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ORIGINAL PAPER

Investigating the compatibility of the biocontrol agent *Clonostachys rosea* IK726 with prodigiosin-producing *Serratia rubidaea* S55 and phenazine-producing *Pseudomonas chlororaphis* ToZa7

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Abstract This study was carried out to assess the compatibility of the biocontrol fungus *Clonostachys rosea* IK726 with the phenazine-producing *Pseudomonas chlororaphis* ToZa7 or with the prodigiosin-producing *Serratia rubidaea* S55 against *Fusarium oxysporum* f. sp. *radicis-lycopersici*. The pathogen was inhibited by both strains in vitro, whereas *C. rosea* displayed high tolerance to *S. rubidaea* but not to *P. chlororaphis*. We hypothesized that this could be attributed to the ATP-binding cassette (ABC) proteins. The results of the reverse transcription quantitative PCR showed an induction of seven genes (*abcB1*, *abcB20*, *abcB26*, *abcC12*, *abcC12*, *abcG8* and *abcG25*) from subfamilies B, C and G. *In planta* experiments showed a significant reduction in foot and root rot on tomato plants inoculated with *C. rosea* and *P. chlororaphis*. This study demonstrates the potential for combining different biocontrol agents and suggests an involvement of ABC transporters in secondary metabolite tolerance in *C. rosea*.

Keywords ABC transporter · Biocontrol · *Clonostachys rosea* · Prodigiosin · *Pseudomonas chlororaphis* · *Serratia rubidaea*

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Introduction

Clonostachys rosea (Schroers, Samuels, Seifert and Gams) is an effective antagonist against fungal pathogens of agricultural and horticultural crops, e.g., *Bipolaris sorokiniana* (Saccardo in Sorokin, Shoemaker), *Fusarium culmorum* (Saccardo) (Knudsen et al. 1995; Jensen et al. 2000, 2002), *Pythium* spp. (Møller et al. 2003), *Alternaria* spp. (Jensen et al. 2004), *Botrytis cinerea* (Pers.: Fr.) (Li et al. 2004) and *Fusarium* spp. (Luongo et al. 2005). The strain "IK726" is an effective biocontrol agent (BCA) against several important plant pathogens and promotes plant growth (Knudsen et al. 1995; Jensen et al. 2000). The analysis of *C. rosea* IK726 genome revealed a decreased number of chitinolytic enzymes (Tzelepis et al. 2015) compared to other mycoparasites like *Trichoderma* species (Seidl-Seiboth et al. 2014). Moreover, differences between *Trichoderma* sp. and *C. rosea* have been also observed in the transcription patterns of chitinase genes during fungal–fungal interactions (Gruber et al. 2011a, b; Tzelepis et al. 2015). These data indicate a possible different mode of action between *Trichoderma* sp. and *C. rosea* IK726.

The latter is also able to effectively colonize roots in competition with other microbes and antagonize other fungi through direct parasitism and is highly tolerant towards several chemical compounds and shows no known non-target effects (Johansen et al. 2005; Jensen et al. 2007; Karlsson et al. 2015).

Pseudomonas chlororaphis ToZa7 (Guignard and Sauvageau) and *Serratia rubidaea* S55 (Stapp) were both isolated in Greece, and their antagonistic activity against *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Jarvis and Shoemaker) (hereafter mentioned as Forl) was proven (Kamou et al. 2015). *S. rubidaea* strain S55 produces the antibiotic prodigiosin (2-methyl-3-pentyl-6-methoxy-prodigiosin) (reported here) and *P. chlororaphis* ToZa7 produces the broad spectrum antibiotic phenazine-1-carboxamide (PCN) and hydrogen cyanide (HCN). Both bacterial strains produce proteases, siderophores and promote growth of tomato plants (Kamou et al. 2015). Since *C. rosea* IK726 and the bacterial strains could have different and compatible modes of action, a possible combination of the fungus and *P. chlororaphis* ToZa7 or *S. rubidaea* S55 could provide more effective biocontrol effects. In order to assess this possibility, it is necessary to know whether *C. rosea* IK726 can tolerate the antifungal metabolites produced by the bacterial strains.

Fungal ABC transporters also play important roles in interactions with biocontrol fungi. The role of ABC transporter proteins in the active efflux of toxic compounds is highlighted in several studies. Induction of ABC transporter genes has been observed upon exposure to antibiotics (Schoonbeek et al. 2002; Schouten et al. 2008). An important prerequisite for a BCA to be successful is its ability to tolerate chemical fungicides and pesticides, and/or other BCAs (Jensen et al. 2007). ABC transporters are reported to protect biocontrol fungi, such as *Trichoderma* spp. (Ruocco et al. 2009) and *C. rosea* (Dubey et al. 2014; Kosawang et al. 2014), against secondary metabolites produced by the fungal prey and against fungicides. The *C. rosea* IK726 genome contains 86 ABC transporters, with particularly high numbers in subfamilies B (multidrug resistance, MDR) and G (pleiotropic drug resistance, PDR) (Karlsson et al. 2015). In a potential combination of fungal and bacterial BCAs, fungal ABC transporters could play an important role by maintaining non-toxic intracellular levels of bacterial antifungal compounds (Del Sorbo et al. 2000; Schoonbeek et al. 2002).

The fungal ATP-binding cassette (ABC) transporter proteins bind and hydrolyse ATP in order to produce the necessary energy to transport solutes over the plasma membrane. ABC transporters are responsible for the efflux of a large number of toxicants, such as antibiotics produced by other microorganisms, synthetic fungicides and even plant defence compounds (Del Sorbo et al. 2000; Coleman and Mylonakis 2009). Based on a phylogenetic analysis of fungal ABC proteins, they are classified into eight subfamilies (from A to H) plus a "non-classified" group (Kovalchuk and Driessen 2010). A typical ABC transporter (full size) is consisted of four stable core domains, two cytoplasmic nucleotide binding domains (NBDs) and two membrane-associated transmembrane domains (TMDs). Half-size transporters possess only one NBD with a single TMD domain (Coleman and Mylonakis 2009; Lamping et al. 2010). Several transporters from subfamilies B (full size) and G are associated with MDR or PDR. Additional members of subfamilies B (half size) and C (multidrug resistance-associated protein, MRP) are involved in transport of toxins out of cells, or in secondary metabolite transport (Coleman and Mylonakis 2009).

The objective of this study was to investigate whether *P. chlororaphis* ToZa7 and *S. rubidaea* S55 could be combined in consortia with *C. rosea* IK726 for use in biocontrol of foot and root rot disease on tomato, for a sustainable agriculture. Other objectives were to identify the antibiotic prodigiosin produced by *S. rubidaea* S55 and to investigate the tolerance of *C. rosea* IK726 against secreted bacterial metabolites and thus the antibiotics that the two bacterial strains produce, by measuring fungal growth rate, conidial germination and biomass in the presence of the bacteria. Furthermore, the putative mechanism behind the tolerance of *C. rosea* against secreted bacterial compounds was investigated by analysing the expression patterns of 12 putative ABC transporter genes in *C. rosea*.

Materials and methods

Strains and cultural practice

Clonostachys rosea IK726 and Forl (isolate ZUM 2407/IPO-DLO) were routinely kept on potato dextrose agar (PDA, BD Difco) plates at 25 °C. *C. rosea* conidia were harvested as a sterile aqueous suspension that was passed through glass wool filters to exclude mycelium. Forl was grown in liquid Czapek Dox Broth (Duchefa Biochemie), for 5–7 days, at 25 °C, on a rotating incubator, at 150 rpm, and conidia were separated from mycelium by filtering through Miracloth (Calbiochem, USA). The conidial concentration of the two fungi was determined each time with a haemacytometer (Thoma, Blaubrand GmbH, Germany, 0.1 mm × 0.0025 mm^2). Stock cultures of *P. chlororaphis* ToZa7 (Netherlands Culture Collection of Bacteria, NCCB isolate number 100413) and *S. rubidaea* S55 (NCCB isolate number 100416) were cultured on Luria–Bertani (LB, Bertani 1951) agar plates at 25 °C.

Dual cultures and spore germination bioassays

Two confrontation bioassays and two spore germination bioassays were conducted: (1) *P. chlororaphis* and *S. rubidaea* versus *C. rosea*, and (2) *P. chlororaphis* and *S. rubidaea* against Forl. Dual culture bioassays were performed by streaking the bacteria (2.5 cm colony) at 1 cm from the edge of a 9-cm LB agar Petri dish and positioning agar plugs (4 mm diameter) of the fungi in the centre of the plate, at 3 cm distance from the bacteria. The fungi growing in the absence of the bacteria were used as control. The inhibition ratio was calculated as: Inhibition Ratio % $= (C - E)/C * 100$ %, where C represents the average diameter of the colonies of the control groups and E stands for the average diameter of the colonies of the experimental groups. Generally, if the inhibition ratio exceeded 20 %, the tested fungus was considered inhibited. Plates were incubated for 12 days at 25 °C in darkness in five replicates, and the experiment was repeated twice.

Regarding the spore germination bioassay, the interaction was tested in 9-cm Petri dishes which were pre-inoculated with fungal conidia at a concentration of 10^4 spores ml^{-1}. Two bacterial colonies (2.5 cm) were streaked, at two opposite spots, 2 cm from the edge of each plate. All dishes were incubated at 25 °C in darkness for 12 days and were regularly examined macroscopically for the formation of inhibition zones around the bacterial colonies. Inhibition zone was determined each time as the average of two measurements, with a vernier calliper, of the zone diameter from the bacterial colony to the edge of the mycelial growth. Five replicates were included in each spore germination bioassay, and the experiment was repeated twice.

Bacterial culture filtrates preparation and fungal biomass measurement

P. chlororaphis and *S. rubidaea* culture filtered supernatants, used in the biomass measurement experiment, were prepared by centrifugation of 24 or 48 h LB liquid cultures, at 5000 rpm, for 10 min and filter sterilization using a 0.20-μm cellulose acetate membrane (VWR, Radnor, PA). Forl and *C. rosea* were separately inoculated and incubated on a rotary shaker (200 rpm) at 25 °C, in 100 ml of these culture filtrates, and dry fungal biomass was recorded 4 dpi.

Prodigiosin identification

One hundred millilitres of 48 h liquid culture of *Serratia rubidaea* S55 was centrifuged at 6000 rpm for 10 min. The supernatant was collected and extracted with equal volume of diethyl ether and concentrated to 1 ml. A Surveyor HPLC system associated with a TSQ Quantum Discovery MAX triple quadrupole mass spectrometer (Thermo Electron Corporation, San Jose, CA, USA) was used for LC–MS/MS detection. Chromatography was carried out on a HyPURITY C18 150 × 2.1, 5 μm column (Thermo Scientific). Mobile phase consisted of 0.1 % formic acid in a water: acetonitrile 40:60, v/v mixture. Mobile phase flow rate was 0.2 mL/min. The chromatography column was thermostated at 25 °C. Injection volume was set at 10 μL. The mass spectrometer was operated in the selected reaction monitoring (SRM) mode. The ionization of the analytes was accomplished by electrospray ionization (ESI) in the positive mode. Data acquisition was performed by the Xcalibur software (Thermo Electron Corporation). The transitions 324.3 → 252.0 and 324.3 → 309.3 were used for quantization and confirmation purposes, respectively. A prodigiosin reference standard (Santa Cruz Biotechnology, Santa Cruz, CA) was used for peak identification.

Gene expression analysis

Regarding the transcription analysis of the putative ABC transporter genes, *C. rosea* was inoculated in culture filtrates and derived from *P. chlororaphis* or *S. rubidaea*. Culture filtrates were made by the above bacterial cultures, incubated on potato dextrose broth (PDB, BD Difco) for 24 and 72 h and filter-sterilized using a 0.45 mm cellulose acetate membrane (VWR). Then, 25 ml medium was re-inoculated with *C. rosea* mycelia grown previously on PDB for 5 days on a rotary shaker at 25 °C. *Clonostachys rosea* mycelia grown in 25 ml of PDB were used as a control. After 1 and 4 h of incubation, mycelia were harvested, frozen in liquid nitrogen and freeze-dried overnight. Approximately 50 mg of mycelia was homogenized in liquid nitrogen. Total RNA was extracted using the Qiagen RNeasy kit (Qiagen, Hilden, Germany). Residual traces of DNA were removed by DNase I (Fermentas, St. Leon-Rot, Germany) treatment. RNA quality was determined with Agilent Bioanalyzer (RNA 6000 Nanochip, Agilent Technologies), and 1 μg of total RNA was reverse transcribed in a total volume of 20 μl using Maxima First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany).

Gene-specific primers designed by Karlsson et al. (2015) were used. The gene encoding actin (*act*) was used as a reference gene in *C. rosea* (Mamarabadi et al. 2008; Zapparata 2014). Expression of 12 *C. rosea* putative ABC transporter genes representing subfamilies B, C and G was measured by quantitative reverse transcriptase PCR (RT-qPCR) using the SsoFast™ EvaGreen® supermix (Bio-Rad, Hercules, CA). QPCR reactions were carried out in an iQ5 qPCR System (Bio-Rad, Hercules, CA) as described previously by Tzelepis et al. (2012), and melt curve analysis was conducted in order to assess specific amplification. Transcript levels were quantified in five biological replicates, each

based on two technical replicates. Data analysis was carried out with relative quantification, using the $2^{-\Delta\Delta C}$T method (Livak and Schmittgen 2001), and data normalization was achieved using the expression levels of the reference gene. Serial dilutions of *C. rosea* genomic DNA were used to determine PCR amplification efficiencies for primer pairs.

In planta experiments

The effects of combining *C. rosea* with *P. chlororaphis* or *S. rubidaea* in consortia to control tomato foot and root rot (hereafter mentioned as TFRR) caused by Forl were evaluated in a pot experiment, as described by Kamou et al. (2015) with minor adjustments. The tomato seeds were pre-germinated, and the conidial concentration of Forl was adjusted to 10^4 spores ml^{-1}. *C. rosea* was also coated in the seeds as described above, using an 1:1 v/v mixture of conidia in water (10^4 spores ml^{-1}) and 4 % aqueous solution of methyl cellulose. Combination of the two BCAs was done following the same method using a 1:1:2 v/v/v mixture of a bacterial cell suspension in PBS, at a concentration of approximately 1×10^7, ($O.D._{620}$ value 0.7), a suspension of conidia in water (10^4 spores ml^{-1}) and 4 % aqueous solution of methyl cellulose. Eighteen plants were used per treatment and were grown in a growth chamber at 22 °C, with adequate luminescence (14 h), and after 6 weeks disease severity was estimated using a disease index scale, as described by Kamou et al. (2015). During this period, the presence and viability of used microorganisms were confirmed every two weeks, by re-isolation on LB agar plates. The experiment was repeated three times.

Statistical analysis

Treatments in the *in planta* experiments (disease index comparisons) were compared as follows: in case of a significant result, according to the Kruskal–Wallis test (non-parametric one-way ANOVA), the pair-wise differences were tested with a series of Mann–Whitney tests. In non-parametric hypothesis testing procedures, the significance level (*P* value) was computed with the Monte Carlo simulation method utilizing 10,000 re-sampling circles (Mehta and Patel 1999). Data from gene expression analysis were analysed by analysis of variance (ANOVA), based on the completely randomized design (CRD), and mean values were computed from five replicates. Following a significant ANOVA *F* test, the differences between treatments' mean values were compared with the Tukey's test. The significance level in all hypothesis testing procedures was predetermined at $P \leq 0.05$. All statistical analyses were performed with the SPSS v 19.0 software (SPSS Inc., Chicago, IL). The corresponding results of each ANOVA (F-values, df and *P* values) and of Kruskal–Wallis test (Chi-square values, *df* and *P* values) are displayed in Supplementary Table S1.

Results and discussion

Interest in the ability of fungi to tolerate toxic metabolites or chemicals through efficient cell detoxification ability is increased during the past two decades (Del Sorbo et al. 2000; Schoonbeek et al. 2002; Zwiers et al. 2003; Coleman and Mylonakis 2009). A further step in this research is taken nowadays since researchers also focus on biocontrol agents' responses towards endogenous and exogenous compounds (Ruocco et al. 2009; Kosawang et al. 2014; Dubey et al. 2014). This rising interest in BCAs could be explained by the need to establish a more sustainable and environmental friendly agriculture, or the need to find an alternative way to control plant pathogens when the chemical approach is not sufficient. As stressed by Jensen et al. (2007), a successful BCA should be able to tolerate technological products, such as chemical formulation, or commercialized or natural BCAs. In this study, we investigated whether a combination of BCAs could be feasible.

Both bacterial strains demonstrated a significant antifungal activity since they produce a variety of potential antifungal metabolites including HCN, siderophore compounds and proteases, while *P. chlororaphis* also produces the phenazine derivative PCN (Kamou et al. 2015). As shown in the present study, *S. rubidaea* produces the tripyrrole antibiotic, prodigiosin (2-methyl-3-pentyl-6-methoxyprodigiosin). The retention time of prodigiosin was 3.38 min. A concentration of 0.4339 mg of prodigiosin was obtained from 100 ml of cultured broth. This antibiotic is known for its antimicrobial, immunosuppressive and anticancer properties (Williamson et al. 2005).

The fungal species *C. rosea* is known for its biocontrol activity and has been studied for its ability to interact with different substances such as fungicides and secreted metabolites, including some mycotoxins (Jensen et al. 2007; Kosawang et al. 2014; Dubey et al. 2014). In the current study, a series of in vitro assays was performed in order to test the tolerance of mycelium and spores of *C. rosea*, in comparison with tolerance of Forl, to *P. chlororaphis* and *S. rubidaea*. Dual culture tests showed that Forl was significantly more inhibited by *S. rubidaea* (40.53 %) compared to *C. rosea* (17.43 %). The latter expressed a significant higher tolerance to *S. rubidaea* compared to the effect that *P. chlororaphis* had on its growth (33.15 % inhibition) (Fig. 1a). Spore germination of both fungi was significantly more inhibited by *P. chlororaphis* than by *S. rubidaea*. The most severe inhibition was measured in the interaction of *C. rosea* IK726 and *P. chlororaphis* (32.44 %). *S. rubidaea* showed a similar effect on the conidial germination of both

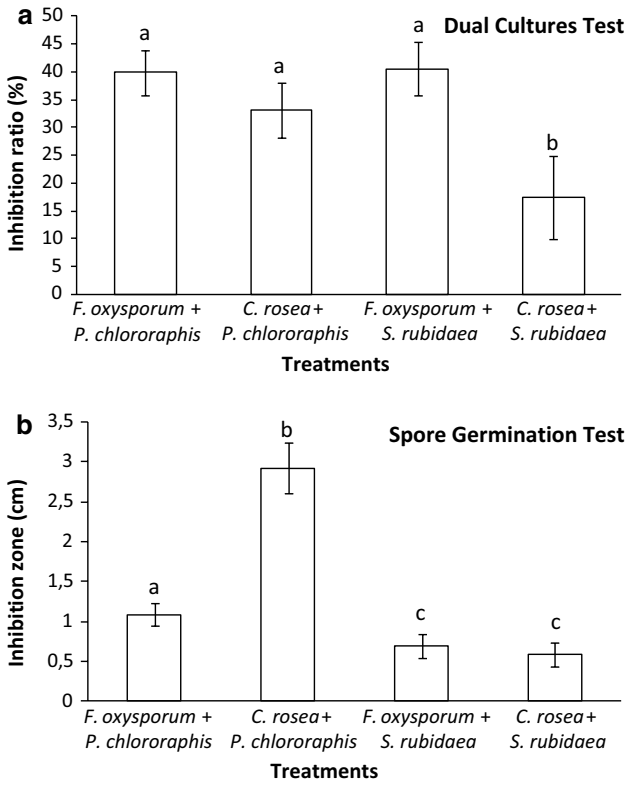

Fig. 1 **a** Effects of *Pseudomonas chlororaphis* ToZa7 and *Serratia rubidaea* S55 on the fungal growth of *Fusarium oxysporum* f. sp. *radicis-lycopersici* and *Clonostachys rosea* IK726. Dual cultures were incubated at 25 °C in darkness for 12 days. Inhibition ratio was calculated as: Inhibition Ratio % = $(C - E)/C * 100$ %, (C average diameter of the control colonies, E average diameter of the experimental colonies). **b** Effects of *Pseudomonas chlororaphis* ToZa7 and *Serratia rubidaea* S55 on the conidial germination ability and fungal growth of *Fusarium oxysporum* f. sp. *radicis-lycopersici* and *Clonostachys rosea* IK726. Petri dishes were pre-inoculated with fungal conidia at a concentration of 10^4 spores/ml. After 12 days of incubation at 25 °C in darkness, inhibition zones, around the bacterial colonies, were measured. (*a*, *b*) *Error bars* represent the standard deviation based on five technical replicates. Different letters (*a*, *b*) indicate statistically significant differences according to Tukey's test ($P \leq 0.05$)

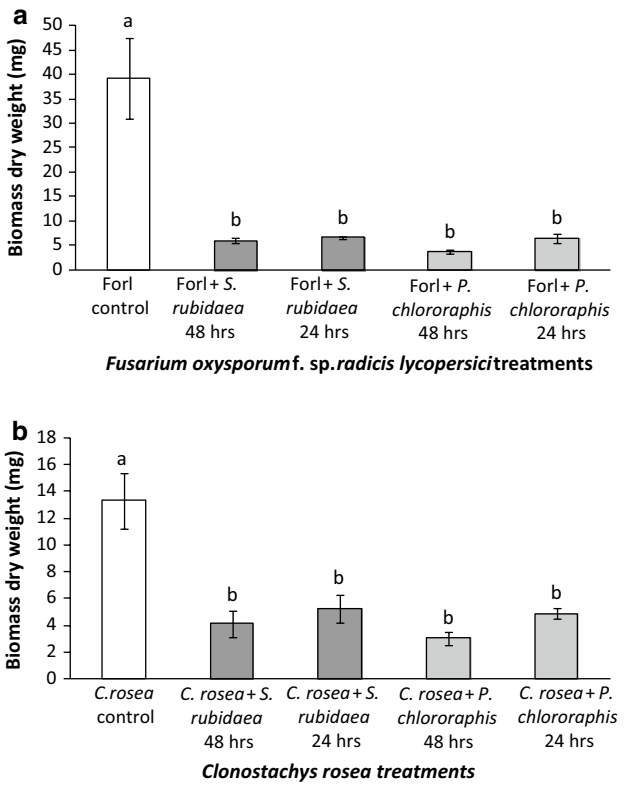

Fig. 2 Effect of 24 and 48 h culture filtrates of *Pseudomonas chlororaphis* ToZa7 (*light grey*) and *Serratia rubidaea* S55 (*grey*) **a** on the biomass weight of *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Forl) (*white*) and **b** on the biomass weight of *Clonostachys rosea* IK726 (*white*). *Error bars* represent the standard deviation based on three technical replicates. Different letters (*a*, *b*) indicate statistically significant differences according to Tukey's test ($P \leq 0.05$)

Forl and *C. rosea* (7.77 and 6.56 %, respectively) (Fig. 1b). These results demonstrate that *C. rosea* is more tolerant to metabolites produced by *S. rubidaea* than to those produced by *P. chlororaphis*. On the other hand, the inhibition caused to Forl by the same bacterium was greater than the one caused to *C. rosea* and this indicates that *C. rosea* is able to tolerate these substances more efficiently than Forl.

Mycelial biomass production of fungal strains was measured in culture filtrates of *P. chlororaphis* and *S. rubidaea* 24 h or 48 h post-inoculations, in order to test the tolerance of *C. rosea* and Forl to the secreted metabolites. The reduction in fungal biomass in 24 and 48 h culture filtrates of both *P. chlororaphis* and *S. rubidaea* was significantly stronger in Forl. The reduction ranged from 83.09 %, for *S. rubidaea* 24 h culture filtrate, to 90.38 %, for *P. chlororaphis* 48 h culture filtrate, as compared to the control (Fig. 2a), and this shows the strong inhibitory effect of these strains against this plant pathogen. The reduction caused to *C. rosea* biomass, compared to the control, was not as strong. The highest reduction (77.27 %) was measured in 48 h culture filtrate of *P. chlororaphis* (Fig. 2b). A similar significant reduction in growth was also observed when combining the beneficial fungus *T. atroviride* (P. Karst) with wild-type strains of *P. fluorescens* (Migula) (Lutz et al. 2004). At this point, it is important to stress out that the inhibition was caused by compounds that were already present in the bacterial culture supernatants and these are expected to be HCN, siderophore compounds, proteases and the antibiotics PCN, for *P. chlororaphis* and prodigiosin, for *S. rubidaea*.

The ability of *C. rosea* to tolerate toxic compounds, including metabolites produced by other microorganisms, could be attributed to cell detoxification mechanism and efflux of toxins from the BCA cell. Based on the results of various studies indicating an involvement of ABC

transporters during the interaction of BCAs with other pathogens or fungicides (Marra et al. 2006; Ruocco et al. 2009; Kosawang 2013; Kosawang et al. 2014; Dubey et al. 2014, Karlsson et al. 2015), we decided to investigate gene expression of ABC transporter efflux pumps during interaction with two rhizobacterial strains. Indeed, our data from RT–qPCR analysis showed that seven different ABC transporter genes were induced by the 24 and 72 h culture filtrates of the bacteria. More specifically, *abcB1*, *abcB26* and *abcB20* from subfamily B, *abcC14* and *abcC12* from subfamily C and *abcG8* and *abcG25* from subfamily G were induced as compared to their respective controls.

A putative pheromone transporter gene (*abcB1*) in *C. rosea* showed induction after treatment with the 24 and the 72 h culture filtrates of *S. rubidaea* and a 12.9-fold ($P < 0.001$) induction was observed 4 hpi (hours post-inoculation) in the 72 h culture filtrate compared to control (Fig. 3a). A putative mitochondrial peptide transporter gene (*abcB26*) was up-regulated 8.4-fold ($P < 0.001$) 4 hpi in the 72 h culture filtrate of *S. rubidaea* compared to control samples. Moreover, 11.21-fold and 8.3-fold induction of the *abcB26* was observed 1 and 4 hpi in the 72 h culture filtrate of *P. chlororaphis* compared to control ($P < 0.001$) (Fig. 3a). The 24 h culture filtrate of *S. rubidaea* caused a twofold induction of the same gene 1 hpi ($P = 0.017$) (Fig. 3a). Induction of this gene coincides with the expression of putative secondary metabolites transporter genes *abcC12* and *abcC14*. In this case, ATP molecules could be necessary to activate the efflux pumps, and in order to secrete the metabolites *C. rosea* could have produced in response to the presence of bacterial enzymes that are present in the culture filtrates. *Clonostachys* sp. is known to produce and exude, argadin and argifin, two cyclic pentapeptides which inhibit chitinases (Arai et al. 2000). The transcription patterns of the putative MDR gene *abcB20* (subfamily B) increased 1 hpi in 72 h *P. chlororaphis* culture filtrate ($P < 0.001$), but no induction was observed 4 hpi comparing to control. Exposure to the 72 h culture filtrate of *S. rubidaea* caused a 4.8-fold increase 4hpi compared to control ($P = 0.027$) (Fig. 3a). An explanation could be that there is a need for the *abcB20* ABC transporter protein production as a response to a possible antibiotic and/or HCN accumulation in *P. chlororaphis* 72 h culture filtrate. The rapid decrease to control levels could mean that *abcB20* proteins act as a first level of defence that is replaced by more efficient degradation mechanisms, as it is demonstrated in the case of the BcatrB efflux pump in *B. cinerea* (Schouten et al. 2008).

Expression of the putative secondary metabolite transporter genes *abcC14* and *abcC12* (subfamily C) was up-regulated rapidly after exposure to the 72 h culture filtrates of *S. rubidaea* and *P. chlororaphis*. More specifically, after 4 h exposure to *S. rubidaea* 72 h culture filtrate, *abcC14* exhibited a 5.9-fold ($P < 0.001$) induction (Fig. 3b). The 72 h culture filtrate of *P. chlororaphis* caused a 7.5-fold ($P < 0.001$) up-regulation after 1 h of incubation and then rapidly reduced to the control levels. *AbcC12* was induced 21.8-fold and 15.6-fold ($P < 0.001$), after 1 and 4 h of incubation in the 72 h culture filtrate of *S. rubidaea*, respectively (Fig. 3b). This finding could suggest that *C. rosea* is secreting compounds either related to a detoxification mechanism, or to an inhibition mechanism. More specifically, *C. rosea* may be using efflux pumps to secrete compounds to inhibit or detoxify antifungal metabolites. It has already been shown that *Clonostachys rosea* possesses a detoxification mechanism which converts zearalenone (ZEN), a mycotoxin, produced by *Fusarium* sp., to a non-toxic product (Takahashi-Ando et al. 2002, 2004).

Two genes, *abcG8* and *abcG25*, belonging to subfamily G and putatively involved in PDR, were both induced by the 24 h culture filtrate of *S. rubidaea* S55. After 4 h exposure to the latter, *abcG8* was up-regulated 1.7-fold ($P = 0.052$). *AbcG25* was induced fourfold after 1 h of incubation in the same filtrate ($P = 0.004$) and eightfold after 4 h ($P = 0.002$) (Fig. 3c). We postulate that the fact that this rapid induction, after 1 h of incubation, occurs only for the case of *S. rubidaea* suggests that it could be specifically induced by a compound produced by *S. rubidaea* only, and not by *P. chlororaphis*.

To summarize, when looking at the overall picture, these results suggest that the higher tolerance that *C. rosea* demonstrates towards *S. rubidaea* compared to *P. chlororaphis* could partly be justified by the co-regulation of the genes aforementioned. Moreover, the expression patterns corroborate the results of the in vitro experiments, which also showed that *C. rosea* IK726 is comparatively more tolerant to *S. rubidaea* S55 and a successful combination of these two biocontrol agents could be possible. On the other hand, Karlsson et al. (2015) demonstrated that the combination of *C. rosea* IK726 with other *P. chlororaphis* strains is compatible. The up-regulation of genes *abcB1*, *abcB20*, *abcB26*, *abcC12*, *abcC14* and *abcG8*, during the interaction of *C. rosea* IK726 with *P. chlororaphis*, suggests that in these cases, the detoxification mechanism was active and could lead to a certain tolerance of *C. rosea* towards the toxic compounds of several *P. chlororaphis* strains (Karlsson et al. 2015).

Interestingly, the *in planta* experiments demonstrated that the treatment against Forl, where *C. rosea* IK726 is combined with *P. chlororaphis* ToZa7 significantly reduces the disease severity (56.25 %), to the same level as when *C. rosea* IK726 acts alone or in combination with *S. rubidaea* S55 (Fig. 4). *P. chlororaphis* has been reported to cause a 45 % reduction in disease severity, when applied alone on tomato, under the same conditions (Kamou et al. 2015). These results show that a consortium of *C. rosea*

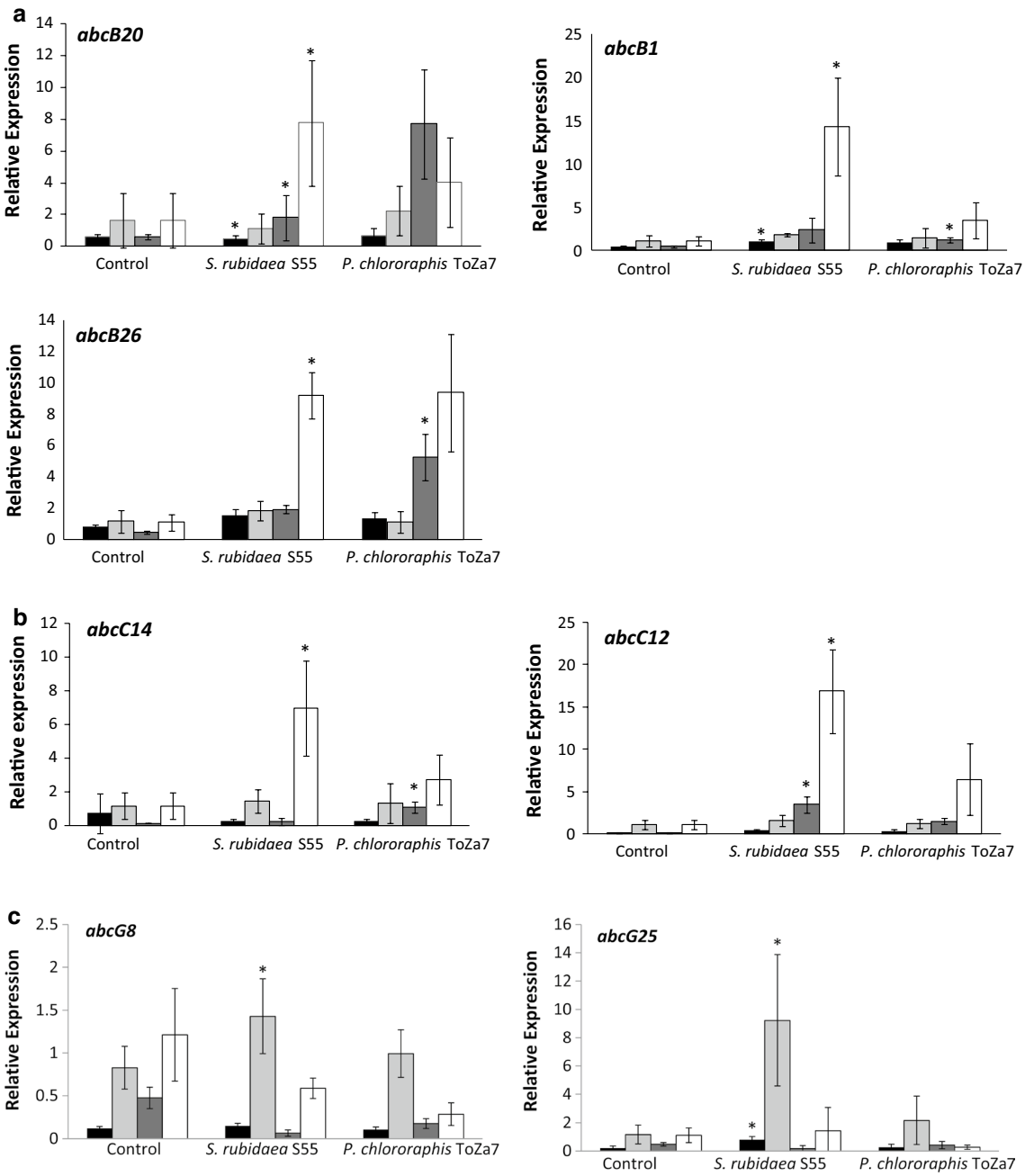

Fig. 3 **a** Expression analyses of three subfamily B *Clonostachys rosea* IK726 ABC transporter genes (*abcB1*, *abcB26* and *abcB20*) after induction with 24 and 72 h culture filtrate of *Pseudomonas chlororaphis* ToZa7 and *Serratia rubidaea* S55 for 1 and 4 h. **b** Expression analyses of two subfamily C *C. rosea* IK726 ABC transporter genes (*abcC14* and *abcC12*) after induction with 72 h culture filtrate of *P. chlororaphis* ToZa7 and *S. rubidaea* S55 for 1 and 4 h. **c** Expression analyses of two subfamily G *C. rosea* IK726 ABC transporter genes (*abcG8* and *abcG25*) after induction with 24 h culture filtrate of *P. chlororaphis* ToZa7 and *S. rubidaea* S55 for 1 and 4 h. *Black* Treatment of 24 h culture filtrates, 1 h post-inoculation (hpi). *Light grey* Treatment of 24 h culture filtrates, 4 hpi. *Dark grey* Treatment of 72 h culture filtrates, 1 hpi. *White* Treatment of 72 h culture filtrates, 4 hpi. Data analysis was performed using the $2^{-\Delta\Delta C}$T method. *Error bars* represent the standard deviation based on five biological replicates. An *asterisk* indicates a significant difference of expression in comparison with control treatment, according to Tukey's test (for *P* values see Supplementary Table S1)

IK726 with the phenazine-producing *P. chlororaphis* ToZa7 is also possible and reduces the disease severity successfully. Duffy et al. (1996) also demonstrated that the combination of a phenazine and HCN producing *P. chlororaphis* strain with the BCA *T. koningii* (Oudemans) is feasible even when microorganisms are applied simultaneously. Similar results were found during interaction of *C. rosea* IK726 with another PCN producing strain, *P.*

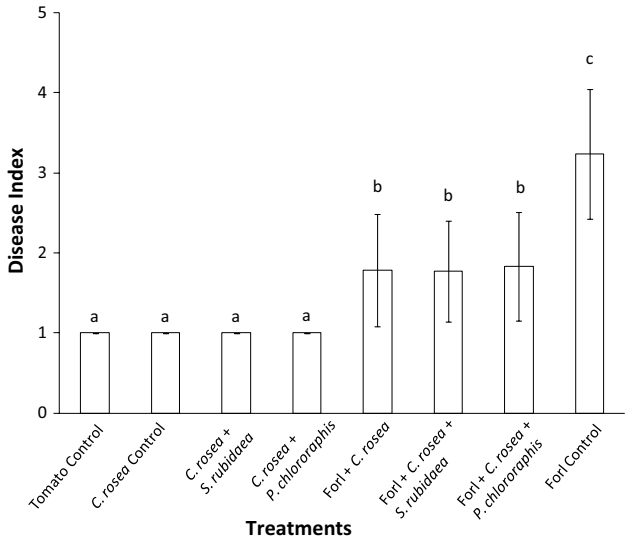

Fig. 4 Effect of *Clonostachys rosea* IK726, *Pseudomonas chlororaphis* ToZa7 and *Serratia rubidaea* S55on TFRR severity caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* on tomato, in pots under controlled conditions. Tomato control plants were not inoculated with any microorganism. Forl control plants were inoculated with the pathogen without biocontrol agent treatment. Disease was assessed after 6 weeks. The experiment was repeated three times. *Different letters* indicate significant differences according to the Mann–Whitney test at $P \leq 0.05$. *Error bars* represent standard deviation, for the first four error bars are not plotted since all measurements are equal to 1

chlororaphis PCL1391 (Tzelepis and Lagopodi 2011). One possible explanation could be that Forl hyphae are used as a source of nutrients since observations showed that some bacteria, such as *P. fluorescens* WCS365 and *P. chlororaphis* PCL1391, extensively colonize them (Bolwerk et al. 2003) and *C. rosea* hyphae have been observed to parasitize Forl hyphae (Karlsson et al. 2015). Our study should broaden the perspective regarding the combinations of BCAs for biocontrol and may contribute to the understanding of the mechanisms that result in a high tolerance of *C. rosea* IK726 to *S. rubidaea* S55 in vitro, by underlining the importance of the ABC transporter genes in the fungus. As reported, the majority of the induced genes are predicted, based on their phylogenetic placement, to be involved in secondary metabolite efflux.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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