Curr Genet (2016) 62:619–641
DOI 10.1007/s00294-016-0567-5

ORIGINAL ARTICLE

The SRAP based molecular diversity related to antifungal and antioxidant bioactive constituents for biocontrol potentials of *Trichoderma* against *Sclerotium rolfsii* Scc.

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Received: 16 November 2015 / Revised: 30 December 2015 / Accepted: 10 January 2016 / Published online: 23 January 2016

Abstract The study was performed to examine 11 isolates of *Trichoderma* for their bio-control potentials against *Sclerotium rolfsii* Sacc. causing stem rot in groundnut. The antagonists *Trichoderma* were subjected to sequence related amplified polymorphism (SRAP) based molecular diversity analysis and compared with their hardness to *S. rolfsii* with respect to secretary antifungal and antioxidant profile. *T. virens* NBAII Tvs 12 evident highest (87.91 %) growth inhibition of test pathogen followed by *T. koningii* MTCC 796 (67.03 %) at 7 days after inoculation (DAI). Microscopic study confirmed biocontrol mechanism as mycoparasitism for Tvs 12 and antibiosis for MTCC 796. The growth inhibition of test pathogen was significantly negatively correlated with sclerotia formation and lipid peroxidation during antagonism due to release of secretary bioactive antioxidants by antagonists to terminate oxidative burst generated by *S. rolfsii* and causing inhibition of sclerotium formation. The GC–MS profile identified antifungal and antioxidant constituents hexadecane, 1,2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester, 1-hexadecanesulfonyl chloride, and octadecane in potent antagonists Tvs 12; and nonacosane and octadecane in MTCC 796 along with two novel compounds 1-pentadecene and 1-heneicosyl formate for biocontrol activity. Molecular diversity of *Trichoderma* isolates associated with antagonistic activity was assessed by SRAP markers. The 115 primer combinations generate total 1328 amplified products of which, 1095 are shared polymorphic and 199 are unique polymorphic. The 15 SRAP combinations produced 18 bands to diagnose best antagonist Tvs 12 and 13 SRAP combinations generated 19 unique bands for identification of MTCC 796. The mycoparasitic antagonist Tvs 12 would be the best antagonist and released unique antifungal and antioxidant constituents to combat pathogen infection. The SRAP based genetic diversity indicates Tvs12 strain clustered with *T. viride* NBAII Tv23 and shared only 52 % similarity with other isolates of *Trichoderma.* The SRAP similarities explained substantial diversity (19–68 %) across *Trichoderma* isolates.

Keywords *Sclerotium rolfsii* · *Trichoderma* · Biocontrol activity · GC–MS profile · SRAP diversity · Molecular diagnosis

Introduction

The stem rot disease incited by *S. rolfsii* is a serious soil borne disease in groundnut (*Arachis Hypogaea* L.). A *S. rolfsii* infection may cause an average 25 % reduction in groundnut yield (Mayee and Datar 1988). Various management strategies have been adopted to prevent *S. rolfsii* infections in groundnut crop. Many seed dressing fungicides are reported to be effective against stem rot of groundnut but very little is known about successful exploitation of biocontrol agents on disease management. Biological control of stem rot caused by *S. rolfsii* using *Trichoderma* is a cheap and better way to sustain the crop production.

Communicated by M. Kupiec.

Electronic supplementary material The online version of this article (doi:10.1007/s00294-016-0567-5) contains supplementary material, which is available to authorized users.

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Trichoderma is one of the most important filamentous fungi common in soil and root ecosystems and used as an effective biocontrol agents for soil borne fungal plant pathogens and some species are also known for their abilities to enhance systemic resistance to plant diseases (Gajera et al. 2015). The *Trichoderma* antagonists release a variety of compounds that induce localized or systemic resistance responses, and this explains lack of pathogenicity to plants. The plant root microorganism associations cause substantial changes to the plant proteome and metabolism. *Trichoderma* control plant diseases through various mechanisms such as antibiosis, competition, suppression, mycoparasitism, induced resistance, hypovirulence and predation (Mayee and Datar 1988; Tseng et al. 2008).

Screening and characterization of *Trichoderma* from different geographical locations provide diversity which can be exploited further for the control of plant diseases. There are various well-established molecular markers that had been widely used for diversity analysis in *Trichoderma* and other fungi including isozyme variation (Royse and May 1982), DNA sequences in internal transcribed spacer region (ITS1) (Sokol et al. 1999), restriction fragment length polymorphism (RFLP) (Abang et al. 2002), amplified fragment length polymorphism (AFLP) (O'Neill et al. 1997) and random amplified polymorphic DNA (RAPD) (Agrawal and Kotasthane 2009; Graham et al. 1994). However, inter simple sequence repeats (ISSR) and simple sequence repeats (SSR) markers are reliable, but are rarely reported for *Trichoderma* characterization. The marker technology sequence related amplified polymorphism (SRAP) is consistent and repeatable than RAPDs, and are less time-consuming compared to AFLPs, can be used for studying molecular diversity in *Trichoderma*.

Li and Quiros (2001) developed a SRAP for the amplification of open reading frames (ORFs). It is based on two-primer amplification. The primers are 17 or 18 nucleotides long and consist of the following elements. Core sequences, which are 13–14 bases long, where the first 10 or 11 bases starting at the 5′ end, are sequences of no specific constitution ("filler" sequences), followed by the sequence CCGG in the forward primer and AATT in the reverse primer. The core is followed by three selective nucleotides at the 3′ end. The filler sequences of the forward and reverse primers must be different from each other and can be 10 or 11 bases long. SRAP combines simplicity, reliability, moderate throughput ratio and facile sequencing of selected bands. Further, it targets coding sequences in the genome and results in a moderate number of co-dominant markers.

All *Trichoderma* strains could not work equally against specific soil borne disease as *Trichoderma* antagonists have different mechanisms of pathogen recognition. In the backdrop of this scenario, the aim of the present study was (1) to evaluate the antagonistic activity of *Trichoderma* isolates as a biocontroller against pathogen *Sclerotium rolfsii*, causing stem rot of groundnut (2) to observe mechanism of antagonist for growth inhibition of *S. rolfsii* using microscopy, (3) to identify antifungal and antioxidant bioactive constituents present in best antagonist *Trichoderma* for biocontrol activity, and (4) to assess molecular diversity analysis of antagonist *Trichoderma* using SRAP markers and identification of unique strain specific markers to diagnose best antagonists.

Materials and methods

Sources and maintenance of microbes

Culture of 11 isolates of *Trichoderma* including one local isolates [(1) *T. harzianum* NBAII Th1; (2) *T. harzianum* NRRL 13879; (3) *T. harzianum* NRRL 20565; (4) *T.harzianum* Local; (5) *T. viride* NBAII Tv23; (6) *T. viride* NRRL 6418; (7) *T. virens* NBAII Tvs12; (8) *T. hamantum* NBAII Tha 1; (9) *T. koningii* MTCC 796; (10) *T. pseudokoningii* MTCC 2048; (11) *T. species* NRRL 5242] were procured either from Indian Type Culture Collection (ITCC, indicating accession number with NBAII), New Delhi; Microbial Type Culture Collection (MTCC), Chandigarh; or Agricultural Research Service Culture Collection (NRRL), Illinois, USA. The *Trichoderma* strains are available at their respective culture collection centre for future uses. One local isolate of *T. harzianum* was collected from culture collection of Department of Plant Pathology, Junagadh Agricultural University, Junagadh. The phytopathogen *S. rolfsii* were isolated from infected groundnut root by hyphal tip method (Sinclair and Dhingra 1985) and maintained on PDA by storing it under refrigeration (4 °C). Phytopathogen isolated from infected groundnut root was sent for deposition and identification at Indian type culture collection (ITCC), Division of plant pathology, IARI, New Delhi and they identified the pathogen as *Sclerotium rolfsii* (identity number 9107.13).

In vitro antagonism of *Trichoderma* against *S. rolfsii* and microscopic characterization during antagonism

The *Trichoderma* isolates were evaluated for their antagonistic activity against the *S. rolfsii* pathogen by dual culture assay on PDA plate (Dennis and Webster 1971). The time of inoculation of pathogen was decided based on its growth rate with respect to antagonist. The radial growth of the pathogen was measured at 7 DAI from control and antagonists plate and percent inhibition was calculated using the formula given by Vincent (1927). Total sclerotia formed from *S. rolfsii* during antagonism along with control plate at 7 DAI were counted. The experiment was conducted in three independent replications; and mean values of index

of antagonism and sclerotial count of test pathogen were pointed up in graphical representation depicting standard deviation (bar) between replications.

Determination of growth rates, mechanism of inhibition and mycoparasitism were observed on PDA media using light microscope. Thin layer of PDA was prepared on microscopic slide under aseptic condition. The antagonist and pathogen were inoculated on same slide at opposite pole and slides were incubated at 28 °C for 7 days. The antagonistic activity was observed under binocular microscope attached with image analyzer (Nicon). Phase contrast images of antagonism were taken using 10× and 100× lenses.

Lipid peroxidation of *S. rolfsii* and total lipid estimation

Lipid peroxidation in the periphery of the mycelial colony of *S. rolfsii* both in the presence and absence of the antagonist at 7 DAI was measured as the amount of thiobarbituric acid reactive substances (TBARS) determined by the thiobarbituric acid (TBA) reaction (Heath and Packer 1968). Lipid peroxides were measured in chloroform extracts of total lipids as malondialdehydes (MDA) formed from the decomposition of lipid hydroperoxides during the heating stage of the assay (Georgiou 1997). The mycelia of *S. rolfsii* (0.5 g) from inhibition zone of antagonists plate were harvested and suspended in 0.5 ml chloroform for lipid extraction. For total phospholipid determination, 0.1 ml of the chloroform suspension of extracted total lipids were vacuum-dried and phosphorus were estimated from the dry lipid residue using Bartlett method (Bartlett 1959). The known aliquot (0.4 ml) of the chloroform suspension of extracted total lipids from mycelial samples were vacuum dried and re-suspended in a 1 ml 0.1 M NaCl, 0.5 ml 25 % (v/v) HCl and 0.5 ml 1 % (w/v) TBA (in 0.05 M NaOH) for lipid peroxidationl. The mixture was incubated for 15 min in a 100 °C waterbath. The developed chromogen was extracted with 1.5 ml isobutanol and the absorbance was read at 532 nm with an UV–Visible spectrophotometer. The value for non-specific absorption at 600 nm was subtracted from the 532 nm reading. Lipid peroxides as MDA-TBA adduct were estimated as mM. MDA M^{-1} of total phospholipids using a molar extinction coefficient of 155 mM^{-1} cm^{-1} and represent in a graphical illustration with means of three determinations. The molar extinction coefficient is parameter defining how strongly a substance absorbs light at a given wavelength per molar concentration.

Bioactive constituents from antagonist *Trichoderma* by GC–MS

The *S. rolfsii* was grown in a 250 ml potato dextrose broth at 28 °C. It was then incubated while being shaken at 150 rpm in a 500 ml Erlenmeyer flask for 7 days. Mycelia were collected by centrifugation at 3000 rpm for 10 min at room temperature. The mycelia were washed twice using 50 ml sterile deionized water and then boiled for 20 min twice to obtain deactivated mycelia, which were then stored at −4 °C until use as carbon source (Tseng et al. 2008).

The best *Trichoderma* antagonists (*T. virens* NBAII Tvs12; *T. koningii* MTCC 796) and least (*T. viride* NRRL 6418) were grown on synthetic media (SM) containing 0.1 % glucose or 0.1 % inactive cell wall of pathogen *S. rolfsii* as carbon sources, 1.4 g $(NH4)_2$ SO_4, 0.2 gm KH_2PO_4, 6.9 g $NaH_2PO_4.H_2O$, 0.3 gm $MgSO_4{\cdot}7H_2O$ for release of bioactive compounds and incubated for 7 days in shaker incubator at 28 °C (125 rpm). The content was centrifuged for 15 min at 8000 rpm and supernatant collected followed by filtration with Whatman-1 to remove fungal mycelia. The mycelia free culture filtrates were extracted with ethyl acetate at volume ratio of 1:1 by use of a separating funnel. The extract was passed through a pad of anhydrous sodium sulphate to remove excess water and thereafter evaporated to dryness using a rotary vacuum evaporator (Siddiquee et al. 2012).

The extracts were used for GC–MS profile (Keszler et al. 2000). The GC–MS analysis was carried out with fused silica capillary column (RT × 5 MS 30 m length, 0.25 µm diameter, 0.25 mm thickness), operating in electron impact mode at 70 eV, and helium (99.999 %) was used as carrier gas at a constant flow of 1 ml min^{-1} and an injection volume of 1 µl was employed (split ratio of 10:1), injector temperature 280 °C; ion-source temperature 230 °C. The column over temperature was programmed from 80 °C (isothermal for 7 min), with an increase of 6–290 °C/min, ending with a 35 min isothermal at 280 °C. Interpretation of mass spectra of GC–MS was done using the database of national institute standard and technology (NIST) having more than 2,82,000 patterns. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular formula, molecular weight and biological activity of the compounds of the test materials were ascertained.

PCR-SRAP analysis

Total genomic DNA was extracted using CTAB method from all 11 isolates of *Trichoderma* and pathogen *S. rolfsii* (Narayanasamy and Saravana 2009). The purity of DNA was checked in 0.8 % agarose gel electrophoresis and found single intact band. DNA was quantified using agarose gel electrophoresis. The quantity of genomic DNA isolated from 11 *Trichoderma* strains and *S. rolfsii* was measured in piccodrop (Picodrop PET01) and 20 ng/µl DNA concentration was maintained for PCR-SRAP amplification. The

Table 1 List of SRAP primers used in the study

Sr. no.	Primer	Primer sequence (5′–3′)	T_m value (°C)	GC %
1	me 1 F	TGAGTCCAAACCG-GATA	50.4	47
2	me 2 F	TGAGTCCAAACCG-GAGC	55.2	59
3	me 3 F	TGAGTCCAAACCG-GAAT	50.4	47
4	me 4 F	TGAGTCCAAACCG-GACC	55.2	59
5	me 5 F	TGAGTCCAAACCG-GAAG	52.8	53
6	me 6 F	TGAGTCCAAACCG-GACA	52.8	53
7	me 7 F	TGAGTCCAAACCG-GACG	55.2	59
8	me 8 F	TGAGTCCAAACCG-GACT	52.8	53
9	me 9 F	TGAGTCCAAACCG-GAGG	55.2	59
10	me 10 F	TGAGTCCAAACCG-GAAA	50.4	47
11	me 11 F	TGAGTCCAAACCG-GAAC	52.8	53
12	me 12 F	TGAGTCCAAACCG-GAGA	52.8	53
13	me 13 F	TGAGTCCAAACCG-GAGT	52.8	53
14	me 14 F	TGAGTCCAAACCG-GATG	52.8	53
15	me 15 F	TGAGTCCAAACCG-GATC	52.8	53
16	me 16 F	TGAGTCCAAACCG-GATT	50.4	47
17	em 1 R	GACTGCGTACGAAT-TAAT	53.2	40
18	em 2 R	GACTGCGTAC-GAATTTGC	57.1	39
19	em 3 R	GACTGCGTACGAATT-GAC	57.3	50
20	em 4 R	GACTGCGTAC-GAATTTGA	57.3	50
21	em 5 R	GACTGCGTACGAAT-TAAC	49.1	39
22	em 6 R	GACTGCGTACGAATT-GCA	53.7	50
23	em 7 R	GACTGCGTAC-GAATTCCA	53.7	50
24	em 8 R	GACTGCGTAC-GAATTCAC	51.4	44
25	em 9 R	GACTGCGTAC-GAATTCAG	51.4	44
26	em 10 R	GACTGCGTAC-GAATTCAT	53.7	50
27	em 11 R	GACTGCGTAC-GAATTCTA	53.7	50
28	em 12 R	GACTGCGTAC-GAATTCTC	53.7	50
29	em 13 R	GACTGCGTAC-GAATTCTG	53.7	50
30	em 14 R	GACTGCGTAC-GAATTCTT	51.4	44
31	em 15 R	GACTGCGTACGAATT-GAT	51.4	44
32	em 16 R	GACTGCGTACGAATT-GTC	53.7	50

SRAP was used for molecular characterization of *Trichoderma* and pathogen *S. rolfsii* for studying genetic diversity and similarity across 11 *Trichoderma* strains which differed in their antagonistic activity. Molecular characterization of Trichoderma using PCR-SRAP was done as method given by Li and Quiros (2001). The amplification of 256 combinations of 16 forward and 16 reverse SRAP primers (Table 1) were screened using one *Trichoderma* strain resulted to 141 primer combinations gave positive amplification. Therefore, these 115 primer combinations used for further diversity analysis across 11 *Trichoderma* isolates and test pathogen.

The PCR reaction mixture (15 µl) contained 1.5 µl 10× Taq Buffer, 0.3 µl 1U/µl Taq polymerase, 1.2 µl 10 mM/µl dNTP mix, 1.2 µl Primer, 1.2 µl Genomic DNA and 9.6 µl Nuclease free water. The reaction mixtures were heated to 96 °C for 4 min, then subjected to 5 cycles of 1 min at 94 °C; 1.15 min at 35 °C and 2 min at 72 °C, then subjected to 30 cycles of 1 min at 94 °C; 1.15 min at 50 °C and 2 min at 72 °C and a final extension for 10 min at 72 °C (Li and Quiros 2001). The amplification products were separated in 2 % (w/v) agarose gel in 1× TBE buffer and visualized by staining with 0.1 % ethidum bromide. The PCR-SRAP repeated twice and consistence bands were considered for analysis. The bands were recorded as presence (1) and absence (0) across 11 *Trichoderma* strains and pathogen *S. rolfsii* in binary matrix and utilized for polymorphic pattern and phylogenetic relationship.

Statistical analysis

Antgonistic (biocontrol) activities of 11 *Trichoderma* isolates against phytopathogen *S. rolfsii* (viz., growth inhibition of test pathogen, Sclerotium count and lipid peroxide products) were carried out in three independent experiments and data were analyzed for standard deviation between antagonists (Fisher and Yates 1948).

GC–MS profile of beat and least antagonists were carried out in duplicate and mean values were presented. Variations among 11 *Trichoderma* strains and pathogen *S. rolfsii* across the SRAP primers used in the present study were evaluated from pairwise comparison for the proportion of shared bands amplified (Nei 1987). The similarity coefficients were calculated by using the Jaccard's similarity coefficient and cluster analysis by UPGMA using NTSYSpc-2.02i software developed by Rohlf (1998). A polymorphic information index (PIC) for SRAP profiles was calculated as $PIC = 1 - p^2 - q^2$, where, p is band frequency and q is no band frequency (Ghislain et al. 1999). The PIC values were then used to calculate a SRAP primer index (SPI), which was generated by adding up the PIC values of all the markers amplified by the same primer (Anderson et al. 1993).

Results

Antagonist activity and microscopic characterization of *Trichoderma* inhibiting *S. rolfsii*

Growth inhibition of *S. rolfsii* during in vitro interaction with biocontrol agents *Trichoderma* was observed at 7 DAI (Fig. 1). The growth of test pathogen was significantly reduced at 7 DAI (Fig. 2). Percent growth inhibition of pathogen *S. rolfsii* was found significantly higher in *T. virens* NBAII Tvs12 (T7) followed by *T. koningii* MTCC 796 (T9), *T. viride* NBAII Tv23 (T5), *T. harzianum* NBAII Th1 (T1) and *T. species* NRRL 5242 (T11) at 7 DAI. The strain Tvs 12 was found to be best antagonist inhibiting 87.91 % growth inhibition of test pathogen followed by MTCC 796 with 67.03 % inhibition of test pathogen.

The intersection effect between antagonists and test pathogen were examined under microscope at 7 DAI (Fig. 1). The Tvs 12 (T7) isolates overgrew completely on the pathogen with mycoparasitism as observed in microscopic observations. The strong antagonist Tvs 12 (T7) grew over mycelia of test pathogen with surround coiling and hock like structure formation followed by disintegration and disruption of mycelia of pathogen. Thus, the T7 antagonist (i.e. interaction between *Trichoderma* strain *T. virens*—NBAII Tvs12 and *S. rolfsii)* has best antagonistic activity followed by T9 antagonist (i.e. interaction between *Trichoderma* strain *T. koningii*—MTCC 796 and *S. rolfsii*) as compared to the other biocontrol agents. The best antagonist *T. virens*—NBAII Tvs 12 strain inhibited the growth of pathogen by using mode of action mycoparasitism whereas other isolates including second best antagonist *T. koningii*—MTCC 796 exhibited strong antibiosis and formed about 2–4 mm zone of inhibition after 7 days and the same were confirmed in microscopic examinations.

Lipid peroxidation and sclerotium count of *S. rolfsii* during antagonism with *Trichoderma* isolates

The lipid peroxidation of total lipids of test pathogen *S. rolfsii* during antagonism was examined at 7 DAI and lipid peroxides as MDA-TBA adduct were found highest 25.3 mM MDA M^{-1} of total phospholipids in absence of antagonists *Trichoderma* (control plate) with formation of maximum number (85) of sclerotia. The interactions of *S. rolfsii* with best antagonists *T. virens*—NBAII Tvs12 and *T. koningii*—MTCC 796 diminished lipid peroxidation of test pathogen mycelia at inhibition zone without sclerotial formation compared to least antagonists acquaintances (Fig. 2). The growth inhibition of test pathogen was significantly negatively correlated ($P_{0.001}$) with number of sclerotia ($r = -0.7236$) and lipid peroxides ($r = -0.8248$) in antagonists plate. However, lipid peroxidation was positively correlated ($r = 0.5490$) with sclerotial formation. It indicates that lipid peroxidation is an essential phenomenon to elevate cellular oxidative processes for sclerital differentiation and promote growth of test pathogen *S. rolfsii*. However, best biocontrol agents secreted antioxidant bioactive constituents in the antagonists plate during antagonism which cease oxidative burst and stop the development of the test pathogen to the undifferentiated mycelial stage and thereby restrain growth of *S. rolfsii.*

Comparative evaluation of bioactive compounds from antagonist *Trichoderma*

The best (*T. virens* NBAII Tvs12 and *T. koningii* MTCC 796) and least (*T. viride* NRRL 6418) antagonists *Trichoderma* were grown in synthetic media containing inactive cell wall of pathogen as carbon sources to release bioactive constituents under influence of pathogen cell wall (Figs S1–S4). The GC–MS profile of SM containing glucose and cell wall of pathogen without inoculation of antagonists *Trichoderma* were also preformed as negative control and the compounds detected common in inoculated and without inoculation of *Trichoderma* were eliminated before comparison of best and least antagonists.

Total 30 bioactive compounds were identified in best antagonist strain NBAII Tvs12 (T7) when inoculated with SM containing glucose as normal growth. However, replacing the glucose with cell wall of pathogen in SM altered the metabolic constituents of antagonist and about 27 bioactive compounds were identified. The antagonist T7 evident maximum growth inhibition of test pathogen through mycoparasitism. The antagonist *T. koningii* MTCC 796 (T9) inhibited growth of pathogen differently with development of inhibition zone and secreted 19 bioactive compounds using cell wall of pathogen as carbon source. The comparative GC MS profile of bioactive compounds found

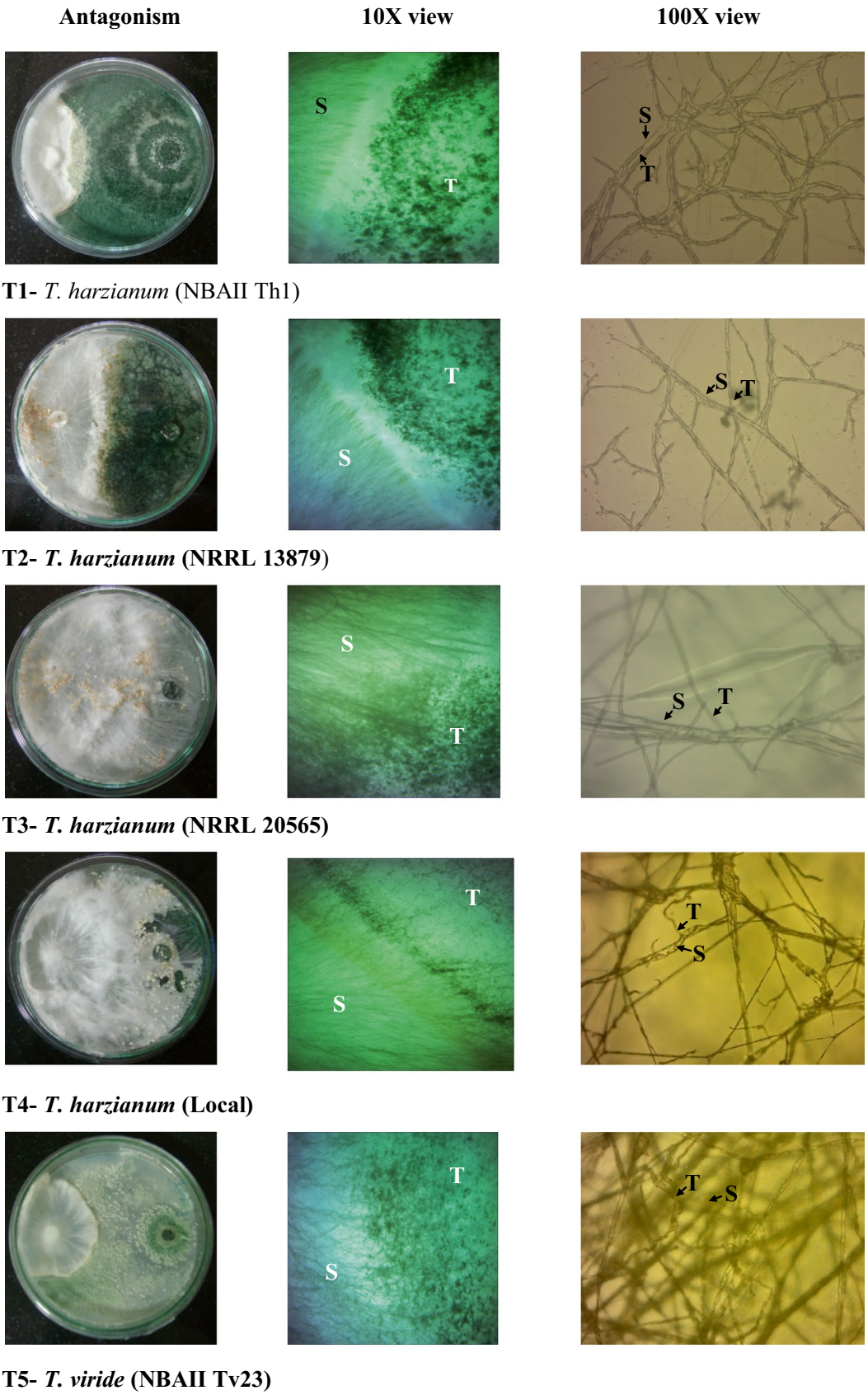
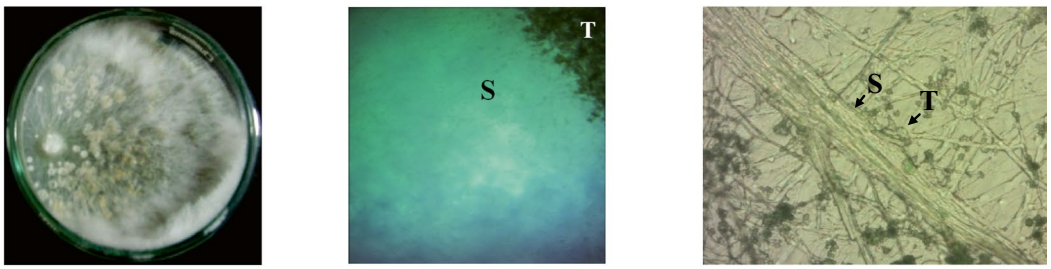
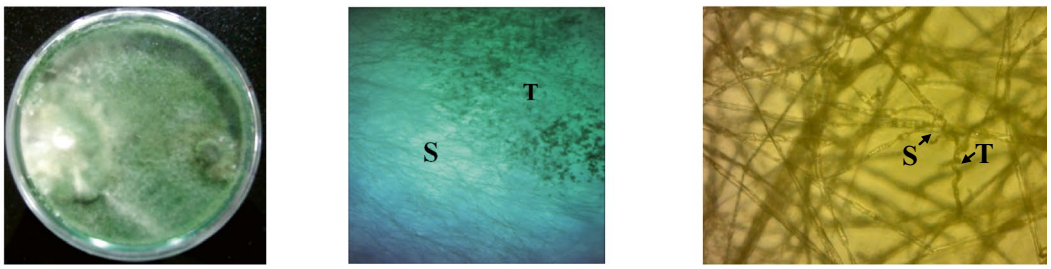


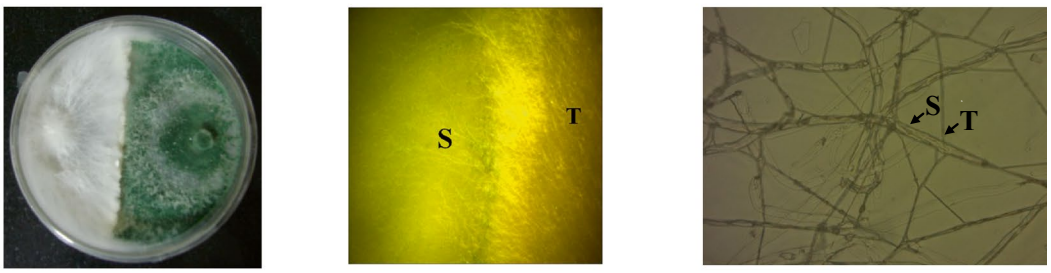
Fig. 1 Microscopic characterization of *Trichoderma* isolates for in vitro growth inhibition of pathogen *S. rolfsii* at 7 DAI under 10× and 100× (*left side*—test pathogen, *right side—Trichoderma* isolates, *T—Trichoderma* and *S—S. rolfsii*)



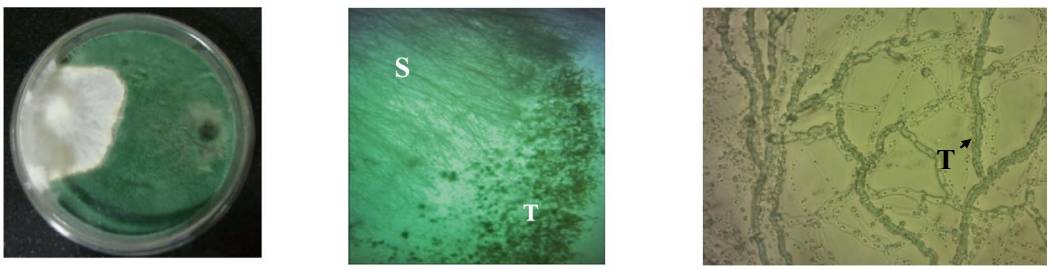
T6- *T. viride* (NRRL 6418)



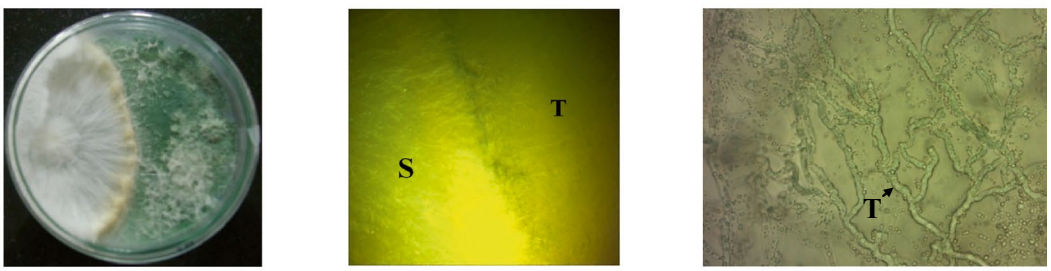
T7- *T. virens* (NBAII Tvs12)



T8- *T. hamantum* (NBAII Tha1)



T9- *T. koningii* (MTCC 796)



T0- *T. pseudokoningii* (MTCC 2048)

Fig. 1 continued

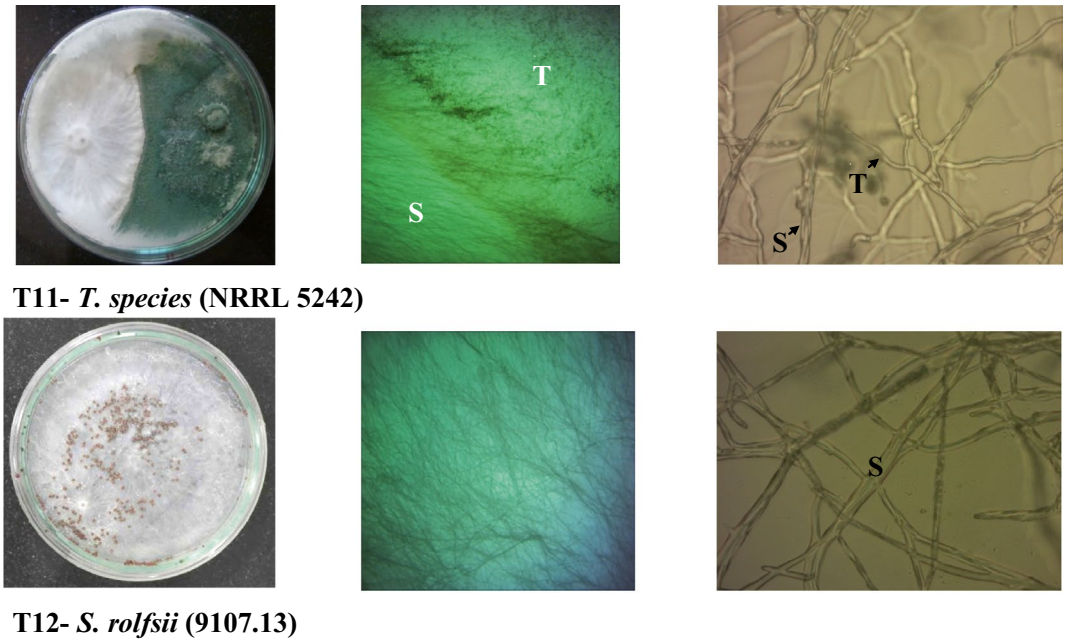


Fig. 1 continued

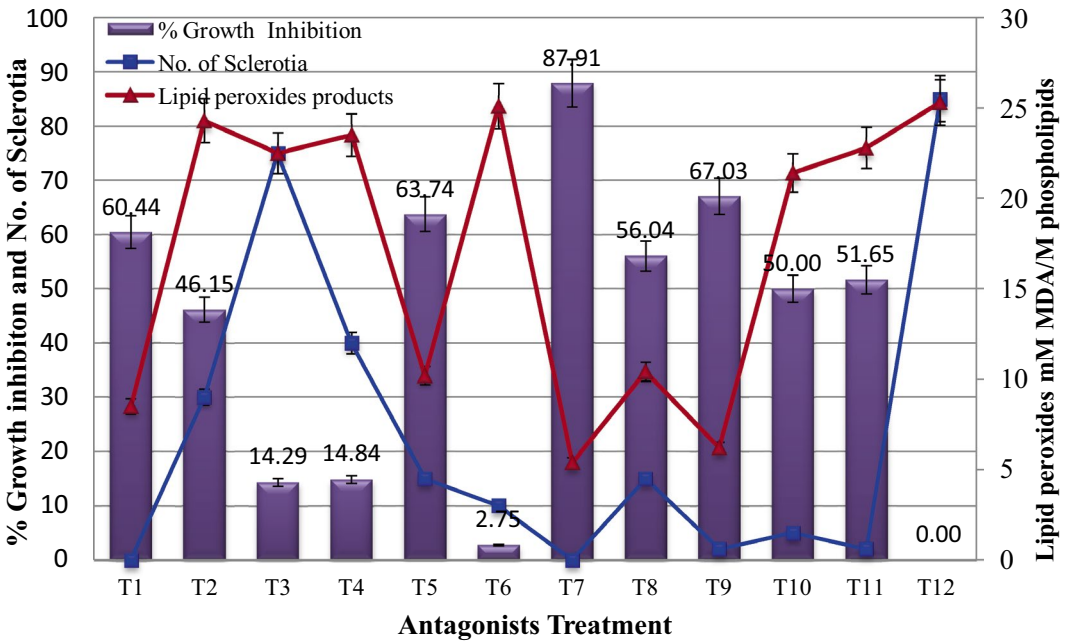


Fig. 2 Percent growth inhibition, sclerotium count and lipid perixidation of *S. Rolfsii* during in vitro antagonism with *Trichoderma* strains at 7 DAI (*bars* indicate standard deviations between three experiments). T1 = *T. harzianum* (NBAII Th1) × *S. rolfsii*; T2 = *T. harzianum* (NRRL 13879) × *S. rolfsii*; T3 = *T. harzianum* (NRRL 20565) × *S. rolfsii*; T = *T.harzianum* (Local) × *S. rolfsii*; T5 = *T. viride* (NBAII Tv23) × *S. rolfsii*; T6 = *T. viride* (NRRL 6418) × *S. rolfsii*; T7 = *T. virens* (NBAII Tvs12) × *S. rolfsii*; T8 = *T. hamantum* (NBAII Tha 1) × *S. rolfsii*; T9 = *T. koningii* (MTCC 796) X *S. rolfsii*; T10 = *T. pseudokoningii* (MTCC 2048) × *S. rolfsii*; T11 = *T. species* (NRRL 5242) × *S. rolfsii*; T12 = *S. rolfsii* control (9107.13)

in best and least inhibitory antagonists along with molecular formula, molecular weight and biological activity are depicted in Table 2.

The compounds contributing more than 2 % area as relative abundance were considered as major bioactive constituents. Major bioactive constituents in the best

Table 2 Comparative GC–MS profile for secretary bioactive compounds from best and least *Trichoderma* antagonists inoculated with synthetic media containing cell wall of pathogen

Sr.no.	RT	Compound identified	Molecular formula	MW	Relative abundance (% area)				NIST similarity (%)	Biological activity
					G + T7	CW + T7	CW + T9	CW + T6		
1	7.496	1-Heptanol, 2-propyl-	$C_{22}H44O_2$	158	1.29	–	–	–	84	–
2	13.42	cis-11-Hexadecenal	$C_{16}H_{30}O$	29.5	–	–	–	1.59	63	–
3	13.882	Dehydroacetic Acid	$C_8H_8O_4$	168	1.23	–	–	–	91	–
4	14.09	1-Tridecene	$C_{13}H_{26}$	182	5.3	–	–	–	96	–
5	16.137	2,5-Cyclohexadiene-1,4-dione, 2,6-bis (1,1-dimethylethyl)	$C_{14}H_{20}O_2$	220	–	–	1.25	–	81	–
6	18.418	1-Pentadecene	$C_{15}H_{30}$	210	–	3.55	–	–	95	–
7	18.539	1-Dodecene	$C_{12}H_{24}$	168	1.78	–	–	–	96	–
8	18.546	Nonadecane	$C_{19}H_{40}$	268	1.28	–	–	–	94	Antimicrobial and cytotoxic (Akpuaka et al. 2013)
9	20.207	3-Buten-2-one, 4-(3-hydroxy-6, 6-dimethyl-2-methylenecyclohexyl)-	$C_{13}H_{20}O_2$	208	1.2	–	–	–	66	–
10	20.457	3-(1-Methylhept-1-enyl)-5-methyl-2,5-dihydrofuran-2-one	$C_{13}H_{20}O_2$	208	–	1.06	–	–	70	–
11	21.769	alpha.-Bisabolol	$C_{15}H_{26}O$	78	8.29	–	–	–	22.2	–
12	23.1	Heptadecane	$C_{17}H_{36}$	240	–	–	–	1.62	23.1	–
13	24.675	Piperazine, 1-ethyl-4-(4-piperidyl)-	$C_{11}H_{23}N_3$	197	–	0.5	–	–	49	–
14	24.84	9-[3-Hydroxypropyl]hypoxanthine	$C_8H_{10}N_4O_2$	194		1.31	–	–	53	–
15	25.023	Octadecane	$C_{18}H_{38}$	254	–	1.44	1.69	1.3	90	Hypocholesterolemic (Manorenjitha et al. 2013)
16	25.447	Pentadecanoic acid	$C_{15}H_{30}O_2$	242	5.71	–	–	–	71	Antioxidant (Roy et al. 2011)
17	25.914	1-Heneicosyl formate	$C_{22}H_2O$	341	–	10.23	8.15	–	88	–
18	25.983	Triacontane, 1-bromo	$C_{30}H_{61}Br$	500	1.03	–	–	–	78	Antimicrobial and cytotoxic (Akpuaka et al. 2013)
19	26.006	Nonacosane	$C_{29}H_6O$	409	–	–	1.31	–	78	Antibacterial (Akpuaka et al. 2013)
20	26.025	Hexadecane	$C_{16}H_{34}$	226	–	1.76	–	–	88	Pesticide, nematicide, antioxidant activity, antiandrogenic, hemolytic and 5-alpha reductase inhibitor (Akpuaka et al. 2013)
21	27.504	1-Hexadecanol	$C_{16}H_{34}O$	242	1.77	–	–	–	81	–
22	28.46	Oleic acid	$C_{18}H_{34}O_2$	282	–	–	–	2.08	78	–
23	28.771	Octadecanoic acid	$C_{18}H_{36}O_2$	284	–	2.55	–	4.00	91	Hypocholesterolemic, antifungal, antitumoractivity and antibacterial, antioxidant (Manorenjitha et al. 2013; Govindappa et al. 2014; Roy et al. 2011)

Table 2 continued

Sr.no.	RT	Compound identified	Molecular formula	MW	Relative abundance (% area)				NIST similarity (%)	Biological activity
					G + T7	CW + T7	CW + T9	CW + T6		
24	29.136	9-Tricosene, (*Z*)-	$C_{23}H_{46}$	90	5.41	–	–	–	32.2	–
25	32.1	13-Tetradecen-1-ol acetate	$C_{18}H_{34}O_2$	254	–	–	–	2.97	65	–
26	32.116	9-Hexacosene	$C_{23}H_{46}$	90	2.42	–	–	–	33.2	–
27	32.65	3,7-Diazabicyclo[3.3.1]nonane, 9,9-dimethyl-	$C_9H_{18}N_2$	154	1.37	–	–	–	39	Antifungal and antibacterial (Akpuaka et al. 2013)
28	33.242	D-Streptamine	$C_{21}H_{39}N_5O_8$	489	0.9	–	–	–	33	–
29	33.492	1,3,5-Triazin-2-amine, 4,6-dichloro-*N*,*N*-diethyl	$C_7H_{10}Cl_2N_4$	220	2.42	–	–	–	31	–
30	33.833	*N*-(*O*-Ethyl fluorophosphoryl)-*N*′,*N*′-diethyl-acetamidine	$C_8H_{18}FN_2O_2P$	224	0.97	–	–	–	33	–
31	33.95	2-Butanol, 4-(2,2-dimethyl-6-methylene-cyclohexylidene)-	$C_{13}H_{22}O$	194	1.93	–	–	–	47	–
32	34.104	Cedranol<5-Neo->DB5-2058	$C_{15}H_{26}O$	36	1.7	–	–	–	22.2	–
33	34.369	Silane, trichlorodecyl-	$C_{10}H_{21}Cl_3Si$	274	1.34	–	–	–	40	–
34	34.49	1, 2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester	$C_{16}H_{22}O_4$	278	–	3.95	–	–	93	Antifungal, antibacterial, antiviral and antioxidant (Akpuaka et al. 2013)
35	34.681	2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl]cyclohex-1-en-1-carboxaldehyde	$C_{23}H_{32}O$	324	5.99	–	–	–	74	–
36	34.859	1-Hexadecanesulfonyl chloride	$C_{16}H_{33}ClO_2S$	324	–	4.39	–	–	92	Antifungal, antibacterial and antioxidant activities (Akpuaka et al. 2013)

NIST National Institute Standard and Technology, *RT* retention time, *MW* molecular weight, *G* glucose, *CW* cell wall of *S. rolfsii*, *T7 T. virens* (NBAII Tvs12), *T9 T. koningii* (MTCC 796), *T6 T. viride* (NBAII Tv23), − not detected

Tvs12 (T7) (SM + glucose) are alpha-bisabolol (8.29 %), 2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl]cyclohex-1-en-1 carboxaldehyde (5.99 %), pentadecanoic acid (5.71 %) and 9-tricosene (5.41 %). The key compounds in T7 (SM + pathogen cell wall) were evident 1-heneicosyl formate (10.23 %), 1-hexadecanesulfonyl chloride (4.39 %), 1,2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester (3.95 %) and 1-pentadecene (3.55 %). The major compounds in T9 (SM + Cell wall) were found to be 1-heneicosyl formate (8.15 %) and octadecane (1.69 %). Total 20 compounds were identified in least antagonists *T. viride* NRRL 6418 (T6) (SM + pathogen cell wall). The foremost compounds in the T6 were found to be octadecanoic acid (4.00 %), 13-tetradecen-1-ol acetate (2.97 %) and oleic acid (2.08 %).

Molecular diversity of *Trichoderma* isolates using SRAP markers

The genomic DNA of 11 *Trichoderma* and test pathogen *S. rolfsii* were utilized for molecular diversity analysis using SRAP marker. Total 115 SRAP primer combinations amplified to generate the 1328 bands across 11 isolates *Trichoderma* and pathogen *S. rolfsii* (Table 3). The SRAP-22 primer produced maximum number of 21 bands, while SRAP-15 produced minimum number of 1 band. Out of 1328, 1291 bands were polymorphic and 32 bands were monomorphic. Among the 1291 polymorphic bands, 1095 alleles were shared polymorphic within two or more isolates, while 199 bands were unique-polymorphic. The percent polymorphism obtained for SRAP primers were ranged from 61.50 to 100 % with an average value of 97.00 % per primer. The PIC values for SRAP marker ranged from 0.58 (SRAP-88) to 0.94 (SRAP-22 and SRAP-242) with an average value of 0.86 per primer and SPI differed from 2.33 (SRAP-88) to 19.76 (SRAP-22).

Based on PIC and SPI, SRAP-22 and SRAP-242 are most informative markers for polymorphism of genomic DNA of *Trichoderma* strains. Among 115 SRAP primers, 80 primers showed the amplification of specific unique bands. Maximum 10 unique bands were produced by SRAP-151. The SRAP 26 amplified specific unique bands of 232 bp to diagnose best antagonists *T. virens* NBAII Tvs12; and 1133 and 125 bp for *T. koningii* MTCC 796 (Fig. 3). Total 18 unique bands were produced by 15 SRAP primers to identify best antagonist strain Tvs12 However, MTCC 796 were identified by 19 unique bands produced from 13 SRAP primer pairs (Table 4).

The dendrogram was constructed using UPGMA based Jaccard's similarity coefficient and found in the range from 19 to 68 % based on SRAP data across 11 *Trichoderma* strains and pathogen *S. rolfsii* (Fig. 4). The 11 isolates of *Trichoderma* and *S. rolfsii* were grouped into two main clusters: cluster A and cluster B and shared 26 % similarity. The cluster A comprised of two clusters, cluster A1and cluster A2. while Cluster B contains pathogen *S. rolfsii.* Cluster A1 was further subdivided into sub clusters, cluster A1(a) and cluster A1(b). Cluster A1(a) consisted of *T. harzianum* NBAII Th1, *T. viride* NBAII Tv23 and *T. harzianum* NBAII Tha1 while cluster A1(b) contained *T. koningii* MTCC 796, *T. pseudokoningii* MTCC 2048 and *T.species* NRRL 5242. Cluster A2 was further subdivided into sub clusters, cluster A2(a) and cluster A2(b). Cluster A1(a) consisted of *T. harzianum* NRRL 13879 and *T. harzianum* Local. Test pathogen out grouped from all *trichoderma* isolates and share minimum 19 % similarity. however best antagonist *T. virens* NBAII Tvs12 grouped with *T. viride* NBAII Tv23 and shared 52 % similarity with other isolates of *Trichoderma.*

Discussion

Different biocontrol agents can be used for the control of stem rot disease in groundnut including bacteria, fungi and actinomycetes. The most important biocontroller belongs to the genus *Trichoderma*. In vitro antagonism of 11 *Trichoderma* isolates with *S. rolfsii* indicated maximum (87.91 %) growth inhibition of test pathogen with *T. virens* NBAII Tvs 12 followed by *T. koningii*–MTCC 796 (67.03 %) at 7 DAI. The microscopic depiction of antagonist during interaction confirmed mycoparasitism as mode of action for potential Tvs 12 and antibiosis for MTCC 796 strain. The unique antifungal and antioxidant constitutes are released by dominant antagonists Tvs 12 and MTCC 796 to combat the test pathogen.

Similar to present study, Anand and Reddy (2009) studied biocontrol potential of 42 *Trichoderma* strains, isolated from rhizosphere soil samples from cultivated lands, against *S. rolfsii*. On day 5, maximum percentage reduction of the pathogen growth over control indicated T30 (43.22 %) and T6 (40.0 %) to be the best performers while T13 (−41.22 %) recorded the least. Pan and Bhagat (2007) used dual culture technique for screening isolates of *Trichoderma* against five most widely occurring soil-borne pathogenic fungi viz. *R. solani*, *S. rolfsii*, *M. phaseolina*, *F. oxysporium* and *Pythium* spp. Their study revealed that *Trichoderma* isolates not only differed in their reaction against different pathogens but antagonists themselves differed in the reaction against any single phytopathogen, required 3–7 days after incubation to attain S1 phase (the antagonist completely overgrown on the growth of pathogen). This may be due to variability in antagonistic isolates isolated from different ecological niches. The pathogens *Pythium* spp. and *R. solani* were highly affected by all *Trichoderma* isolates (*T. harzianum*, *T. viride*, *G. virens*),

Table 3 Polymorphism obtained with different SRAP primers generated from 11 isolates of *Trichoderma* and *S. rolfsii*

Sr. no.	SRAP primers	Primer combination	Band size (bp)	Total no. of allele/bands	Poly-morphic bands			Mono-morphic Bands	% Polymor-phim	PIC	SPI
					S	U	Total bands				
1	SRAP-1	me 1–em 1	202–1788	11	9	2	11	0	100.0	0.90	9.86
2	SRAP-2	me 1–em 2	139–2523	14	12	0	12	2	85.7	0.88	12.39
3	SRAP-7	me 1–em 7	101–2158	19	15	2	17	2	89.5	0.92	17.54
4	SRAP-8	me 1–em 8	116–2094	13	11	0	11	2	84.6	0.88	11.49
5	SRAP-19	me 2–em 3	104–3137	18	15	3	18	0	100.0	0.92	16.64
6	SRAP-22	me 2–em 6	108–2003	21	19	2	21	0	100.0	0.94	19.76
7	SRAP-23	me 2–em 7	100–2854	16	16	0	16	0	100.0	0.93	14.82
8	SRAP-26	me 2–em 10	101–1818	14	7	7	14	0	100.0	0.89	12.50
9	SRAP-33	me 3–em 1	100–2052	16	14	2	16	0	100.0	0.91	14.63
10	SRAP-39	me 3–em 7	190–2379	10	8	2	10	0	100.0	0.87	8.69
11	SRAP-41	me 3–em 9	102–1325	4	2	1	3	1	75.0	0.67	2.69
12	SRAP-42	me 3–em 10	101–1991	8	2	4	6	1	87.5	0.67	5.32
13	SRAP-50	me 4–em 2	150–1751	5	4	1	5	0	100.0	0.76	3.80
14	SRAP-54	me 4–em 6	135–1332	12	6	6	12	0	100.0	0.88	10.56
15	SRAP-55	me 4–em 7	147–2057	14	9	5	14	1	92.9	0.86	12.10
16	SRAP-56	me 4–em 8	112–2240	14	12	2	14	0	100.0	0.91	12.75
17	SRAP-57	me 4–em 9	111–2046	14	12	1	13	0	100.0	0.91	12.68
18	SRAP-63	me 4–em 15	136–1056	9	3	6	9	0	100.0	0.77	6.93
19	SRAP-64	me 4–em 16	101–1460	9	6	3	9	0	100.0	0.81	7.28
20	SRAP-65	me 5–em 1	112–1589	5	4	1	5	0	100.0	0.75	3.77
21	SRAP-72	me 5–em 8	119–564	5	2	3	5	0	100.0	0.57	2.84
22	SRAP-73	me 5–em 9	109–1421	15	12	1	13	2	86.7	0.90	13.52
23	SRAP-76	me 5–em 12	147–1457	11	7	4	11	0	100.0	0.87	9.52
24	SRAP-77	me 5–em 13	100–1417	14	10	3	13	1	92.9	0.89	12.42
25	SRAP-79	me 5–em 15	104–2124	9	5	3	8	0	100.0	0.82	7.38
26	SRAP-81	me 6–em 1	116–511	9	2	5	7	1	88.9	0.73	6.61
27	SRAP-88	me 6–em 8	129–865	4	2	1	3	0	100.0	0.58	2.33
28	SRAP-109	me 7–em 14	301–660	6	4	2	6	0	100.0	0.79	4.71
29	SRAP-111	me 7–em 16	103–601	5	5	0	5	0	100.0	0.73	3.66
30	SRAP-116	me 8–em 4	123–676	3	2	2	4	0	100.0	0.63	1.88
31	SRAP-119	me 8–em 7	109–1777	9	3	5	8	0	100.0	0.74	6.63
32	SRAP-121	me 8–em 9	131–2218	9	9	0	9	0	100.0	0.85	7.62
33	SRAP-122	me 8–em 10	109–3451	10	8	2	10	0	100.0	0.84	8.36
34	SRAP-131	me 9–em 3	102–2355	16	16	0	16	0	100.0	0.93	14.87
35	SRAP-132	me 9–em 4	102–2537	16	16	0	16	0	100.0	0.92	14.75
36	SRAP-133	me 9–em 5	121–2048	14	12	2	14	0	100.0	0.91	12.73
37	SRAP-134	me 9–em 6	134–1605	17	17	0	17	0	100.0	0.93	15.83
38	SRAP-135	me 9–em 7	131–2457	16	14	2	16	0	100.0	0.91	14.60
39	SRAP-136	me 9–em 8	102–2279	13	12	0	12	1	92.3	0.89	11.51
40	SRAP-137	me 9–em 9	108–2195	14	11	3	14	0	100.0	0.91	12.71
41	SRAP-141	me 9–em 13	104–2298	7	7	0	7	0	100.0	0.80	5.63
42	SRAP-142	me 9–em 14	110–2351	13	10	3	13	0	100.0	0.90	11.72
43	SRAP-144	me 9–em 16	105–2060	12	12	0	12	0	100.0	0.90	10.83
44	SRAP-146	me 10–em 2	116–1949	15	15	0	15	0	100.0	0.92	13.78
45	SRAP-147	me 10–em 3	111–2105	13	13	0	13	0	100.0	0.91	11.78
46	SRAP-148	me 10–em 4	126–2901	14	12	2	14	0	100.0	0.91	12.78
47	SRAP-150	me 10–em 6	108–2407	16	16	0	16	0	100.0	0.92	14.75

Table 3 continued

Sr. no.	SRAP primers	Primer combination	Band size (bp)	Total no. of allele/bands	Poly-morphic bands			Mono-morphic Bands	% Polymor-phim	PIC	SPI
					S	U	Total bands				
48	SRAP-151	me 10–em 7	116–2419	15	1	10	11	4	61.5	0.83	10.73
49	SRAP-152	me 10–em 8	113–2345	12	8	3	11	1	91.7	0.85	10.17
50	SRAP-153	me 10–em 9	112–2684	7	2	3	5	2	71.4	0.72	5.03
51	SRAP-155	me 10–em 11	115–1380	9	9	2	9	0	100.0	0.81	7.27
52	SRAP-158	me 10–em 14	157–1159	8	5	2	7	1	87.5	0.75	5.99
53	SRAP-159	me 10–em 15	106–2085	11	7	4	11	0	100.0	0.85	9.34
54	SRAP-160	me 10–em 16	101–1432	10	9	1	10	0	100.0	0.87	8.65
55	SRAP-161	me 11–em 1	112–1322	8	4	4	8	0	100.0	0.78	6.26
56	SRAP-162	me 11–em 2	147–2125	5	4	1	5	0	100.0	0.65	3.24
57	SRAP-164	me 11–em 4	122–1595	12	12	0	12	0	100.0	0.89	10.68
58	SRAP-165	me 11–em 5	116–1675	8	3	3	6	2	75.0	0.77	6.17
59	SRAP-167	me 11–em 7	110–3237	9	7	1	8	1	88.9	0.80	7.21
60	SRAP-168	me 11–em 8	112–1571	9	4	4	8	1	88.9	0.78	6.98
61	SRAP-169	me 11–em 9	105–1189	13	12	0	12	1	92.3	0.88	11.50
62	SRAP-172	me 11–em 12	128–1043	13	11	1	12	1	92.3	0.88	11.49
63	SRAP-173	me 11–em 13	136–1877	18	17	1	18	0	100.0	0.92	16.53
64	SRAP-174	me 11–em 14	125–2506	15	15	0	15	0	100.0	0.92	13.79
65	SRAP-175	me 11–em 15	110–1894	15	15	0	15	0	100.0	0.90	11.76
66	SRAP-177	me 12–em 1	110–2308	13	13	0	13	0	100.0	0.91	11.84
67	SRAP-178	me 12–em 2	171–1601	14	14	0	14	0	100.0	0.91	12.69
68	SRAP-179	me 12–em 3	124–1799	13	11	2	13	0	100.0	0.89	11.63
69	SRAP-181	me 12–em 5	133–1823	12	9	2	11	1	91.7	0.87	10.46
70	SRAP-182	me 12–em 6	165–1665	10	9	1	10	0	100.0	0.87	8.73
71	SRAP-183	me 12–em 7	115–1521	13	13	0	13	0	100.0	0.91	11.87
72	SRAP-184	me 12–em 8	103–1761	14	13	1	14	0	100.0	0.91	12.71
73	SRAP-185	me 12–em 9	112–1256	12	11	1	12	0	100.0	0.90	10.85
74	SRAP-186	me 12–em 10	177–1908	14	12	2	14	0	100.0	0.90	12.65
75	SRAP-187	me 12–em 11	151–2212	8	6	2	8	0	100.0	0.85	6.77
76	SRAP-189	me 12–em 13	116–2231	13	12	1	13	0	100.0	0.90	11.71
77	SRAP-190	me 12–em 14	100–1943	14	13	1	14	0	100.0	0.91	12.68
78	SRAP-191	me 12–em 15	105–2011	13	13	0	13	0	100.0	0.91	11.82
79	SRAP-192	me 12–em 16	101–1772	11	11	0	11	0	100.0	0.89	9.74
80	SRAP-194	me 13–em 2	104–2141	13	12	1	13	0	100.0	0.90	11.75
81	SRAP-195	me 13–em 3	179–1976	14	14	0	14	0	100.0	0.91	12.76
82	SRAP-197	me 13–em 5	114–2161	13	12	1	13	0	100.0	0.91	11.81
83	SRAP-198	me 13–em 6	202–1817	8	7	1	8	0	100.0	0.85	6.78
84	SRAP-199	me 13–em 7	116–2014	13	13	0	13	0	100.0	0.90	11.72
85	SRAP-200	me 13–em 8	116–2215	11	9	2	11	0	100.0	0.86	9.41
86	SRAP-201	me 13–em 9	145–1481	9	7	2	9	0	100.0	0.84	7.58
87	SRAP-202	me 13–em 10	112–1763	11	9	1	10	1	90.9	0.86	9.44
88	SRAP-218	me 14–em 10	167–1570	9	7	2	9	0	100.0	0.85	7.69
89	SRAP-220	me 14–em 12	125–1796	7	7	0	7	0	100.0	0.83	5.78
90	SRAP-221	me 14–em 13	118–1941	12	12	0	12	0	100.0	0.90	10.81
91	SRAP-222	me 14–em 14	103–2464	14	14	0	14	0	100.0	0.91	12.76
92	SRAP-223	me 14–em 15	128–1671	12	10	2	12	0	100.0	0.90	10.85
93	SRAP-224	me 14–em 16	162–1947	15	15	0	15	0	100.0	0.91	13.63
94	SRAP-226	me 15–em 2	117–2678	13	9	4	13	0	100.0	0.90	11.66

Table 3 continued

Sr. no.	SRAP primers	Primer combination	Band size (bp)	Total no. of allele/bands	Poly-morphic bands			Mono-morphic Bands	% Polymor-phim	PIC	SPI
					S	U	Total bands				
95	SRAP-228	me 15–em 4	113–2112	9	9	0	9	0	100.0	0.89	7.98
96	SRAP-229	me 15–em 5	120–2724	12	9	2	11	1	91.7	0.82	9.86
97	SRAP-231	me 15–em 7	125–2434	12	12	0	12	0	100.0	0.89	10.63
98	SRAP-232	me 15–em 8	127–1952	12	10	2	12	0	100.0	0.89	10.69
99	SRAP-233	me 15–em 9	110–1462	13	12	1	13	0	100.0	0.90	11.71
100	SRAP-234	me 15–em 10	159–2209	7	6	0	6	1	85.7	0.91	6.34
101	SRAP-235	me 15–em 11	301–2170	12	12	0	12	0	100.0	0.78	9.33
102	SRAP-236	me 15–em 12	100–1489	12	10	2	12	0	100.0	0.87	10.48
103	SRAP-237	me 15–em 13	137–1949	10	10	0	10	0	100.0	0.87	8.74
104	SRAP-238	me 15–em 14	147–1553	10	8	3	10	0	100.0	0.84	8.40
105	SRAP-240	me 15–em 16	101–1620	8	4	4	8	0	100.0	0.82	6.53
106	SRAP-242	me 16–em 2	108–3133	19	17	2	19	0	100.0	0.94	17.78
107	SRAP-243	me 16–em 3	103–3006	12	8	4	12	0	100.0	0.90	10.76
108	SRAP-244	me 16–em 4	114–2904	10	8	2	10	0	100.0	0.88	8.83
109	SRAP-245	me 16–em 5	136–2808	8	4	4	8	0	100.0	0.83	6.67
110	SRAP-246	me 16–em 6	133–2778	13	12	1	13	0	100.0	0.91	11.87
111	SRAP-250	me 16–em 10	502–1036	4	2	2	4	0	100.0	0.69	2.78
112	SRAP-252	me 16–em 12	114–3082	13	10	3	13	0	100.0	0.86	11.24
113	SRAP-254	me 16–em 14	109–2943	10	10	0	10	0	100.0	0.88	8.79
114	SRAP-255	me 16–em 15	102–2918	11	8	3	11	0	100.0	0.89	9.82
115	SRAP-256	me 16–em 16	124–2158	14	12	2	14	0	100.0	0.91	12.79
Total				1328	1095	199	1291	32	–	–	–
Average				11.54	9.52	1.73	11.54	0.28	97.00	0.86	10.10

SRAP Primer Index (SPI) = total number of bands × PIC

S shared, *U* unique, *T* total polymorphic bands, *PIC* polymorphism information content

whereas *S. rolfsii* and *M. phaseolina* were comparatively less inhibited.

Joshi et al. (2008) evaluated the antagonistic potential of seventeen fungal isolates (15 *Trichoderma harzianum* and two *Fusarium solani* isolates) in vitro and in glasshouse against two important stages (sclerotia and mycelium) in the infection cycle of three plant pathogens viz., *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *Sclerotium rolfsii*. Out of the 17 isolates, only three isolates (S-1, S-6 and S-12) of *T. harzianum*, exhibited significantly higher sclerotial parasitism and hyphal inhibition activity against all the three pathogens.

The antagonism between *Trichoderma* strain and test pathogen were examined under microscope at 7 DAI. The best antagonist Tvs 12 (T7) showed mycoparasitism on the pathogen while *T. koningii*—MTCC 796 exhibited strong antibiosis and formed about 2–4 mm zone of inhibition after 7 days. Similar to present study, Yogendra and Singh (2002) studied the effect of *Trichoderma* based biocontrol agents, viz. *T. viride* and *T. harzianum* on the growth of *Sclerotium rolfsii* in vitro. *T. harzianum* exhibited strong mycoparasitism and covered 100 % colony growth on the pathogen, where as *T. viride* showed strong antibiosis and formed 2–3 mm zone of inhibition after 6 days of incubation in dual culture. *T. harzianum* and *T. longibrachiatum* inhibited the in vitro growth and produced coiling around mycelium of *Sclerotium rolfsii* resulting in lysis of hyphae.

Gajera et al. (2012) reported pathogen-specific mechanism of antagonists *Trichoderma* for biocontrol activity. *T. koningii* MTCC 796 was capable of overgrowing and degrading *M. phaseolina* mycelia, coiling around the hyphae with apressoria and hook-like structures. Formation of apressoria-like structures enabled the hyphae of *Trichoderma* spp. to firmly attach to the surface of its host mycelium. However, some antagonists (*T. viride* NBAII Tv 23, *T. hamatum* NBAII Tha 1) used different mechanisms against *M. phaseolina* just touched the hyphae without coiling. Whereas, *T. pseudokoningii* showed spore around pathogen not attached to hyphae. Present study agreed with the results suggesting all *Trichoderma* strains could not work equally against specific soil borne disease as various

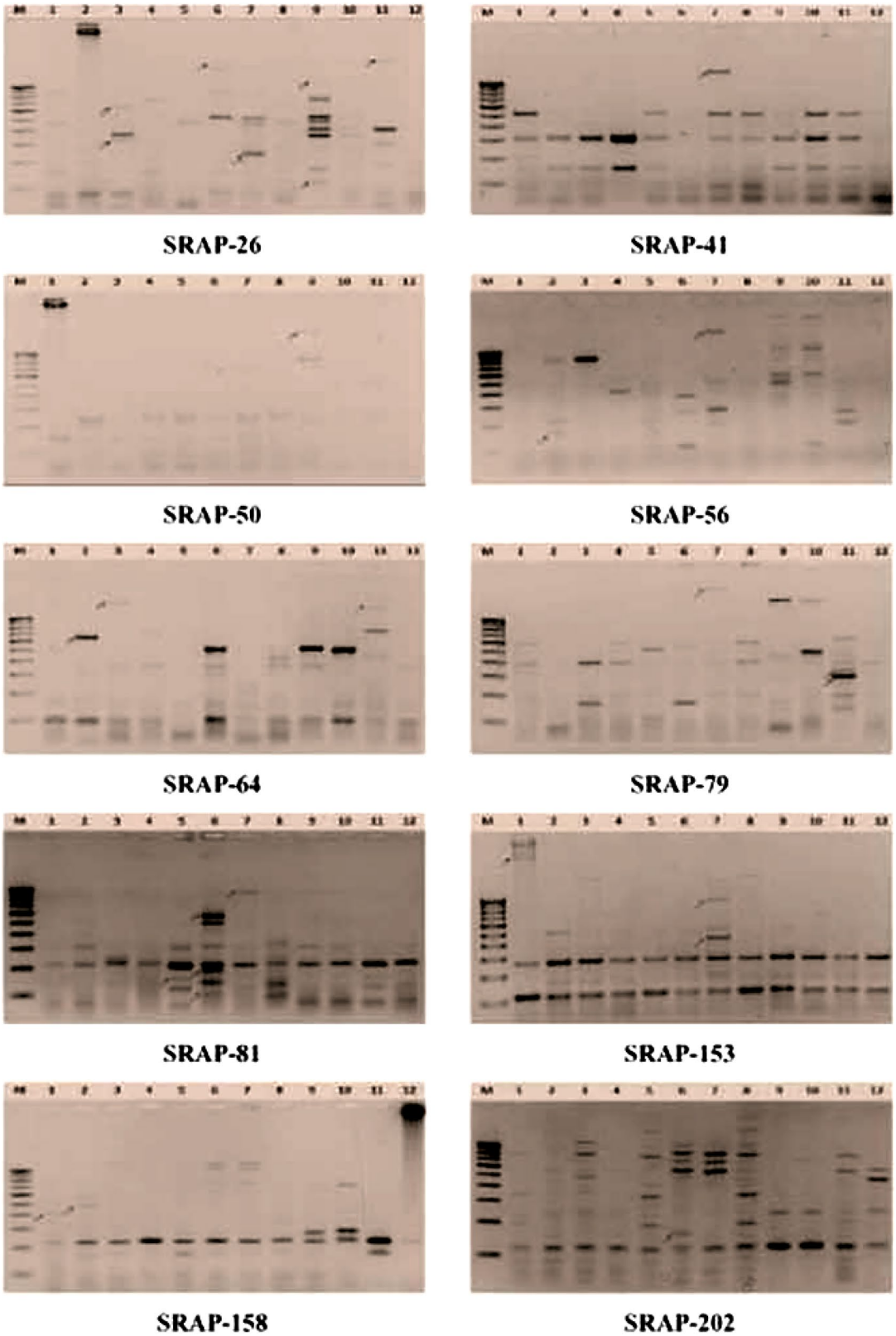


Fig. 3 SRAP profile of antagonist and test pathogen using different SRAP primers [M = DNA Marker (100 bp); 1, *T. harzianum* (NBAII Th1); 2, *T. harzianum* (NRRL 13879); 3, *T. harzianum* (NRRL 20565); 4, *T.harzianum* (Local); 5, *T. viride* (NBAII Tv23); 6, *T. viride* (NRRL 6418); 7, *T. virens* (NBAII Tvs12); 8, *T. hamantum* (NBAII Tha 1); 9, *T. koningii* (MTCC 796); 10, *T. pseudokoningii* (MTCC 2048); 11, *T. species* (NRRL 5242); 12, *S. rolfsii* (9107.13)]

Table 4 *Trichoderma* isolates and pathogen *S. rolfsii* specific unique markers generated by SRAP primers

Primer	Band size (bp)											
SRAP	*T. harzianum* NBAII Th1	*T. harzianum* NRRL 13879	*T. harzianum* NRRL 20565	*T.harzianum* Local	*T. viride* NBAII Tv23	*T. viride* NRRL 6418	*T. virens* NBAII Tvs12	*T. hamantum* NBAII Tha 1	*T. koningii* MTCC 796	*T. pseu-dokoningii* MTCC 2048	*T. species* NRRL 5242	*S. rolfsii* 9107.13
SRAP-1	–	–	–	–	–	–	–	–	–	–	202	865
SRAP-7	–	–	272	–	–	–	478	–	–	–	–	–
SRAP-19	–	–	–	1731	–	–	–	–	–	–	190	144
SRAP-22	–	108	–	310	–	–	–	–	–	–	–	–
SRAP-26	–	–	636	–	–	1709	232	–	1133	–	1745	–
	–	–	303	–	–	–	–	–	125	–	–	–
SRAP-33	–	–	157	–	–	–	–	–	–	–	1787	–
SRAP-39	–	–	289	488	–	–	–	–	–	–	–	–
SRAP-41	–	–	–	–	–	–	1325	–	–	–	–	–
SRAP-42	–	–	–	–	–	240	–	–	–	–	1991	–
	–	–	–	–	–	–	–	–	–	–	913	–
	–	–	–	–	–	–	–	–	–	–	474	–
SRAP-50	–	–	–	–	–	–	–	–	1751	–	–	–
SRAP-54	–	893	1188	–	–	–	–	–	138	–	–	–
	–	738	441	–	–	–	–	–	–	–	–	–
	–	–	379	–	–	–	–	–	–	–	–	–
SRAP-55	–	–	–	–	–	–	–	407	1323	–	1244	–
	–	–	–	–	–	–	–	–	956	–	–	–
	–	–	–	–	–	–	–	–	719	–	–	–
SRAP-56	–	152	–	–	–	–	1593	–	–	–	–	–
SRAP-57	–	–	–	1341	–	–	–	–	–	–	–	–
SRAP-63	–	–	–	–	–	1038	–	–	–	–	162	338
	–	–	–	–	–	744	–	–	–	–	–	176
	–	–	–	–	–	432	–	–	–	–	–	–
SRAP-64	–	662	1460	–	–	–	–	–	–	–	1322	–
SRAP-65	–	–	–	–	–	–	–	–	1589	–	–	–
SRAP-72	–	–	217	–	–	564	–	449	–	–	–	–
SRAP-73	–	–	–	–	–	–	–	–	–	–	–	762
SRAP-76	–	–	–	340	–	147	–	–	–	–	296	–
	–	–	–	202	–	–	–	–	–	–	–	–
SRAP-77	–	121	1240	–	–	–	–	–	–	–	–	–
	–	–	171	–	–	–	–	–	–	–	–	–

Table 4 continued

Primer	Band size (bp)											
SRAP	*T. harzianum* NBAII Th1	*T. harzianum* NRRL 13879	*T. harzianum* NRRL 20565	*T.harzianum* Local	*T. viride* NBAII Tv23	*T. viride* NRRL 6418	*T. virens* NBAII Tvs12	*T. hamantum* NBAII Tha 1	*T. koningii* MTCC 796	*T. pseu-dokoningii* MTCC 2048	*T. species* NRRL 5242	*S. rolfsii* 9107.13
SRAP-79	–	–	–	–	–	–	2124	–	–	–	318	–
	–	–	–	–	–	–	–	–	–	–	281	–
SRAP-81	–	–	–	–	143	572	959	–	–	–	–	–
	–	–	–	–	–	172	–	–	–	–	–	–
	–	–	–	–	–	100	–	–	–	–	–	–
SRAP-88	–	–	–	–	–	–	–	–	511	–	–	–
SRAP-109	–	–	–	–	–	–	–	–	526	–	660	–
SRAP-116	–	–	–	–	–	–	–	190	–	–	–	–
	–	–	–	–	–	–	–	123	–	–	–	–
SRAP-119	353	261	205	–	–	–	–	–	–	1777	–	–
	–	109		–	–	–	–	–	–	–	–	–
SRAP-122	–	–	109	–	–	–	–	–	–	–	595	–
SRAP-133	–	–	–	–	–	172	–	–	–	–	198	–
SRAP-135	–	161	2457	–	–	–	–	–	–	–	–	–
SRAP-137	–	–	194	–	–	–	873	–	–	–	361	–
SRAP-142	–	–	831	884	–	–	–	–	–	–	638	–
SRAP-148	–	400	–	–	–	–	–	2901	–	–	–	–
SRAP-151	2419	897	326	697	–	–	1298	–	500	–	–	1843
		–	–	352	–	–	–	–	–	–	–	657
		–	–	178	–	–	–	–	–	–	–	–
SRAP-152		–	463	–	–	–	–	–	–	–	143	2345
SRAP-153	2684	–	–	–	–	–	1040	–	–	–	–	–
		–	–	–	–	–	460	–	–	–	–	–
SRAP-155	398	–	–	–	–	–	273	–	–	–	–	–
SRAP-158	376	467	–	–	–	–	–	–	–	–	–	–
SRAP-159	–	–	373	–	–	–	–	2085	–	–	–	–
	–	–	–	–	–	–	–	1777	–	–	–	–
	–	–	–	–	–	–	–	1370	–	–	–	–
SRAP-160	172	–	–	–	–	–	–	–	–	–	–	–
SRAP-161	1322	–	–	–	–	–	590	–	152	–	–	–
	–	–	–	–	–	–	463	–	–	–	–	–
SRAP-162	–	–	–	–	–	–	–	445	–	–	–	–
SRAP-165	–	–	163	–	593	140	–	–	–	–	–	–

Table 4 continued

Primer	Band size (bp)											
SRAP	*T. harzianum* NBAII Th1	*T. harzianum* NRRL 13879	*T. harzianum* NRRL 20565	*T.harzianum* Local	*T. viride* NBAII Tv23	*T. viride* NRRL 6418	*T. virens* NBAII Tvs12	*T. hamantum* NBAII Tha 1	*T. koningii* MTCC 796	*T. pseu-dokoningii* MTCC 2048	*T. species* NRRL 5242	*S. rolfsii* 9107.13
SRAP-167	–	–	–	–	–	3237	–	–	–	–	–	–
SRAP-168	112	–	–	–	–	–	–	1308	–	–	330	–
	–	–	–	–	–	–	–	846	–	–	–	–
SRAP-172	–	–	–	–	–	–	163	–	–	–	–	–
SRAP-173	–	–	–	–	–	–	–	1435	–	–	–	–
SRAP-179	–	–	1601	–	–	–	–	–	–	–	794	–
SRAP-181	–	124	1799	–	–	–	–	–	–	–	–	–
SRAP-182	–	–	362	–	–	–	–	–	–	–	–	–
SRAP-184	–	–	–	–	–	–	–	–	–	–	1012	–
SRAP-185	–	–	–	–	–	345	–	–	–	–	–	–
SRAP-186	–	–	–	–	–	–	–	–	–	–	1908	–
	–	–	–	–	–	–	–	–	–	–	281	–
SRAP-187	–	–	–	–	–	–	–	–	–	–	2091	–
	–	–	–	–	–	–	–	–	–	–	1799	–
SRAP-189	–	201	–	–	–	–	–	–	–	–	–	–
SRAP-190	–	–	162	–	–	–	–	–	–	–	–	–
SRAP-194	–	223	–	–	–	–	–	–	–	–	–	–
SRAP-197	–	–	–	–	–	–	–	208	–	–	–	–
SRAP-198	–	–	272	–	–	–	–	–	–	–	–	–
SRAP-200	–	2215		–	–	116	–	–	–	–	–	–
SRAP-201	–	212	145	–	–		–	–	–	–	–	–
SRAP-202	–			–	–	155	–	–	–	–	–	–
SRAP-218	–	1012		–	–	394	–	–	–	–	–	–
SRAP-223	–		1671	–	–		–	–	325	–	–	–
SRAP-226	–	1699	–	–	–	–	–	–	1440	–	–	–
	–	–	–	–	–	–	–	–	1034	–	–	–
	–	–	–	–	–	–	–	–	383	–	–	–
SRAP-229	–	–	–	–	–	–	1608	–	–	–	–	–
	–	–	–	–	–	–	971	–	–	–	–	–
SRAP-232	–	1952	–	1015	–	–	–	–	–	–	–	–
SRAP-233	–	–	–	–	–	462	–	–	–	–	–	–
SRAP-236	–	–	1489	–	–	535	–	–	–	–	–	–

Table 4 continued

Primer	Band size (bp)											
SRAP	*T. harzianum* NBAII Th1	*T. harzianum* NRRL 13879	*T. harzianum* NRRL 20565	*T.harzianum* Local	*T. viride* NBAII Tv23	*T. viride* NRRL 6418	*T. virens* NBAII Tvs12	*T. hamantum* NBAII Tha 1	*T. koningii* MTCC 796	*T. pseu-dokoningii* MTCC 2048	*T. species* NRRL 5242	*S. rolfsii* 9107.13
SRAP-238	–	866	–	–	–	–	–	–	–	–	1553	–
	–	147	–	–	–	–	–	–	–	–	–	–
SRAP-240	–	127	–	284	–	–	–	–	–	–	–	–
	–	101	–	177	–	–	–	–	–	–	–	–
SRAP-242	–	–	–	108	–	–	1507	–	–	–	–	–
SRAP-243	–	–	600	–	–	–	–	–	–	–	203	158
	–	–	–	–	–	–	–	–	–	–	139	–
SRAP-244	–	–	–	–	2904	356	–	–	–	–	–	–
SRAP-245	–	1414	–	–	–	–	–	–	213	–	136	–
	–	821	–	–	–	–	–	–	–	–	–	–
SRAP-246	–	–	–	–	2778	–	–	–	–	–	–	–
SRAP-250	–	–	–	–	–	–	–	1036	–	–	502	–
SRAP-252	–	–	320	–	–	–	–	–	1343	–	–	–
	–	–	–	–	–	–	–	–	114	–	–	–
SRAP-255	–	–	–	–	2918	456	–	–	–	–	–	–
	–	–	–	–	389	–	–	–	–	–	–	–
SRAP-256	–	–	–	–	2158	–	210	–	–	–	–	–
Total	8	26	30	14	7	21	18	15	19	1	31	9

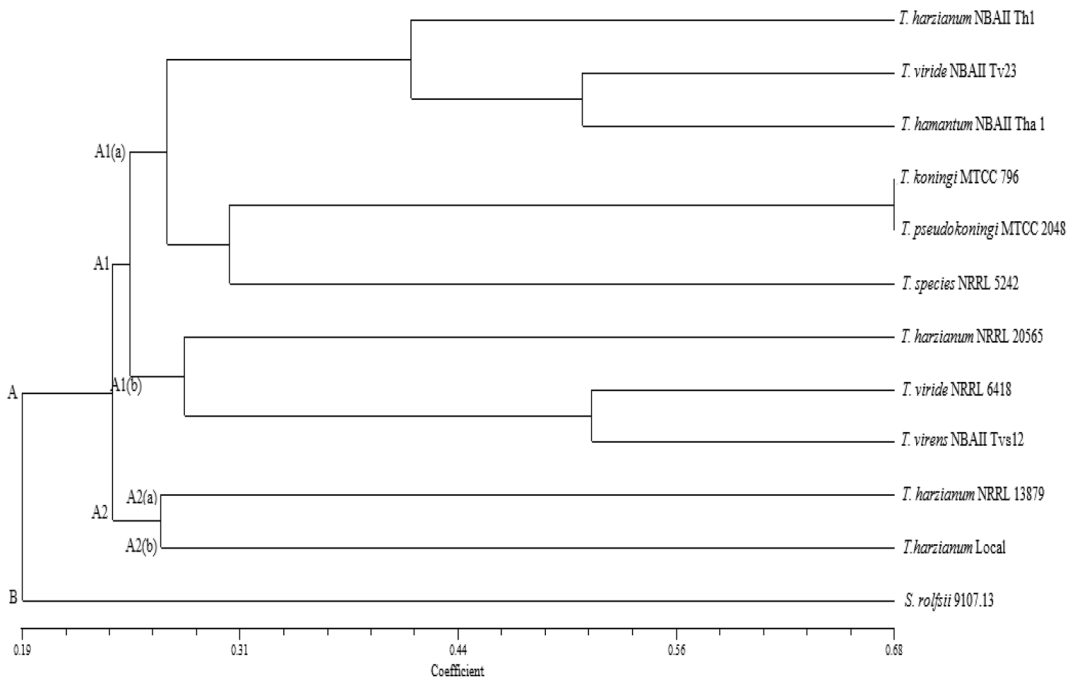


Fig. 4 Dendogram depicting the phylogenetic relationship among 11 isolates of *Trichoderma* and pathogen *S. rolfsii* based on the SRAP data

Trichoderma antagonists have different mechanisms of pathogen recognition.

Lipid peroxidation has been implicated in a large number of cellular processes including cell proliferation and differentiation in fungi. The filamentous fungi exhibit a marked increase in lipid peroxidation during differentiation. The *S. rolfsii* causes noticeable increase in lipid peroxidation during its differentiation state (Georgiou 1997). The lipid peroxidation is a indicator of high oxidative stress and lipofuscins were identified as lipid peroxidation products in all fungal representatives of sclerotial differentiation. The sclerotial biogenesis in *S. rolfsii* was accompanied by the accumulation of high levels of lipid peroxidation products and it is a well-established oxidative stress indicator. Present study evident that lipid peroxidation elevated in absence of antagonists *Trichoderma* with formation of maximum sclerotia by *S. rolfsii* and promote growth of test pathogen. The best antagonists Tvs12 and MTCC 796 strains diminished mycelial lipid peroxidation of test pathogen without formation of sclerotia compared to least antagonists. The growth inhibition of test pathogen was significantly negatively correlated with sclerotial formation and lipid peroxidation in antagonists plate due to release of secretary bioactive antioxidants to terminate oxidative burst generated by *S. rolfsii* and causing inhibition of sclerotium formation. The role of antioxidant beta-carotene in sclerotial metamorphosis is supported by the finding that when it is administered in the growth medium at concentrations that do not inhibit growth, it causes a concentration-dependent reduction of oxidative stress (lipid peroxidation) of *S. rolfsii*, *S. minor*, and *S. sclerotiorum* undifferentiated mycelia, and an equally proportional reduction of sclerotial differentiation (Georgiou et al. 2006).

The bioactive constituents of best strains Tvs12 and MTCC 796; and least *T. viride* NRRL 6418 antagonists were identified using GC–MS profile. The compounds identified in the study are saturated hydrocarbons (C7–C30), cyclohexane, cyclopentane, fatty acids, alcohols, esters, sulfur-containing compounds, simple pyrane and benzene derivatives. Similarly, Siddiquee et al. (2012) carried out separation and identification of volatile compounds from liquid cultures of *Trichoderma harzianum* by GC–MS using three different capillary columns. They were identified more than 278 volatile compounds which are in analogous nature to present study.

The antagonist Tvs12 evident maximum growth inhibition of test pathogen through mycoparasitism and identified 27 bioactive constituents while MTCC 796 evident 19 bioactive compounds using cell wall of pathogen as carbon source. The nonadecane, triacontane, 1-bromo and 3,7-diazabicyclo[3.3.1]nonane, 9,9-dimethyl were found unique in best Tvs12 (SM + glucose), which were reported for antimicrobial and cytotoxic effect (Akpuaka et al. 2013). The Tvs12 strain under the influence of pathogen cell wall evident to produce hexadecane which were reported for pesticide, nematicide, antioxidant, hypocholesterolemic, antiandrogenic activity, hemolytic and 5-alpha reductase inhibitor; octadecanoic acid for antifungal,

antibacterial, hypocholesterolemic, antitumoral and antioxidant activities (Akpuaka et al. 2013, Govindappa et al. 2014; Roy et al. 2011) and 1,2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester and 1-hexadecanesulfonyl chloride for antifungal, antibacterial, antiviral and antioxidant activities (Manorenjitha et al. 2013). The nonacosane was noticed in MTCC 796 (SM + pathogen cell wall) for antibacterial activity (Akpuaka et al. 2013). However, octadecane found in best MTCC 796 antagonists with higher relative abundance (1.69 %) followed by Tvs 12 (1.44 %) and least NRRL 6418 (1.30 %) when inoculated in SM containing pathogen cell wall and reported for hypocholesterolemic activity (Manorenjitha et al. 2013). The novel compounds 1-pentadecene (3.55 %) was evident only in best antagonists Tvs 12 strain while 1-heneicosyl formate was found in Tvs 12 (10.23 %) and MTCC 796 (8.15 %) strains inoculated with SM containing pathogen cell wall for biocontrol activity.

Present study evidenced that secretary antioxidants like hexadecane, 1,2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester, 1-hexadecanesulfonyl chloride, pentadecanoic acid and octadecanoic acid released by antagonists ((Tvs 12 and MTCC 796) cause *S. rolfsii* to form very thick mycelia without sclerotia during interaction compared to control plate having sole test pathgen *S. rolfsii*. The results are supported with antioxidants *N*-acetylcysteine (Patsoukis and Georgiou 2008), ascorbic acid (Georgiou et al. 2003), hydroxyl radical scavengers (Georgiou et al. 2000), beta-carotene (Georgiou et al. 2001) when they are supplemented in the growth medium of *S. rolfsii*. The best antagonists (Tvs 12 and MTCC 796) secreted vast bioactive antioxidants which act as a damping them into the growth medium and causing inhibition of sclerotium formation by *S. rolfsii*. The high antioxidant concentrations inhibited sclerotial differentiation in any sclerotium producing phytopathogenic filamentous fungi including *S. rolfsii*, and thereby restrain the ability *of S. rolfsii* to proliferate via sclerotium formation. Meanwhile, these antioxidant at high concentrations could arrest growth and halt the development of the fungus to the undifferentiated mycelial stage, where mycelia are more vulnerable to degrade by antagonists (Georgiou et al. 2006; Papapostolou and Georgiou 2010). Therefore, antioxidants secretary antagonists act as an antifungal alternatives to traditional fungicides.

Bansode et al. (2011) reported SRAP markers as a useful tool for genetic diversity and phenetic relationship among 80 isolates of *Trichoderma*. Total 43 pairs of SRAP primers were used for diversity analysis of 83 *Trichoderma* isolates belonging to 4 different species. These 43 pairs of primers produced 595 amplified products among which 587 were polymorphic. These SRAP primers produced 97.98 % polymorphism. However, present study demonstrated better genetic diversity (0.19–0.68 Jaccard similarity coefficient) for *Trichoderma* and identified unique bands for best biocontrollers Tvs 12 and MTCC 796. Total 115 primer combinations generating 1328 amplified products. Budak et al. (2004) used SRAP markers for estimation of genetic diversity and phenetic relationship in natural and domesticated populations of turfgrass. Their initial screening showed SRAP is highly polymorphic and more informative when compared with AFLP, RAPD and SSR markers. They applied SRAP markers to obtain an overview of genetic diversity and phenetic relationships present among cool season (C3) and warm season (C4) turfgrass species and their relationship with other Gramineae species were tested. They found widespread genetic variation among C3 and C4 turfgrass species. However, present study suggested genetic variation and phylogenetic relationship between 11 *Trichoderma* isolates which were differentially potential for antagonistic biocontrol activity against phytopathogen *S. rolfsii*.

Further, the SRAP markers were reported for various application of molecular biology viz. construction of a detailed linkage map for *Brassica oleracea* (Okazaki et al. 2007; Gao et al. 2007), mapping QTL for cotton fibre quality (Lin et al. 2005), the genes for lateral branch traits in cucumber (Wang et al. 2005), constructing a linkage map of cotton (Zhongxu et al. 2003), examine comparative genetics of different species (Fernando et al. 2006), tagging of the gene for resistance to *fusarium* wilt in eggplant (Mutlu et al. 2008).

Trichoderma virens NBAII Tvs 12 is evidently best antagonist inhibiting 87.91 % of growth of test pathogen *S. rolfsii* followed by *T. koningii*—MTCC796 (67.03 % inhibition). The best antagonists Tvs 12 inhibited growth of pathogen through mycoparasitism and continued to grow over pathogen without formation of inhibition zone during antagonism. However, *T. koningii*—MTCC 796 suppressed the growth of pathogen with formation of inhibition zone (strong antibiosis) and did not overgrow on pathogen. The growth inhibition of test pathogen was negatively correlated with sclerotium formation and lipid peroxides products in antagonists plate. The GC–MS profile of best Tvs 12 antagonist identified 27 bioactive constituents while MTCC 796 evident 19 bioactive compounds using cell wall of pathogen as carbon source. The molecular diversity analysis using SRAP marker clearly distinguished antagonist *Trichoderma* and test pathogen with minimum 19 % similarity. However, within *Trichoderma* antagonists, the clustering pattern were found according to biocontrol activity of *Trichoderma* rather than species.

Conclusion

Among the 11 isolates of *Trichoderma*, mycoparasitic *T. virens* NBAII Tvs 12 was the best bioagent inhibited maximum growth of fungal pathogen *S. rolfsii*. The best antagonists Tvs 12 inhibited growth of pathogen through mycoparasitism and continued to grow over pathogen without formation of inhibition zone during antagonism. However, *T. koningii*—MTCC 796 suppressed the growth of pathogen with formation of inhibition zone (strong antibiosis) and did not overgrow on pathogen. The growth inhibition of the test pathogen was negatively correlated with sclerotium formation and lipid peroxides products in antagonists plate. The antagonists secretary antifungal and antioxidant bioactive constituents responsible for disintegration and disruption of pathogen cell wall were identified with GC–MS profile. The SRAP based molecular analysis indicates significant genetic diversity (19–68 %) present in the *Trichoderma* antagonists and the best antagonist Tvs 12 were distinguished with 18 unique fragments generated by 15 SRAP markers. The Tvs 12 strain might have a significant role in the control of stem rot disease by reducing the virulence of *S. rolfsii* in the groundnut rhizosphere.

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