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Studies on the nutritional requirement and *in vitro* synthesis of mycorrhiza of *Cedrus deodara* with *Rhizopogon himalayensis*

Lal Singh[1], Ashwani Tapwal[2], J.S. Thakur[3] and T.N. Lakhanpal[4*]
[1]*Himalayan Research Group, Chotta Shimla 171002, Himachal Pradesh, India*
[2]*Himalayan Forest Research Institute, Shimla 171013, Himachal Pradesh, India*
[3]*Govt. College Banjar, Kullu 175123, Himachal Pradesh, India*
[4]*Department of Biosciences, Himachal Pradesh University, Shimla 171005, Himachal Pradesh, India*
**Corresponding author Email: tezlakhanpal@rediffmail.com*
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ABSTRACT

Investigations on *in vitro* cultures of *Rhizopogon himalayensis* Mujic *et al.* (syn.: *Trappeindia himalayansis*), a mycobiont of *Cedrus deodara* discovered first in the N.W. Himalayas, were carried out on twelve solid and four liquid media to determine its requirement of carbon, nitrogen, trace elements, vitamins and growth regulators. Out of these Pridham Yeast Malt Dextrose Agar and Glucose Asparagine solution, respectively were evaluated as the best solid and liquid mediums for the growth of this mycobiont. For investigations, the basal media was amended with different concentrations of selected nutrient sources. The dry weight of mycelium was recorded and compared with control. The results revealed that out of sixteen carbon sources evaluated maximum growth of mycelium was observed in basal medium having sucrose as the carbon source. Among the twenty four organic, inorganic and complex nitrogen sources, arginine supported the maximum mycelial growth as compared to others. The amendment of the basal media with different trace elements had no significant effect on the mycelial growth of the mycobiont. Among vitamins and growth hormones, ascorbic acid and gibberellic acid, respectively registered enhanced growth of *R. himalayensis*. *In vitro* ectomycorrhizal synthesis between *R. himalayansis* and *C. deodara* was achieved and the synthesized mycorrhiza resembled the natural one in almost all aspects.

Keywords: *Rhizopogon himalayensis, Trappeindia himalayensis*, *Cedrus deodara*, hypogeous, mycobiont, nutritional requirement, N.W. Himalaya.

INTRODUCTION

Cedrus deodara (Roxb. ex D. Don) G. Don, commonly known as Deodar or Himalayan cedar, is a valuable timber tree of India. Natural regeneration of *C. deodara* is influenced by seedling conditions, germination and climatic conditions. In good seed years, abundant seedlings grow in the neighborhood of seed bearing trees but usually they fail to establish. Many reasons have been ascribed for this poor regeneration in nature but one major reason appears to be non-mycorrhization or poor mycorrhization of seedlings under natural conditions. Hence inoculation of seedlings with specific mycorrhizal associate appears to be a possible alternative to help the seedling establishment and survival. Mycorrhiza is well known to help the inoculated seedlings in their establishment, survival and growth (Harley and Smith, 1983; Goltapeh *et al.,* 2008; Plassard and Dell, 2010; Shi *et al.*, 2017; Kumar and Atri, 2017). *C. deodara* was documented to form ectomycorrhizal association with a hypogeous edible fungus, *Rhizopogon himalayensis* Mujic *et al.*, first collected and described from the N.W. Himalaya (Himachal Pradesh). The fungus was initially identified as *Octaviania densa* (Rodw.) Cunn. by S.L. Miller (USA) through the courtesy of Dr. S.L. Stephenson, W. Virginia, USA. To begin with some preliminary studies were published under the same name (Singh and Lakhanpal, 1988). James Trappe and M. Castellano (personal communication, 1990) authenticated it to be a new genus and species and Castellano *et al.* (2012) published it as *Trappeindia himalayansis,* a new taxon. After molecular and phylogenetic analysis now it has found its placement in *Rhizopogon himalayensis* (Mujic *et al.,* 2019) which is considered to be representing a unique lineage of *Rhizopogon*.

Nomenclature riddle apart, the fungus has an interesting anecdote. It was first brought to the last author by a colleague Dr. Tej Partap who collected it from Kasol near Manikaran in the Kullu valley in 1986 where local inhabitants gather it for their personal consumption since early times from the natural *Cedrus deodara* forests. They consumed it either raw after peeling off the peridium or after roasting it in burning ash and eating it like potatoes. Subsequently it has been collected from the same as well other areas from deodar forests (Singh, 1992; J.S. Thakur, personal communication).

In this article the basic nutritional requirements of the mycobiont and preliminary observations on *in vitro* mycorrhizal synthesis are being presented. The systematic account of the fungus has already been published (Castellano *et al.*, 2012),

METHODOLOGY

1. Collection of the materials: The sporophores of this fungus usually start appearing during spring, in the month of March after the snow melts, and can be collected up to the end of April. Being an entirely hypogeous mushroom, for locating the fructifications the soil in the vicinity of *C. deodara* trees was gently raked with the help of a forest raker. The sporocarps were found organically attached to roots as beads to a string. These roots were detached from the tree along with the sporocarps, and then these were gently washed in running water 2-3 times so as to remove the extraneous debris. Subsequently the roots were fixed in Formaline Acetic Acid (FAA)** for 24 hours and then thoroughly rinsed in running water and preserved in 70% alcohol for undertaking morpho-anatomical studies. The collected sporocarps were dried usually in normal sunshine.

** Formaldehyde 40% = 5.00 mL, Ethyl Alcohol 70% = 90 mL, Acetic Acid= 5.00 mL

2. Morpho-anatomical studies of the natural mycorrhiza: Morpho-anatomical studies of mycorrhizae were undertaken following Zak (1971). For this purpose both hand and microtome sectioning was done following Johansen (1940). The colour of the Hartig net and mantle was observed in unstained sections. For bringing clarity in cellular details, the sections were stained in Cotton Blue and Safranin- Fast green combinations.

Isolation of mycobiont and screening of media for its culturing: The mycobiont was isolated from fresh sporocarps and mycorrhizal roots on potato dextrose agar (PDA) at 22 ± 1°C. To find out the best media for growth, the mycobiont was grown on twelve solid media[1] and four liquid media[2] at 30 ±1 °C for 10 days and growth was measured.

3. Optimization of nutritional requirements of the mycobiont: The nutritional requirements of the mycobiont were investigated by raising cultures in liquid basal medium (Glucose Asparagine Solution) at 30 ± 1°C for 10 days.

3.1 Effect of Carbon (C) and Nitrogen (N): To study the effect of carbon sources on mycelial growth, 30 g dextrose in basal medium was replaced by different carbon sources on the basis of their molecular weight. The polysaccharides were used at the rate of 5 g/L. Similarly different nitrogen sources were used in equivalent quantities so as to replace nitrogen provided by 1 g of Asparagine of the basal medium. Complex nitrogen source (pepton) was used at the rate of 1 g/L (Saini and Suppal, 1977). An amount of 50 mL of each medium was taken in 250 mL flasks, autoclaved and inoculated with 8 mm mycelial disc having 0.6 mg mycelial load. The flasks were incubated at 30 ± 1°C for 10 days and growth was measured on dry weight basis of the mycelium.

3.2 Effect of vitamins: Seven vitamins[3] in three concentrations each were used to determine their effect on vegetative growth of the mycelium. The stock solutions of all the vitamins except biotin, were prepared in double glass distilled water and stored at 2-5°C in refrigerator. The stock solution of biotin was prepared in 5 mL of 50 per cent ethanol and volume was made up with double glass distilled water. The basal medium was purified by adding 5 g/L of activated charcoal and filtering through Whatman filter paper number 1 following Mathur *et al*. (1950). An amount of 50 mL of each vitamin substituted medium was sterilized in 250 mL flasks by steaming for one hour for three successive days. Inoculation was done using 8 mm culture disc bearing 0.7 mg mycelial load and flasks were incubated at 30 ± 1°C for ten days. The growth was recorded on dry weight basis of the mycelium.

3.3 Effect of trace elements: Five trace elements[4] in three concentrations, each were used to study their effect on mycelial growth. The stock solutions were prepared in double glass distilled water. The medium was purified by adding 1 g/L of Calcium carbonate (Steinberg, 1950). Two controls, one containing no trace element and the other containing a mixture of all the trace elements in their respective concentrations, were kept. Mycelial disc of 8 mm size bearing 0.7 mg mycelial load was inoculated in the flask containing 50 mL medium. The inoculated flasks were incubated at 30 ± 1°C and growth was recorded by taking into account dry weight of the mycelium after 10 days of incubation.

3.4 Effect of growth regulators: To study the effect of growth regulators on vegetative growth of mycelium, five growth regulators[5] in five concentrations each were used. The stock solutions of all the growth regulators, except gibberellic acid (GA), were prepared in double glass distilled water and stored at 5± 1°C in a refrigerator. GA was first dissolved in 10 mL acetone and then the required dilutions were prepared. For inoculation 8 mm mycelial disc bearing 0.6 mg mycelial load was used in each flask containing 50 mL the medium. The flasks were incubated at 30 ± 1°C and the growth was recorded on the basis of dry weight of the mycelium obtained after 10 days of incubation.

4. *In vitro* synthesis of mycorrhiza: *In vitro* synthesis of mycorrhiza between *Cedrus deodara* and *Rhizopogon himalayensis* was carried out following Molina and Palmer (1982). In two litre flask 880 mL of vermiculite and 20 mL of peat moss was added, saturated with 600 mL of Pridham Yeast Malt Dextrose Agar and autoclaved for 2 hours. The seeds of *C. deodara* were surface sterilized with 30% H_2O_2 and germinated on water agar. The germinating seed was fully embedded into the substrate and inoculated with 8 mm discs bearing average mycelial load of 0.7 mg of *R. himalayensis*. After this growth and development of mycorrhiza was recorded.

RESULTS

Cedrus deodara is a large, evergreen tree attaining a height of about 76 m and girth of about 14 m. The old trees usually have flattened tops, but young trees are conical in appearance (**Fig. i**). The short roots of limited growth are usually infested by mycorrhizal fungi.

1. Morpho-anatomical features of the *C. deodara* mycorrhiza: The mycorrhizal root arises in *C. deodara* as a lateral outgrowth from the mother root. Young mycorrhizal roots are creamish white initially, then turn brown, dark brown and ultimately black with age. They are racemosely branched initially and on further branching assume a coralloid appearance (**Fig. ii**). The coralloid roots are highly coiled and brittle when dead. They lack root hairs and average diameter of mycorrhizal roots ranges from 0.5-1.50 mm. The mycorrhizal roots exhibit typical ectomycorrhizal anatomy, *viz*. thin mantle

1 Pridhan Yeast Malt Dextrose Medium, Malt Yeast Agar Extract, Malt Agar, PDA, Yestal PDA, Horse Gram Extract, Pea Extract, Czapek's Dox Agar, Coriander Extract, Maize Grain Extract, Wheat Grain Extract, Glocose Yeast Agar.

2 Glocose Asparagine, Czapeks-dox Solution, Dimmick Medium, Asthana and Hawker's Media.

3 Biotin; Nicotinic Acid (Niacine); Riboflavin; Folic Acid, Choline Chloride; Thiamine Hydrochloride; Ascorbic Acid. All in 3 concentrations i.e. 25, 50 & 100 mg/L

4 Iron ($FeSO_4.7H_2O$), Manganese, ($MnSO_4.H_2O$), Zinc ($ZnSO_4.7H_2O$), Molybednum ($(NH_4)_6Mo_7O_{24}4H_2O$), Boron (H_3BO_3). All in 3 concentrations i.e. 1, 2 & 3 mg/L.

5 Gibberellic Acid (GA), Indole Acetic Acid (lAA), Indole Butyric Acid (IBA), Naphthalene Acetic Acid (NAA), Kinetin. All in 5 concentrations i.e. 1, 5, 10, 20 & 40 mg/L.

and characteristic Hartig net (**Fig. iii**). In due course the mantle disintegrates and disappears in mature roots. The intracellular hyphae in the cortex are altogether absent.

2. Isolation and culturing of mycobiont: Mycobiont was isolated on PDA from the fresh fruit body of *Rhizopogon himalayansis* as well as from natural mycorrhizal short roots. The comparison of both cultures revealed similar microscopic and cultural characteristics. *R. himalayansis* is an edible hypogeous mushroom which grows in the rhizospheric region of *C. deodara*. Fruit bodies were completely subterranean, but these usually get exposed by trampling of soil by animals or by the erosion of the above lying soil due to rain (**Fig. iv**).

The culture of *R. himalayansis* initiated growth within 3-4 days of inoculation and attained 5-6 cm colony diameter within 10 days of incubation. Colony was radially furrowed, mat cottony white, margin even, colony surface tomentose, which became dirty white with age (**Fig. v**). The submerged

Figs.: i. *Cedrus deodara* tree, ii. Natural mycorrhizal roots, iii. T.S. of natural mycorrhizal root, iv. Sporophores of *R. himalayensis,* v. *R. himalayansis* in culture on solid media, vi. *In vitro* synthesis of mycorrhiza, vii. Mycorrhizal roots under low magnification, viii. Mycorrhizal roots under high magnification ix. T.S. of mycorrhizal root.

mycelium in liquid medium also appeared dirty white. Distinct odour and colour change in liquid medium was not observed. Hyphae were 2.0-4.5 µm in diameter, cylindrical and hyaline, slightly yellowish in KOH and light yellowish brown in Melzer's reagent and there was no change in colour in ferrous sulphate ($FeSO_4$) solution. Surface encrustations and clamp connections in mycelia were altogether absent. Among the tested solid and liquid media, Pridham Yeast Malt Dextrose Agar (PYMDA) and Glucose Asparagine solution were recorded as the best media for the growth of mycobiont. The culture isolated from sporophores and from mycorrhizal roots exhibited similar culture characteristics and behaviour in liquid and solid media. The growth of the fungus was optimum at 30± 1°C, at pH of 7.00. Therefore, further studies were conducted only in glucose asparagine solution at 30±1°C.

3. Nutritional requirements of *R. himalayansis*: The nutritional requirements of *R. himalayensis* were investigated by raising its cultures in liquid media supplemented with different nutrient sources (carbon, nitrogen, trace elements, vitamins and growth hormones). The mean mycelial dry weight was recorded and compared for significance by t-test.

3.1. Carbon nutrition: Sixteen carbon sources were tried for assessing the growth response of *R. himalayansis* in culture. In tested carbon sources, except for sugar acids (oxalic acid, tartaric acid, succinic acid and citric acid) significantly higher growth of mycobiont was observed ($p \leq 0.05$). Maximum growth of mycelium was observed in basal medium substituted with sucrose followed by growth in maltose (135.86 mg), dextrose (125.33 mg), xylose (124.45 mg), lactose (116.83 mg) and galactose (101.13 mg). The mycelial dry weight in rest of the carbon sources was below 100 mg, but higher in comparison to control (57.40 mg) (**Table-1**).

3.2. Nitrogen nutrition: The basal medium was substituted

Table-1: Dry weight yield of *Rhizopogon himalayansis* grown in basal media containing different carbon and nitrogen sources.

Carbon Source	Dry weight of mycelium (mgs)	Nitrogen Source	Dry weight of mycelium (mgs)
Dextrose	125.33 (1.28)*	Ammonium nitrate	53.10 (0.55)
Fructose	63.70 (1.04)	Ammonium tartrate	60.83 (1.04)
Xylose	124.45 (1.20)	Ammonium oxalate	63.73(1.92)
Sucrose	265.00 (3.37)	Sodium nitrate	59.23(1.36)
Maltose	135.86 (1.41)	Urea	53.73(1.53)
Lactose	116.83 (0.58)	Peptone	67.03(2.05)
Mannose	96.00 (1.10)	Arginine	77.76 (1.87)
Galactose	101.13 (1.62)	Alanine	43.23(1.06)
Glycogen	86.33 (2.30)	Cystine	63.53(2.15)
Cellulose	77.50 (1.34)	Glutamic Acid	54.30 (3.12)
Starch	67.00 (1.38)	Leucine	64.66(1.95)
Mannitol	35.70 (1.17)	Histidine	55.93(1.00)
Oxalic Acid	-	Lysine	50.96 (0.83)
Tartaric Acid	-	Methionine	43.23 (0.32)
Succinic Acid	-	Proline	59.10 (1.00)
Crtric Acid	-	Threonine	58.10 (0.36)
Control	57.40 (1.51)	Tyrosine	61.63(1.51)
		Valine	60.60 (1.21)
		Tryptophan	56.66 (0.72)
		Aspartic Acid	-
		Glycine	52.13 (1.33)
		Phenylalanine	67.11 (1.22)
		Serine	45.40 (2.52)
		Asparagine	62.40 (0.78)
		Control	57.30 (1.37)

*Figures in parenthesis are standard deviations.

Table-2: Dry weight yield of *Rhizopogon himalayansis* grown in basal media containing different concentrations of trace elements

S. No.	Trace Element Source	Dry weight of mycelium (mgs)		
		1 mg/L	2 mg/L	5 mg/L
1.	Boron H_3BO_3	55.26 (3.60)*	47.80 (4.07)	42.32 (3.04)
2.	Iron $FeSO_47H_2O$	46.73 (3.85)	57.76 (1.33)	51.53 (0.35)
3.	Manganese $MnSO_47H_2O$	52.66 (3.05)	46.20 (0.00)	51.81 (3.90)
4.	Zinc $ZnSO_47H_2O$	52.70 (0.17)	52.60 (0.65)	46.43 (1.60)
5.	Molybedenum $(NH_4)_6Mo7O24H_2O$	43.63 (1.80)	49.20 (1.70)	50.70 (1.21)
6.	Mixture of trace elements	44.40 (3.55)	39.86 (1.33)	45.66 (0.58)
7.	Control	55.90 (1.05)	55.90 (1.05)	55.90 (1.05)

*Figures in parenthesis are standard deviations.

with twenty four different nitrogen sources in separate sets of experiments and dry weight of mycelium was recorded. Among the tested nitrogen sources, 13 nitrogen sources enhanced the growth of fungus in comparison to control (**Table-1**). Significantly higher growth ($p \leq 0.05$) was recorded in the media substituted with ammonium tartrate, ammonium oxalate, peptone, arginine, cystine, leucine, proline, tyrosine, valine, phenylalanine and asparagine as nitrogen source. In other sets of experiments, less growth was recorded as compared to control. The media substituted with aspartic acid as nitrogen source did not support the growth of the fungus. Maximum growth was recorded in media containing arginine (77.76 mg) followed by phenylanine (67.11 mg) and peptone (67.03 mg).

3.3. Trace element nutrition: Five different trace elements in three concentrations (1 mg/L, 2 mg/L, and 5 mg/L) were tested to determine their effect on the mycelial growth of *R. himalayansis*. It has been observed that the amendment of media with selected trace elements has not significantly altered the mycelial growth of mycobiont. The dry weight of the mycelia was either less or at par with control (**Table-2**).

3.4. Vitamin nutrition: Seven different vitamins in three concentrations (25 mg/L, 50 mg/L and 100 mg/L) were tested to investigate their effect on the growth of *R. himalayansis* (**Table-3**). The amendment of media with vitamins exerted varied response on the growth of *R. himalayensis*. At 25 mg/L concentration of vitamin in basal media, the growth of mycobiont was found to be higher than control (54.13 mg) only in media supplemented with riboflavin (38.72 mg) and mixture of vitamins (54.55 mg). Similarly, at 50 mg/L concentration, higher growth was recorded in media supplemented with choline chloride (57.23 mg). The differences in the growth were non-significant ($p \leq 0.05$).

Table-3: Dry weight yield of *Rhizopogon himalayansis* grown in basal media containing different concentrations of vitamins

S. No.	Vitamin	Mycelium weight (mgs)		
		25 mg/L	50 mg/L	100 mg/L
1.	Biotin	49.31 (2.83)*	45.90 (3.46)	56.98 (0.72)
2.	Ascorbic Acid	46.88 (2.15)	45.88 (3.47)	91.66 (1.50)
3.	Nicotinic Acid	52.23 (2.68)	42.99 (1.80)	77.15 (0.92)
4.	Folic Acid	49.66 (0.49)	49.33 (3.05)	68.33 (1.98)
5.	Choline Chloride	51.16 (1.40)	57.23 (2.89)	56.86 (1.27)
6.	Riboflavin	38.72 (2.73)	51.73 (1.54)	34.92 (4.34)
7.	Thiamine Hydrochloride	55.33 (2.08)	46.16 (2.00)	45.25 (9.33)
8.	Mixture	54.55 (0.50)	52.52 (2.59)	80.58 (1.49)
9.	Control	54.13 (2.80)	54.15 (2.83)	54.15 (2.83)

*Figures in parenthesis are standard deviations.

While at the 100 mg/L concentration of vitamins in media, significantly higher growth of mycobiont was recorded ($p \leq 0.05$) in media amended with ascorbic acid (91.66 mg), nicotinic acid (77.15 mg), folic acid (68.33 mg) and mixture of vitamins (80.58 mg). The growth of mycobiont in other sets of experiments was found to be either less or at par with control (54.15 mg).

3.5. Effect of growth hormones: Five growth hormones in five different concentrations (1 mg/L, 5 mg/L, 10 mg/L, 20 mg/L and 40 mg/L) were supplemented in basal media and mean dry weight of mycelium was recorded. The result revealed higher growth of mycobiont in all treatments except for naphthalene acetic acid at the concentrations of 5, 20 and 40 mg/L, where less growth was recorded (**Table-4**). The dry weight of *R. himalayensis* was significantly higher ($p \leq 0.05$) in media supplemented with 5 mg/L gibberellic acid (135.27 mg) followed by growth in kinetin (130.45 mg) at 5 mg/L concentration and gibberellic acid (129.48 mg) at 10 mg/L concentration as compared to control (56.23 mg).

4. *In vitro* synthesis of mycorrhiza: *In vitro* mycorrhizal synthesis was carried out in 2 liter flasks. Aseptically germinated seedlings were introduced into the flasks and inoculated with 8 mm disc of mycobiont culture. The mycorrhizal synthesis was carried out successfully in four months. Ectomycorrhizal formation was observed well with all the roots of *C. deodara* seedlings. The mycorrhizal roots arise as monopodial branches and upon further branching they became coralloid. The colour also changes from creamish white to dark brown. The Hartig net is well developed and mantle gets obliterated with age. After fungal penetration, the colour of cortical cells changes to light yellow in the beginning and later on to dark brown. The

Table-4: Dry weight yield of *Rhizopogon himalayansis* grown in basal media containing different concentrations of growth hormones.

S. No.	Growth hormone	Dry weight of mycelium (mgs)				
		1 mg/L	5 mg/L	10 mg/L	20 mg/L	40 mg/L
1.	Gibberellic Acid	81.28 (4.67)*	135.27 (5.02)	129.48 (3.65)	99.01 (5.28)	88.66 (6.11)
2.	Indole Acetic Acid	69.40 (4.13)	66.73 (4.60)	60.36 (1.45)	58.38 (3.50)	57.66 (2.51)
3.	Indole Butyric Acid	76.80 (2.46)	105.00 (3.65)	110.80 (1.25)	95.08 (4.98)	79.82 (2.58)
4.	Naphthalene Acetic Acid	45.42 (2.47)	57.76 (2.81)	60.35 (2.06)	48.82 (3.24)	41.17 (1.00)
5.	Kinetin	82.98 (2.79)	130.45 (1.62)	123.2 (2.87)	113.66 (5.68)	97.81 (2.59)
6.	Mixture	81.42 (3.19)	122.83 (2.46)	103.33 (4.08)	99.77 (4.96)	85.72 (5.87)
7.	Control	56.23 (1.04)	56.23 (1.04)	56.23 (1.04)	56.23 (1.04)	56.23 (1.04)

*Figures in parenthesis are standard deviations.

ectomycorrhiza synthesized *in vitro* resembles the natural ectomycorrhiza in morphology and anatomy. The mycobiont was reisolated and compared with the initial culture and these were observed to be identical, verifying the ectomycorrhizal association between *C. deodara* and *R. himalayensis* (**Fig. vi-ix**).

DISCUSSION

The present studies corroborate the findings of Bakshi *et al.* (1968) that the mycorrhizal roots of *C. deodara* are racemosely branched initially, and corralloid on later branching. Anatomically the mycorrhizal roots possess a characteristic Hartig net but an ephemeral and not well defined mantle. In the initial stages of mycorrhization, the roots are covered with a few white fungal hyphae which are not organized into a well defined sheath. This is in consonance with the observation of Harley and Smith (1983) who also reported that mantle may be as thick as the cortex or it may be just of few scattered hyphae on the outer surface of roots.

In the ectomycorrhizal association, the host plant supplies carbon to the mycobiont and in the present study best growth of the mycobiont was recorded in basal medium containing sucrose followed by maltose as has been reported by Molina and Palmer (1982). As has been observed presently, the ectomycorrhizal fungi are reported to prefer simple carbohydrates as carbon source (Harley and Smith, 1983; Hampp and Schaeffer, 1999). As reported in the present studies, Lazarević *et al.* (2016) also recorded significant differences in the growth and development of a number of ectomycorrhizal fungi when grown on different sources of carbon and nitrogen at varied temperature and pH levels under *in vitro* conditions.

Results on physiological requirements in the present study are supported by Bakshi (1974) for *Scleroderma bovista*, *Astraeus hygrometricus* and *Cenococcum graniforme* and by Molina and Palmer (1982). Thapar (1989) documented maximum mycelial growth of these fungi in the medium containing glucose, sucrose and maltose as the carbon sources. Similarly cellobiose, maltose, trehalose and sucrose among disaccharides have been reported to support satisfactory growth of many isolates of mycorrhizal fungi (Molina and Palmer, 1982; Thapar, 1989).

In the present study, the sugar acids (oxalic acid, tartaric acid, succinic acid and citric acid) were observed to be inhibitory to the mycelial growth of *R. himalayansis*. Earlier, a few organic acids or their salts (citric, fumaric, malic, propionic and succinic acids) are reported to support growth of a few isolates whereas acetates have been reported to be inhibitory (Ferry and Das, 1968; Giltrap and Lewis, 1981; Lahio, 1970; Lamb, 1974; Norkrans, 1950; Palmer and Hacskaylo, 1970).

Out of the twenty four organic, inorganic and complex nitrogen sources evaluated, maximum growth of *R. himalayansis* was obtained in the basal medium containing the organic source arginine supporting the observations of Melin (1925), Molina and Palmer (1982), Dames *et al.* (1999) and Lazarević *et al.* (2016).

During the present studies the fungus has been observed to show specific preference for amino acid nitrogen sources like asparagine followed by phenylalanine, cystine and leucine. Bakshi (1974) also reported similar results for nitrogen utilization by different fungi. Contrary to this, Lundeberg (1970) reported inhibitory effect of asparagine on *Paxillus involutus*, *Amanita citrina, A. muscaria* and some strains of boletii. However, Lahio (1970) reported preference for glutamic acid as a nitrogen source for *Paxillus involutus*.

Out of the five trace elements used, only boron (l mg/L), iron (2 mg/L) and manganese (l mg/L) supported good mycelial growth of *R. himalayansis* statistically equal to control. It has been pointed out that essentiality of trace elements has not been determined for ectomycorrhizal fungi (Palmer, 1971; Molina and Palmer, 1982). However, the trace elements have been reported to play a significant role in increasing and decreasing the growth of cultures of cultivated fungi such as *Pleurotus* sp. (Delmas and Mamoun, 1981; Jandaik and Kapoor, 1976).

The growth of *R. himalayansis* was significantly higher in 100 mg/L of nicotinic acid, folic acid and mixture of different vitamins. The other vitamins in different concentrations, individually or in mixture retarded the growth of mycelium as compared to control. As in case of trace elements, the role of vitamins is well documented in cultivated mushrooms than in the ectomycorrhizal fungi (Sugimori *et al.,* 1971; Voltz, 1972). Molina and Palmer (1982) reported that many ectomycorrhizal fungi are heterotrophic for thiamine and some for biotin but that none of the vitamins is regularly added to semi synthetic media. They suggested that both these should be added if the fungus is being cultured for the first time, that's why the vitamin requirement of the fungus was studied.

Little is Known about the effect of growth hormones on the ectomycorrhizal fungi. Molina and Palmer (1982) stressed that stimulation of growth by growth regulators is quite frequent and that inoculation of single compound and natural mixture of casein hydrolysate frequently increases the growth but rarely to a significant level. In the present study except for naphthalene acetic acid, all growth hormones exerted stimulatory effect on the growth of mycelium.

In vitro synthesis of ectomycorrhiza between *C. deodara* and *R. himalayansis* was carried out by slightly modifying the methodology of Molina and Palmer (1982). The *in vitro* synthesized mycorrhiza was identical to the naturally occurring one, confirming the ectomycorrhizal association. Mycorrhizal synthesis was carried out successfully in four months. This is the first experimental confirmation of *in vitro* ectomycorrhizal synthesis between *R. himalayansis* and *C. deodara*.

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