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Research Article

Enhancing the shelf life of *Trichoderma* species by adding antioxidants producing crops to various substrates

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Abstract: *Trichoderma* is one of the efficient biocontrol agents due to its high reproductive capacity, ability to survive under unfavorable conditions, efficiency in nutrient utilization, capacity to modify the rhizosphere, strong aggregativeness against the pathogenic fungi and efficiency in promoting plant growth and defense mechanisms. Therefore, the present investigation is carried out as an alternative practical and safe approach for mass multiplication of *Trichoderma* on different agro based media. Among them wheat straw and farmyard manure were found to be the best solid media supplemented with 10% wheat flour.The highest population count of *Trichoderma* species was observed in wheat straw. Antioxidant producing crops were also added to this carrier medium at a rate of 5g/kg in order to enhance the shelf life of propagules of *Trichoderma* species. Maximum population count wasobserved in soybean, maize and brown rice.

Keywords: *Trichoderma*, biological control, mass multiplication and antioxidant crops

Introduction

The genus *Trichoderma* belongs to class Ascomycota and order Hypocreales is a filamentous fungus widely distributed in the soil, plant material, decaying vegetations and wood. Species of this genus are of great economic importance, as they serve as a source of enzymes, antibiotics, plant growth promoters, xenobiotic degraders and most commercial biofungicides (Ozbay and Newman, 2004). *Trichoderma* spp. are considered as potential biocontrol and growth promoting agents for many crop plants (Savazzini *et al*., 2009). *Trichoderma* populations can be established relatively easily in different types of soil and can continue to persist at detectable levels for months. *Trichoderma* spp. arebiocontrol agents against varioussoil borne plant pathogens and can easily colonize in plant rhizosphere and help to promote the plant growth (Verma *et al*., 2007). They also helps in increasing nutrient uptake from soil (Vurro *et al*., 2001), reduce the toxic metabolites produced by other rhizospheric microorganisms or pesticides (Lanzuise, 2002), stimulate plants for producing chemical defenses compound and induce resistance in the plants (Howell *et al.,* 2000), induce mycoparasitism or directly attackother pathogenic fungi (Lo *et al.,* 2000) and improve root system and plant growth (Harman, 2000). *Trichoderma* spp. have evolved numerous mechanisms that are involved in inhibiting other fungi and enhance plant and root growth.

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These mechanisms include antibiosis, competition for space and nutrients (Elad *et al*., 1999), mycoparasitism (Haran *et al*., 1996), production of inhibitory compounds, inactivation of the pathogen's enzymes (Roco and Perez, 2001) and induced resistance (Kapulnik and Chet, 2000).

The biocontrol activity of *Trichoderma* is due to an enzyme,chitinase, which is responsible for disintegration of cell wall of phytopathogens (Anand and Reddy, 2009). Fungal diseases aremain obstacles for obtaining high yield incommercial cultivation and therefore, fungicides are applied to control these diseases. Due to the emergence of fungicide resistant strains and also regarding the health of public and environmental impacts of these chemicals,the fungicides are being replaced by biocontrol agents. During the past fewdecades, several potential biocontrol organisms have been isolated, characterized and commercialized, and thus, biocontrol of plantdiseases has received more consideration in plant disease control (Shali *et al*., 2010). Therefore, keeping in view its importance several coworkers had used agriculture waste material for its mass multiplication and shelf life enhancement. 3% jaggery and 10% wheat flour had been used as nutritional supplements for enhancing conidial yield of *Trichoderma* spp. Coir pith + neem cake (1: 1) at 35% and 45% moisture gave longer shelf life for *Trichoderma* propagules. Pre boiled sorghum grains, coir pith and neem cake (1: 1), cow dung + neem cake (1: 1) and wheat flour 10% maintained maximum inoculum density. While mycelium growth was observed on sorghum grains on the third day of incubation and it covered entire surface of the substrate with green sporulation in 6 days (Prasad *et al.,* 2002). Highest population of *Trichoderma harzianum* was recorded in molasses yeast medium followed by broken sorghum grains, whole sorghum grains and broken maize grains after 15 and 30 days (Kumar and Palakshappa, 2009). Different formulations of *T. harzianum* formed by using six different type of substrates namely Spent Mushroom Compost (SMC), Farmyard Manure (FYM), Vermicompost (VC), Sorghum Grain (SG), Wheat Grain (WG) and Broken Maize Grain (BMG), Spent Mushroom Compost (SMC) gave higher level of population count as well found efficient in controlling *Rhizoctoniasolani* causing collar rot disease of cowpea (Singh *et al.*, 2014).

In the present study, different *Trichoderma* spp. were multiplied on various solid media by adding antioxidant crops in order to enhance the shelf life of *Trichoderma*.

Materials and Methods

Isolation and identification of *Trichoderma* spp. The rhizospheric samples were collected randomly from different locations of districts of Himachal Pradesh (India) and were well mixed to form a single composite sample for the isolation of residential antagonistic microorganisms particularly *Trichoderma* spp. The isolations were made by serial dilution method described by Johnson in 1957. From the composite sample one gram of soil was added aseptically to 100ml of sterilized distilled water in 250ml of flask.The soil was thoroughly mixed by constant shaking on stirrer homogenizer and subsequently serial dilutions were made from this solution up to 1×10^7. Simultaneously, potato dextrose agar (PDA) medium was prepared through the addition of rose bengal (0.03g/l), chloramphenicol (0.4g/l), and streptomycin sulfate (0.03g/l) after autoclaving the medium, pH 6 and poured into sterilized Petri plates aseptically. There after 1 ml of soil diluent was spreaded uniformly on Potato Dextrose Agar (PDA). These Petri plates were kept for incubation at 25 ± 1 °C for about 120 hours in case of fungi. The emerging colonies of *Trichoderma* spp. thus obtained were picked and transferred to PDA slants. Cultures were purified by single spore isolation in case of fungi and later maintained on PDA slants. Identification of fungi was done on the basis of morphological characters as described by Gilman (1957) in his book "A manual of soil fungi" and "A revision of the genus *Trichoderma*" by Rifai (1969).

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Effect of natural antioxidants (crops) on best solid medium.

Effect of different natural antioxidants producing crops was studied on viability of *Trichoderma* spp. on the best carrier medium. In order to know the best antioxidant sustaining maximum growth of biocontrol agents, seven different antioxidants namely Maize (grains), Ginger (rhizome), Turmeric (rhizome), Soybean (seeds), Sunflower (seeds), Brown Rice (grains) and Green Tea (leaf) were evaluated for population count of biocontrol agents.

The antioxidants were cleaned and their seeds and rhizomes were converted into powdered form. Then, 100 g of each best carrier solid medium containing antioxidants @ 5g per kg, were filled in polypropylene bags, plugged and autoclaved at 15lb psi for 30 min for two consecutive days. Each bag was inoculated with 4 bits (4mm) of biocontrol agents as mentioned above separately, replicated thrice and was incubated at 25 ± 1 °C in BOD incubator for 10 days. Population count (cfu/g × 10^4) of all the potential biocontrol agents was recorded after two months interval.

Results and Discussion

Morphological characters of *Trichoderma* isolates

The isolated species of *Trichoderma* were morphologically characterized on the basis of colony color, reverse color, colony edge, mycelia, color, conidial size and growth rate (Fig. 1). It was evident from the Table 1 that the colony color varied from snow white to white and light green, green, dark green to dirty green whereas reverse color of some isolates was orange while some represented no color and only one was yellowish. Colony edge was also observed which varied from smooth, effused to raised type. There was no variation seen in mycelial color of all the isolates, under microscope it was observed to be hyaline only. Presence of water droplets on the surface of mycelium was prevalent in almost all the isolates but more prominent in *T. hamatum, T. virens and T. viride*. The average growth diameter of the colony was 8-9 cm in 5 days with full green colored sporulation. However, the length and width varied from 5-10 × 5-7µm in all the isolates. On the basis of morphological description and their comparison with the keys given in "A revision of the genus *Trichoderma*" by Rifai (1969), the isolates were identified as *T. viride, T. hamatum, T.virens, T.polysporum, T.harzianum, T.piluliferum*, and *T. koningii*.

Druzhinina and Kubicek (2005) studied the species concepts and biodiversity in *Trichoderma* by aggregating the morphological, physiological and genetic studies. Samuels (2006) described the systematics, the sexual stage and the ecology of *Trichoderma* and mentioned in his study that the morphology of *Trichoderma* is not only limited to a few characters but many species may be included in this genus due to theirgeographical distribution. The macro and microscopic characters of *Trichoderma* spp., the major and remarkable macroscopic features in species identification were the colony features, including diameter after 7 days, color of conidia, mycelial color, colony reverse, colony texture and shape whereas microscopic characteristics were identified on the basis of conidial head; conidia shape, roughness and vesicle serration (Lunge and Patil, 2012). The growth patterns and sporulation patterns were varied among different *Trichoderma* isolates recorded by Kumar and Garampalli (2013). He also noticed that conidial wall patterns and shape were rough and subglobose among *T. harzianum,* while they were smooth and globose to ovoid among *T. viride.*

Effect of natural antioxidants (crops) on population count of *Trichoderma* spp.

In order to enhance the growth and population count of *Trichoderma* spp; natural antioxidants producing crops were added to the best solid medium i.e. wheat straw + wheat flour and their effect was noticed and presented in Table 2.

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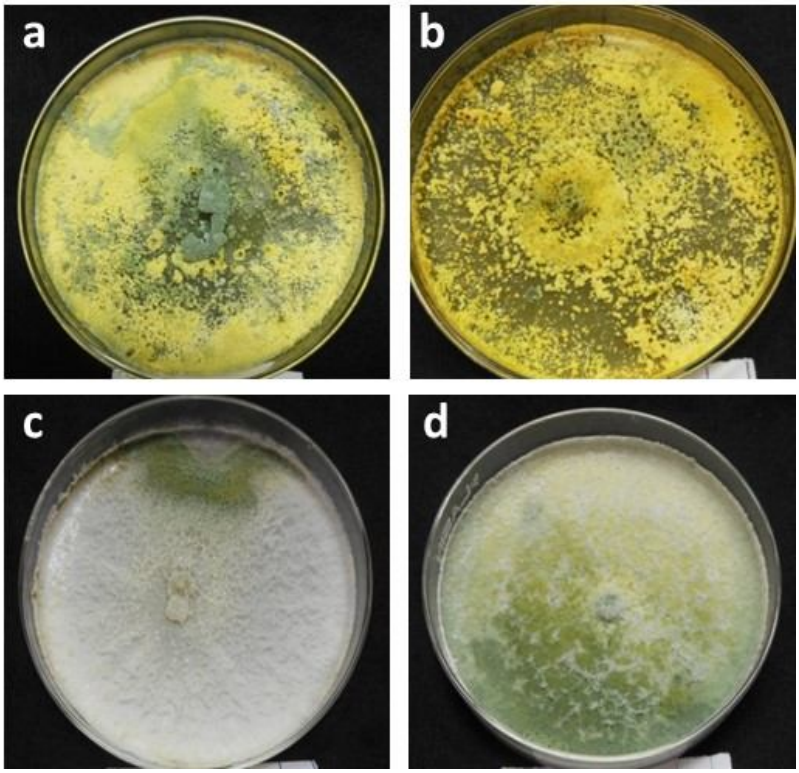

Figure 1 Cultures of *Trichoderma* isolates. (a) *T. harzianum*, (b) *T. viride*,(c) *T. virens*, (d) *T. hamatum*.

Table 1 Morphological descriptors used for the characterization of native isolates of *Trichoderma* spp.

Name of Strain	Colony color	Reverse color	Colony edge	Mycelial color	Growth diameter (cm)	Conidial size (µm)		Species identified
						Length	Width	
Trichoderma sp. (S1)	Green	Colorless	Smooth	White	7-8	5	5	*T. viride*
Trichoderma sp. (S2)	Dark Green	Colorless	Raised	White	7-8	5	5	*T. harzianum*
Trichoderma sp. (S3)	Light Green	Orange	Raised	White	7-8	7	5	*T. hamatum*
Trichoderma sp. (S4)	Green	Orange	Smooth	White	7-8	5	5	*T. virens*
Trichoderma sp. (S5)	Dark Green	Orange	Smooth	White	8-9	7	7	*T. polysporum*
Trichoderma sp. (SR1)	Light Green	Colorless	Smooth	White	8-9	5	5	*T. harzianum*
Trichoderma sp. (SR2)	Light Green	Orange	Smooth	White	8-9	7	5	*T. viride*
Trichoderma sp. (SR3)	Dirty Green	Yellowish	Raised	White	8-9	7	7	*T. koningii*
Trichoderma sp. (SR4)	Snow White	Colorless	Effuse	White	8-9	10	5	*T. piluliferum*
Trichoderma sp. (B1)	Dark Green	Orange	Smooth	White	7-8	5	5	*T. polysporum*

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Table 2 Effect of natural antioxidants (crops) on population count of *Trichoderma* spp. multiplied on wheat straw supplemented with wheat flour (10%).

Natural antioxidants (Crops)	Population count (cfu/g × 10^4) after two month interval								
	Trichoderma species								
	T. harzianum (S2)	*T. viride* (S1)	*T. hamatum* (S3)	*T. virens* (S4)	*T. polysporum* (S5)	*T. viride* (SR2)	*T. harzianum* (SR1)	*T. piluliferum* (SR4)	Mean
Soybean (seeds)	301 (2.48)	290.3 (2.46)	294.7 (2.47)	284.7 (2.45)	271.0 (2.43)	251.0 (2.40)	248.3 (2.39)	220.3 (2.34)	270.2 (2.43)
Maize (grains)	271.0 (2.43)	251.0 (2.40)	265.0 (2.42)	244.7 (2.39)	231.0 (2.36)	230.3 (2.36)	228.3 (2.36)	224.0 (2.35)	245.6 (2.39)
Brown Rice (grains)	281.0 (2.45)	256.0 (2.41)	260.3 (2.42)	241.0 (2.38)	237.0 (2.38)	234.0 (2.37)	228.3 (2.36)	227.0 (2.36)	243.2 (2.39)
Green tea (leaves)	251.0 (2.40)	234.0 (2.37)	240.3 (2.38)	231.3 (2.36)	225.3 (2.35)	220.0 (2.34)	219.0 (2.34)	218.3 (2.34)	234.8 (2.37)
Ginger (rhizome)	221.3 (2.35)	212.3 (2.33)	220.3 (2.34)	212.0 (2.33)	204.0 (2.31)	201.0 (2.30)	199.0 (2.29)	197.7 (2.29)	229.9 (2.36)
Turmeric (rhizome)	261.3 (2.42)	241.0 (2.38)	250.3 (2.39)	234.7 (2.37)	227.7 (2.36)	222.0 (2.35)	220.7 (2.34)	221.0 (2.34)	208.5 (2.32)
Sunflower (seeds)	200.3 (2.30)	183.0 (2.26)	191.0 (2.28)	170.3 (2.23)	163.7 (2.21)	165.7 (2.22)	160.0 (2.04)	151.3 (2.18)	173.2 (2.24)
Overall mean	255.3 (2.40)	246.0 (2.39)	238.2 (2.37)	231.2 (2.36)	222.8 (2.34)	217.7 (2.34)	214.8 (2.33)	208.5 (2.32)	

$CD_{0.05}$ Antioxidants = 0.002.
Isolates = 0.002.
Media × Strain = 0.006.
Figures in parenthesis are logx transformed values.

The perusal of the data revealed that among all the crops tested soybean supported the maximum population count (270.2) followed by maize (245.6), rice (243.2), green tea (234.8), ginger (229.9), turmeric (208.5) and sunflower (173.2) in the descending order of their performance on the substrate supplemented with natural antioxidants. Though maize and rice were statistically at par. However, *T. harzianum* (255.3) yielded maximum population count with regards to the strainwise performance followed by *T. viride* (246.0), *T. hamatum* (238.2) and *T. virens* (231.2) while the strain *T. polysporum* had least colony count.

With regards to interaction between *Trichoderma* sp. and antioxidant crops it was revealed that *T. harzianum* performed well in soybean (301.0) followed by *T. hamatum* and *T. viride*, though the performance of same species was found to be better on rice as well as maize as compared to other crops and recorded least in term of colony count on sunflower.

Similar findings were illustrated by Sathiyaseelan *et al*. (2009) who reported that the survivibility of *Trichoderma* spp. was better in soybean oil as it has enhanced its shelf life to greater extent. Khandelwal *et al*. (2012) also evaluated that the maximum population count was supported by pulses followed by rice and wheat. Wheat, grain of sorghum, wheat and pulse and rice bran were used for mass production by Saju *et al*. (2002) which supported better shelf life of *Trichoderma* spp. as well as performed better in controllingcollar rot disease of cowpea caused by *Rhizoctoniasolani* thereby promoting growth of plants. Jeyarajan (2006) also used various cereal grains like, sorghum, millets and ragi as substrates for mass production of *Trichoderma* spp., these substrates were then coated with *Trichoderma* spp and used for treating seeds.

Conclusion

The current study assures that the wheat straw supplemented with wheat flour along with natural antioxidant crops (soybean, maize and rice) can be recommended as suitable

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substrate for mass multiplication of *Trichoderma* spp. that has usefulness in IDM programme. This could be aneasy and cost effective method of mass multiplication of *Trichoderma* spp. This way at least the adverse effect of chemicals such as inducing resistance in the pathogens, residual effect, deterioration of soil and water pollution can be minimized to great extent.

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افزایش ماندگاری گونه‌های *Trichoderma* از طریق افزودن محصولات آنتی اکسیدان به بسترهای مختلف

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چکیده: جنس *Trichoderma* به‌دلیل ظرفیت تولید مثل بالا، توانایی بقا در شرایط نامساعد، کارایی در استفاده از مواد غذایی، توانایی تغییر ریزوسفر، تجمع زیاد در مقابل قارچ‌های بیماری‌زای گیاهی و کارایی در تحریک رشد گیاه و مکانیسم‌های دفاعی، یکی از عوامل کنترل زیستی کارآمد به‌شمار می‌رود. از این‌رو پژوهش حاضر به‌منظور دستیابی به روش عملی و بی‌خطر برای تکثیر انبوه *Trichoderma* روی محیط‌های مختلف مبتنی بر محصولات کشاورزی انجام شد. از میان ترکیبات مختلف، کاه و کلش گندم و کود گاوی که با ده درصد آرد گندم غنی شده‌اند، به‌عنوان بهترین محیط جامد شناخته شدند. بیش‌ترین جمعیت گونه‌های *Trichoderma* در محیط کاه و کلش گندم شمارش شد. محصولات تولیدکننده مواد آنتی اکسیدانت نیز به مقدار ۵ گرم/کیلوگرم به این محیط اضافه شد تا عمر واحدهای تکثیری گونه‌های *Trichoderma* را افزایش دهند. حداکثر جمعیت شمارش شده در محیط حاوی سویا، ذرت و برنج قهوه‌ای مشاهده شد.

واژگان کلیدی: تریکودرما، کنترل زیستی، نکثیر انبوه و محصولات آنتی اکسیدانت

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