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Management of castor gray mold using trichoderma species

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Abstract
Castor graymold caused by *Botryotinia ricini* is one of the most destructive diseases of castor causing heavy yield losses. Ecologically sustainable approaches towards tackling diseases are gaining importance owing to ill-effects of chemical pesticides. One among them is biological control through application of fungal and bacterial antagonists that has wide range applicability against plant diseases. Biological control using antagonistic microorganisms is a potential eco-friendly and sustainable approach, and the effective bioagents can be applied for the efficient management of castor gray mold to ensure better returns. Keeping in view the importance of disease in castor production, the present investigation was carried out to identify the *Trichoderma* species against *B. ricini* under *in vitro* and glasshouse conditions. The antagonistic activity of twelve *Trichoderma* isolates was evaluated against *Amphobotrys ricini* under laboratory and greenhouse conditions. Among the *Trichoderma* isolates tested, *Trichoderma asperellum* isolate 1, *T. harzianum* isolate 5 and *T. asperellum* isolate 3 recorded significantly highest percent inhibition of mycelial growth of *A. ricini*. These *Trichoderma* isolates were found to have high chitinase and glucanase activity compared to other isolates tested. Further, greenhouse experiments have revealed the efficacy of *T. harzianum* isolate 1, *T. asperellum* 2, *T. harzianum* isolate 4 and *T. harzianum* isolate 3 in reducing graymold severity. The antagonistic isolates were found to have high chitinase and glucanase activity.

Keywords: *Amphobotrys ricini*, castor, chitinase, glucanase, gray mould, *Trichoderma*

1. Introduction
Castor (*Ricinus communis* L.), is an important non-edible oilseed crop. It contains 50-55% oil and plays a vital role in Indian vegetable oil economy. It is mainly grown in tropical and sub-tropical climate. Castor is known to suffer from many fungal and bacterial diseases at different crop growth stages. Among these, one of the most destructive diseases of castor, primarily infecting inflorescence and racemes, is gray mold, caused by the fungus, *Amphobotrys ricini* (N.F. Buchw.) Hennebert. Yield losses are extensive due to this disease and are a major threat to commercial cultivation of the crop. Seed yield loss up to 100% was reported from India (Soares, 2012) [17].

Plants are surrounded by a diverse range of organisms in their environment, including bacteria, fungi, oomycetes, nematodes, insects and viruses. Although some of these organisms may have a negative impact on the plant, others may exert beneficial effects by enhancing the general fitness of the plant and/or by suppressing plant disease. Research on such biocontrol organisms has intensified during recent decades and their importance has increased as a part of integrated management practices to reduce chemical pesticide use (Glare *et al*., 2012) [9].

Over the past century, growers have relied heavily on the use of chemicals to control diseases caused by *Amphobotrys* species. Fungicide resistance coupled with current public concern for both the environment & pesticide residues in food has highlighted the need for alternative methods for disease control and biological control is one such approach. Biological control may result from direct or indirect interactions between the beneficial microorganisms and the pathogen (Benítez *et al*., 2004; Viterbo *et al*., 2007) [3, 19]. A direct interaction may involve physical contact and synthesis of hydrolytic enzymes, toxic compounds or antibiotics as well as competition. An indirect interaction may result from induced resistance in the host plant, the use of organic soil amendments to improve the activity of antagonists against the pathogens (Viterbo *et al*., 2007) [19].

The genus *Trichoderma* comprises a great number of fungal strains with biocontrol capacity. They have adapted to diverse environmental conditions and are often the most frequently isolated fungi from soil (Harman *et al*., 2004) [8]. They are prolific producers of extracellular proteins, including enzymes that degrade cellulose and chitin (Nagy *et al*., 2007) [14]. Moreover, their capacity to reduce plant disease has been studied intensively, with both direct and indirect effects on plant pathogens (Lorito *et al*., 2010) [10]. Their high reproductive capacity, ability to survive under unfavourable conditions and high nutrient utilization efficiency contribute to their success as biocontrol agents (Benitez *et al*., 2004) [3].
There are several reports on the use of *Trichoderma* species as biological agents against plant pathogens (Harman *et al*., 2004) [8]. *Trichoderma* species (*T. harzianum, T. viride, T. virens* etc.) have been used as biological control agents against a wide range of pathogenic fungi like *Rhizoctonia, Pythium, Botrytis, Fusarium, Phytophthora*, etc. (Benítez *et al*., 2004; Zeilinger and Omann, 2007) [3, 20]. *T. harzianum* Th4d and *T. asperellum* Tv5 were most effective in inhibiting the pathogen. The growth inhibition was attributed to the production of mycolytic and defense related enzymes by the *Trichoderma* species (Navaneetha *et al*., 2015) [15]. The present investigation was undertaken to evaluate the effect of Trichoderma species against *A. ricini* under laboratory and greenhouse conditions.

2. Materials and Methods

The present experiments were carried out in the Department of Plant Pathology at College of Agriculture, Rajendranagar and Division of Crop Protection, Indian Institute of Oilseeds Research (IIOR), Rajendranagar, Hyderabad, Telangana in the year 2015 period of six months**.**

2.1 Evaluation of *Trichoderma* spp. against *A. ricini* under laboratory conditions

Twelve *Trichoderma* isolates obtained from the Department of Plant Pathology, College of Agriculture, Rajendranagar, Hyderabad were screened for antagonism against *A. ricini* by dual culture method (Mortan and Sproube, 1955) [13]. Per cent inhibition of mycelial growth of *A. ricini* was calculated as per the formula of Vincent (1927) [18].

2.2 Determination of chitinase and glucanase activity of potential antagonists

Plate assay for determining the chitinase activity was conducted using 500 ml mineral salt solution, 500 ml of distilled water, 0.02% yeast extract, 15 g of agar and 2.4% of purified chitin (N-acetyl glucosamine from crab shells, Sigma chemical Co., St. Louis) as described by Campbell and Williams (1951) [5]. The assay for determining the activity of glucanase was conducted using the same medium as described for chitinase activity, but substituting the chitin with 0.5% (w/v) laminarin.

Assay procedure

All plates containing chitin and glucan were inoculated with the antagonists and were incubated at room temperature for 4-6 days and then flooded with 1% congo red solution in water. The stain was removed after 30 min and plates were destained with 1M Nacl in buffer solution for 15 min (Hagerman and Butler, 1985) [7]. Clear zones developed in the opaque agar around the colonies indicated the degradation of the substrate. The isolates were classified into different groups based on the zone of clearance (Low chitinase/glucanase activity - $\leq$0.5 cm, medium chitinase/glucanase activity - 0.51 to 0.99 cm and high chitinase/glucanase activity- $\geq$1.0 cm). Plates which were not flooded with any of the stains described above, served as control.

2.3 Evaluation of *Trichoderma* spp. against *A. ricini* under green house conditions

The *Trichoderma* spp. were screened for their biocontrol potential against *A. ricini* using detached spike assay in a closed polythene humid chamber. Racemes/ spikes were collected from castor plants (var. DCH-519) and were kept in conical flasks containing 2% sucrose solution and are arranged on a green house bench in a randomized complete block design (RCBD) and replicated thrice. Racemes were sprayed with conidial suspension (10^7conidia/ml) of *Trichoderma* spp. and inoculum (10^6conidia/ml) of *A. ricni* was sprayed 24 hours after pathogen inoculation. Racemes sprayed with water alone or inoculated with spore suspension (10^6 conidia/ml) of *A. ricini* alone served as healthy and inoculated controls. Treated racemes were transferred to polythene humid chamber with fogging devices in which temperature and humidity were maintained at 22 ± 2^0C and relative humidity at 90%. Per cent infected capsules in each treatment were calculated when maximum number of capsules was infected in control using the formula:

$$\text{Per cent infected capsules} = \frac{\text{Number of infected capsules in a raceme}}{\text{Total number of capsules in a raceme}} \times 100$$

The disease progress in various treatments was assessed using the disease severity scale given by Mayee and Datar *et al*. (1996) [11] when maximum disease severity is observed in inoculated control.

3. Results and Discussion

3.1 Evaluation of *Trichoderma* spp. against *A. ricini* under *in vitro* conditions

Five isolates of *Trichoderma asperellum* and seven isolates of *T. harzianum* obtained from the Department of Plant Pathology, College of Agriculture, Rajendranagar were screened for their antagonistic potential against *A. ricni* by dual culture method. The perusal of the data in Table 1 indicated that all the *Trichoderma* isolates tested were highly effective in inhibiting the mycelial growth of *A. ricini*. Among the *Trichoderma* isolates tested, *Trichoderma asperellum* isolate 1, *T. harzianum* isolate 5 and *T. asperellum* isolate 3 (77.96, 77.41 and 77.04 per cent, respectively) recorded maximum inhibition of mycelial growth of *A. ricini* which were found statistically on par with each other. These were followed by *T. harzianum* isolate 4 with mycelial growth inhibition of 75.56 per cent.
Trichoderma harzianum isolate 2, *T. asperellum* 2 and *T. asperellum* 4 were statistically on par with each other and recorded an inhibition of mycelial growth by 68.15, 67.60 and 65.92 per cent, respectively. These were followed by *T. harzianum* isolate 3, *T. harzianum* isolate 7 and *T. asperellum* isolate 5 with mycelial growth inhibition of 65.00, 62.59 and 62.22 per cent. Least inhibition of mycelial growth of test fungus was observed with *T. harzianum* isolate 6 (56.85%) and *T. harzianum* isolate 1 (58.52%) which were statistically on par with each other

The present investigations are in agreement with the findings of Raoof *et al.* (2003) [16] who tested the efficacy of different strains of *Trichoderma* spp. against *Botrytis ricini* under *in vitro* conditions. Highest inhibition of the test pathogen was observed in *T. asperellum* treatment followed by *T. koningii* under dual culture assays and the inhibition of pathogen by *Trichoderma* spp. was observed 72 hours after incubation and after 96 hours, the hyphae of *Trichoderma* overgrew on *B. ricini*. Similarly, the effectiveness of *T. viride* and *T. harzianum* was established by Bhattiprolu and Bhattiprolu (2006) [4].

Navaneetha *et al*. (2015) [15] conducted extensive studies on the efficacy of *Trichoderma* species against *A. ricini* and found that *T. harzianum* Th4 and *T. asperellum* Tv5 were most effective in inhibiting the pathogen. The growth inhibition was attributed to the production of mycolytic enzymes by the *Trichoderma* species.

Table 1: *In vitro* evaluation of *Trichoderma* spp. in reducing the mycelial growth of *A. ricini*

Sl. No.	Treatment	Per cent inhibition of mycelial growth
1	*Trichoderma asperellum* isolate 1	77.96 (61.98)*
2	*Trichoderma asperellum* isolate 2	67.60 (55.28)
3	*Trichoderma asperellum* isolate 3	77.04 (61.36)
4	*Trichoderma asperellum* isolate 4	65.92 (54.27)
5	*Trichoderma asperellum* isolate 5	62.22 (52.05)
6	*Trichoderma harzianum* isolate 1	58.52 (49.89)
7	*Trichoderma harzianum* isolate 2	68.15 (55.62)
8	*Trichoderma harzianum* isolate 3	65.00 (53.71)
9	*Trichoderma harzianum* isolate 4	75.56 (60.35)
10	*Trichoderma harzianum* isolate 5	77.41 (61.61)
11	*Trichoderma harzianum* isolate 6	56.85 (48.92)
12	*Trichoderma harzianum* isolate 7	62.59 (52.27)
CD (P=0.05)		1.521
SEm (±)		0.518
CV (5%)		1.613

*Figures in the parenthesis are angular transformed values

3.2 Screening of *Trichoderma* isolates for chitinase production on solid medium supplemented with colloidal chitin

Chitinase activity exhibited by 12 *Trichoderma* isolates was determined by the diameter of the clear zone developed around the colonies indicating the degradation of colloidal chitin to N-acetyl glucosamine and the results are presented in Table 2. The *Trichoderma* isolates, *T. asperellum* isolate 1, *T. asperellum* isolate 3 and *T. harzianum* isolate 5 represented high chitinase activity group with 1.43, 1.23 and 1.63 cm zone of clearance on medium amended with colloidal chitin. Four isolates (*T. asperellum* isolate 4, *T. asperellum* isolate 5, *T. harzianum* isolate 4, *T. harzianum* isolate 6, *T. harzianum* isolate 7) expressed medium chitinase activity. Low chitinase activity was observed in the isolates, *T. asperellum* isolate 2, *T. harzianum* isolate 1, *T. harzianum* isolate 2, *T. harzianum* isolate 3, *T. harzianum* isolate 6 and *T. harzianum* isolate 7.

Trichoderma species are known to produce chitinolytic enzymes and are most effective agents of biological control of plant diseases (Agarwal and Kotasthane, 2012) [1]. In the present investigation, high chitinolytic strains of *Trichoderma*, *T. asperellum* isolate 1, *T. asperellum* isolate 3 and *T. harzianum* isolate 5 have shown maximum inhibition of mycelial growth of *A. ricini* compared to medium or low chitinolytic isolates under *in vitro* conditions.

Table 2: *In vitro* evaluation of *Trichoderma* spp. for chitinase production on solid medium supplemented with colloidal chitin

Sl. No.	Isolate	Zone of clearance (cm)
1	*Trichoderma asperellum* isolate 1	1.43*
2	*Trichoderma asperellum* isolate 2	0.48
3	*Trichoderma asperellum* isolate 3	1.23
4	*Trichoderma asperellum* isolate 4	0.53
5	*Trichoderma asperellum* isolate 5	0.75
6	*Trichoderma harzianum* isolate 1	0.38
7	*Trichoderma harzianum* isolate 2	0.35
8	*Trichoderma harzianum* isolate 3	0.40
9	*Trichoderma harzianum* isolate 4	1.00
10	*Trichoderma harzianum* isolate 5	1.63
11	*Trichoderma harzianum* isolate 6	0.60
12	*Trichoderma harzianum* isolate 7	0.70

*Mean of four replications

3.3 Screening of *Trichoderma* isolates for glucanase production on solid medium supplemented with laminarin

Glucanase activity was determined for twelve isolates of *Trichoderma* and it was observed that a clear zone developed around the colonies on medium amended with laminarin. The data in Table 3 showed that *T. asperellum* isolate 1 produced maxuimum β-1,3-glucanase activity with 1.18 cm zone diameter followed by *T. asperellum* isolate 3 (0.95 cm) and *T. harzianum* isolate 5 (0.83 cm). Among the test isolates, the least β-1,3-glucanase activity was recorded in *T. harzianum* isolate 1 (0.38 cm).

Trichoderma asperellum isolate 1 represented high β-1,3-glucanase group followed by *T. asperellum* isolate 3, *T. harzianum* isolate 5, *T. harzianum* isolate 7, *T. harzianum* isolate 3 and *T. asperellum* isolate 4 which represented medium β-1,3-glucanase group. However the remaining isolates (*T. asperellum* isolate 2, *T. asperellum* isolate 5, *T. harzianum* isolate 1, *T. harzianum* isolate 2 and *T. harzianum* isolate 6) represented low β-1,3-glucanase group.

The extracellular enzymes produced by *Trichoderma* isolates can be correlated with the antagonism. Elad *et al*. (1982) [6] reported that the isolates of *T. harzianum* differ in their ability to suppress plant pathogens based on the levels of mycolytic enzymes produced by them. Results from the present study revealed that most of the isolates have shown good enzymatic activities. *Trichoderma* directly attacks the plant pathogen by excreting lytic enzymes such as chitinases, β-1,3-glucanases, proteases and also with the production of volatile and non-volatile metabolic compounds.

Table 3: *In vitro* evaluation of *Trichoderma* spp. for glucanase production on solid medium supplemented with laminarin

Sl. No.	Isolate	Zone of clearance (cm)
1	*Trichoderma asperellum* isolate 1	1.18*
2	*Trichoderma asperellum* isolate 2	0.50
3	*Trichoderma asperellum* isolate 3	0.95
4	*Trichoderma asperellum* isolate 4	0.53
5	*Trichoderma asperellum* isolate 5	0.50
6	*Trichoderma harzianum* isolate 1	0.38
7	*Trichoderma harzianum* isolate 2	0.45
8	*Trichoderma harzianum* isolate 3	0.78
9	*Trichoderma harzianum* isolate 4	0.73
10	*Trichoderma harzianum* isolate 5	0.83
11	*Trichoderma harzianum* isolate 6	0.43
12	*Trichoderma harzianum* isolate 7	0.78

*Mean of four replications

3.4 Effect of *Trichoderma* spp. on castor gray mold severity under green house conditions

Five *T. asperellum* isolates and seven *T. harzianum* isolates were evaluated under green house conditions for their efficacy against gray mold of castor using detached spike technique. The results are presented in Table 4. The perusal of the data indicated that all the treatments have significantly reduced the per cent infected capsules compared to inoculated control except *T. harzianum* isolate 2 and *T. harzianum* isolate 5. The per cent infected capsules were minimum in racemes pre-inoculated with *Trichoderma* isolates (Plate 1), *T. harzianum* isolate 1 (5%), *T. asperellum* 2 (6.67%), *T. harzianum* isolate 4 (6.67%) and *T. harzianum* isolate 3 (12%) which were statistically on par with each other. These were followed by *T. asperellum* isolate 4 (16.33%) which is significantly at par with *T. asperellum* isolate 5 (20.67%). There was no significant difference between the raceme sprays with T10 (25.0%) and *T. harzianum* isolate 7 (29.00%). More than fourty per cent of the capsules were infected in racemes sprayed with *T. asperellum* isolate 3 (41.67%) and *T. asperellum* isolate 1 (50.0%). The per cent infected capsules were maximum in racemes sprayed with *T. harzianum* isolate 2 (86.0%) and *T. harzianum* isolate 5 (91.33%), which were on par with inoculated control. The efficacy of various treatments in terms of disease control is depicted in Fig.1.

The results are in accordance with Raoof *et al*. (2003) [16] who tested the efficacy of different strains of *Trichoderma* spp. against *Botrytis ricini* under green house conditions using detached spike method. It was observed that spraying of *T. asperellum* on castor racemes reduced the disease up to 45 per cent. Similar results were also obtained by Bhattiprolu and Bhattiprolu (2006) [4] under field conditions by spraying castor spikes with *T. viride* (10^6 spores ml^{-1}) immediately after the disease appearance. Antagonism of three *Trichoderma* species (*T. harzianum*, *T. viride* and *T. longibrachiatum*) was evaluated against *Botrytis fabae* and *B. cinerea*, the causal agents of chocolate spot of Faba bean (*Vicia faba* L.) in Algeria. Among the *Trichoderma* isolates tested, *T. harzianum* and *T. longibrachiatum* were shown to over grow the colony of *Botrytis*, whereas *T. viride* was known to suppress the *Botrytis* species by antibiosis (Bendahmane *et al*., 2012) [2]. Research on such biocontrol organisms has intensified during recent decades and their importance has increased as a part of integrated management practices to reduce chemical pesticide use (Glare *et al*., 2012) [9].

Table 4: Greenhouse evaluation of *Trichoderma* species in reducing castor gray mold disease caused by *A. ricini*

S. No.	Treatments	Mean per cent infected capsules	Severity rating
1	*Trichoderma asperellum* isolate 1	50.00 (44.98)	6
2	*Trichoderma asperellum* isolate 2	6.67 (14.75)	3
3	*Trichoderma asperellum* isolate 3	41.67 (40.15)	6
4	*Trichoderma asperellum* isolate 4	16.33 (23.50)	4
5	*Trichoderma asperellum* isolate 5	20.67 (26.75)	4
6	*Trichoderma harzianum* isolate 1	5.00 (12.74)	3
7	*Trichoderma harzianum* isolate 2	86.00 (68.14)	9
8	*Trichoderma harzianum* isolate 3	12.00 (20.08)	4
9	*Trichoderma harzianum* isolate 4	6.67 (14.75)	3
10	*Trichoderma harzianum* isolate 5	91.33 (73.79)	9
11	*Trichoderma harzianum* isolate 6	25.00 (29.67)	4
12	*Trichoderma harzianum* isolate 7	29.00 (32.39)	5
13	Control	94.00 (75.92)	9
CD (P=0.05)		8.00	
SEm (±)		2.73	
CV (5%)		12.85	

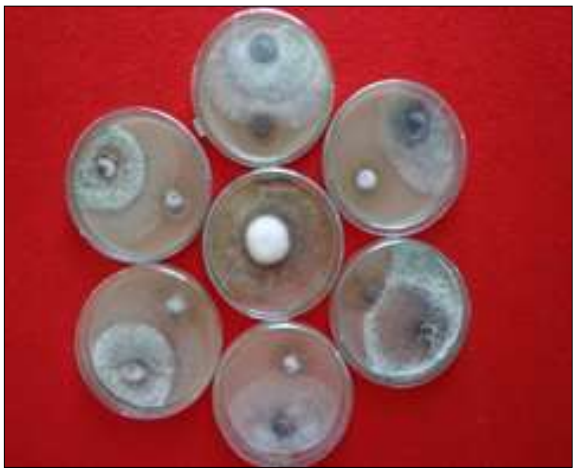

Plate 1: Antagonistic activity of *Trichoderma* spp. against *Botryotinia ricini*

Plate 2: Effect of *Trichoderma asperellum* (Isolate 2) on castor gray mold disease under green house conditions

Plate 3: Chitinase activity of *Trichoderma asperellum* (Isolate1)

Plate 4: Glucanase activity of *T. harzianum* isolate 5 & *T. asperellum* isolate 3

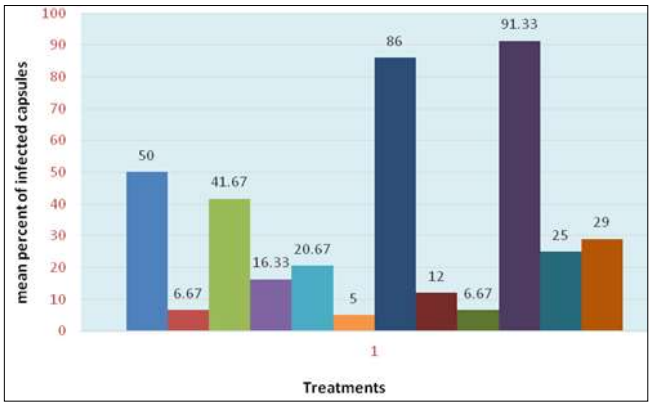

Fig 1: Effect of trichoderma spp. On castor gray mold severuty under green house conditions

4. Conclusion

To conclude, the *Trichoderma* isolates, *T. harzianum* isolate 1, *T. asperellum* 2 and *T. harzianum* isolate 4 were identified as potential bioagents in management of gray mold of castor with high or medium chitinase and glucanase activity.

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