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

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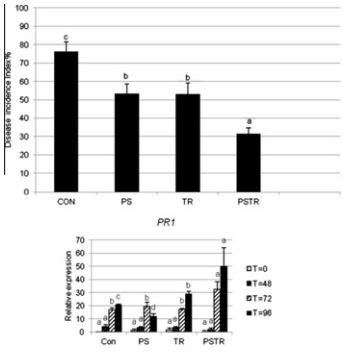
HIGHLIGHTS

- *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.

GRAPHICAL ABSTRACT



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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

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(ISR) (Harman et al., 2004a; Kloepper et al., 1980; Marx, 2004; Van Loon et al., 1998; Vinale et al., 2008). Rhizosphere colonization by certain plant growth-promoting rhizobacteria (PGPR) and fungi (PGPF) can elicit ISR (Bent, 2006; De Vleesschauwer and Höfte, 2009; Harman et al., 2004a; Kloepper et al., 1992; Shoresh et al., 2010; Van Loon et al., 1998; Van Wees et al., 2008). ISR triggered by PGPFs and PGPRs confers a broad-spectrum resistance that is effective against different types of attackers (De Vleesschauwer 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 Vleesschauwer and Höfte, 2009; Van Loon et al., 1998). 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 Vleesschauwer 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, peptaibols, swollenin, 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 koningiopsis* 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 (Lorito et al., 2010). Enhanced efficacy of mixtures of PGPR and PGPF strains against plant diseases have been reported (Harish et al., 2009; Jetiyanon and Kloepper, 2002; Leeman et al., 1996; Shanmugam et al., 2011), 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 as 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 cucumerinim* (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 $MgSO_4$ 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 °C. 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. *radicis 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 (Nubel 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 $MgCl_2$ (Invitrogen®), 2 μl of 2 mM dNTPs (Invitrogen®), 2 μl forward and reverse primer (Biolegio10 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 °C followed by 35 cycles of 30 s at 95 °C, 30 s at 55 °C, and 1 min at 72 °C, followed by 5 min at 72 °C. *Trichoderma* isolate Tr6 was identified by sequencing of ITS1, 5.8S, and ITS2 *rRNA* with primer pair ITS1 and ITS4 (5′-TCCGTAGGTGAACCTGCGG-3′ and 5′-TCCTCCGCTTATTGATATGC-3′) (Chaverri et al., 2003; Samuels, 2006; White et al., 1990). DNA extraction was carried out by DNasy Qiagene 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 1× 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 (*Cucumes 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 twice with a 24 h interval. Plants were grown in a growth chamber with 70% relative humidity, at 21 °C, and an 8 h photoperiod, as described previously (Pieterse et al., 1996).

2.4. Microbial inoculum preparation

Inoculum of the *Trichoderma* isolates and Forc strain F42 were prepared by culturing on potato dextrose agar (PDA) for 7 (*Trichoderma*) and 14 (Forc) days at 28 °C. Conidia were collected from the agar plates, filtered through glass wool and suspended in 10 mM $MgSO_4$. The inoculum concentration was adjusted to a final density after counts in a haemocytometer. For stem inoculation, spore suspensions of Forc were prepared in half strength potato dextrose broth (PDB) at a density of 10^7 spores/ml. For soil drench, spore suspensions were prepared in 10 mM $MgSO_4$ and added to the soil to a final inoculum density of 10^5 cfu per g soil. *B. cinerea* was cultured on half strength PDA for 2 weeks at 21 °C. Spore suspensions were prepared in half strength PDB (10^5 spores/ml) and incubated for 2 h at room temperature prior to inoculation.

Suspensions of the *Pseudomonas* strains were obtained from overnight cultures incubated at 28 °C on KB agar. Bacterial cells were scraped of the plates in 10 mM $MgSO_4$, centrifuged at 5000 rpm for 10 min, and resuspended in 10 mM $MgSO_4$. The density of the suspension was set at 10^9 colony forming units (cfu)/ml by measuring the OD at 660 nm spectrophotometrically.

2.5. Inoculation of plants

Soil was inoculated with *Trichoderma* (10^3 spores/g) or *Pseudomonas* (10^3 cfu/g) by mixing the suspension into the soil after which seedlings were transplanted into the treated soil. Alternatively, seedlings were transplanted into the soil and three days before challenge inoculation with the pathogen, *Trichoderma* (10^5 spores/g of soil) or *Pseudomonas* (10^7 cfu/g of soil) were applied as a soil drench.

Cucumber plants were inoculated with Forc F42 in different ways. In the split root experiments the soil was drenched three day after introduction of the biocontrol agents with Forc F42 in a final density of 10^5 spores per gram of soil (Liu et al., 1995). For stem inoculation, a 15 μl droplet of a suspension (10^7 spores /ml in half strength PDB) was placed on the stem of seedlings at the two true leaves stage. Using a scalpel blade an incision was made in the stem through the 15 μl droplet resulting in rapid uptake of the conidia into the xylem (Duijff et al., 1993). For inoculation of *Arabidopsis* with *B. cinerea*, 5 μl droplets of a pregerminated spore suspension (10^5 spores/ml in half strength PDB) were placed on four to six leaves of each plant (24 plants for each treatment). Inoculated plants were grown at 100% relative humidity and disease symptoms were scored after three days.

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 in 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 10^3 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 $MgSO_4$ 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 natamycin (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′-GTCAAAATACTGGGAAGATC-3′ and 5′-TTTGAGGTAGGAAGTGTAGT-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′-ATTATCACCACCCTTTCGCAGT-3′ and 5′-CCGTATGCGAATCAGAACACAT-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 EP0452/EP0451) according to the manufacturer's instructions.

2.11. qPCR

The following primers were used: *EF 1-α*, forward 5′-CTGTGCTGTCCTCATTATTG-3′ and reverse 5′-AGGGTGAAAGCAAGAAGAGC-3′; *CHIT1*, forward 5′-TGGTCACTGCAACCCTGACA-3′and reverse 5′-AGTGGCCTGGAATCCGACT-3′; *CACS*, forward 5′-TGGGAAGATTCTTATGAAGTGC-3′ and reverse 5′-CTCGTCAAATTTACACATTGGT-3′; *β-1,3-Glucanase*, forward 5′-TCAATTATCAAAACTTGTTCGATGC-3′ and reverse 5′-AACCGGTCTCGGATACAACAAC-3′; *PAL1*, forward 5′-ATGGAGGCAACTTCCAAGGA-3′ and reverse 5′-CCATGGCAATCTCAGCACCT-3′; *PR1*, forward 5′-TGCTCAACAATATGCGAACC-3′ and reverse 5′-TCATCCACCCACAACTGAAC-3′; *LOX1*, forward, 5′-CTCTTGGGTGGTGGTGTTTC-3′ and reverse 5′-TGGTGGGATTGAAGTTAGCC-3′ (Migocka and Papierniak, 2011; Shoresh et al., 2005; Wan et al., 2010). Primers for *LOX1* and *PR1* were designed using primer3 (http://frodo.wi.mit.edu/primer3/input.htm). PCR products for primers were between 100–200 bp with TM = 60 °C. Primers for Arabidopsis genes were *PR1*, Forward 5′-CTCGGAGCTACGCAGAACAACT-3′ and reverse 5′-TTCTCGCTAACCCACATGTTCA-3′; *PDF1.2*, forward, 5′-TTTGCTGCTTTCGACGCAC-3′ and reverse 5′-CGCAAACCCCTGACCATG-3′; and *b-Actin2*, forward 5′-AGTGGTCGTACAACCGGTATTGT-3′ and reverse 5′-GATGGCATGAGGAAGAGAGAAAC-3′.

PCR reactions were carried out in 96-well plates (20 µl per well). The reaction mixture contained 2 µl of diluted cDNA, 10 µl SYBR® Green mix (AB Applied Biosystems, Foster, CA) and 8 µl primers mix (Biolegio 1 pmol). Efficiency of primers was calculated by dilution method. Quantitative real time PCR was performed using the GeneAmp 7900HT Sequence Detection System (AB Applied Biosystems) at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. Genes *EF 1-α* and *CACS* were used as reference genes in the cucumber experiments. In *Arabidopsis* experiments the b-*Actin*2 gene was used as reference gene. Relative expression of genes of interest was calculated according to Pfaffi (Pfaffl, 2001).

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 [57]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 10^3 cfu per g of soil for both Tr6 and Ps14 controlled disease more effectively than higher densities of 10^5 and 10^7 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

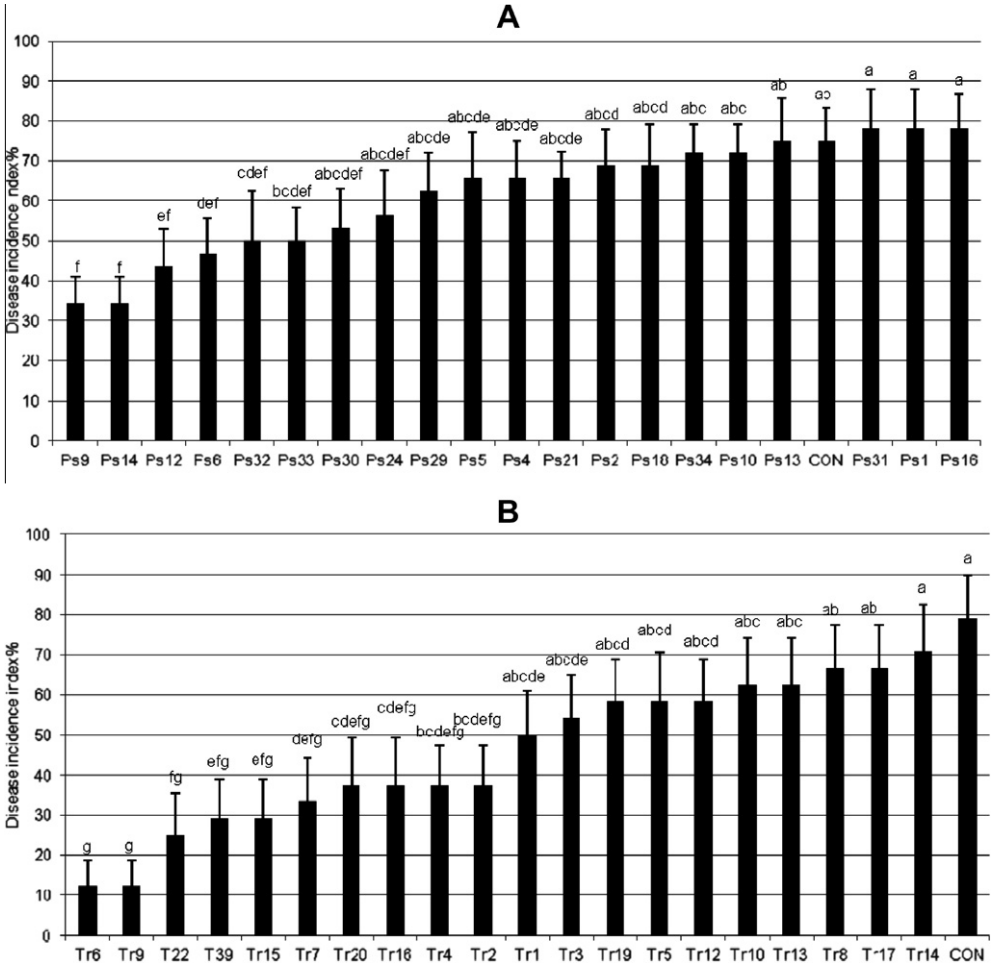

Fig. 1. Control of *Fusarium* stem and root rot disease by (A) *Pseudomonas* and (B) *Trichoderma* strains that were isolated from cucumber rhizosphere. The pathogen and the potential biocontrol strains were applied and kept spatially separated by using a split root system. Bars with different letters are significantly different (Duncan post hoc test $P < 0.05$).

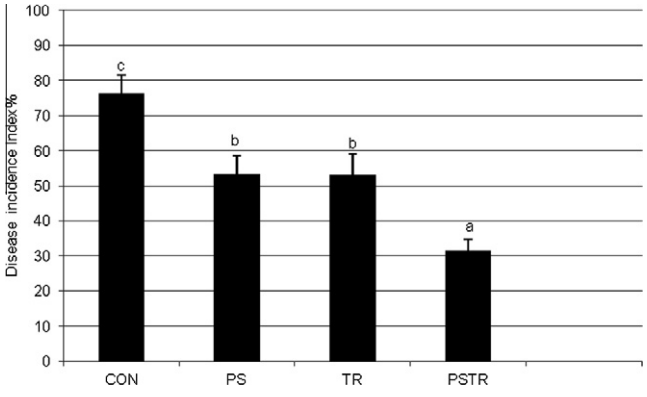

Fig. 2. Control of *Fusarium* stem and root rot disease by individual and combined application of *T. harzianum* Tr6 (TR) and *Pseudomonas* sp. Ps14 (PS). The biocontrol agents and the pathogen were applied and maintained spatially separated by inoculating the biocontrol strains on the roots and inoculating the pathogen into the stem. Bars with different letters are significantly different (Duncan post hoc test, $P < 0.05$).

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

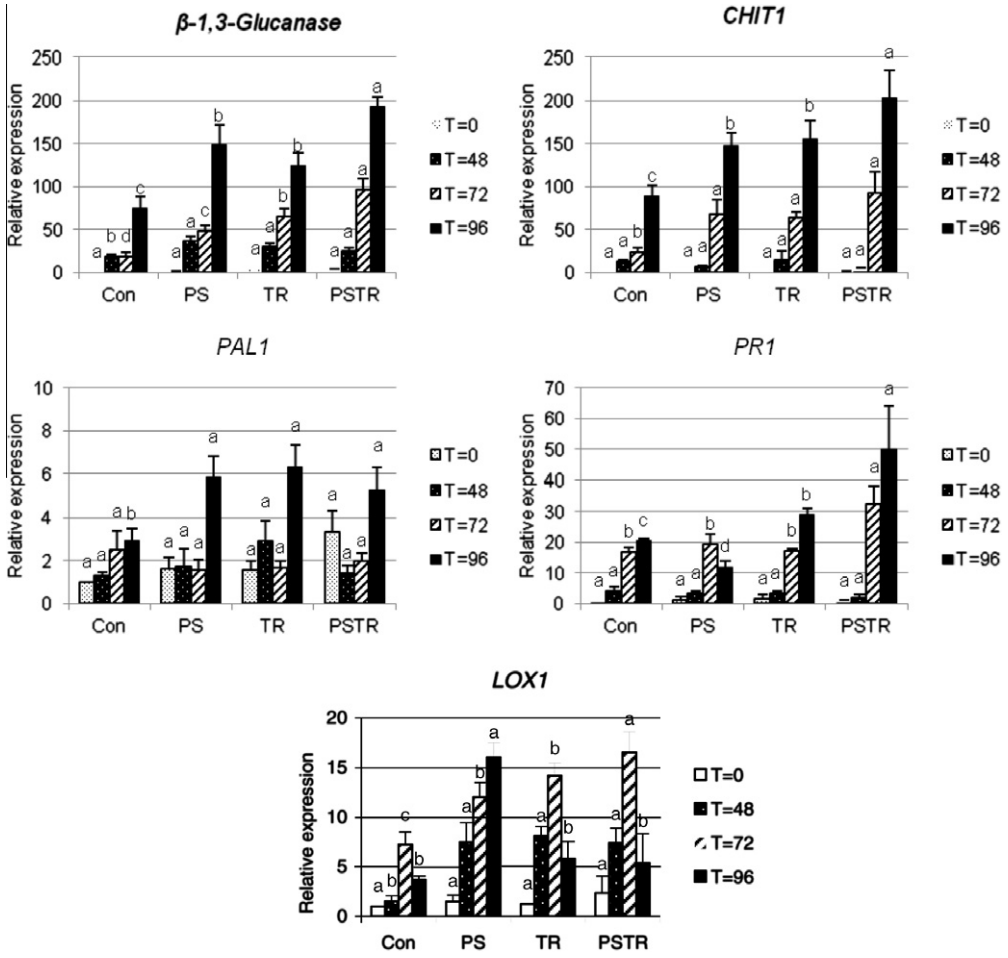

Fig. 3. Relative expression of: *β-1,3-Glucanase, CHIT1, PAL1, PR1 and LOX1* genes in stems of cucumber grown in soil treated with *Pseudomonas* sp. Ps14 (PS), *T. harzianum* Tr6 (TR) and their combination (PSTR) at different time points after stem inoculation with *Fusarium*. For each time point bars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

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).

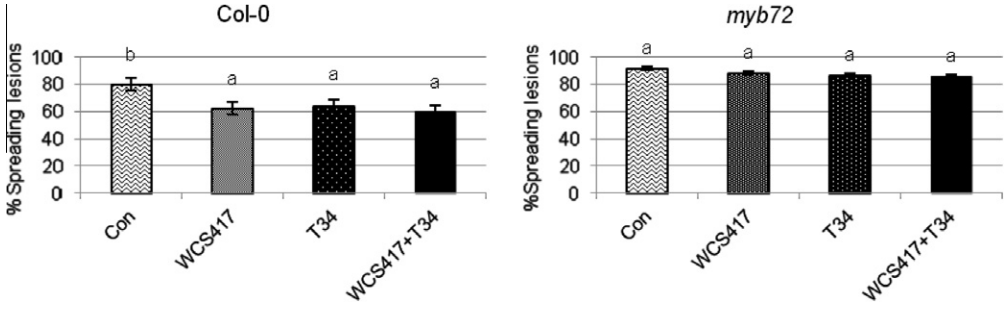

Fig. 4. Effects of root colonization by *T. asperellum* T34, *P. fluorescens* WCS417r and their combination on disease development by *B. cinerea* inoculated on the leaves of *A. thaliana* Col-0 and its mutant *myb72*. Numbers of spreading lesions were counted three days after inoculation with the pathogen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

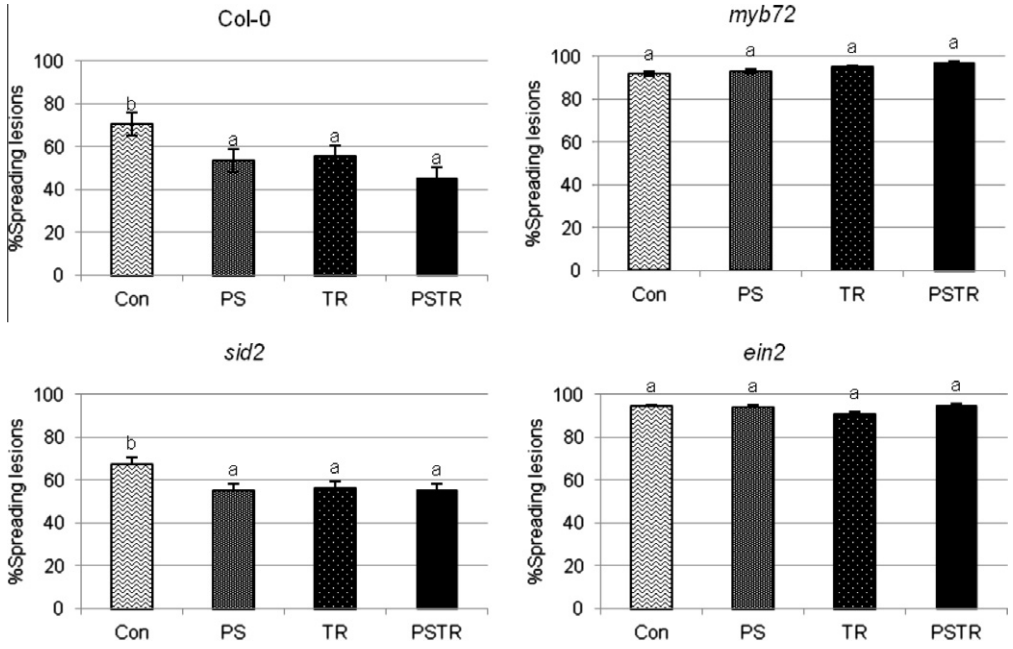

Fig. 5. Effects of root colonization by *Trichoderma* sp. Tr6, *Pseudomonas* sp. Ps14 and their combination on disease development by *B. cinerea* inoculated on the leaves of wild type *A. thaliana* Col-0 and mutants *sid2, myb72* and *ein2*. Numbers of spreading lesions were counted three days after inoculation with the pathogen and data were analyzed by logistic regression. Bars with different letters are significantly different ($P < 0.05$).

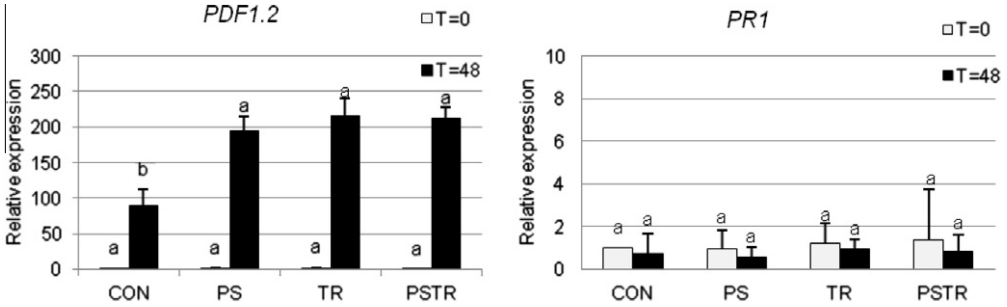

Fig. 6. Relative expression of *PDF1.2* and *PR1* at 0 and 48 h after inoculation of *A. thaliana* Col-0 leaves with *B. cinerea*. For each time point bars with different letters are significantly different (Duncan post hoc test, $P < 0.05$).

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

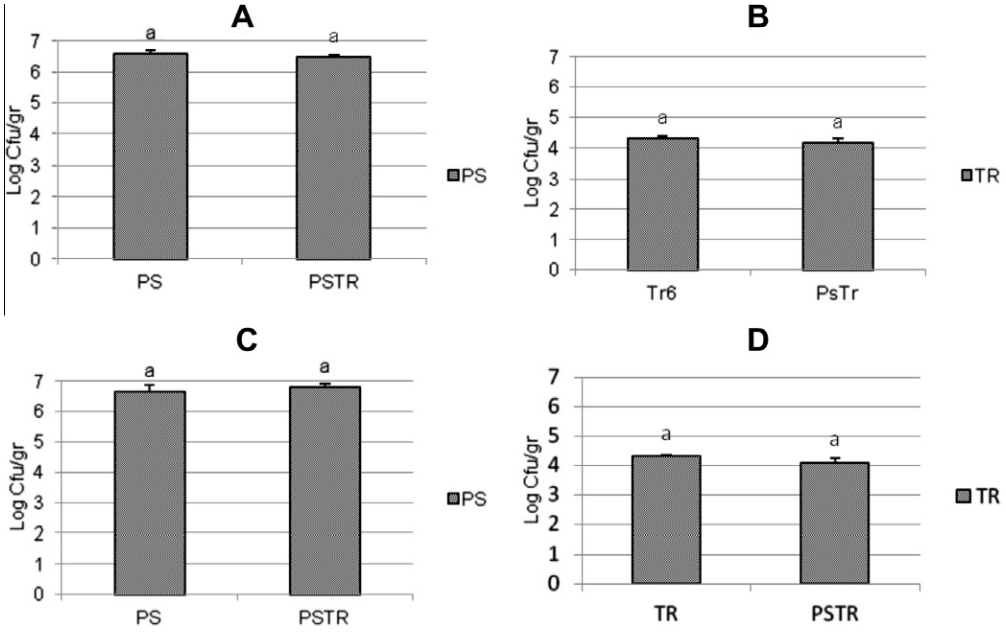

Fig. 7. Colonization of *Pseudomonas* sp. Ps14 (A and C) and *Trichoderma* sp. Tr6 (B and D) in the rhizospheres of cucumber (A and B) and *A. thaliana* (C and D) at 4 weeks after introduction of these microorganisms in the soil. For each time point bars with different letters are significantly different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

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 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 elicit 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.

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