Journal of Root Crops, 2018, Vol. 44 No. 2, pp. 53-60
Indian Society for Root Crops
ISSN 0378-2409, ISSN 2454-9053 (online)

Comparative Analysis of Antimicrobial Activities of 43 *Trichoderma* Isolates Against *Sclerotium rolfsii,* the Pathogen Causing Collar Rot Disease in Elephant Foot Yam

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Abstract

Elephant foot yam (*Amorphophallus paeoniifolius* (Dennst.) Nicolson) is an important tuber crop which is popular as a food security crop as well as a remunerative cash crop. The crop is preferred in tropical and sub-tropical regions due to its high production potential (50–80 t ha^{-1}), market acceptability and lucrative economic returns. Collar rot caused by *Sclerotium rolfsii* is the most destructive and predominant disease causing great crop loss. Application of *Trichoderma* spp is recommended as the eco-friendly strategy to combat the crop loss. However, all *Trichoderma* isolates may not perform equally against specific soil borne pathogens as *Trichoderma* antagonists have different mechanisms of pathogen recognition. The present study was conducted with an objective of identifying most potent isolates against *Sclerotium rolfsii* from the collection of *Trichoderma* isolates (43 nos.) obtained from tuber crop ecosystem during the period 2010-2018. The differential antagonistic potential of the isolates were assessed by adopting three *in vitro* screening methods viz., direct confrontation, antibiosis test based on production of diffusible inhibitory metabolites and production of volatile compounds. In dual culture method, percentage inhibition of mycelial growth of pathogen varied from 9.44% (isolate T26) to 82.32% (isolate T32). The percentage inhibition ranged from 3.70% (isolate T31) to 100% (30 isolates had 100% inhibition) in antibiosis test based on production of diffusible inhibitory metabolites by isolates against pathogen. Comparatively low inhibition was noticed with volatile compounds produced by isolates against pathogen. Percentage inhibition of mycelial growth of pathogen varied from 15.38% (isolate T26) to 42.96% (isolate T31). To identify the most potent isolates, additive inhibition effect by the isolates were calculated and statistically analysed by SAS statistical software (SAS2010 - SAS Institute Inc., Cary, North Carolina, USA.). Based on the additive effect as well as results obtained from different methods, it was concluded that the isolates, T38, T36, T32, T40 and T6 have excellent potential to be used as bio-control agents to mitigate the crop loss caused by *S. rolfsii.* These isolates may be tested in field conditions for their field performance as well as for survival in soil before recommending to farmers.

Key words: Elephant foot yam, *Trichoderma, Sclerotium rolfsii*, collar rot

Introduction

Elephant foot yam (*Amorphophallus paeoniifolius* (Dennst.) Nicolson) is an important tuber crop of tropical and sub-tropical countries because of its high production potential (50–80 t ha^{-1}), market acceptability and lucrative economic returns (Misra, 2005). Elephant foot yam (EFY) is an excellent and cheap source of carbohydrate, protein, vitamin B6, fiber and omega 3 fatty acids. They are rich in potassium, magnesium and phosphorous, trace minerals like selenium, zinc and copper (Santosa et al., 2017). The crop is being used in various pharmacological preparations because of their potential as analgesic, anti-

inflammatory, CNS depressant, antihelmintic, antibacterial, antifungal, antioxidant, antitumour, hepatoprotective, immunomodulatory and cytotoxic agent (Dey et al., 2012; Singh and Wadhwa, 2014).

Like all crops, EFY is also susceptible to many pathogens. Collar rot disease caused by *Sclerotium rolfsii* is the most destructive and prevalent disease in all *A. paeonifolius* growing areas causing yield loss up to 100% (Misra, 1997; Gogoi et al., 2002; Kumar et al., 2017). *S. rolfsii* is one of the most destructive soil borne pathogen causing great damage to many crops particularly of tropics and subtropics, where temperature is adequately high which favour the growth and survival of the fungus (Dasgupta and Mandal, 1989). Several methods are employed to mitigate the disease including the use of chemical fungicides (Theertha et al., 2017). However, the indiscriminate use of these chemicals has imposed serious environmental impacts (Cook and Baker, 1983). The development of resistance in pathogens to these chemicals is another major threat (Dekker and Georgopoulos, 1982). Also, organic growers have limited options for managing collar rot since most of the effective fungicides, fumigants and seed treatments are synthetic, toxic and potentially polluting (Veena et al., 2013) Therefore, developing an eco-friendly approach for managing collar rot is the need of the hour and bio-control is one such approach. The use of microorganisms as bio-control agents has provided a very promising alternative and less hazardous method for plant disease control (Cook, 1985).

Among the investigated biological control agents, *Trichoderma* species have attracted special position due to the particular biological characteristics since the early 1930s (You et al., 2016). *Trichoderma* which are soil-borne, free-living and non-pathogenic fungi are important in controlling several phytopathogens and promote plant growth by multiple modes of action including systemic resistance, antibiosis, enhanced nutrient efficiency and mycoparasitism (Benitez et al., 2004; Mathys et al., 2012). It can colonize the rhizosphere as well as roots of many plants and are often added to soils to increase crop yields and control soil borne pathogen. In India alone, more than 250 *Trichoderma* based formulations are sold commercially (Lee et al., 2016). *Trichoderma* species are prominent bio-control agents used to control *S. rolfsii* (Rao et al., 2004). Various mechanisms which support the striking performance of *Trichoderma* species during antagonism with plant pathogens are mycoparasitism, antibiosis, competition for nutrients and space, modification of the growing region, and/or stimulating plant growth and plant defense mechanisms (Benitez et al., 2004; Druzhinina et al., 2011). Understanding the variability of *Trichoderma* strains in terms of antagonistic potential, biological and biochemical activities are necessary to improve the selection of different isolates as bio-control agents (Consolo et al., 2012; Sharma et al., 2009). Application of *Trichoderma* is recommended to manage collar rot in elephant foot yam. Many *Trichoderma* isolates were collected from the rhizosphere region of tuber crops from different parts of the country. Present study aimed to select the best isolates with multiple mode of action against the pathogen from the 43 *Trichoderma* isolates maintained at microbial repository, Division of Crop Protection, ICAR-Central Tuber Crops Research Institute.

Materials and methods

Sources of *Trichoderma* isolates

Forty-three isolates (T1 to T43) of *Trichoderma* obtained from tuber crops ecosystem in different parts of India (Kerala, Karnataka, Andhra Pradesh and Odisha) and maintained at Microbial repository, ICAR-CTCRI were used for the study. The details of locations from where the isolates were obtained are given below (Table 1).

Isolation of *Sclerotium rolfsii*

The elephant foot yam plants (variety- Gajendra) showing typical symptoms of collar rot were collected from experimental field of ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram. The diseased portion from the collected plants was thoroughly washed under running tap water to remove surface debris and contaminants. The tissues infected with pathogen were cut out from the leading edge of lesion, and subjected to dip in 1% sodium hypochlorite for five minutes followed by washing with sterile distilled water and dried using sterile filter paper. The pathogen was isolated by hyphal tip method (Sinclair and Dhingra, 1985) from dry small pieces of root plated onto PDA media and incubated at 28°C. The cultures were transferred periodically on other PDA plate and repeated

until pure culture was obtained. The pure culture was maintained in PDA plates.

Screening of *Trichoderma* isolates against *Sclerotium rolfsii*

The differential antagonistic potential of all isolates were assessed by adopting three *in vitro* screening methods viz., dual culture method (Skidmore and Dickinson, 1976), antibiosis test based on production of diffusible inhibitory metabolites (Dennis and Webster, 1971a) and antibiosis test based on production of volatile compounds (Dennis and Webster, 1971b).

Dual culture/direct confrontation method

The inhibitory action of isolates was assessed by co-inoculating mycelial discs of *Trichoderma* and *S. rolfsii* in a single PDA plate. Mycelial discs of 5mm diameter were taken from the actively growing culture of each *Trichoderma* isolate. The mycelial discs were placed in PDA plates leaving 3cm from periphery of the plates. Similarly, mycelial discs were cut from actively growing culture of *S. rolfsii* and placed it against *Trichoderma* by leaving a distance of 3cm between the mycelial discs. Three replicates were maintained for each isolate and the mycelial growth of *S. rolfsii* was measured at an interval of 24h. The plates inoculated with *S. rolfsii* alone served as the control. On day 3, mycelial growth of *S. rolfsii* completely covered 90 mm petri-dishes in control plates. The radial growth of *S. rolfsii* was measured, and percentage of inhibition was calculated in relation to growth of the controls as follows:

$$I = (C-T/C) \times 100$$

Where, I = percent inhibition; C = radial growth of pathogen (in mm) alone in the control plate; T = radial growth of pathogen (in mm) in the presence of *Trichoderma* isolates (Edington et al., 1971).

Antibiosis test based on production of diffusible inhibitory metabolites by isolates against the pathogen

This test was performed to evaluate the role of diffusible inhibitory metabolites produced by *Trichoderma* isolates on controlling the growth of *S. rolfsii*. In this method, *Trichoderma* discs were inoculated centrally on sterile cellophane membrane placed over the PDA medium in Petri dishes. The *Trichoderma* inoculated plates were incubated for 2 days at 28 ± 2^{o}C. The cellophane membranes along with mycelial growth of *Trichoderma* isolates were removed aseptically using sterile forceps. Mycelial discs (5mm) of *S. rolfsii* were cut from actively growing cultures and were inoculated in the centre of the plates where *Trichoderma* was grown earlier on cellophane membrane. Plates without the prior inoculation of *Trichoderma* under the same above mentioned conditions served as the control. Mycelial growth of *S. rolfsii* was measured at 24 h interval. The percentage inhibition of *S. rolfsii* was calculated in relation to growth of the controls as follows:

$$I = (C-T/C) \times 100$$

Where, I = percent inhibition; C = radial growth of pathogen (in mm) alone in the control plate; T = radial growth of pathogen (in mm) on PDA plates with prior inoculation of *Trichoderma* isolates.

Antibiosis test for production of volatile compounds by isolates against pathogen

The isolates were screened by adopting this method to evaluate the potential of volatile compounds produced by *Trichoderma* isolates on controlling the growth of *S. rolfsii*. The PDA plates were separately inoculated centrally with mycelial discs of *Trichoderma* isolates and *S. rolfsii*. The lids of the petri plates inoculated with *Trichoderma* were replaced by the bottom portion of the plates with *S. rolfsii* inoculum. The plates were sealed using an adhesive tape and incubated for 2-3 days. The radial growth of *S. rolfsii* was measured at an interval of 24 h. The percentage inhibition of *S. rolfsii* was calculated in relation to growth of the controls as follows:

$$I = (C-T/C) \times 100$$

Where, I = percent inhibition; C = radial growth of pathogen (in mm) alone in the control plate; T = radial growth of pathogen (in mm) on PDA plates placed over the *Trichoderma* inoculated bottom dish.

Additive effect

To identify the most potent isolates, additive/cumulative inhibition effect of the isolates were calculated and based on the rank, the isolates were selected.

Statistical analysis

The data were statistically analysed using SAS statistical software (SAS 2010 – SAS Institute Inc., Cary, North Carolina, USA).

Results and Discussion

A total of 43 *Trichoderma* isolates were evaluated against *S. rolfsii*. Three different methods viz., dual culture method/direct confrontation method, antibiosis test based on production of diffusible inhibitory metabolites and volatile compounds by isolates were adopted to select the most potent isolates. The percentage inhibition of mycelial growth of *S. rolfsii* by *Trichoderma* isolates in three different methods of screening is given Table 1.

Dual culture method

All 43 isolates tested could restrict the mycelial growth of *S. rolfsii* (Table 1 and Fig. 1). The isolates had differential inhibition potential by suppressing the mycelial growth of the pathogen. Maximum percentage inhibition was shown by the isolate T32 (82.32%). It was followed by the isolates viz., T36 (75.85%), T38 (74.13%), T34 (69.38%) and T40 (62.73%). However, these isolates did not vary significantly in their ability to inhibit mycelial growth of pathogen. Most of the isolates exhibited high inhibition towards the collar rot pathogen.

Dual culture method was used in studying antagonism of different species of *Trichoderma* by many workers (Sharma et al., 2009; Srivastava et al., 2012; Singh et al., 2013; Reddy et al., 2014; Gogoi et al., 2015; Yuzana and Thein, 2018).

Antibiosis test based on production of diffusible inhibitory metabolites

Most of the isolates showed 100% inhibition of mycelial growth by this method. In this screening method, 69.70% of the isolates (30 isolates) had 100% inhibition of pathogen. It suggests the involvement of lytic enzymes and other metabolites in pathogen suppression. Lowest inhibition was shown by the isolate T31 (3.70%) followed by the isolate T26 (5.93%). The isolate T26 had the least inhibition in dual culture method also. Nath et al. (2014) and John et al. (2015) also used antibiosis test based on production of diffusible inhibitory metabolites method to screen *Trichoderma* isolates against tuber crop pathogens. During the bio-control action of *Trichoderma* spp., antibiotics were found to play an important role.

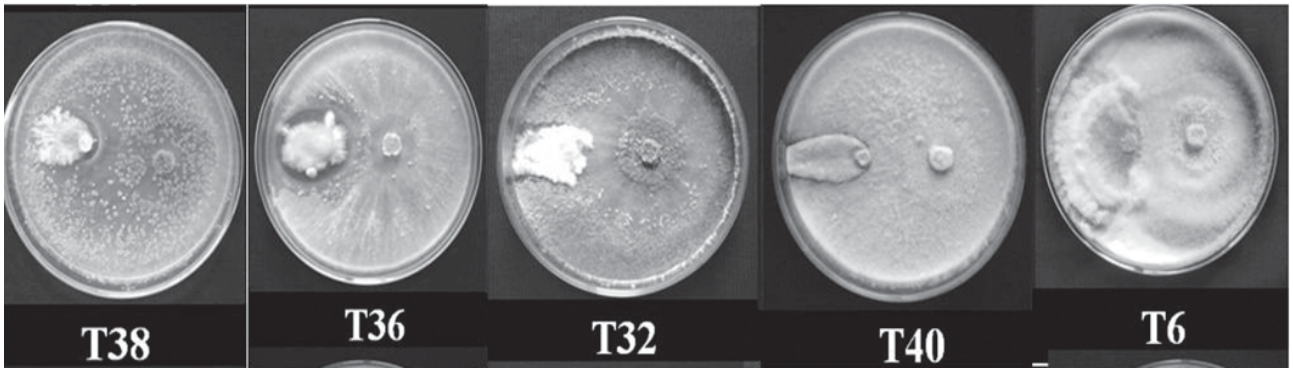

Fig. 1. Mycelial growth inhibition of *S. rolfsii* by *Trichoderma* isolates (top five isolates)

Out of 43 isolates, 74.40% of the isolates had more than 50% inhibition. The majority of isolates (27 isolates) showed 50% to 60% inhibition (Fig. 2). The least percentage inhibition was shown by the isolateT26 (9.44%). A curve in the contact region of pathogen and *Trichoderma* was observed in majority of isolates. The isolate T31 was an exception by showing straight line at the contact region.

Various methods are being considered to determine the potency of *Trichoderma* isolates against various pathogens (Hirpara et al., 2017). To study the direct effect of fungi on each other, inoculating them on a single PDA plate was recommended (Skidmore and Dickinson, 1976).

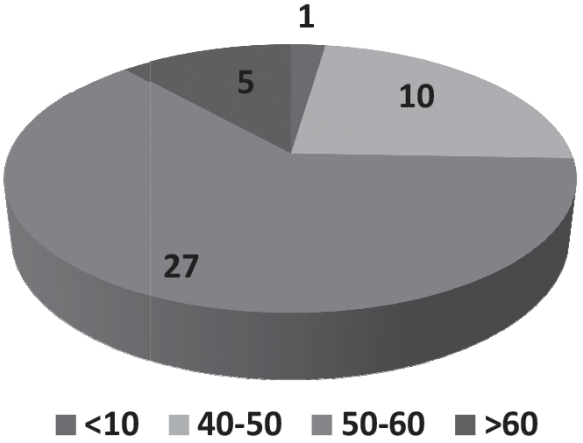

Fig. 2. Number of *Trichoderma* isolates showing specified inhibition (%) on dual culturing

Table 1. Mycelial growth inhibition of *S.rolfsii* by different *Trichoderma* isolates under different screening methods

Isolate code	Mycelial growth inhibition (%)		
	Dual culture	Metabolite production	Volatile production
T1	49.98 (0.52)^{ef*}	100.00(1.57)^{a}	24.96(0.25)^{abcdefg}
T2	53.00 (0.56) ^{def}	100.00(1.57)^{a}	36.70(0.38) ^{abcdef}
T3	54.29 (0.57) ^{def}	100.00(1.57)^{a}	22.41(0.23) ^{bcdefg}
T4	45.67(0.47) ^{ef}	100.00(1.57)^{a}	17.82(0.18) ^{efg}
T5	54.29 (0.58) ^{def}	100.00(1.57)^{a}	19.86(0.20) ^{cdefg}
T6	58.17 (0.62) ^{def}	100.00(1.57)^{a}	37.19(0.38) ^{abcde}
T7	48.25(0.50) ^{ef}	100.00(1.57)^{a}	29.12(0.30) ^{abcdefg}
T8	52.13(0.55) ^{ef}	51.11(0.74)^{bcde}	27.77(0.28) ^{abcdefg}
T9	48.68(0.51) ^{ef}	80.56(0.94)^{bc}	24.18(0.24) ^{abcdefg}
T10	49.98(0.52) ^{ef}	59.72(0.64)^{cdefg}	30.46(0.31) ^{abcdefg}
T11	57.74 (0.62)^{def}	100.00(1.57)^{a}	25.47(0.26) ^{abcdefg}
T12	46.10(0.48)^{ef}	100.00(1.57)^{a}	24.45(0.25) ^{abcdefg}
T13	49.55(0.52) ^{ef}	100.00(1.57)^{a}	28.02(0.28) ^{abcdefg}
T14	57.74(0.62) ^{def}	100.00(1.57)^{a}	30.07(0.31) ^{abcdefg}
T15	54.29(0.57) ^{def}	100.00(1.57)^{a}	36.19(0.37) ^{abcdef}
T16	53.86(0.57) ^{def}	100.00(1.57)^{a}	30.58(0.31) ^{abcdefg}
T17	54.29(0.57) ^{def}	100.00(1.57)^{a}	24.63(0.25) ^{abcdefg}
T18	56.88(0.61) ^{def}	100.00(1.57)^{a}	24.41(0.25) ^{abcdefg}
T19	51.27(0.54) ^{ef}	100.00(1.57)^{a}	25.53(0.26) ^{abcdefg}
T20	55.15(0.58) ^{def}	100.00(1.57)^{a}	19.25(0.19) ^{defg}
T21	58.17(0.62) ^{def}	41.85(0.44)^{ef}	24.18(0.24) ^{abcdefg}
T22	51.27(0.54) ^{ef}	62.78(0.70)^{cde}	35.02(0.36) ^{abcdefg}
T23	48.25(0.50) ^{ef}	33.33(0.34)^{fg}	35.02(0.36) ^{abcdefg}
T24	44.80(0.46) ^{f}	67.04(0.76)^{bcde}	40.16(0.41) ^{ab}
T25	51.27(0.54) ^{ef}	47.22(0.50)^{ef}	19.25(0.19) ^{defg}
T26	9.44(0.10) ^{g}	5.93(0.06)^{g}	15.38(0.15) ^{g}
T27	57.74(0.62) ^{def}	52.78(0.56)^{def}	35.95(0.37) ^{abcdef}
T28	59.0(0.63)3 ^{cdef}	100.00(1.57)^{a}	22.39(0.23) ^{bcdefg}
T29	58.17(0.62) ^{def}	86.67(1.06)^{b}	30.34(0.31) ^{abcdefg}
T30	52.13(0.55) ^{ef}	100.00(1.57)^{a}	21.46(0.22) ^{bcdefg}
T31	50.41(0.53) ^{ef}	3.70(0.53)^{def}	42.96(0.44) ^{a}
T32	82.32(0.97) ^{a}	100.00(1.57)^{a}	19.59(0.20) ^{cdefg}
T33	53.86(0.57) ^{def}	100.00(1.57)^{a}	17.25(0.17) ^{fg}
T34	69.38(0.77) ^{abcd}	100.00(1.57)^{a}	25.43(0.26) ^{abcdefg}
T35	48.68(0.51) ^{ef}	100.00(1.57)^{a}	35.64(0.37) ^{abcdef}
T36	75.85(0.86) ^{ab}	100.00(1.57)^{a}	27.43(0.28) ^{abcdefg}
T37	55.15(0.59) ^{def}	100.00(1.57)^{a}	17.42(0.18) ^{fg}
T38	74.13(0.84) ^{abc}	100.00(1.57)^{a}	38.94(0.40) ^{abc}
T39	56.88(0.61) ^{def}	76.92(0.88)^{bcd}	25.93(0.26) ^{abcdefg}
T40	62.73(0.68) ^{bcde}	100.00(1.57)^{a}	37.94(0.39) ^{abcd}
T41	53.03(0.56) ^{def}	100.00(1.57)^{a}	34.43(0.35) ^{abcdefg}
T42	57.62(0.61) ^{def}	100.0(1.57)^{a}	31.93(0.33) ^{abcdefg}
T43	54.03 (0.57) ^{def}	100.00(1.57)^{a}	30.93(0.31) ^{abcdefg}

*Values in parentheses are values after arcsine transformation. Means with at least one letter common are not statistically significant using TUKEY's Honest Significant Difference.

Antibiosis test based on production of volatile compounds by isolates against pathogen

The inhibitory action by volatile metabolites was found to be relatively less. Thirty six isolates could inhibit the mycelial growth significantly. The volatiles of isolates T4, T5, T20, T25, T26, T33 and T37 could not restrict the mycelial growth of *S. rolfsii*. Isolate T31 had maximum percentage inhibition (42.96%) followed by the isolate T24 (40.16%). The least inhibition was with the isolate T26 (15.38%) and this isolate had least inhibition in all three methods adopted for screening the isolates. Among 43 isolates, 18 isolates had more than 30% inhibition of mycelial growth. The volatile compounds produced by *Trichoderma* spp. are physiologically active and it takes part in signaling between microbes (Srinivasa et al., 2017). Various volatile and non volatile secondary metabolites have also been characterized from *Trichoderma* spp. with high antagonistic potential and plant defense promoting activities (Brotman et al., 2010).

Screening results helps to clearly differentiate the isolates based on its antagonistic potential. In the present study, maximum number of *Trichoderma* isolates were collected from the rhizosphere of tuber crop ecosystems in Kerala. Out of 43 isolates, 15 isolates were collected from different fields of ICAR-CTCRI, Sreekariyam. In dual culture, 73.30% of these isolates showed more than 50% inhibition which ranged from 45.67% (isolateT4) - 74.13% (isolate T38). In antibiosis test based on production of diffusible inhibitory metabolites, 86.60% of isolates had 100% inhibition against the test fungus. The inhibition varied from 41.85% (isolate T21) – 100%. Eleven isolates were collected from Pathanamthitta district of Kerala. In this, nine isolates were collected after 2018 flood in Kerala from the rhizosphere of tuber crops in Pathanamthitta district. In dual culture, 63.60% of the isolates showed more than 50% inhibition and which ranged from 9.44% (isolate T26) – 58.17% (isolate T21). Similarly, 36.30% of these isolates had 100% inhibition against the test fungus in antibiosis test based on production of diffusible inhibitory metabolites. The inhibition varied from 3.70% (isolate T31) to 100%. In antibiosis test based on production of volatile compounds by the isolates against pathogen, 53.30% and 63.60% of CTCRI isolates and of Pathanamthitta isolates respectively had more than 30% inhibition. Thus the isolates under study exhibited high variability in their antagonistic potential irrespective of their place of collection or method used for evaluation. The result indicates the need for the use of suitable isolate for the management.

Competition for nutrients or space, production of lytic enzymes, inactivation of the pathogen's enzymes and parasitism are the direct mechanisms exhibited by *Trichoderma* isolates to elicit its bio-control action (Harman, 2006). Maximum inhibition was recorded with the method, production of diffusible metabolites. It suggests the involvement of lytic enzymes and other metabolites in pathogen suppression. The efficiency of *Trichoderma* spp. to manage plant diseases is mainly because of their direct antagonistic effects on the fungal pathogen and mainly their ability to produce lytic enzymes (Benítez et al., 2004; Viterbo et al., 2002). Harman et al. (2004) suggested the key role of antibiosis or production of lytic enzymes during antagonism. The least inhibition was recorded with volatile metabolites. John et al. (2015) reported the inhibition of *S. rolfsii* by volatile metabolites as non significant.

Selection of *Trichoderma* isolates

Trichoderma exhibits numerous mechanisms during pathogen suppression. Hence to select the most potent isolates for further study, additive inhibition effect of the isolates were considered (Table 2). On considering the additive effect of three screening methods, the isolate T38 had maximum inhibition (213.07) followed by the isolates T36 and T32 with scores of 203.28 and 201.90 respectively. The isolates that ranked top 10 may be checked for their field performance in terms of disease

Table 2. The ranking of *Trichoderma* based on cumulative effect shown by the isolates

Isolate code	Cumulative inhibition	Rank
T38	213.07	1
T36	203.28	2
T32	201.91	3
T40	200.67	4
T6	195.36	5
T34	194.81	6
T15	190.48	7
T2	189.70	8
T42	189.55	9
T14	187.81	10

suppression as well as survival in the soil. Based on the field performance of the 10 isolates, most potent isolate may be utilized in bio-intensive management of collar rot in elephant foot yam.

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