrol agents did eliciting ISR in *Arabidopsis*, their combination was not more effective in disease suppression. In accordance to this ISR by both Ps14 and Tr6 depended on MYB72 but it was independent of SA signaling. Following this line of reasoning the enhanced disease suppression by the combination of Ps14 and Tr6 in cucumber is most likely due to activation of different signaling pathways by these micro-organisms. Developing mutants in cucumber affected in specific defense signaling pathways is necessary to explore this phenomenon more in depth.

**Acknowledgments**

This project was financially supported by University of Jiroft.

**References**


**Fig. 7.** Colonization of *Pseudomonas* sp. Ps14 (A and C) and *Trichoderma* sp. Tr6 (B and D) in the rhizospheres of cucumber (A and B) and *A. thaliana* (C and D) at 4 weeks after introduction of these microorganisms in the soil. For each time point bars with different letters are significantly different (Duncan post hoc test, P < 0.05).