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Characterization of Biocontrol Agents Isolated From Temperate Region of India

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Trichoderma **species are potential fungal bio-control agents used against a wide range of soil borne plant pathogens. In the present study a total of 20** ***Trichoderma*** **isolates viz., AT1, AT2, AT3, AT4, AT5, AT6, AT7, BT1, BT2, BT3, BT4, BT5, BT6, BT7, BT8, BT9, BT10, BT11, BT12, BT13 were isolated from vegetable fields of Kashmir valley and their efficacy was tested by using various biochemical tests. Thirteen isolates of** ***Trichoderma*** **viz., AT1, AT2, AT3, AT4, AT5, AT6, AT7, BT1, BT7, BT8, BT10, BT11 and BT12 were found to be positive for ammonia production. Similarly twelve isolates viz., AT2, AT3, AT7, BT1, BT3, BT4, BT7, BT8, BT10, BT11, BT12 and BT13 were found to be positive as far as the chitinase activity is concerned. In the IAA production assay maximum IAA was produced by isolate BT9 (6.605 µg mL^{-1}) followed by BT6 (5.278 µg mL^{-1}), while minimum IAA was produced by isolate AT1 (1.538 ìg mL^{-1}). Only five isolates viz., AT1, AT2, AT3, AT4 and AT5 metabolized lactose and sucrose while seven isolates viz., AT1, AT2, AT3, AT4, AT5, AT6 and AT7 were found to metabolize maltose. Hydrocyanic acid (HCN) was observed to be produced by only three isolates of** ***Trichoderma*** **viz., AT3, AT5 and AT7.** ***Trichoderma*** **isolate AT3 qualifying most of the biochemical tests were morphological characterized as** ***Trichoderma harzianum.***

Keywords: *Trichoderma,* Biochemical Characterization, Morphological Characterization.

The practices of integrated agricultural management, where chemicals are either replaced or minimized by by-products, are the most suitable option (Cavalcante *et al*., 2008). Biological control agents (BCAs) are by-products based on microorganisms that cause harmful alterations to plant pathogens by chemical or physical processes (Vinale *et al.,* 2008). BCA differ from chemical agents in that to be effective, they need to grow and successfully colonize and therefore they need to be applied in high and frequent quantities (Lo *et al*., 1998). Fungi from the genus Trichoderma spp. have a long history of successful control of plant diseases (Benitez *et al.,* 2004). *Trichoderma* play an important role not only in controlling the plant diseases but also in increasing growth and yield of the plants (Chadha *et al.*, 2015), so different biochemical attributes leading to its effective use against various pathogens are of paramount importance. There is a vast number of biochemical being released by *Trichoderma* species to bring about suppression of a huge number of phytopathogens and these biochemicals may be released constitutively or may be induced by the pathogen presence (Aarti and Meenu, 2015; Rawat and Tewari, 2011) .

What we observe and define as biocontrol may be the culmination of a number of different mechanisms working synergistically to achieve disease control. Our knowledge of the complexity of these systems is currently limited by our ability to perceive them, and a great deal of research will have to be undertaken in order to

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fathom exactly what is taking place during the biocontrol process. As with so many other aspects of science, basic knowledge about the mechanisms involved in the biocontrol process will be of immense value to that intent on developing new methods for utilizing biocontrol agents. With this aim present study was undertaken to study the various biochemicals involved in the process of plant growth promotion by *Trichoderma* species.

MATERIALS AND METHODS

Sample Collection

Rhizospheric soil samples were collected from various commercially grown chilli fields and kitchen gardens of district Anantnag (Bangidar, BagiWanpoh, Danter, Harnag) and Baramulla (Delina, Arampora, Palhalan and Johama) of Kashmir valley. Twenty (20) samples from each district were randomly collected, out of which 10 were taken from commercially grown chilli fields and 10 from local kitchen gardens.

Biochemical Characterization

Trichoderma species isolated from soil samples were characterized for carbohydrate metabolization (Bakker and Schipper, 1987), phosphate solubilization (Pikovskaya, 1948), ammonia (Bakker and Schipper, 1987), chitinase (Okay *et al.*, 2008), HCN (Bakker and Schipper, 1987) and IAA production (Brick *et al.*, 1991).

Morphological Characterization

Distinct cultural and morphological characteristics were observed for identification, and the plates were stored at 4 °C. Cultural and morphological characteristics which were studied include colony growth rate, colony colour, reverse colour, colony edge, mycelial form, conidiophore branching, conidial colour and presence or absence of chlamydospores (Shahid *et al.*, 2013).

RESULTS AND DISCUSSION

Isolation of *Trichoderma* species from vegetable fields

A total of 20 (7 from Anantnag and 13 from Baramulla) *Trichoderma* isolates (plate 1) were isolated from 40 randomly collected rhizospheric soil samples from various commercially grown chilli fields and kitchen gardens of district Anantnag and Baramulla of Kashmir valley by using *Trichoderma* specific medium. Nomenclature given to these *Trichoderma* isolates, the collection sites and source of soil samples is detailed in Table 1. Similarly *Trichoderma* species were isolated from chilli rhizosphere by Wani *et al.* (2014) and the technique used for isolation of *Trichoderma* species is in agreement with chaudhari *et al.* (2011) and Khandelwal *et al.* (2012).

Screening of *Trichoderma* isolates for ammonia production, chitinase activity, HCN production and phosphate solubilisation

During ammonia production test thirteen isolates (AT1, AT2, AT3, AT4, AT5, AT6, AT7, BT1, BT7, BT8, BT10, BT11 and BT12) were found to be positive and seven isolates (BT2, BT3, BT4, BT5, BT6, BT9 and BT13) were found negative for ammonia production (Table 2). These findings are in agreement with the earlier reports (Aarti and Meenu, 2015). Similar findings were reported by Chadha *et al.* (2015) in *Mucor hiemalis*, *Aspergillusniger*and *Fusarium moniliforme*. Ammonia production by *T. harzianum*, KT6, SE6, KT28 and BRT11 has widely been documented (Rawat and Tewari, 2011) as means to offer nitrogen to plant and culminate the pathogens in the vicinity as a result of its toxicity. The production of lytic enzymes by *Trichoderma* species is known as one of the major mechanisms for biocontrol activity against phytopathogenic fungi (Lorito *et al.*, 1994). Chitinase activity is one of the important beneficial character exhibited by *Trichoderma* species as chitinases are known to contribute to the biocontrol properties of *Trichoderma atroviride* (Limon *et al.*, 1999; Woo *et al.*, 1999; Viterbo *et al.*, 2001). This might be due to the reason that chitinases attack directly on the fungal structural components (Sela-Buurlage *et al.*, 1993), while checking chitinase activity twelve isolates (AT2, AT3, AT7, BT1, BT3, BT4, BT7, BT8, BT10, BT11, BT12 and BT13) were found to be positive and eight isolates (AT1, AT4, AT5, AT6, BT2, BT5, BT6 and BT9) were found negative (Table 2). This is in agreement with earlier reports (Sharaf *et al.*, 2012). Lytic enzymes like chitinases and β-1,3-glucanases, proteases and cellulases are potential mechanism associated with the ability of *Trichoderma* to control phytopathogens (Harighi *et al.*, 2007).

HCN production is also an important trait found in various soil micro-organisms as it indirectly promotes plant growth by controlling

Table 1. Details of *Trichoderma* isolates isolated from rhizospheric soils of chilli fields

District	Location	Isolate name	Source
Anantnag	Danter	AT1	Kitchen garden
	Bangidar	AT2	Commercial field
	Bangidar	AT3	Commercial field
	Harnag	AT4	Commercial field
	Harnag	AT5	Kitchen garden
	BagiWanpoh	AT6	Commercial field
	BagiWanpoh	AT7	Commercial field
Baramulla	Delina	BT1	Kitchen garden
	Delina	BT2	Kitchen garden
	Delina	BT3	Kitchen garden
	Arampora	BT4	Commercial field
	Arampora	BT5	Commercial field
	Arampora	BT6	Commercial field
	Arampora	BT7	Commercial field
	Palhalan	BT8	Commercial field
	Palhalan	BT9	Commercial field
	Palhalan	BT10	Commercial field
	Johama	BT11	Kitchen garden
	Johama	BT12	Kitchen garden
	Johama	BT13	Kitchen garden

some soil borne diseases (Kremer and Souissi, 2001; Siddiqui *et al.*, 2006), while screening for HCN only three isolates (AT3, AT5 and AT7) were found positive and rest were found negative. This is in agreement with earlier reports (Aarti and Meenu., 2015). The metabolite HCN production by fungal isolates SE6, KT28 and BRT11 has widely been reported as a possible mechanism of disease control (Rawat and Tewari 2011). Similar results were obtained by Ngoma *et al.* (2013) in case of bacterial isolates. While in case of phosphate solubilisation test, none of the isolate was found to be positive. *Trichoderma* species are widely known for releasing phosphatases into soil and release the otherwise unavailable phosphate forms from tricalcium phosphate primirily (Saraf *et al.*, 2013). Phosphate solubilizing efficiency of different isolates of *T. harzianum* was observed by Tallapragada and Gudimi (2011) on Sperber's medium with modifications and it was observed that many of the isolates of Trichoderma isolates do not solubilize phosphate.

Table 2. Screening of various isolates of *Trichoderma* species for ammonia production, chitinase activity, HCN production and Phosphate solubilisation

S. No.	Isolates	Ammonia production	Chitinase activity	HCN production	Phosphate solubilisation
1	AT1	+	-	-	-
2	AT2	+	+	-	-
3	AT3	+	+	+	-
4	AT4	+	-	-	-
5	AT5	+	-	+	-
6	AT6	+	-	-	-
7	AT7	+	+	+	-
8	BT1	+	+	-	-
9	BT2	-	-	-	-
10	BT3	-	+	-	-
11	BT4	-	+	-	-
12	BT5	-	-	-	-
13	BT6	-	-	-	-
14	BT7	+	+	-	-
15	BT8	+	+	-	-
16	BT9	-	-	-	-
17	BT10	+	+	-	-
18	BT11	+	+	-	-
19	BT12	+	+	-	-
20	BT13	-	+	-	-

Table 3. Cultural and morphological characteristics of *Trichoderma* isolates

Name of the isolate	Colony color	Colony edge	Common characters among all isolates
AT1	Dark green	Wavy	Conidial color: GreenConidiophore branching: Highly branched
AT2	Dark green	Wavy	regularMycelial form Floccose to ArachnoidReverse color :
AT3	Dark green	Wavy	ColorlessColony growth rate (cm/day) : 8-9 in 3
AT4	Dark green	Wavy	daysMycelial color : Watery white
AT5	Dark green	Wavy	
AT6	Dark green	Wavy	
AT7	Dark green	Wavy	
BT1	Light green	Wavy	
BT2	Light green	Wavy	
BT3	Light green	Smooth	
BT4	Light green	Smooth	
BT5	Light green	Smooth	
BT6	Light green	Wavy	
BT7	Light green	Wavy	
BT8	Light green	Wavy	
BT9	Light green	Smooth	
BT10	Light green	Smooth	
BT11	Light green	Smooth	
BT12	Light green	Smooth	
BT13	Light green	Smooth	

Table 4. Carbohydrate metabolisation by various isolates of *Trichoderma* species

S. No.	Isolates	Carbohydrate metabolisation				
		Glucose	Fructose	Lactose	Sucrose	Maltose
1	AT1	+	+	+	+	+
2	AT2	+	+	+	+	+
3	AT3	+	+	+	+	+
4	AT4	+	+	+	+	+
5	AT5	+	+	+	+	+
6	AT6	+	+	-	-	+
7	AT7	+	+	-	-	+
8	BT1	+	+	-	-	-
9	BT2	+	+	-	-	-
10	BT3	+	+	-	-	-
11	BT4	+	+	-	-	-
12	BT5	+	-	-	-	-
13	BT6	+	-	-	-	-
14	BT7	+	+	-	-	-
15	BT8	+	+	-	-	-
16	BT9	+	+	-	-	-
17	BT10	+	+	-	-	-
18	BT11	+	+	-	-	-
19	BT12	+	+	-	-	-
20	BT13	+	+	-	-	-

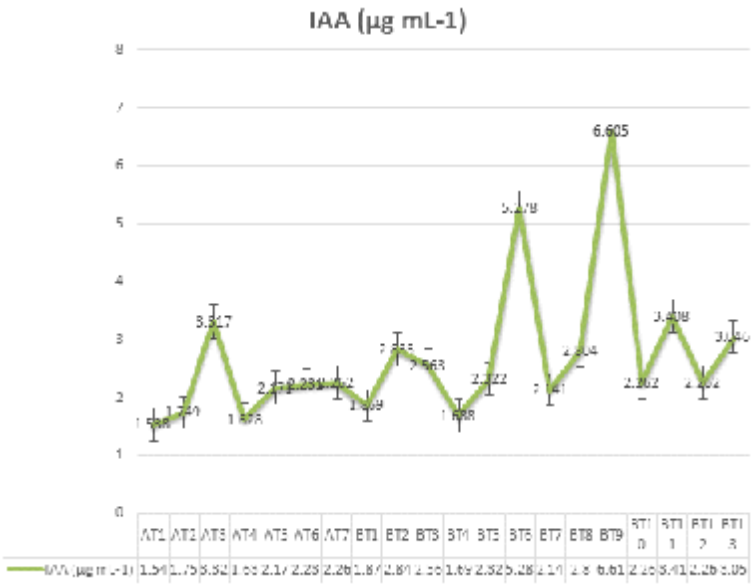

Fig. 1. Production of IAA by various isolates of *Trichoderma* species

Production of IAA by various isolates of *Trichoderma* specie

Microbial synthesis of the phytohormone auxin (indole-3-acetic acid/indole acetic acid/IAA) has been known for a long time. It is reported that 80% of microorganisms isolated from the rhizosphere of various crops possess the ability to synthesize and release auxins as secondary metabolites (Patten and Glick, 1996). During IAA production test all the isolates were found to produce IAAhowever their production amount varied considerably. Maximum IAA was produced by isolate BT9 (6.605 ìg mL^{-1}) followed by BT6 (5.278 ìg mL^{-1}), BT11 (3.408 ìg mL^{-1}) and AT3 (3.317 ìg mL^{-1}) while minimum IAA was produced by isolate AT1 (1.538 ìg mL^{-1}) (Fig. 1). Resende *et al.* (2014) also reported the production of IAA by various isolates of *Trichoderma* and the amount of IAA produced varied from as low as 1.21ìg mL^{-1} and as high as 2.18 ìg $mL^{-1}$and thus supporting our findings. Similar findings were recorded by Aarti and Meenu (2015), Gravel *et al.* (2007) and Badawi *et al.* (2011) in *Trichoderma* species.

Carbohydrate metabolization by various isolates of *Trichoderma* species

During these tests, it was found that all the isolates metabolized glucose. Similarly all the isolates metabolized Fructose except isolates BT5 and BT6. It was found that only five isolates (AT1, AT2, AT3, AT4 and AT5) metabolized lactose and sucrose while seven isolates (AT1, AT2, AT3, AT4, AT5, AT6 and AT7) were found to metabolize maltose (Table 3). Kubicek *et al.* (2003) also detected the species-specific metabolic properties of *Trichoderma*. The carbon sources supported best growth in all species detected were: d-mannitol, *N*-acetyl-d-glucosamine, l-erythritol, glycerol, fructose, fucose, l-arabinose, d-galactose, and xylitol and thus authenticatingour findings further even though there were some isolates lacking the ability to metabolize lactose and sucrose may be as a result of certain enzymatic complications. Similar findings were observed by Monga (2001) and Mehta *et al.* (2012).

Cultural and morphological characterization of *Trichoderma* isolate (AT3)

Although cultural and morphological characteristics of all the isolates were studied, our main focus was on the potential *Trichoderma* isolate (AT3). The characteristics which were studied include colony growth rate, colony colour, reverse colour, colony edge, mycelial form, conidiophore branching, conidial colour and presence or absence of chlamydospores (Table 4). After studying these characteristics *Trichoderma* isolate (AT3) was found to resemble *Trichoderma harzianum*.

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