Effects of ultraviolet irradiation on the *in vitro* antagonistic potential of *Trichoderma* spp. against soil-borne fungal pathogens

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A B S T R A C T

Development of new effective biocontrol agents is largely based on the antagonistic capacity of candidate agents against targeted pathogens *in vitro*. Different mechanisms contribute to such capacity, including the activity of cell wall-degrading enzymes, secretion of antimicrobial secondary metabolites, growth vigour and resistance to exogenous and endogenous toxins. In this study, a series of laboratory experiments were designed to improve the antagonistic activities of *Trichoderma* spp. against two plant fungal pathogens, *Sclerotium rolfsii* and *Rhizoctonia solani*. A simple but efficient mutagenesis programme was carried out using ultraviolet light to induce modifications in the genetic structure of two *Trichoderma* biocontrol agents, *T. virens* and *T. asperellum*. The obtained mutants were subjected to **a)** initial screening for media-permeable antifungal metabolites using the cellophane membrane-based method, and **b)** selected mutants were subjected to a series of antagonistic tests. Results revealed that the antagonistic potential of selected mutants was significantly improved against the two plant pathogens. Genetic stability test results indicated that the UV-derived mutant Tv3, maintained its elevated performance after 12 rounds of sub-culture. Gene expression analysis for five antagonism-associated genes were examined using real-Time PCR. Results revealed that the gene expression of two genes, chitinase 33, a cell wall degrading enzyme and, polyketide synthase, which is responsible for polyketide biosynthesis, a class of secondary metabolites with antimicrobial roles, were significantly upregulated in one of the mutated *T. virens* strains. Results of our *in vitro* antagonistic studies along with our molecular analysis indicate that the UV mutagenesis could be an effective strategy to improve *Trichoderma* antagonistic potential.

1. Introduction

Repetitive and intensive growth of crops in the same agricultural areas creates ecological systems prone to spread of disease epidemics. Biological control, or simply biocontrol, is a plant protection strategy that involves using biological control agents (BCAs) to regulate existing pest populations. Among the widely used BCAs are *Trichoderma* spp. for their biocontrol activities and beneficial plant interactions (Benítez et al., 2004; Nawrocka and Małolepsza, 2013). Members of the fungal genus *Trichoderma* (*Ascomycota, Hypocreales, Hypocreaceae*) are ubiquitous and commonly encountered as soil inhabitants, plant symbionts, saprotrophs, and mycoparasites. Certain species have been used for controlling diverse plant diseases and/or industrial enzyme production (Benítez et al., 2004; Mukherjee et al., 2013). Antibiosis is a biocontrol mechanism in which different biologically active molecules are produced by the antagonist and act synergistically to kill or suppress the growth of competing microorganisms. These molecules include volatile and non-volatile

metabolites with antimicrobial activities. Mycoparasitism is another biocontrol mechanism in which *Trichoderma* spp., following successful recognition of competing fungi, launch a direct attack leading to the death of competing fungi or limiting their growth (Li et al., 2018; Seidl et al., 2009). Resistance to exogenous toxins, priming plant immunity against plant pathogens, ability to perceive and attack to kill or suppress other fungi are all factors contributing to the commercial success of *Trichoderma* BCAs (Mukherjee et al., 2013). However, despite extensive research on *Trichoderma* BCAs and their wide and very promising applications, biocontrol mechanisms are not yet fully understood. Inconsistent field efficacy hampers wider biocontrol applications and invites more research to fully understand the underlying mechanisms. Meanwhile, the antagonistic potential of BCAs can be further improved through various approaches. Mutagenesis is one such approach that induces diversification of the genetic structure of targeted organisms. Induced changes could either be random or targeted depending on the selected mutagenesis technique. By successfully screening and analysing

the obtained mutants, biocontrol-associated traits could be significantly improved.

In the present study, we used a UV-based mutagenesis programme to enhance the *in vitro* activities of the biocontrol-associated traits of *Trichoderma* spp. UV-induced mutants were obtained by exposure to UV light followed by screening of mutants and evaluation of their antagonistic potential against the soil-borne fungal pathogens *Sclerotium rolfsii* and *Rhizoctonia solani*. The expression levels of biocontrol-associated genes were examined using RT-PCR to test whether the mutagenesis programme had an impact on the expression of these genes. The genetic stability of the obtained mutants was also tested after 12 rounds of subculture on potato dextrose agar (PDA).

2. Materials and methods

2.1. Fungal strains and growth conditions

The following *Trichoderma* spp. were used as wild-type strains before the mutagenic programme: a) *T. asperellum* ICC 012 from the commercial biocontrol product BIO-TAM® 2.0 (Isagro, Morrisville, NC, United States) and b) *T. virens*, an isolate previously identified and characterized with promising BCA potential (Alfiky and Eldenary, 2018). Both WT strains displayed very promising antagonistic activities against several plant pathogens and were selected to demonstrate the efficacy of UV mutagenic programs as strain improvement strategy. Soil-borne fungal pathogens *Sclerotium rolfsii* and *Rhizoctonia solani* were provided by the Microbiological Resources Center (MIRCEN, Cairo). Wild type (WT) fungal cultures and selected UV-derived mutants were preserved as spore suspensions in 20% glycerol in cryogenic vials at −80 °C and were activated by inoculating PDA (Difco, USA) plates.

2.2. UV mutagenesis

Fungal spores were collected from a one-week-old culture by flooding the surface of sporulated PDA culture with sterile water at room temperature. The liquid was gently swirled to dislodge the spores, which were transferred to sterile 50 mL Falcon tubes. The conidia were diluted in water to a concentration of 10^5 per mL and aseptically transferred to sterile Petri plates. The spore suspension in the Petri plate (without the lid to prevent shielding) was subjected to UV light using a Philips TUV 15 W SLV/25 lamp, which was placed at a distance of 25 cm from treated spores. All UV irradiations were performed in a custom-built UV chamber at a wavelength of 254 nm for different time intervals (5, 10, 15 and 20 min) with stirring. For each time interval, only one plate with fresh diluted spore suspension was placed in the chamber for the required irradiation time; this was done to prevent any light from reaching the plates during transfer. Treated spores were kept in the dark during and after UV exposure for at least one hour to prevent photoreactivation. Following the treatment, UV-treated spores were serially diluted and plated on PDA plates containing 0.1% Triton X-100 as a colony restriction factor, which caused the fungus to grow in small colonies (Kallinen, 2016). Surviving spores developed into small mutant colonies that were picked, transferred to PDA plates and incubated at 25 °C. The obtained mutants were screened for their antimicrobial properties.

2.3. Screening and selection of mutants

2.3.1. Preliminary evaluation of mutants using a cellophane membrane assay

WT and all UV-derived *Trichoderma* spp. mutants were screened for their antifungal activity against *R. solani* using a cellophane membrane (CM) assay for the antifungal activities of their media-permeable metabolites. In this technique, a sterile CM (autoclaved while submerged in distilled water) of the same diameter as a Petri plate was overlaid on PDA medium using sterile forceps. A disc of mutant *Trichoderma* spp. was inoculated at the centre of the membrane and maintained at 28 °C for 3

days. Following incubation, the *Trichoderma* culture along with the membrane was carefully removed from the plate, and a disc of *R. solani* was centrally inoculated in the plate and maintained at 28 °C for 7 days. The colony diameter of *R. solani* was measured with a ruler and compared to the control treatment in which *R. solani* was cultured on fresh PDA plates. This technique provided a rapid, easy-to-perform and versatile method for evaluating a large number of mutants in a relatively short time and cost-effective manner without the need for advanced equipment or sophisticated procedures. Selected mutants based on this assay were then screened for variation in their antifungal activities using the dual culture (DC) technique, culture filtrate (CF) assay and sandwich system for volatile interactions.

2.3.2. Dual culture test

The dual culture (DC) technique was used to investigate the outcome of direct interaction by plating both mutant *Trichoderma* cultures and plant pathogens *S. rolfsii* and *R. solani* on the same PDA plate (90 mm) at the opposite end. The antagonistic activity of *Trichoderma* spp. was determined 7 days post-inoculation (dpi) by measuring the radial growth of *S. rolfsii* and *R. solani* in the direction of the *Trichoderma* isolates. Pathogen growth inhibition (I) was calculated using formula (C-T)/C X 100, where C refers to the growth of the pathogen in the control plate, and T refers to the growth of the pathogen with *Trichoderma* in the treatment plate. PDA plates inoculated with *S. rolfsii* or *R. solani* alone were used as controls. All plates were incubated at 28 °C.

2.3.3. Culture filtrate assay

The CF assay aimed to examine the antifungal properties of the culture filtrate of *Trichoderma* mutants against the previously mentioned pathogens. The assay was carried out by inoculating 100 ml potato dextrose broth (PDB) in 250 ml Erlenmeyer flasks with mycelium discs from the margins of actively growing *Trichoderma* cultures. Inoculated flasks were maintained at 28 °C at a shaking speed of 125 RPM for 7 days. Non-inoculated PDB maintained under the same conditions were used as the control treatment. The CF for each mutant was prepared in two consecutive steps: **i)** filtration through Whatman filter paper to remove mycelia and **ii)** through a micropore disposable membrane with a pore size of 0.22 μm (VWR, USA) to remove any fungal spores. All prepared CFs were tested for the absence of colony forming units. Under aseptic conditions, 10 ml of CF was transferred to an empty sterile 9 cm Petri plate and mixed with 10 ml of melted PDA (at 45 ± 5 °C) to obtain a final concentration of 50% (V/V). Once cooled and solidified, plates were inoculated at the centre with a 5 mm mycelium plug of *S. rolfsii* or *R. solani* and incubated at 28 °C. Mycelial growth inhibition was calculated based on measurements taken from control and treatment plates when the fungal growth fully covered the control plate (without the CF).

2.3.4. Sandwich system for volatile interactions

Sandwiched Petri plates, a setup previously described and used for volatile organic compound-mediated interactions (Dennis and Webster, 1971; Li et al., 2018), was used to test whether the amount/activity or both of volatile antifungal organic compounds produced by *Trichoderma* spp. was affected by the mutagenic treatment. After inoculating *Trichoderma* mutants, *S. rolfsii* and *R. solani* on PDA plates, *S. rolfsii* or *R. solani* plates were placed on top of a *Trichoderma* plate, sealed with three layers of Parafilm (PM-996, USA) and incubated at 28 °C. Plates with *S. rolfsii* and *R. solani* were sandwiched with uninoculated PDA plates as a control treatment. The colony diameters of *S. rolfsii* and *R. solani* were measured at 5 dpi and compared to control plates.

2.4. RNA extraction and reverse transcription

RNA was isolated 3 days post inoculation from the WT *T. virens* and its UV-derived mutant Tv3, which was cultured on a CM, using a Qiagen RNeasy kit (Qiagen, Valencia,CA, USA) according to the manufacturer's instructions. The concentration of purified RNA was measured using

Table 1
Primers used for qPCR in *T. virens*.

Gene	Function*	Primer sequences (5′-3′)**		Amplicon size (bp)
Chitinase 33 (*Chit*)	CWD	F	TTGCTCGCTCTTGTTCCTTC	105
		R	TGCTTTGTGAGTTGGCTGAG	
β-1,3-Endoglucanase (*Bgn*)	CWD	F	ACAAGCTCAACCACGCATAC	136
		R	GGCATGTCGTCCTTGTTGTT	
Subtilisin-like protease prb1 (*Prb*)	PD	F	CCTCGGTAATCCTTGACGGT	126
		R	TAATAGCGGTAGTCCAGGCG	
Polyketide synthase (Pks)	SM	F	GTCATGTGTGCCCAACAAAG	147
		R	AAACCGCCAAGATGTAGCAC	
Terpene synthase (*Tps*)	VOC	F	TCAGACGGGCCTTGAATAAG	151
		R	TCTTCTCCTCTGCGACATTG	
β-Actin (*Act*)	HKG	F	TGGCACCACACCTTCTACAA	108
		R	CTGGGTCATCTTCTCACGGT	

* CWD, cell wall-degrading enzyme. PD, protein degradation. SM, secondary metabolism. VOC, volatile organic compound biosynthesis. HKG, housekeeping gene.
** Based on gene ID in the reference genome of *T. virens* Gv29-8 v2.0.

NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific). Purified RNA was treated with DNase I (Ambion). One microgram of purified RNA was reverse transcribed to cDNA in a 20 μL reaction using the RevertAid first-strand cDNA synthesis kit following the manufacturer's instructions (Thermo Fisher Scientific).

2.5. Quantitative PCR analysis for antagonism-associated genes

Primers used for this analysis (Table 1) were designed using Primer 3 (http://primer3.ut.ee/) and were based on sequences of selected genes retrieved from the reference genome of *T. virens* Gv29-8 (V2.0), which is available at http://genome.jgi.doe.gov/. Real-time PCRs were performed using a Rotor-Gene Q 5-plex thermal cycler (Qiagen). All reactions were performed in triplicate. Each amplification mixture (final volume of 25 μL) consisted of 12.5 μL SYBR green PCR master mix (Bio-Rad), 100 nM each of forward and reverse primers and 2.5 μL diluted cDNA (1:100). Thermal cycling conditions were performed as previously described by (Steiger et al., 2010). Sterile water was used as a no DNA control, and RNA following DNase treatment was used as a non-reverse transcription control to confirm the absence of genomic DNA. The gene encoding β-actin was used as a reference gene to normalize gene expression data (Li et al., 2018; Seidl et al., 2009). Calculations for relative expression of individual genes were performed following the method published by (Pfaffl, 2001).

2.6. Statistical analysis

Each experiment (except for the mutagenic treatment) included three biological replicates and was repeated at least twice. Data were analysed using SPSS 16.0 (SPSS Inc., Chicago, IL). All data were calculated and statistically analysed using one-way ANOVA and Duncan's multiple range tests ($P \leq 0.05$).

3. Results and discussion

3.1. Mutagenic treatment

To induce diversification in the genetic material of WT *Trichoderma* spp. UV light was used for its mutagenic effect on DNA structure. While targeted mutagenesis techniques are becoming widely available for filamentous fungi (Nødvig et al., 2015), the ability to introduce genome-wide random modifications also provides an invaluable option for genetic improvement (Bose, 2014). The results showed that increasing exposure time (UV dose) was negatively associated with spore's survival rate (Fig. 1). Following UV exposure, 229 mutants were obtained, 121 of *T. virens* and 108 of *T. asperellum*. Morphological changes in growth features were evident in a number of obtained mutants and included changes in colony appearance, colony colour, sporulation rate and pigmentation. This is in agreement with (Ikehata and Ono, 2011; Pfeifer et al., 2005) who reported that different UV treatments can induce distinct mutagenic consequences. Photoreactivation, excision repair and post-replication repair are cellular repair systems that provide a probable explanation for why UV-surviving mutants were able to withstand the stress induced in the form of DNA damage as a result of UV light mutagenesis (Ganesan, 1974; Seeberg et al., 1995; Yasui et al., 2003). Excessive damage in the form of multiple strand brakes, numerous DNA cross-linkages, and cyclobutane pyrimidine dimers, beyond the ability of these systems to repair, leads to cell death (Lin and Wang, 2001). All the obtained mutants were subjected to the CM assay to evaluate their media-permeable antifungal activities (data not shown here), and top five mutants of both *T. virens* and *T. asperellum* displaying the highest antifungal activity against *R. solani* were selected for further *in vitro* evaluations (Table 2).
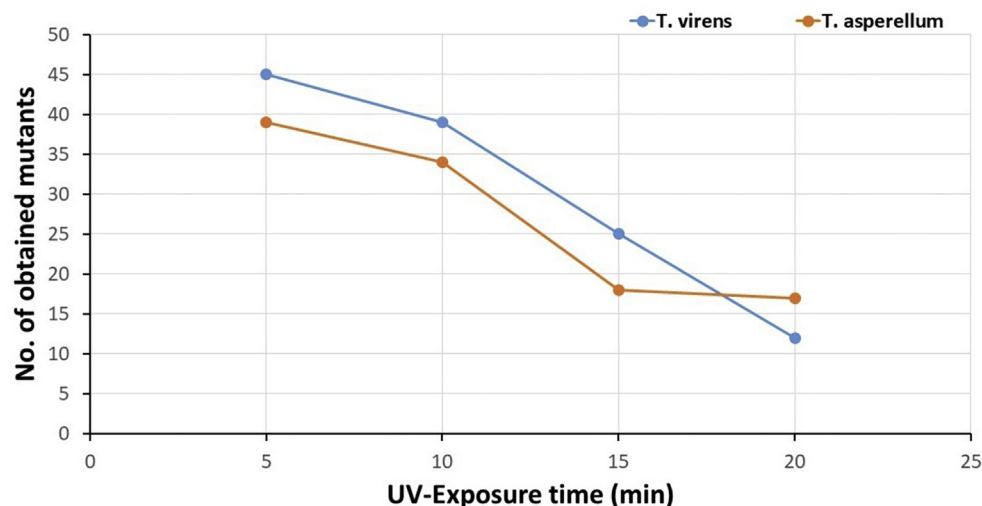

Fig. 1. Relationship between UV dose and number of obtained mutants.

Table 2

The antifungal activities of media-permeable metabolites produced by WT and the top five mutants of *T. virens* and *T. asperellum*.

Fungal strain/ mutant	CMA activity* (%)	Fungal strain/ mutant	CMA activity* (%)
T. virens WT	100[a]**	*T. asperellum* WT	100[a]**
Tv1	123[b]	Ta1	129[b]
Tv2	135[c]	Ta2	138[c d]
Tv3	127[b]	Ta3	132[b c]
Tv4	156[e]	Ta4	141[d]
Tv5	144[d]	Ta5	136[b c d]

* CMA, cellophane membrane assay. Ta, *T. asperellum*. Tv, *T. virens*. WT, wild type.

** Values presented are the means of three Petri plates. Means with different superscripted letters in the same column are significantly different based on Duncan's multiple range test of one-way ANOVA, P < 0.05.

3.2. Antagonistic potential of selected mutants

Data obtained by the CM assay provided an efficient screening strategy for the selection of better antagonistically performing mutants. To validate this screening method, the top five mutants were subjected to the DC test, CF assay and sandwich system for volatile interaction with plant pathogens. The results obtained from the DC test (Fig. 2) are presented in Table 3, which showed that all five selected *T. virens* mutants were either statistically equal to or significantly better than the WT when tested against *S. rolfsii* and *R. solani,* with mutant Tv3 scoring the highest growth inhibition at 76.6% and 78.3%, respectively. Similarly, all five selected *T. asperellum* mutants were either equal to or significantly better than their original wild type when challenged with the pathogens. Mutant Ta3 scored 67.4% growth inhibition against *S. rolfsii* as the highest performing mutant, and mutant Ta2 performed the best against *R. solani,* with a growth inhibition value of 56.3% (Fig. 3,I).

Furthermore, the CF assay showed similar results to those obtained from the CM assay (Table 3). All five selected *T. virens* mutants were
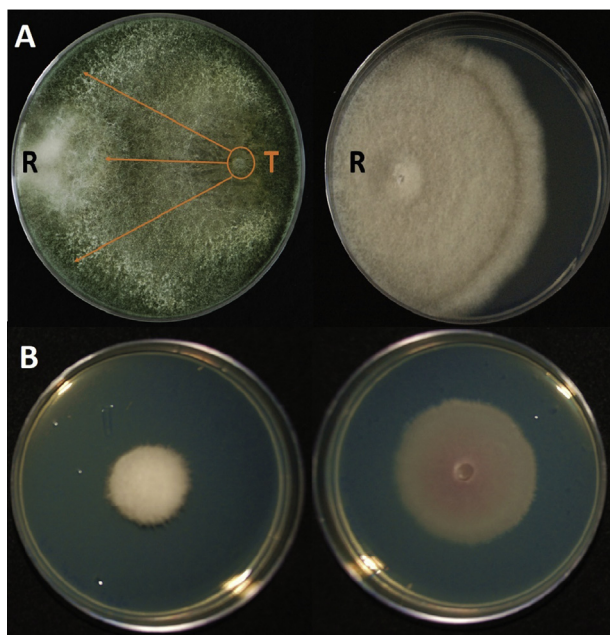
Fig. 2. Representative of antagonistic tests between *Trichoderma* spp. and fungal plant pathogens. (A) Dual culture (DC) assay between *T. virens* (T) and *R. solani* (R). Notice *Trichoderma*'s powerful encroachment towards its competitor to seize control of available growth space and niche, preventing its competitor from extending its growth beyond the contact line. (B) Culture filtrate (CF) assay for media-permeable metabolites. The plates on the right are control treatments, and the plates on the left are treatment plates.

Table 3

Antagonistic potential of WT and selected *T. virens* and *T. asperellum* mutants against *S. rolfsii* and *R. solani*.

Fungal strain/ mutant	Mycelial growth inhibition* (%)		Fungal strain/ mutant	Mycelial growth inhibition* (%)	
	S. rolfsii	*R. solani*		*S. rolfsii*	*R. solani*
Dual culture test					
T. virens WT	51.5[a]**	63.1[b]	*T. asperellum* WT	41.4[a]	47.2[a]
Tv1	50.8[a]	57.5[a]	Ta1	46.4[b]	52.1[b c]
Tv2	56.3[b]	68.1[c]	Ta2	50.6[c]	56.3[c]
Tv3	76.6[e]	78.3[d]	Ta3	67.4[e]	49.6[a b]
Tv4	64.4[c]	61.3[b]	Ta4	41.0[a]	48.6[a b]
Tv5	69.8[d]	67.7[c]	Ta5	56.7[d]	51.0[a b]
Culture filtrate assay					
T. virens WT	31.6[a]	45.3[a]	*T. asperellum* WT	25.0[a]	35.3[a]
Tv1	36.3[b]	48.3[b]	Ta1	27.3[a b]	39.6[b]
Tv2	43.3[c]	56.3[d]	Ta2	31.6[c]	45.3[c]
Tv3	38.6[b]	53.0[c]	Ta3	29.0[b c]	41.3[b c]
Tv4	61.6[e]	69.6[f]	Ta4	37.3[d]	54.0[d]
Tv5	55.3[d]	61.6[e]	Ta5	30.3[b c]	43.6[b c]
Sandwich system for volatile interactions					
T. virens WT	36.6[a]	41.6[a b]	*T. asperellum* WT	39.6[a b]	31.3[a]
Tv1	34.0[a]	43.3[b c]	Ta1	42.6[b]	32.6[a]
Tv2	37.6[a]	41.3[a b]	Ta2	39.6[a b]	29.6[a]
Tv3	43.6[b]	46.6[c]	Ta3	40.3[a b]	31.6[a]
Tv4	35.6[a]	39.3[a]	Ta4	38.0[a b]	33.3[a]
Tv5	36.3[a]	39.0[a]	Ta5	41.6[a b]	31.6[a]

* Values presented are the means of three replicates.

** Means with the different superscripted letters in the same column for each antagonistic test are significantly different based on Duncan's multiple range test of one-way ANOVA, P ≤ 0.05.

significantly better than the WT. The growth inhibition of pathogens ranged between 36.3% and 61.6% for *S. rolfsii* and 48.3% and 69.6% for *R. solani* compared to 31.6% and 45.3%, respectively, for their WT parents. Mutant strain Tv4 displayed the highest antifungal activity against both pathogens through its secreted metabolites. In a similar manner, *T. asperellum* mutants were either equal to (one of the mutants) or significantly better (four of the mutants) than their WT parent when their CFs were tested against *S. rolfsii*. The growth inhibition of *S. rolfsii* ranged between 27.3% and 37.3%. Furthermore, the same *T. asperellum* mutants significantly outperformed their parent when tested against *R. solani* using the CF assay. Growth inhibition of *R. solani* ranged between 39.6% and 54%. Mutant strain Ta4 showed the highest antagonistic potential against both pathogens using the CF assay under our test conditions (Fig. 3,II).

Our third antagonistic technique was the sandwich system for volatile interactions between *Trichoderma* spp. and plant pathogens. This experimental setup prevents physical contact and exposure to secreted and media-diffused metabolites between the antagonist and its interaction partner. This setup was first reported by (Dennis and Webster, 1971) and has been used recently to study volatile fungal interactions between *Trichoderma* biocontrol agents and *Fusarium oxysporum* (Li et al., 2018). Data obtained from this experiment are presented in Table 3. Colony diameters of plant pathogens *S. rolfsii* and *R. solani* co-cultivated with WT and mutant *Trichoderma* spp. were measured, and growth inhibition was calculated using the aforementioned formulae. Statistical analysis of the obtained results did not show any significant difference between the inhibitory effect of the volatiles from WT *T. asperellum* and its mutants on either *S. rolfsii* or *R. solani*. The inhibitory effect of mutant Tv3 only outperformed the WT *T. virens* and its other mutants when co-cultivated with *S. rolfsii*. VOCs from this same mutant exhibited a distinctively elevated inhibitory effect on *R. solani* growth (Fig. 3,III). Volatile organic compounds (VOCs) are ideal infochemical candidates for microbial communications that can take many forms, usually inhibition, but recognition and stimulation have also been reported (Li et al., 2018; Wheatley, 2002).

In summary, Tv3 scored the highest inhibition against both *S. rolfsii* and *R. solani* in both the DC assay and the sandwich system for volatile interactions, while Tv4 was the best *T. virens* UV-derived mutant against both pathogens in the CF assay. On the other hand, Ta3 was the best *T. asperellum*-derived mutant against *S. rolfsii* but not against *R. solani*. Ta4 was the best against both pathogens in the CF assay. None of the mutants selected based on the CM assay and further evaluated through other antagonistic tests were statistically inferior to their original WTs, suggesting the effectiveness of this technique as a mutant screening strategy for antifungal activity.

Trichoderma spp. in general show promising antagonistic potential against a wide range of plant pathogens, including bacteria (Leelavathi et al., 2014), fungi (Chet and Inbar, 1994; Hermosa et al., 2012) and oomycetes (Naseby et al., 2000). Several species in this genus secrete metabolites with antimicrobial properties (Mukherjee et al., 2012; Schuhmacher et al., 2007; Zeilinger et al., 2016), and volatile metabolites are among those that have been identified (Amin et al., 2010; Meena et al., 2017). 6-Pentyl-α-pyrone, 3-octanone and 1-octen-3-ol are all volatile compounds from *Trichoderma* spp. that have fungistatic and fungicidal effects (Mutawila et al., 2015; Wheatley et al., 1997). Moreover, this phenomenon of antagonism through volatile organic

compounds is not limited to *Trichoderma* but rather extends to other fungal and bacterial species (Audrain et al., 2015; Minerdi et al., 2009). Fungal volatile metabolites, including those from *Trichoderma* spp., have been shown to affect plant health and development directly and indirectly in numerous ways (Kottb et al., 2015; Li et al., 2016; Morath et al., 2012).

3.3. Expression levels of antagonism-associated genes in Trichoderma

To investigate whether UV mutagenesis affected the expression of known potential antagonistic genes, we analysed the gene expression of five genes in both WT *T. virens* and its mutant Tv3, which was selected due to its elevated antagonistic potential (Fig. 4). Selected genes belonged to different functional groups, including: cell wall-degradation, protein degradation, secondary metabolism and volatile organic compound biosynthesis. Our results indicated that gene expression levels for three genes were not affected by the UV treatment, while two of the genes showed statistically significant upregulation in the mutated strain compared to its wild type. Chitinase 33, an endochitinase gene encoding a cell wall-degrading extracellular enzyme, was found to be upregulated three-fold in Tv3. Chitinase plays roles in mycoparasitism processes,
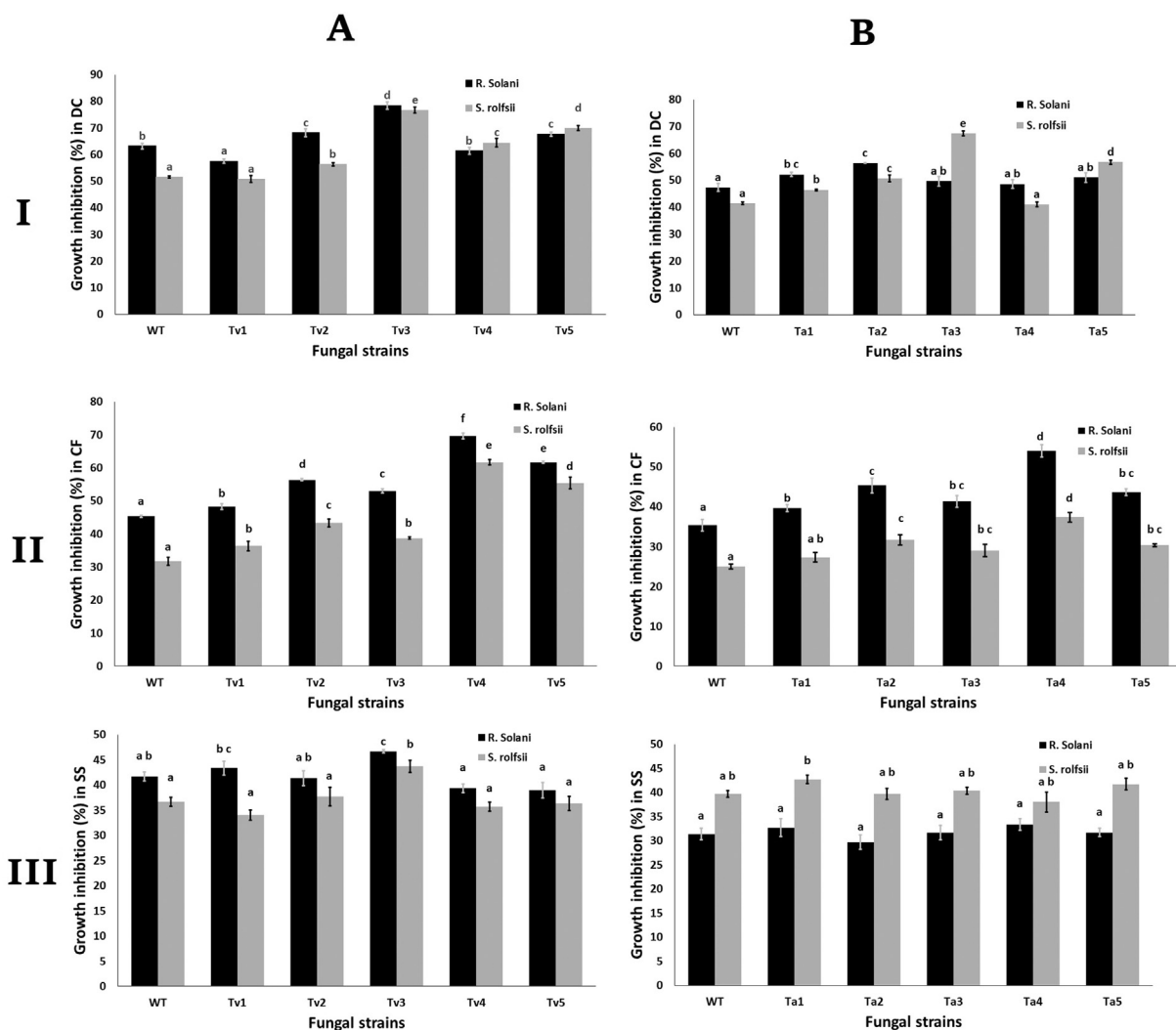
Fig. 3. Growth inhibition of *S. rolfsii* and *R. solani* when challenged with (A) WT and mutant *T. virens* and (B) WT and mutant *T. asperellum* using (I) the dual culture technique (DC), (II) the culture filtrate (CF) assay at 50% concentration and (III) the sandwich system (SS) for volatile interactions. The values presented in the figure correspond to the means of data from three replicates. Vertical bars indicate standard errors. Columns marked with different letters for each pathogen indicate significant differences between fungal strains/mutants based on Duncan's multiple range test of one-way ANOVA at $P < 0.05$.

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