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Evaluation of different substrates for mass multiplication of *Trichoderma* species

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Abstract
The present study was conducted to isolate efficient *Trichoderma* species which not only are being used for the management of plant diseases but also for increasing the production of vegetables. Twenty isolates of *Trichoderma* viz., AT1, AT2, AT3, AT4, AT5, AT6, AT7, BT1, BT2, BT3, BT4, BT5, BT6, BT7, BT8, BT9, BT10, BT11, BT12, BT13 were isolated from various commercial and kitchen gardens of chilli from district Anantnag and Baramulla of Kashmir valley. Among *Trichoderma* isolates, AT3 was found best in almost all the biochemical tests and characterized as *Trichoderma harzianum*. Among various substrates viz., charcoal, ash, sawdust, cowdung, vermicompost, maize seeds, wheat seeds, paddy seeds, dal weed and talc used for mass multiplication of *Trichoderma harzianum*, maximum growth was retained on maize seeds and wheat seeds with 38×10^7 cfu/g, followed by cowdung (37.5×10^7) and talc (37×10^7) cfu/gram of formulation. Maximum growth was retained at 10°C in comparison with 20 and 30°C. Among supplements molasses was found to be the best compared to yeast extract and glycerol. It is concluded from the present study that *Trichoderma* isolate (AT3) isolated from commercial field of Bangidhar Anantnag retains maximum cfu on maize seeds at 10°C and by using molasses as a supplement.

Keywords: Isolation, Biocontrol agent, Cowdung, Vermicompost, Maize seeds

Introduction
The biocontrol activity of *Trichoderma* is of immense importance not only to agriculture and its crops but also to the environment as it does not accumulate in the food chain and thus does no harm to the plants, animals and humans (Monte and Llobell, 2003; Perveen and Bokhari, 2012; Reena *et al.*, 2013) [21, 26, 27]. A major limitation of biocontrol by *Trichoderma* strains is the production of inoculum on large scale. Cowdung and decomposed poultry refuse are noted as excellent, low-cost and easily available substrates for growth of *Trichoderma harzianum* (Sawant and Sawant, 1996) [32]. Once active strains have been identified with the *in-vitro* assays, a further selection must be done by studying other factors such as tolerance of high or low temperatures, suitability for formulation as foliar sprays and/or soil enhancement, spore viability in stored and field conditions, shelf-life and inoculum efficacy under commercial conditions. Formulation and shelf-life are of prime importance for commercial use of any biological agent. There is abundant literature on the use of conventional synthetic media like glucose, cellulose, soluble starch and molasses to produce *Trichoderma* species (Gupta *et al.*, 1997) [11]. However, the cost of these raw materials for commercial production of biocontrol agents is one of the major limitations behind the restricted use. To overcome the cost limitation, many researchers have successfully used substrates like composted coir pith, coffee (Rukmani and Mariappan, 1993) [28], coffee wastes and poultry manures (Sawant *et al.*, 1995) [33], neem cake, coir pith, farmyard manure (FYM) and decomposed coffee pulp (Saju *et al.*, 2002), well decomposed FYM, dried cowdung, gobar gas slurry (GGS), sorghum grain floor, wheat bran, groundnut shell, molasses, sawdust, wheat straw, mushroom bed straw, neem cake, peat soil, fly ash and talc (Sangle and Bambawale, 2005) [30], vegetable wastes, fruit wastes, crop wastes, FYM and poultry manure (Simon, 2011) [38], vegetable waste, fruit juice waste, sugarcane baggase, rotten wheat grains (Babu and Pallavi, 2013) [3] for mass multiplication of *Trichoderma* species. Keeping in view the importance of *Trichoderma* in disease management and the use of various substrates for its mass multiplication, the present study was carried out to evaluate various locally available substrates for mass multiplication of *Trichoderma* species.

Material and Methods
Sample Collection
Rhizospheric soil samples were collected from various commercially grown chilli fields and

kitchen gardens of district Anantnag (Bangidar, Bagi Wanpoh, 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. The soil samples were collected in polyethylene bags, labeled with information of collection sites and origin of samples. Then, the samples were brought to laboratory for isolation of biocontrol agent *Trichoderma* species.

Isolation of *Trichoderma* species

Trichoderma species were isolated from soil samples by using multiple tube dilution plate technique (Johnson, 1957) [15]. 8 test tubes (25 ml) were taken and 9 ml sterilized distilled water was poured into each test tube. 1 g of soil sample was weighed and transferred into the first test tube containing 9 ml sterilized distilled water. It gives the dilution of 10^{-1}. 1 ml of soil suspension was transferred from 10^{-1} dilution into 2nd test tube in order to get dilution of 10^{-2}. Similarly 1 ml of suspension was serially transferred from dilution 10^{-2} to 10^{-3}, 10^{-3} to 10^{-4}, 10^{-4} to 10^{-5}, 10^{-5} to 10^{-6}, 10^{-6} to 10^{-7} and 10^{-7} to 10^{-8}. From dilution 10^{-8}, 1 ml was poured into Petri plates containing 20 ml of *Trichoderma* specific medium (Elad and Chet, 1983) [10]. The plates were incubated at 28°C in BOD incubator for 4-7 days. The plates were observed daily. Visible fungal colonies were transferred to new Potato Dextrose Agar (PDA) plates and incubated at 28°C for 5 days. 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) [35].

Biochemical characterization to evaluate potential *Trichoderma* isolates

Ammonia production

Freshly grown *Trichoderma* isolates were inoculated in culture tubes containing 8-10 ml peptone water broth and incubated at 25-26°C for 48 hours. Subsequently, Nesseler's reagent (1 ml) was added in each tube. The development of colour from yellow to brownish orange was a positive test for ammonia (Bakker and Schipper, 1987) [6].

HCN production

HCN production of *Trichoderma* isolates was tested qualitatively following the method of Bakker and Schipper (1987) [6]. *Trichoderma* isolates were inoculated on petriplates containing trypticase-soy-agar medium (Annexure-1). A Whatman filter paper soaked in alkaline picric acid solution (2.5 g of picric acid; 12.5 g of Na_2CO_3; 1000 ml of distilled water) was placed in the upper lid of each plate. The plates were incubated at 25-26°C for 4 days. A change in colour of the filter paper from yellow to light brown, brown or reddish-brown was recorded as indication of HCN production.

Estimation of Indole acetic acid (IAA)

The quantitative analysis of indole-3-acetic acid was performed by the method suggested by Brick *et al.* (1991) [7]. *Trichoderma* isolates were inoculated in 10 ml Luria Bertani broth amended with 0.1 gL^{-1} L-Tryptophan and incubated at 25-26°C for 24 hours. The broth was centrifuged at 10,000 rpm for 15 minutes, 2 ml of the supernatant was taken to which 2 ml of Salkowski's reagent (1 ml of 0.05 M $FeCl_3$ in 50 ml of 35% $HClO_4$) was added and left at room temperature for 20 minutes and absorbance was measured at 530 nm using a spectrophotometer.

Chitinase production

Preparation of Colloidal chitin: The practical grade chitin powder was used to prepare the colloidal chitin. Chitin powder (40 g) was dissolved in 500 ml of concentrated hydrochloric acid and continuously stirred at 4 °C for 1 h. After stirring, the hydrolyzed chitin was washed number of times with distilled water in order to remove the acid completely and hence bring the pH in the range of 6-7. As the desired pH was attained, the colloidal chitin was filtered through Whatman filter paper No.1. The sieved colloidal chitin was subsequently collected and stored in the form of a paste at 4°C (Khan *et al.*, 2010) [16]. This colloidal chitin was used at 0.5% of the composition of the medium as the sole carbon source with other minimal salts (1 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, 0.5% crude chitin, and 1 L of distilled water) and agar. The *Trihoderma* cultures were inoculated on the prepared media and the plates were incubated at 25-26°C for 10 days. Isolates with chitinolytic activity grew on the agar while no growth indicates absence of chitinase activity (Okay *et al.*, 2008) [23].

Shelf-life of potential *Trichoderma* isolate on locally available substrates

Different locally available substrates such as charcoal, ash, sawdust, cowdung, vermicompost, maize seeds, wheat seeds, rice seeds, dal weed and talc were used for checking shelf life of potential *Trichoderma* isolate. Mass multiplication of *Trichoderma harzianum* was done in two steps:

1. Stock culture
2. Carrier

Stock culture was developed on maize seeds. Maize seeds were kept over night in water. Thereafter water was drained out and seeds were crushed in a mixer. These crushed maize seeds were then autoclaved. Crushed maize seeds were inoculated with a loop full of *Trichoderma harzianum* and kept in an incubator at 25-26°C for 10 days till 100% colonization take place. For the preparation of carrier, 20 g of above mentioned substrates was taken separately in 100 ml flasks. These carriers were first made into powdered form. These carriers were then supplemented with 5% molasses, 5% yeast extract and 5% glycerol separately and kept overnight. These flasks were than autoclaved at 121°C temperature for 20 minutes. After that these carriers were transferred into sterilized low density polyethylene (LDPE) bags under laminar air flow cabinet. 1 g of stock culture of *Trichoderma harzianum* was added to 20 g of each substrate for making formulations. Three sets of bioformulations were prepared with glycerol (5%) in one set, molasses (5%) in second set and Yeast extract (5%) in third set. The substrates (carriers) were incubated at three different temperatures (10ºC, 20ºC, 30ºC) for shelf-life tests. Colony forming unit (CFU) was counted using three Petri plates for each treatment containing *Trichoderma* specific medium suggested by Sawant and Sawant (1996) [32]. CFU count was checked after every 15 days up to the period of three months.

All the data was analyzed statistically using completely randomized design (CRD) as per the procedure of Gomez and Gomez (1984) [12] at 5% level of probability.

Results

Isolation of *Trichoderma* species

A total of 20 (7 from Anantnag and 13 from Baramulla) *Trichoderma* isolates 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).

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
	Bagi Wanpoh	AT6	Commercial field
	Bagi Wanpoh	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

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

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, BT13) were found negative for ammonia production. Similarly 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. Similarly during HCN production test only three isolates (AT3, AT5, and AT7) were found positive and rest were found negative (Table 2).

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
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	-	+	-

Production of IAA by various isolates of *Trichoderma* species

During IAA production test all the isolates were found to produce IAA however their production amount varied considerably. Maximum IAA was produced by isolate AT3 (6.605 µg mL^{-1}) followed by BT6 (5.278 µg mL^{-1}), BT11 (3.408 µg mL^{-1}) and BT9 (3.317 µg mL^{-1}) while minimum IAA was produced by isolate AT1 (1.538 µg mL^{-1}) (Table 3).

Table 3: Production of IAA by various isolates of *Trichoderma* species

S. No.	Isolates	IAA (µg mL^{-1})
1	AT1	1.538
2	AT2	1.749
3	AT3	6.605
4	AT4	1.628
5	AT5	2.171
6	AT6	2.231
7	AT7	2.262
8	BT1	1.869
9	BT2	2.835
10	BT3	2.563
11	BT4	1.688
12	BT5	2.322
13	BT6	5.278
14	BT7	2.141
15	BT8	2.804
16	BT9	3.317
17	BT10	2.262
18	BT11	3.408
19	BT12	2.262
20	BT13	3.046

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. After studying these characteristics *Trichoderma* isolate (AT3) was found to resemble *Trichoderma harzianum*.

Shelf-life of potential *Trichoderma* isolate on locally available substrates

For carrying out shelf life tests, ten different locally available substrates viz., charcoal, ash, sawdust, cowdung, vermicompost, maize seeds, wheat seeds, rice seeds, dal weed and talc were used for the study. All the substrates retain the shelf life of *Trichoderma harzainum* for three months but it was maximum upto 30 days. Maize seeds retains the maximum shelf life of 50, 48, 46 ($\times 10^7$) cfu/g of formulation at 10, 20 and 30°C; followed by wheat, cowdung and talc (49 $\times 10^7$) cfu at 10°C, cowdung (48 $\times 10^7$) cfu at 20°C, cowdung and talc (46 $\times 10^7$) cfu at 30°C upto 30 days when supplemented with molasses. Thereafter the shelf life of *Trichoderma harzianum* showed the decreased trend but still was highest with maize seeds 39, 31, 22 followed by wheat seeds 38, 30, 21 ($\times 10^7$) cfu/g of formulation at 10, 20 and 30°C upto 90 days (Table 4). More or less similar trend was observed with the shelf life of *Trichoderma harzianum* when supplemented with glycerol and yeast extract (Table 5 and 6). While checking the interaction among different substrates, supplements and temperatures (Table 7), it was found that all the substrates showed good results but the maximum shelf life was retained by maize seed and wheat seed with an overall mean cfu count of 38.2 $\times 10^7$, followed by cowdung with an overall mean cfu count of 37.5 $\times 10^7$, by talc and paddy with an overall mean cfu count of 37 $\times 10^7$, by sawdust with an overall mean cfu count of 36 $\times 10^7$, by vermicompost with an overall mean cfu count of 34 $\times 10^7$, by dalweed with an overall mean cfu count of 32 $\times 10^7$, by charcoal with an overall mean cfu count of 22 $\times 10^7$ while the minimum shelf life was retained by ash with an overall mean cfu count of 15 $\times 10^7$ colony forming units per gram of formulation. Among different supplements molasses was found to be the best with a mean cfu count of 34 $\times 10^7$ followed by yeast extract with a mean cfu count of 32 $\times 10^7$ and glycerol with a mean cfu count of 31 $\times 10^7$ colony forming units per gram of formulation. Further while checking the interaction between different temperatures (10°C, 20°C and 30°C), it was found that maximum shelf life was retained at 10°C with a mean cfu count of 36 $\times 10^7$ followed by 20°C with a mean cfu count of 32 $\times 10^7$ and by 30°C with a mean cfu count of 29 $\times 10^7$ colony forming units per gram of formulation.

Table 4: Shelf life of *Trichoderma harzianum* (AT3) in various substrates supplemented with molasses

Carriers	CFU(×10^7) at 10°C						Mean	CFU(×10^7) at 20°C						Mean	CFU(×10^7) at 30°C						Mean	Overall mean
	15 days	30 days	45 days	60 days	75 days	90 days		15 days	30 days	45 days	60 days	75 days	90 days		15 days	30 days	45 days	60 days	75 days	90 days		
Maize seed	48	50	46	44	41	39	44.6	46	48	43	41	36	31	40.8	44	46	42	39	31	22	37.3	40.9
Paddy seed	46	48	44	42	39	37	42.6	44	46	41	39	34	29	38.8	42	44	40	37	29	20	35.3	38.9
Wheat seed	47	49	45	43	40	38	43.6	45	47	42	40	35	30	39.8	43	45	41	38	30	21	36.3	39.9
Cowdung	47	49	44	43	39	37	43.2	45	48	42	40	34	29	39.6	43	46	41	38	29	20	36.2	39.6
Vermicompost	44	47	41	40	35	33	40.0	42	45	38	35	29	22	35.2	40	43	38	35	26	16	33	36.0
Sawdust	45	48	41	40	35	32	40.2	44	47	40	37	31	23	37.0	42	45	39	37	28	18	34.8	37.3
Dalweed	44	46	40	38	34	31	38.8	40	44	37	34	28	19	33.6	38	42	35	33	24	14	31.0	34.5
Charcoal	32	35	29	25	20	17	26.3	30	33	27	21	16	12	23.2	29	30	25	19	13	0	19.3	22.9
Ash	25	28	22	18	15	12	20.0	23	26	20	16	0	0	14.2	22	24	19	13	0	0	13.0	15.7
Talc	47	49	44	42	38	35	42.5	45	47	42	40	34	29	39.5	43	46	41	37	28	18	35.5	39.2
Mean	42.5	44.9	39.6	37.5	33.6	31.1	38.2	40.4	43.1	37.2	34.3	27.7	22.4	34.2	38.6	41.1	36.1	32.6	23.8	14.9	31.2	

C.D (P≤0.05):

Days : 0.923

Temperatures : 0.928

Carriers : 1.695

Table 5: Shelf life of *Trichoderma harzianum* (AT3) in various substrates supplemented with yeast extract

Carriers	CFU(×10^7) at 10°C						Mean	CFU(×10^7) at 20°C						Mean	CFU(×10^7) at 30°C						Mean	Overall mean
	15 days	30 days	45 days	60 days	75 days	90 days		15 days	30 days	45 days	60 days	75 days	90 days		15 days	30 days	45 days	60 days	75 days	90 days		
Maize seed	45	47	43	41	38	36	41.6	43	45	39	38	33	28	37.6	41	43	39	36	28	19	34.3	37.8
Paddy seed	45	47	42	40	37	34	40.8	42	44	38	37	32	27	36.6	40	42	38	35	27	18	33.3	36.9
Wheat seed	45	46	43	41	38	35	41.3	43	45	40	38	33	28	37.8	41	43	39	36	28	19	34.3	37.8
Cowdung	45	47	42	41	37	35	41.2	43	46	39	36	30	27	36.8	41	44	39	36	27	18	34.2	37.4
Vermicompost	43	45	40	38	34	32	38.6	41	43	37	34	28	20	33.8	38	41	36	33	24	14	31	34.5

Sawdust	43	46	40	38	33	30	38.3	43	45	39	36	29	21	35.5	40	43	37	35	26	16	32.8	35.5
Dalweed	31	43	37	35	31	28	34.2	39	41	35	32	26	17	31.6	36	39	33	31	22	12	28.8	31.5
Charcoal	31	33	28	24	18	14	24.6	29	31	26	20	14	10	21.6	27	29	23	17	11	0	17.8	21.4
Ash	23	25	21	17	13	11	18.3	21	23	18	15	0	0	12.8	20	21	17	12	0	0	11.6	14.2
Talc	44	46	41	39	35	32	39.5	43	45	40	37	32	27	37.3	40	44	38	34	25	15	32.6	36.5
Mean	39.5	42.5	37.7	35.4	31.4	28.7	35.8	38.7	40.8	35.1	32.3	25.7	20.5	32.1	36.4	38.9	33.9	30.5	21.8	13.1	29.1	

C.D (P≤0.05):
Days : 0.932
Temperatures : 0.925
Carriers : 1.690

Table 6: Shelf life of *Trichoderma harzianum* (AT3) in various substrates supplemented with glycerol

Carriers	CFU(×10^7) at 10^{o}C						Mean	CFU(×10^7) at 20^{o}C						Mean	CFU(×10^7) at 30^{o}C						Mean	Overall mean
	15 days	30 days	45 days	60 days	75 days	90 days		15 days	30 days	45 days	60 days	75 days	90 days		15 days	30 days	45 days	60 days	75 days	90 days		
Maize seed	43	45	41	39	36	33	39.5	41	43	37	36	31	26	35.6	39	41	37	34	26	17	32.3	35.8
Paddy seed	43	44	41	38	35	33	39.0	40	42	36	35	31	25	34.8	38	40	36	33	25	16	31.3	35.03
Wheat seed	44	47	42	40	37	35	40.8	41	44	38	37	32	27	36.5	40	42	38	35	27	18	33.3	36.86
Cowdung	44	46	41	39	35	33	39.6	41	44	37	35	29	25	35.2	39	42	37	34	25	16	32.2	35.66
Vermicompost	42	44	39	37	32	30	37.3	39	42	35	33	27	19	32.5	37	40	35	32	23	13	30	33.26
Sawdust	43	45	39	37	33	31	38.o	41	43	37	35	29	21	34.3	38	41	35	33	24	14	30.8	34.36
Dalweed	40	42	37	34	30	27	35.0	37	40	34	31	25	17	30.6	35	38	32	30	22	12	28.2	31.26
Charcoal	29	31	27	24	17	13	23.5	27	29	25	19	13	10	20.5	26	26	22	15	11	0	16.6	20.2
Ash	21	24	20	16	12	11	17.3	20	22	17	14	0	0	12.2	19	20	16	12	0	0	11.2	13.56
Talc	43	45	40	38	36	33	39.2	41	43	37	36	31	26	35.6	39	41	37	33	24	14	31.3	35.36
Mean	39.2	41.3	36.7	34.2	30.3	27.9	34.9	36.8	39.2	33.3	31.1	24.8	19.6	30.8	35	37.1	32.5	29.1	20.7	12	27.7	

C.D (P≤0.05):
Days : 0.852
Temperatures : 0.855
Carriers : 1.561

Table 7: Effect of different supplements on the shelf life of *Trichoderma* based formulations prepared in different substrates

Carriers Supplements	Molasses				Yeast extract				Glycerol				Overall Mean	Temperature		
	10^{o}C	20^{o}C	30^{o}C	Mean	10^{o}C	20^{o}C	30^{o}C	Mean	10^{o}C	20^{o}C	30^{o}C	Mean		10^{o}C	20^{o}C	30^{o}C
Maize seed	44.6	40.8	37.3	40.9	41.6	37.6	34.3	37.8	39.5	35.6	32.3	35.8	38.2	41.9	38.0	34.6
Paddy seed	42.6	38.8	35.3	38.9	40.8	36.6	33.3	36.9	39.0	34.8	31.3	35.0	36.9	40.8	36.7	33.3
Wheat seed	43.6	39.8	36.3	39.9	41.3	37.8	34.3	37.8	40.8	36.5	33.3	36.8	38.2	41.9	38.0	34.6
Cowdung	43.2	39.6	36.2	39.6	41.2	36.8	34.2	37.4	39.6	35.2	32.2	35.6	37.5	41.3	37.2	34.2
Vermicompost	40.0	35.2	33.0	36.1	38.6	33.8	31.0	34.4	37.3	32.5	30.0	33.3	34.6	38.6	33.8	31.3
Sawdust	40.2	37.0	34.8	37.3	38.3	35.5	32.8	35.5	38.0	34.3	30.8	34.4	35.7	38.8	35.6	32.8
Dalweed	38.8	33.6	31.0	34.4	34.2	31.6	28.8	31.5	35.0	30.6	28.2	31.3	32.4	36.0	31.9	29.3
Charcoal	26.3	23.2	19.3	22.9	24.6	21.6	17.8	21.3	23.5	20.5	16.6	20.2	21.5	24.8	22.8	17.9
Ash	20.0	14.2	13.0	15.7	18.3	12.8	11.6	14.2	17.3	12.2	11.2	13.5	14.5	18.5	15	11.9
Talc	42.5	39.5	35.5	39.2	39.5	37.3	32.6	36.4	39.2	35.6	31.3	35.3	37.0	40.4	37.4	33.1
Mean	38.2	34.2	31.2	34.49	35.84	32.14	29.1	32.32	34.9	30.8	27.7	31.12		36.3	32.6	29.3

C.D (P≤0.05):
Carriers (C) : 3.020
Temperatures (T) : 1.656
Supplements (S) : 1.656
Interaction :
S×T : NS
S×C : NS
T×C : NS
S×T×C : NS

Discussion

The present study was planned with an objective to isolate *Trichoderma* species from chilli rhizosphere and biochemical characterization of these isolates in order to get a potential isolate and then evaluating different substrates for mass multiplication of the potential *Trichoderma* isolate. A total of 20 *Trichoderma* isolates 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 following multiple tube dilution plate technique. Similarly *Trichoderma* species were isolated from chilli rhizosphere by Wani *et al.* (2014) [41] and the technique used for isolation *Trichoderma* species is in agreement with chaudhari *et al.* (2011) [9] and Khandelwal *et al.* (2012) [17, 20]. In order to evaluate a potential *Trichoderma* isolate, all the isolates were screened for different biochemical traits. One of the important traits is the production of ammonia. Since ammonia is useful for plant as directly or indirectly. Ammonia production by the *Trichoderma* isolates may influence plant growth indirectly. ACC synthesized in plant tissues by ACC synthase is thought to be exuded from plant roots and be taken up by neighboring micro-organisms. *Trichodrema* may hydrolyze ACC (1-aminocyclopropane-1-carboxylic acid) to ammonia (Ahemad and Kibret, 2014) [2]. With respect to screening for ammonia production, in the present study thirteen isolates (AT1, AT2, AT3, AT4, AT5, AT6, AT7, BT1, BT7, BT8, BT10, BT11 and BT12) were

found to produce ammonia out of twenty isolates. 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, Aspergilus niger* and *Fusarium moniliforme.* 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). In the present study out of twenty isolates twelve (AT2, AT3, AT7, BT1, BT3, BT4, BT7, BT8, BT10, BT11, BT12 and BT13) were found positive for chitinase activity by showing growth on chitin based media. This is in agreement with earlier reports (Sharaf *et al.*, 2012). HCN production is also an important trait found in various soil micro-organisms as it indirectly promotes plant growth by controlling some soil borne diseases (Kremer and Souissi, 2001; Siddiqui *et al.*, 2006). In case of HCN production test only three isolates (AT3, AT5 and AT7) were found to be positive out of twenty isolates. This is in agreement with earlier reports (Aarti and Meenu, 2015). Similar results were obtained by Ngoma *et al.* (2013) in case of bacterial isolates. 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). IAA has been implicated in virtually every aspect of plant growth and development, as well as defense responses. With respect to IAA production all the isolstes were found to produce IAA however their production amount varied considerably. Maximum IAA was produced by isolate AT3 (6.605 µg mL^{-1}) followed by BT6 (5.278 µg mL^{-1}), BT11 (3.408 µg mL^{-1}) and AT3 (3.317 µg mL^{-1}) while minimum IAA was produced by isolate AT1 (1.538 µg mL^{-1}). Similar findings were recorded by Aarti and Meenu (2015), Gravel *et al.* (2007) [13] and Badawi *et al.* (2011) [5] in *Trichoderma* species.

Formulation and shelf-life are of prime importance for commercial use of any biological agent. There is abundant literature on the use of conventional synthetic media like glucose, cellulose, soluble starch and molasses to produce *Trichoderma* species (Gupta *et al.*, 1997) [11]. The main objective of this work has been the development of low cost method for the propagation of the fungi which yields high inoculum levels and thus results in high mass production. Although all the substrates showed good results but the maximum shelf-life was retained by maize seeds followed by wheat seeds at 10°C when supplemented with molasses. This is in agreement with the results recorded by Babu and Pallavi (2013) [3], Shahid *et al.* (2013) [35], Mehta *et al.* (2012) [17, 20], Sargin *et al.* (2013) [31], Tewari and Bhanu (2004) [39], Islam *et al.* (2007), Babu *et al.* (2004) [4]. The possible reason for retention of maximum shelf life by maize seed and wheat seed may be due to their carbohydrate composition, better particle distribution and water absorption capacity (Pandey *et al.*, 2000).

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