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Influence of Soil Nutrition and Rhizobiome Bacteria on the Growth of *Calocybe Indica* (P&C)

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ABSTRACT

In this present investigation, soil nutrient status and the rhizobiome bacterial population of myco-habituated natural soils was analysed. The results were compared with the steamed casing soil used regularly by the growers. The *Calocybe indica* myco-habituated *Cocos nucifera* root zone contained slightly higher level of nitrogen (0.41 g. g^{-1}) and potassium (0.51g. $5g^{-1}$) as compared to the rhizosphere soil of *Delonix regia* (0.46 g. $5g^{-1}$) and regular casing soil (0.41 g. $5g^{-1}$). The amount of phosphorous was slightly higher in *D. regia* rhizosphere soil sample (0.024g. 2.5 g^{-1}) followed by *C. nucifera* (0.021g. 2.5 g^{-1}) as compared to the regular casing soil, which contained 0.018 g. 2.5g^{-1} of soil. In case of bacterial population, ten different bacterial strains have been identified to be present in association with the myco-habitation of *C. indica* strains. The amplified DNA products of 700-800bp were eluted and sequenced. The sequenced nucleotides were blasted with NCBI database. Bacterial species *viz*., *Bacillus megaterium*, *Rhizobium pusense*, *Enterobacter cloacae*, *Sphingobacterium* sp, *Serratia marcescens*, *Pseudomonas hibiscicola*, *Bacillus aryabhattai*, *Bacillus* sp, *Rhizobium* sp, and *Pseudomonas* sp were found to be frequently associated with the mycohabitation of *C.indica*. The results revealed that, the total bacterial population or specific group of bacteria were always higher in the myco-habituated rhizosphere soils of the host trees as compared to the regularly used steamed casing soil.

KEYWORDS

Calocybe Indica, Rhizosphere Soil, Steamed Casing Soil, *Bacillus Megaterium, Pseudomonas.*

Introduction

Casing is an important agronomic practice in the cultivation of any humicolous mushroom and milky white mushroom is not an exception (Krishnamoorthy and Balan, 2015). Furthermore, they have reported that population build-up of pseudomonads was more in clay loam soil. Casing triggers off the change from vegetative to generative phase. Compact casing interfaces impede the diffusion of harmful metabolic gases on mushroom bed surface (Mac Canna, 1983). The casing layer must be sufficiently loose to facilitate primordia emergence (Sassine *et al.*, 2005). Casing soil also protects the compost against desiccation and supports the growth of *Agaricus blazei* and provides anchorage for the developing sporophores (Colauto *et al.*, 2011). Purkayastha (1984-1985) used loam soil and sand (1:1), mixed with calcium carbonate at 12 per cent level (pH 7.0) for casing *C.indica* beds. However, Krishnamoorthy *et al*. (1998) reported increased yield and bio-efficiency, when clay loam soil (pH 8.3) was used for the cultivation of milky mushroom. In addition to physical and chemical properties, the biological properties of the casing soil are known to influence the induction of fruiting bodies in case of *Agaricus bisporus* (Grewal and Rainey, 1991). The required physical and chemical properties of a good casing soil can be high porosity and water holding capacity (WHC), 7.2–8.2 pH, low content of soluble inorganic and organic nutrients and free of disease causing organisms and pests (Taherzadeh and Jafarpour, 2013).

Several bacterial species associated with casing soil and fruiting bodies of mushrooms have been reported. More exclusively, pseudomonads were found to be the dominant group of bacteria within the casing layer during the reproductive stage of mushroom (Miller *et al.*, 1995). *Pseudomonas putida* was the first bacterium identified as an important factor in the initiation and development of sporophores (Eger 1961). *Bacillus megaterium*, *Arthrobacter terregens* and *Rhizobium meliloti* have also been shown to produce metabolites that initiate primordial formation and

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increase the yields in *Agaricus bisporus* cultivation (Park and Agnihotri 1969). Krishnamoorthy *et al.* (1995) analyzed the presence of pseudomonads and actinobactetia in the steamed casing soil and reported that the bacteria favoured the sporophore production of *C. indica* var. APK2. In fact, *Pseudomonas* spp are ubiquitous bacteria in agricultural soils and have many traits that make them well suited as growth-promoting bacteria (Saharan and Nehra, 2011). Even some human pathogenic bacteria *viz.*, *Enterobacter, Pseudomonas*, and red pigmented *Serratia* were also known to be associated with the growth-promoting effects on *Agaricus bisporus* mycelium by the means of phosphate solubilization (Tilak *et al.*, 2005; Zarenejad *et al.*, 2012). It has been observed that certain bacteria could consume the volatile component, 1-octen-3-ol produced by mushroom vegetative mycelium. Such bacteria are involved in the organic and inorganic Pi solubilization and siderophore production (Henry *et al.*, 1991; Zarenejad *et al.*, 2012).

Bacteria and fungi are known to intermingle closely in the mycorrhizosphere and mutually influence their survival and colonization due to the production of carbon volatile components (C1-C10) (Wargo and Hogan, 2006; Minerdi *et al.*, 2008) by diffusion and advection (Minnich and Schumacher, 1993). These volatiles can move through the network of soil pores, since they are active in both gas and liquid phases. They are capable of revitalization after passing through water-saturated pores (Asensio *et al.*, 2008). However, due to their high vapour pressure, volatiles move primarily through vapour diffusion (Minnich and Schumacher, 1993). These processes are mainly, influenced by the inherent chemical properties of the volatiles and physicochemical properties of the surrounding soil. This present study was conducted to know the role of soil nutrition and bacterial association on the growth of milky mushroom *(C. indica*).

Materials and Methods

During survey and collection, *C. indica* isolates were very often found to have mycorrizal association with *Cocos nucifera* (L.) and *Delonix regia* (L.) roots. Hence, it was decided to find out the role of soil nutrition and associated bacteria, in influencing the growth of milky mushroom fungus.

Nutrient Analyses of Mycorrhiza (C. indica) Habituated Rhizosphere Soils

The rhizosphere soils of *C. nucifera* and *D. regia* habituated by *C.indica* were collected, air dried under shade and grounded by wooden roller. The homogenized soil sample was sieved through a 2 mm steel sieve and stored in polyethene bags. Steamed casing soil (clay loam soil with pH 8.3, regularly used by growers) sample was also used in the experiment for comparison. In this case, the mycelium impregnated casing soil at the time of first harvest was used for nutrient analysis after removing the paddy straw debris.

Estimation of Nitrogen

Total nitrogen content of the samples was estimated by 'Kjeldahl method" (Piper, 1966). One gram of the soil samples was taken individually, in 250 mL Kjeldahl flasks. Subsequently, 25 mL of concentrated sulphuric acid was added and after 2 h of cold digestion, the materials were again digested over flame. On completion of digestion (appearance of light green colour), the flasks were transferred to a macro Kjeldahl digestion unit and the ammonia was distilled with the addition of 40 mL of 40 per cent sodium hydroxide. The evolved ammonia was absorbed in 25 mL of 2 per cent boric acid, with mixed indicator (bromocresol green + methyl red) which, was then, titrated against standard sulphuric acid until the pinkish colour turned into green. Nitrogen per cent was calculated based on the titration value.

Estimation of Phosphorous

The method suggested by Olsen *et al.* (1954) was followed. Soil sample weighing, 2.5 g of was added to in 150 mL conical flasks and mixed with 0.5g of activated charcoal. To the mixture, 50 mL of 0.5 M $NaHCo_3$ solution was added and shaken for 30 min. similar process was run for blank without soil. The suspension was filtered through Whatman No.40 paper. Five mL of aliquot extract was added to a 25 mL volumetric flask and acidified with 5N H_2SO_4 and added 4mL with each of ammonium and potassium tartrate solution amended with ascorbic acid. The

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contents in the flash were kept for 10 min. at room temperature (30 ± 2^0C). The intensity of the blue colour developed was read in a spectrometer at 660 nm.

Estimation of Potassium

Soil samples weighing 5 g each were taken separately, in 150mL conical flasks; added with 10 mL of 1N ammonium acetate and shaken for 5 min. The exchangeable potassium was measured by analyzing the filtered extract in an atomic absorption spectrophotometer at 776 mm. The results were recorded in ppm and tabulated.

Assessment of pH and Electrical Conductivity

Individual soil samples weighing 20 g were well shaken with 40 mL of distilled water in a 250 mL conical flask for an hour. Then conductivity of the supernatant (saturation extract of soil) was determined with the help of conductivity meter. The pH was determined on a 1:1 (V/V) soil/water mixture (5 g NCR-13 soil scoop/5 mL DI water). Samples were stirred prior and after 15 min. of equilibration period. Then pH was measured with a Beckman pH meter.

Sample Collection and Isolation of Beneficial Bacteria

For isolating the substrate or mycelium borne beneficial bacteria, sample from the rhizobiome of *C.nucifera* and *D.regia*, as well as steam treated casing soil used for milky mushroom growing were collected. The population of bacteria in the substrate and casing soil was estimated by serial dilution technique using soil extract agar medium (Allen, 1957) and King's B medium (King *et al.,* 1954).

Plating the Rhizobiome Bacteria of Host Plants and Casing Soil

The rhizosphere soil of *C. nucifera* and *D. regia* and casing soil from mushroom beds at the time of first harvest were carefully collected with a sterilized scoop. Five g of each sample was transferred separately to 250 mL conical flasks containing100 mL of sterile distilled water. The flasks were shaken on a rotary shaker for 10 min. Serial dilution up to 10^{-6} were prepared with sterile pipette and the dilutions at 10^{-6} were used for plating the total cfu of associated bacteria. One mL of the aliquot was aseptically pipette out to sterile Petri dishes. The molten agar medium was poured and gently shaken to mix the medium and aliquot. The dishes were incubated at room temperature (30± 2°C). Individual colonies appearing on the plates were counted after 24 h and expressed as number of colony forming units (CFU) per g of soil. The isolated bacteria were identified through 16s rRNA sequencing and verified by BLAST searches in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/).

Isolation of Rhizomorph Associated Bacteria

Conspicuously present thick strands of *C. indica* hyphal filaments adhering to the root surface of *Cocos nucifera* and *Delonix regia* were collected cautiously. The root bits having rhizomorphs were carefully lifted with the help of a sterile camel hair brush and suspended in 10 mM potassium phosphate buffer (pH 7.3) for 20 min to remove the loosely attached hyphae. Fifty mg of mycelial mat, thus separated was carefully washed with sterile distilled water in a Petri plate and vortexed aseptically for 3 min. with 10 mM potassium phosphate buffer admixed with glass beads. The suspension was further sonicated for3 min. using a high intensity ultrasonic liquid processor to separate out the tightly adhering bacteria from the mycelial plane (Kim *et al.,* 2008). One mL of sonicated buffer has been aseptically dispensed to nutrient agar medium or King's B medium following dilution plate technique (Miles and Misra, 1938). The plates were incubated at room temperature (30± 2°C). Pure cultures were obtained by streaking the organisms several times on the growth medium. Frequently growing isolated bacterial colonies have been spotted out by visual observation and pure cultures were made on nutrient media. Molecular identification of the bacteria was done by sequencing of 16s rRNA and verified by BLAST searches in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/).

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Results and Discussion

Soil Nutrient Status

In this present investigation the rhizosphere soil of *C. nucifera* contained slightly higher (0.41 g. g^{-1}) level of nitrogen as compared to the rhizosphere soil of *D. regia* and steamed casing soil regularly used by growers (0.38 g. g of $soil^{-1}$). The potassium content was also found to be more in *C. nucifera* rhizosphere soil (0.51g. $5g^{-1}$) followed by the rhizosphere soil of *D. regia* (0.46 g. $5g^{-1}$) and regular casing soil (0.41 g. $5g^{-1}$). The amount of phosphorous was slightly higher in *D. regia* rhizosphere soil sample (0.024g. 2.5 g^{-1}) followed by the rhizosphere soil of *C. nucifera* (0.021g. 2.5 g^{-1}) as compared to the regular casing soil, which contained 0.018 g. $2.5g^{-1}$ of soil (Table 1). The pH of rhizosphere soils of *C. nucifera* and *D. regia* were 7.8 and 8.2, respectively. The EC was found to be more in the rhizosphere soils of *C. nucifera* (0.10dsm^{-1}) followed by that of regular casing soil (0.08 dsm^{-1}), while it was comparatively lower in the rhizosphere soil of *D. regia* (0.05 dsm^{-1}) **(Table 1).**

Table 1. Myco-habitation of *C. indica* - soil physical properties and major nutrients status

Soil samples	Habitate	pH	EC (ds/m^{-1})	Nutrient contents		
				N (g. g^{-1})	P (g. $2.5g^{-1}$)	K (g. $5g^{-1}$)
Casing soil	Milky mushroom bed casing soil	8.5	0.08	0.38	0.018	0.41
Rhizosphere	*Cocos nucifera*	7.8	0.10	0.41	0.021	0.51
	Delonix regia	8.2	0.05	0.38	0.024	0.46

Krishnamoorthy *et al.* (1995) had reported that *C. indica* had preferred nitrate form of nitrogen rather to ammoniacal nitrogen. Interestingly, Bonfante (2009) reported that plant nitrogen transporters are activated during mycorrhization and the mycobiont releases a substantial quantity of N to its host. Kumar *et al.* (2006) found that neutral pH was more favourable for mushroom growth. Vandana and Mishra (2014) conducted the experiments to find out the effect of casing soil and their physico-chemical properties on pinhead initiation and productivity of *C. indica* and reported that sandy soil having slightly alkaline pH, low nitrogen content, low phosphorus and potassium contents had given the lowest yield with minimum number of fruiting bodies. Evidently, higher nitrogen and phosphorus content in the casing soil resulted in increased yield. Electrical conductivity is a measure of total water soluble salt contents and in a typical commercial mushroom growing medium it would be a about 120-150 milli $mhos.cm^{-1}$ (Maher *et al.*, 2001). They also concluded that the EC level in spent mushroom compost was very closely related to K level in the compost. From the findings it is evident that the presence of EC level of rhizosphere soil of *C. nucifera* was highly correlated with the presence of potassium. Both the pottasium and EC concentration of rhizosphere soil of *C. nucifera* was found to be more. On the other hand, Fidanza and Beyer (2009) also concluded that EC of spent mushroom compost below 13.3 was ideal for early pinhead formation.

Identification of Bacterial Species through 16SrRNA

In this study, the bacterial species found in association with the mycohabitation of host roots, casing soil in mushroom beds and fruiting bodies were identified through 16SrRNA and the results are given in Table 2a and 2b. Ten different bacterial strains have been identified to be in association with the mycohabitation of *C. indica* strains. The amplified DNA products of 700-800 bp were eluted and sequenced. The sequenced nucleotides were blasted with NCBI database. Bacterial species *viz*., *Bacillus megaterium* (CS1, CN3, DR2 and DR5) having 98 per cent similarity matching with KC236804.1; *Rhizobium pusense* having 86 per cent similarity matching with DQ288946 were identified. The isolates CN2 and CN5 belonged to *Enterobacter cloacae* and *Sphingobacterium* sp. respectively.

The isolates DR1 and DR3 were identified to be *Serratia marcescens* and *Pseudomonas hibiscicola*, respectively. The *Bacillus aryabhattai* strain (DR4) was frequently observed in the rhizosphere soil of *D. regia*. The population level of bacteria in terms of CFU and frequency of occurrence in the recommended casing soil as well as in the

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rhizosphere regions of *C. nucifera* and *D. regia* were estimated. The result indicated the frequent occurrence of *Bacillus megaterium* in association with the substrate, mycelium, soil samples and sporophores in all the cases (**Table 2a,b**). However, when the no. of CFU were assessed soil pseudomonads like *Pseudomonas hibiscicola* and an unidentified *Pseudomonas* sp. were found to occur in large numbers (**Table 2a and Figure 1a,b**). The results also revealed that, the total bacterial population or specific group of bacteria were always higher in the myco-habituated rhizosphere soils of the host trees as compared to the regularly used steamed casing soil.

Table 2a. Myco-habitation of *C. indica* - rhizobiome bacteria

Sample	**Habitat specific bacteria**								
	Source	**Bacterial isolates**	**Total no.of colonies ($x10^{-6}$cfu)**	**No.of frequently occurring colonies ($x10^{-6}$cfu)**	**Cultural characters**	**Colony colour**	**Identified species**	**NCBI data base reference**	**Similarity per cent**
Recommended casing soil	Substrate associated mycelium	CS1	17.3	15.5	Slimy undulated growth	white	*Bacillus megaterium*	KC236804.1	98
	Sporophore	CS2	10.2	8.4	Mucoid, slight rhizoid	dirty white	*Rhizobium pusense*	DQ288946.1	86
	Soil with mycelium	CS3	21.5	17.7	Slimy growth with smooth margin	yellowish white	*Pseudomonas* sp.	KY882045.1	97
		CS4	13.4	9.3	Mucoid, undulate	dirty white	*Baciilus*sp.	MF289529.1	98
Rhizosphere soil of *C. nucifera*	Substrate associated mycelium	CN1	16.0	14.5	Slimy undulated growth	dirty white	*Bacillus megaterium*	KC236804.1	99
		CN2	12.4	8.3	Slimy irregular margin	white	*Enterobacterc loacea*	MG274289.1	85
	Sporophore	CN3	20.4	17.4	Slimy undulated growth	dirty white	*Bacillus megaterium*	KC236804.1	99
	Soil with mycelium	CN4	9.1	6.5	Umbonate growth	white	*Rhizobium* sp.	LC368035.1	86
		CN5	12.5	9.4	Slimy, entire margin	white	*Sphingobacterium*	JX102496.1	98
Rhizosphere soil of *D.regia*	Substrate associated mycelium	DR1	20.2	18.5	Pigmented	pinkish	*Serratia marcescens*	MF099813.1	98
		DR2	18.4	15.3	Slimy undulated growth	dull white	*Bacillus megaterium*	KC236804.1	99
	Sporophore	DR3	24.0	21.4	Slimy, transparent, smooth margin	yellow	*Pseudomonas hibiscicola*	KF836437.1	99
	Soil with mycelium	DR4	13.4	8.7	Raised, undulate margin	light purplish	*Bacillus aryabhattai*	KX443710.1	99
		DR5	15.1	11.4	Slimy undulated growth	dirty white	*Bacillus megaterium*	KC236804.1	99

Table 2b. Bacteria frequently found in association

Habitat	***Bacillus megaterium***	*Rhizobium pusense*	*Pseudomonas* sp.	*Baciil us* sp.	*Enterobacter cloacea*	*Rhizobium* sp.	*Sphingobacterium* sp	*Serratia marcescens*	*Pseudomonas hibiscicola*	*Bacillus aryabhattai*

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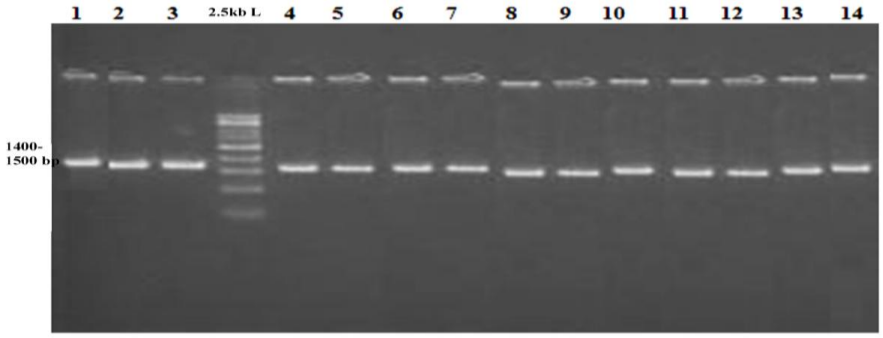
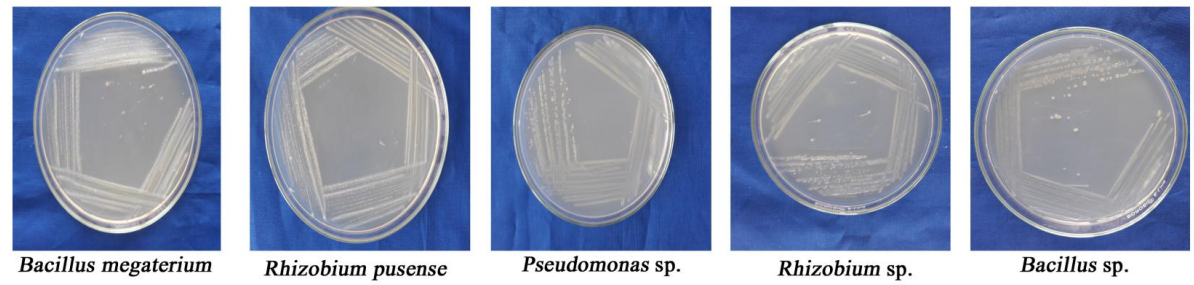
Substrate associated mycelium	√				√			√		√
Sporophores	√	√							√	
Soil with mycelium	√		√	√		√	√			



CS1-*Bacillus megaterium*
CS2-*Rhizobium pusense*
CS3-*Pseudomonas sp.*
CS4-*Baciilus sp*
CN1-*Bacillus megaterium*
CN2-*Enterobacter cloacea*
CN3-*Bacillus megaterium*
CN4- *Rhizobium sp*
CN5-*Sphingobacterium*
DR1-*Serratia marcescens*
DR2-*Bacillus megaterium*
DR3-*Pseudomonas hibiscicola*
DR4-*Bacillus aryabhattai*
DR5-*Bacillus megaterium*

Figure 1a. 16s rRNA amplification of bacterial isolates associated with the mycohabitation of C. indica



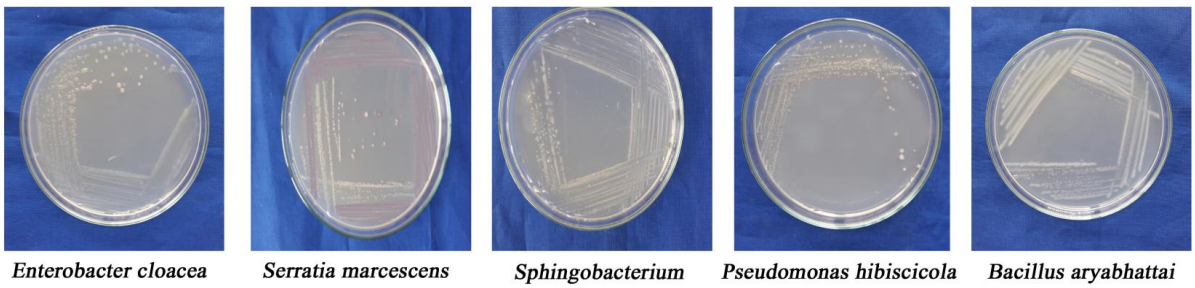


Figure 2b. Bacteria associated with substrate, mycelium and sporophore

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Ingratta and Blom (1979) stated that metabolism of bacterias in the casing material would create changes in lipid metabolism of the mycelium, which may result in the initiation of fruiting bodies. Singh *et al.,* (2007) concluded that steam treated casing soil produced better yield than the chemically treated one with formalin. Cho *et al.* (2008) identified various bacteria (*Agrobacterium*, *Bacillus*, *Erwinia*, *Pseudomonas*, *Ralstonia*, *Sphingobacterium*) from the sterilized and non-sterilized media used for the cultivation of *Pleurotus ostreatus* and they revealed that the population density of bacteria was higher in non-sterilized than in the sterilized media due to the presence of fluorescent *Pseudomonads* and actinobacteria. Since, it has been suggested that fluorescent Pseudomonas promotes the growth of *P. ostreatus*, the density level recorded may have a close relationship with the formation of fruiting body. Jarial (2002) found a positive and significant correlation between the population of *Bacillus megaterium* and *Pseudomonas sp.* isolated from various casing media.

Li-Sen Young *et al.* (2013) identified 34 bacterial species associated with *Agaricus blazeii* mushroom including *Microbacterium arabinogalactanolyticum*, *Bacillus megaterium*, *Serratia marcescens*, *Advenella incenata* and *Ochrobactrum anthropi*. Members of phylum proteobacteria which accommodates *Enterobacter*, *Pseudomonas* and *Serratia* are known to be associated with the plant rhizosphere and are able to exert growth promoting effects of mushrooms (Tilak *et al.,* 2005; Zarenejad *et al.,* 2012). Further, they revealed that 29 per cent of bacterial isolates were able to secrete cellulase, 18 per cent of them conferred phosphate‐solubilization and 14 per cent had showed had nitrogen‐ fixation ability. All these positive characters are very much needed for a mushroom fungus to establish itself strongly at various stages of development. Another explanation was given by Flegg (1989) who stated that the bacteria plays a major role in metabolizing eight carbon (C8) volatile compounds (1-octen-3ol and 2-ethyl-1-hexanol) produced by the mushroom mycelium. The volatile compound 1-octen-3-ol is one of the most important flavor components present in fresh mushrooms. This compound also acts as self-inhibitor at very high spore densities (Chitarra *et al.,* 2005). Noble *et al.* (2009) noticed increased level of total bacterial and pseudomonad populations in the casing soil, 2-ethyl-1-hexanol and 1-octen-3-ol volatile compounds produced by the mycelia was found to be increased.

Conclusion

This study supports the present finding and clearly indicates that the bacterial population in casing layer could contribute to the natural flavor of mushrooms. It was also evident from the current study that, the bacterial population was high in rhizosphere soils when compared to regularly use casing soil. Hence, it is concluded that, the flavor of wild mushrooms will always be more than the cultivated ones.

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