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Characterisation of Selected *Trichoderma* Isolates with Antifungal Activity*

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Sclerotium rolfsii **is a polyphagous pathogen that infects and cause seed/collar rot in more than 500 crop species. The present study was taken up towards developing an eco-friendly, alternate to chemical fungicide. Isolates of *Trichoderma* such as *T. virens, T. harzianum, T. hamatum, T. viride,* and *T. koningii* were evaluated to identify potent isolate. In dual culture assay, among the ten analysed, *Trichoderma* isolates, *T. virens* IABT1002 significantly suppressed (100%) the growth of *S. rolfsii* followed by *T. virens* IABT1010 (95.47%). The production of hydrolytic enzymes such as chitinase and glucanase were high in *T. virens* IABT1002 (90.42 pmol/μg/min) and *T. viride* IABT1044 (596.9 pmol/μg/min) respectively. In inverted plate assay to assay effect of volatiles, *T. harzianum* IABT1041 showed 71.53% of radial growth inhibition of the *S. rolfsii*. The performance of all isolates were evaluated in green house experiment and *T. koningii* IABT1252 had significantly reduced the percent disease infestation from 100% infestation in *S. rolfsii* treated to 55.55% infestation in *T. koningii* IABT1252 treated seeds.**

Key words: *Trichoderma, Sclerotium rolfsii*, Chitinase, Glucanase, Colloidal chitin, Laminarian, N-acetyl-glucosamine.

Trichoderma is an asexual spore forming filamentous fungi belongs to *Hypocreaceae* family, discovered by Persoon[26]. *Trichoderma* occurs worldwide and can be isolated from soil, salt marsh, dead plant materials, living roots, seeds, air and water [25]. The genus *Trichoderma/Hypocrea* consist of approximately 160 species[18] of which *T. virens*, *T. harzianum, T. atroviride, T. viride, T. koningii*, and *T. hamatum* have been found to have biocontrol function. The mycoparasitism property of *Trichoderma* was first time discovered by Weinlding[40] and reported that *T. lingorum* effectively parasitizes *S. rolfsii* and *Rhizoctonia solani* which provoked the idea of use of *Trichoderma* strain as biocontrol agent to control the phytopathogenic fungi. Subsequently, Darpoux[4] observed the capacity of *Trichoderma* to control phytopathogenic fungi. The genus *Trichoderma* has been reported to parasitize various phytopathogenic fungi like *Phythium, Phytophthora erythroseptica, Mucor mucedo, Botrytis cinera, Botrytis fabae, R. solani, Pseuperonospora cubensis, Fusarium, S. rolfsii and Rhizopus oryzae* [3, 7, 8, 10, 12, 15, 16, 19, 32, 34].

Trichoderma possess various mycoparasitism properties such as antibiosis, fungistasis, release of hydrolytic enzymes, production volatile substances, efficient nutrient utilization, fast growth, capacity to modify the rhizosphere, prolific spore production, strong aggressive growth against phtopathogenic fungi[13,16,33] and degradation of pectinase or other enzymes that are essential to the phytopathogen to enter the plant system[42].

Further, in rhizosphere, the communication between *Trichoderma* and plant roots help the fungi to establish endophytic relationship with the plants in a non-pathogenic

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manner[41]. These signals manifest the plant's morphology, physiology and growth characters in a direct or indirect way. *Trichoderma* induces plant defence system to produce jasmonic acid and/or ethylene hormone, which ends up in the accumulation of hydrogen peroxide, various PR proteins, phenolic compounds[23] and modification of cell wall composition like liginification, callose depositions. These interactions promote the root growth, increase the photosynthetic efficiency, increase the micronutrient status (iron and manganese)[1], make healthier canopy of plants and help the plant to overcome the abiotic stresses as well[14, 29, 35]. Hence, *Trichoderma* is known as biopesticide, bioprotectant and biostimulant. *Trichoderma* species can be easily mass multiplied in broth and stored for longer period under appropriate condition. *Trichoderma* based bioformulation covers about 60% of available biofungicides in the market[38]. However, the performance of *Trichoderma* as biological control agent (BCA) depends mainly on different physiological and chemical environment to which they are applied.

Even though the isolates of *Trichoderma* are good in mycoparasitism properties, their behaviour in soil, adaptability, survival and protection of plants from pathogens, are the indispensable parameters which need to be studied to address a potent biocontrol agent. The previous studies in our laboratory identified ten *Trichoderma* isolates with good ability to inhibit *S. rolfsii* in dual culture assay. Therefore, the present study aimed at characterization of these ten *Trichoderma* isolates for various mycoparasitism properties and identification of potent isolate which can control the seed/collar rot of groundnut JL24 caused by *S. rolfsii*.

MATERIALS AND METHODS

Revival of *Trichoderma* isolates and their maintenance

Ten *Trichoderma* isolates were taken from the *Trichoderma* collection maintained at Department of Biotechnology, UAS Dharwad (Table 1). The cultures were maintained on potato dextrose agar (PDA) (200 gm of potato extract, 20 gm dextrose, 100 mg yeast extract, 20 gm agar agar type I and 1000 ml of water).

Isolation of *Sclerotium rolfsii*

The pathogen *S. rolfsii* was collected from the groundnut field in Main Agricultural Research Station UAS, Dharwad. The sclerotial bodies were surface sterilized with 0.5% of sodium hypochlorite for 5 min and thoroughly washed with sterile water for three times. The dried sclerotial body was kept at the centre of sterile PDA plate. After five days of incubation at 28 ^{0}C the sclerotium was confirmed by its morphological appearance and formation of sclerotial bodies after 15 days. The culture was periodically subcultured on fresh PDA plate.

Average linear growth rate

The 5 mm agar plug of the actively growing region of *Trichoderma* was picked from 5 days old culture and placed at the centre of PDA plates. The plates were incubated at 28 ^{0}C with 12 h light and 12 h dark condition until they reached the periphery of the plates. The two perpendicular diameters of each isolates were recorded at 24 h interval and the growth rate was calculated as per the following formula[27].

ALGR= (C3-C1) /T

Where,

ALGR- Average linear growth rate

C3- Growth of isolate on 3rd day

C1-Growth of isolate on 1st day

T - Day interval between two observations

Dual culture assay

The 5 mm agar plug of biocontrol agent and pathogen were collected from actively growing region of 5 days old culture and kept on PDA plate, 5 cm apart. Simultaneously, one control set inoculated with only *S. rolfsii* was maintained. The plates were incubated at 28 ^{0}C for 5 days and at the end of the incubation period, the growth inhibition of pathogen was recorded[5]. Later the per cent growth inhibition was calculated by the following formula[39].

I= (C-T/C) x100

Where,

I- Per cent of growth inhibition

C- Growth of *S. rolfsii* in control plate (mm)

T- Growth of *S. rolfsii* in *Trichoderma* inoculated plate (mm)

The experiment was repeated twice with three replications for each treatment

Inverted plate technique

In order to know whether high antifugal activity of potent isolates was due to the release

of volatile substances, 5 mm of actively growing region of each *Trichoderma* isolates were placed at the centre of PDA plate and simultaneously 5 mm agar plug of *S. rolfsii* was also placed at the centre of another PDA plate. Later, the inoculated plates (bottom plate) of both the cultures (*Trichoderma* and *S. rolfsii*) were kept one above another in such a way that both cultures faced each other and tightly closed with parafilm[6]. During incubation, *S. rolfsii* inoculated plate was kept upper side to prevent the chance of *Trichoderma* spores falling on to *Sclerotium* plate. After five days of incubation, at 28 ^{0}C the radial growth inhibition of *S. rolfsii* was scored[6]. The colony size in each treatment was recorded and per cent inhibition was calculated by below formula.

$I = (C–T/C) \times 100$

Where:

I = Per cent growth inhibition

C = Growth of *S. rolfsii* in control (mm)

T = Growth of *S. rolfsii* in treatment (mm)

Assay for production of hydrolytic enzymes

Preparation of dried mycelia of *Sclerotium rolfsii*

The 5 mm agar plug of *S. rolfsii* was inoculated into 50 ml potato dextrose broth (PDB) and incubated at 28 ^{0}C. After five days of incubation, the mycelial mat was harvested in Laminar air flow chamber and homogenized. The macerated mycelia were dialysed against distilled water for overnight in order to remove glucose moiety, which may interfere in subsequent reaction. The dialysed product was lyophilised and powder was stored in sterile condition at 4 ^{0}C[9].

Induction of *Trichoderma* for hydrolytic enzyme production

For the induction of hydrolytic enzyme production by *Trichoderma* isolates, synthetic medium[30] amended with 0.5% lyophilized *S. rolfsii* culture was used. The 5 mm agar plug from *Trichoderma* isolates was inoculated into 50 ml broth of synthetic medium amended with 0.5% lyophilized *S. rolfsii* culture and incubated in shaker at 28 ^{0}C at 120 rpm. Simultaneously, all the *Trichoderma* isolates were inoculated into synthetic broth lacking *S. rolfsii* separately and served as controls. Synthetic medium with 0.5% lyophilized *S. rolfsii* culture served as absolute control. All the cultures were incubated in 28 ^{0}C at 120 rpm. After 5 days of incubation, the entire culture was centrifuged at 5000 rpm for 10 min at 4 ^{0}C[37]. The supernatant was further clarified by passing through 0.45 µm filter. The glucose moiety that is present in the filtrate was removed by dialysis at 4 ^{0}C. The protein enriched dialysed filtrate was stored at -20 ^{0}C and later used for enzyme assay.

Preparation of colloidal chitin

The colloidal chitin was prepared from chitin flakes (Himedia) according to Roberts and Selintrennikoff [28]. The chitin flakes were broken down into powder form by incubating the 5 gm of chitin flakes in 87.50 ml of HCL for overnight at 4 ^{0}C in shaking condition. Next day, 95% of 500 ml of alcohol was added to overnight mixture and incubation was continued for 12 h at 25 ^{0}C. The mixture was centrifuged at 5000 rpm in 4 ^{0}C for 20 min. The pellet was washed with sterile water until pH reached 7.0. The solution was centrifuged at 5000 rpm 4 ^{0}C for 20 min. Later, the pellet was dried under aseptic condition and dissolved in sterile water to make 5 % stock of colloidal chitin. The solution was stored at 4 ^{0}C for later use.

Chitinase enzyme assay

The induced culture filtrate was used to measure the total protein concentration and to estimate the total hydrolytic enzymes such as chitinase and glucanase production. Initially, total protein was estimated by Lowry's method[20]. The 0.5 ml Mcllvaines's buffer[22], 0.5 ml supernatant and 0.5 ml colloidal chitin was added into a test tube and incubated for 30 min at 50 ^{0}C. Later, the released reducing sugar was measured by adding 0.5 ml of dinitrosalicylic acid (DNSA) reagent[24] and reaction was stopped by heating the tube at boiling water for 10 min. Finally, the volume was made up to 10 ml and the enzyme final product N-acetylglucosamine (NAG) was estimated by measuring the per cent absorbance of light at 540 nm in spectrophotometer (Cary 50 Bio UV/ Visible Spectrophotometer, Australia). The spectrophotometer readings were plotted on N-acetylglucosamine standard graph to find the concentration of chitinase enzyme in the sample. The concentration was expressed in terms of pmol/µg/min.

Glucanase enzyme assay

The 0.5 ml sodium acetate buffer, 0.5 ml induced culture filtrate and 0.5 ml laminarian was added into a test tube and incubated for 30 min in 50 ^{0}C. Later 0.5 ml of DNSA was added and reaction

was stopped by heating the tube at boiling water for 10 min. Finally, the volume was made up to 10 ml and per cent absorbance was recorded at 540 nm. In addition, the standard glucose reaction was maintained. The concentration of enzyme was expressed in terms of pmol/µg/min[36].

***In vivo* experiment**

The 6% *S. rolfsii* giant culture[2] was mixed with potting mixture (soil: sand in 1:1 ratio). The 250 gm of potting mix containing 6% of giant culture was added to cups and incubated at room temperature to facilitate the initial growth of pathogen. After two days, groundnut JL24 seeds were surface sterilized with 0.5% sodium hypochlorite and thoroughly washed with water. These seeds were dipped in *Trichoderma* broth (10^8-10^9spores) and dried in Laminar air flow chamber for half an hour, later used for sowing in cups. Three replicates were used, each replicate consisting of three cups and each cup contained two seeds. *T. harzianum* culture which is a commercial product of Institute of Organic Farming, UAS Dharwad was used as reference strain. After 15 days of sowing, the number of healthy plants and *S. rolfsii* infected seeds (seed rot) were counted. The number of infected seeds out of the total sown seeds in each replication was used to calculate the per cent disease incidence[1].

$$\text{Per cent disease incidence} = \frac{\text{Number of infected plants or seeds}}{\text{Total number of sown seeds}} \times 100$$

Statistical analysis

The experiments were conducted in CRD fashion and all the observations were taken from three replications. The data were statistically analysed by analysis of variance (ANOVA) and means comparisons were made by using Duncan multiple range test (DMRT) at $P<0.05$ test.

RESULTS AND DISCUSSION

Average linear growth rate

The growth rate of biocontrol agent impedes the growth of pathogen by means of space and food availability or physically restricting the growth of pathogen. This parameter is also important while choosing the isolate to use as biocontrol agent[11]. The ten *Trichoderma* isolates differed significantly with respect to linear growth and it ranged from 16.25 to 19.75 mm/day. The highest growth rate was recorded in *T. virens* IABT1010 (19.75 mm/day) followed by *T. viride* IABT1044 and least was recorded in *T. harzianum* IABT1042 (16.25 mm/day) (Fig. 1.).

Direct confrontation assay

The growth inhibition of *S. rolfsii* ranged from 75% (*T. hamatum* IABT1211) to 100% (*T. virens* IABT1002) (Table 2) among the ten *Trichoderma* isolates tested. The inhibition of linear growth of pathogen was significantly high in *T. virens* IABT1002 compared to the remaining *Trichoderma* isolates. After seven days of incubation, the *T. virens* IABT1002 completely overgrew on *S. rolfsii* and no mycelium of pathogen was found on the plate. A few isolates failed to overgrow the pathogen but formed clear inhibition zone (*T. koningii* IABT1252) beyond which *S. rolfsii* did not grow (Fig. 2). Though *T. virens* IABT1002 showed slower growth rate compared to *T. virens* IABT1010, the per cent inhibition of *S. rolfsii* was highest in the presence of *T. virens* IABT1002. *Trichoderma* has several mechanisms through which it controls the fungal plant pathogens[33, 17].

Inverted plate technique

One of the well established biocontrol mechanisms of *Trichoderma* is the release of volatile substances[33]. In this study, different *Trichoderma* isolates showed significant difference for the release of volatile substances. The *T. harzianum* IABT1041 showed maximum (71.53%) growth inhibition of *S. rolfsii* due to the release of volatile substance followed by *T. koningii* IABT1242. The minimum per cent growth inhibition (15.95%) was observed in the presence of *T.*

Table 1. The ten potent *Trichoderma* isolates used in the study

S. No.	*Trichoderma* isolates	Isolates
1	*Trichoderma virens*	IABT1002
2	*Trichoderma virens*	IABT1010
3	*Trichoderma harzianum*	IABT1041
4	*Trichoderma harzianum*	IABT1042
5	*Trichoderma viride*	IABT1044
6	*Trichoderma harzianum*	IABT1046
7	*Trichoderma hamatum*	IABT1211
8	*Trichoderma koningii*	IABT1242
9	*Trichoderma harzianum*	IABT1243
10	*Trichoderma koningii*	IABT1252

harzianum IABT1046 (Table 3).

Hydrolytic enzyme assay

There was a significant difference in the chitinase production on medium supplemented with the *S. rolfsii* mycelia compared to uninduced culture. Among the ten *Trichoderma* isolates significant differences were observed with respect to chitinase production, which ranged from 26.11 pmol/µg/min to 90.42 pmol/µg/min. *T. virens* IABT1002 which showed maximum growth inhibition in dual culture assay also showed highest production of chitinase (90.42 pmol/µg/min) and it was three times high compared to *T. virens* IABT1010 (Fig. 3.). The least production of chitinase enzyme was observed in *T. hamatum* IABT1211 isolate which observed minimum growth inhibition of *S. rolfsii* on dual culture assay. Similarly different *Trichoderma* isolates studied showed significant variation for glucanase production which ranged from 275.12 pmol/µg/min

Table 2. Per cent growth inhibition of *S. rolfsii* when confronted against *Trichoderma* isolates on PDA plate at 28°C

S. No	*Trichoderma* isolates	Isolates no.	Dual culture*
1	*T. virens*	IABT1002	100.00[a]
2	*T. virens*	IABT1010	95.47[b]
3	*T. harzianum*	IABT1041	75.49[ef]
4	*T. harzianum*	IABT1042	75.99[ef]
5	*T. viride*	IABT1044	75.99[ef]
6	*T. harzianum*	IABT1046	86.47[c]
7	*T. hamatum*	IABT1211	75.00[f]
8	*T. koningii*	IABT1242	76.99[e]
9	*T. harzianum*	IABT1243	76.50[ef]
10	*T. koningii*	IABT1252	81.50[d]
11	*S. rolfsii*	SR	0.00[g]
			CV(%)=1.31
			CD(0.05)=1.65

*Values carrying same alphabets does not differ significantly at P<0.05 DMRT test.

Table 3. Per cent growth inhibition of S. rolfsii by volatile substance produced from Trichoderma isolates

S. No	Trichoderma isolates	Isolates no.	Dual culture*
1	T. virens	IABT1002	57.75[c]
2	T. virens	IABT1010	39.93[ef]
3	T. harzianum	IABT1041	71.53[a]
4	T. harzianum	IABT1042	54.39[c]
5	T. viride	IABT1044	35.95[f]
6	T. harzianum	IABT1046	15.95[g]
7	T. hamatum	IABT1211	47.39[d]
8	T. koningii	IABT1242	66.89[b]
9	T. harzianum	IABT1243	42.47[e]
10	T. koningii	IABT1252	47.43[d]
11	S. rolfsii	SR	0.00[h]
			CV(%)=5.48
			CD(0.05)=4.05

*Values carrying same alphabets does not differ significantly at P<0.05 DMRT test.

Table 4. Per cent disease infestation of *S. rolfsii* to groundnut JL24 in presence of *Trichoderma* isolates

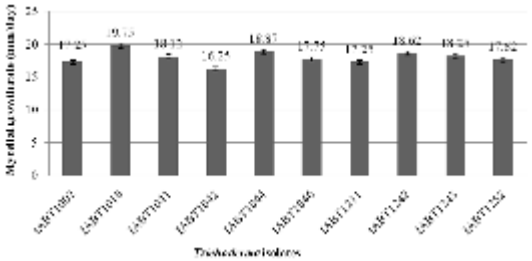
S. No.	Treatments	Per cent disease infestation*
1	*T. virens* IABT1002 treated seeds sown in *S. rolfsii* inoculated soil	94.44^{ab}
2	*T. virens* IABT10101 treated seeds sown in *S. rolfsii* inoculated soil	88.88^{abc}
3	*T. harzianum* IABT1041 treated seeds sown in *S. rolfsii* inoculated soil	88.88^{abc}
4	*T. harzianum* IABT1042 treated seeds sown in *S. rolfsii* inoculated soil	66.67^{de}
5	*T. viride* IABT1044 treated seeds sown in *S. rolfsii* inoculated soil	83.33^{bc}
6	*T. harzianum* IABT1046 treated seeds sown in *S. rolfsii* inoculated soil	77.77^{cd}
7	*T. hamatum* IABT1211 treated seeds sown in *S. rolfsii* inoculated soil	100.00^{a}
8	*T. koningii* IABT1242 treated seeds sown in *S. rolfsii* inoculated soil	88.89^{abc}
9	*T. harzianum* IABT1243 treated seeds sown in *S. rolfsii* inoculated soil	83.33^{bc}
10	*T. koningii* IABT1252 treated seeds sown in *S. rolfsii* inoculated soil	55.55^{e}
11	*T. harzianum* treated seeds sown in *S. rolfsii* inoculated soil	61.11^{e}
12	Untreated seeds sown in healthy soil	0.00^{f}
13	Untreated seeds sown in *S. rolfsii* inoculated soil	100.00^{a}
		CV(%)=11.09
		CD(0.05)=14.16

* Values carrying same alphabets does not differ significantly at P<0.05 DMRT test

to 596.9 pmol/µg/min. The isolate *T. viride* IABT1044 was found to be the highest producer of glucanase (596.9 pmol/µg/min) and in *T. koningii* IABT1252 (275.12 pmol/µg/min), the least (Fig. 4.). However, *T. viride* IABT1044 showed relatively less growth inhibition of *S. rolfsii*. The *T. virens* IABT1002 which showed cent percent inhibition of pathogen produced lesser glucanase than *T. viride* IABT1044, indicating major role of chitinase in pathogen inhibition. In addition, other mechanisms may be operation in *T. virens* IABT1002 to overcome *S. rolfsii*. The ability of *Trichoderma* isolates to produce hydrolytic enzymes varied for different enzymes. *Trichoderma* isolate with high chitinase producing ability was not found to produce high amount of glucanase. Matroudi and associates[21] had recorded *T. harzianum*-8 isolate has double chitinase activity (35 U/mg) against *Sclerotinia sclerotiorum* than compared to glucanase activity (15 U/mg). Similarly, in another isolate PTCC5220 chitinase activity (11 U/mg) is half of the β -1,3 glucanase activity (20 U/mg). The source from where *Trichoderma* was isolated and the substrate used for induction of hydrolytic enzymes may play a role in the ability of a *Trichoderma* to produce hydrolytic enzymes[9].

Green house experiment

In this study, *S. rolfsii* caused pre-emergence rotting of groundnut seeds in cups containing infected soil and the germination was found to be completely affected. However, groundnut seeds germinated normally in cups



*The values labeled over each column is mean of the three replicates and error bars (±one standard error) are also presented.

Fig. 1. Average linear growth rate of ten *Trichoderma* isolates (mm/day)

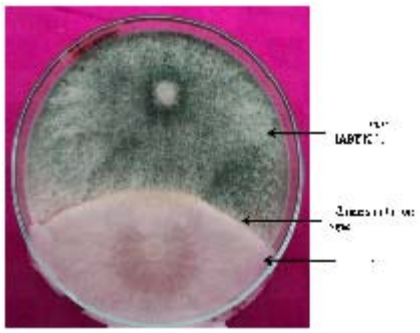
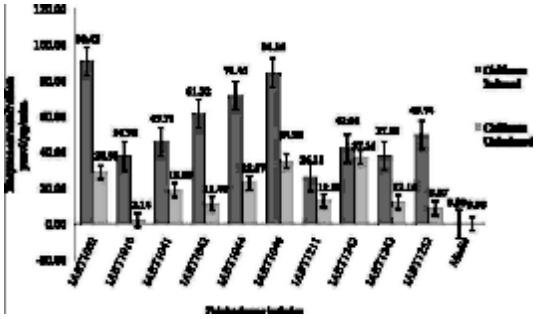


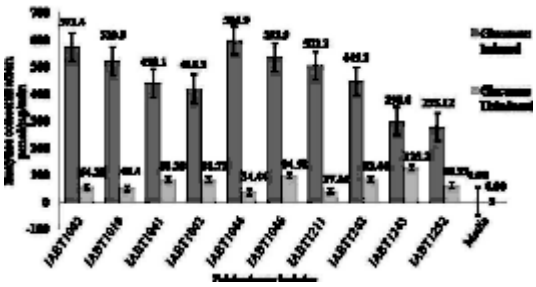
Fig. 2. Formation of growth inhibition zone of *S. rolfsii* during its confrontation with *T. koningii* IABT1252

containing uninfected soil. Further, the seed treatment of groundnut seeds with *Trichoderma* isolates found to decrease the pre- emergence rot caused by *S. rolfsii*. Among the 10 *Trichoderma* isolates used for seed treatment *T. koningii* IABT1252 was found to reduce the pre-emergence seed rot (55.55% of seeds showed rotting). Once germinated groundnut seedlings grew normally even in the presence of *S. rolfsii* (Fig. 5.). However it was noticed that the isolates which performed well under *in vitro* conditions, performed poorly under *in vivo* conditions. For example, *T. virens* IABT1002 and *T. harzianum* IABT1041, significantly suppressed the pathogen growth under dual culture and volatile substance assay respectively but performed poorly under pot experiment (Table 4). Similar difference in performance of *Trichoderma* isolates under *in vitro* and *in vivo* experiments were reported earlier[31]. In addition, in our study we found *T. koningii* IABT1252 positively influenced the seed germination compared to other isolates (result not included).



*Quantified by spectrophotometer (540 nm) and results were depicted above. The values labeled over each column is mean of the three replicates and error bars (±one standard error) are also presented.

Fig. 3. Production of chitinase in *Trichoderma* isolates upon induction by 0.5% lyophilized *S. rolfsii* culture



*Quantified by spectrophotometer (540 nm) and results were depicted above. The values labeled over each column is mean of the three replicates and error bars (±one standard error) are also presented.

Fig. 4. Production of glucanase in *Trichoderma* isolates upon induction by 0.5% lyophilized *S. rolfsii* culture

Fig. 5. Performance of *Trichoderma* isolates against *S. rolfsii* under *in vivo* condition in presence of groundnut JL24 as a host crop

From the above results, it can be concluded that isolate *T. koningii* IABT1252 had significantly controlled the *S. rolfsii* infestation of groundnut crop in green house condition. Further, the isolate need to be evaluated in field to identify its potential and possibility of using this as biological control agent.

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