WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.990
Volume 4, Issue 9, 1188-1198. Research Article ISSN 2277– 7105

PHYSIOLOGICAL AND NUTRITIONAL STUDIES OF SOME ENDOPHYTIC FUNGI OF ACHANAKMAR AMARKANTAK BIOSPHERE RESERVE, CHHATTISGARH

Amit Sharma[1], R. V. Shukla[2] and Shweta Sao[3*]

[1]Asst Prof. Deptt. of Botany, Dr. C. V. Raman University Kota, Bilaspur, Chhattisgarh

[2]Prof. Dept of botany, C M D P G Collage Bilaspur, Chhattisgarh.

[*3]Associate Prof. & Head Dept of Life Sciences, Dr. C. V. Raman University Kota, Bilaspur, Chhattisgarh.

Article Received on
26 June 2015,

Revised on 16 July 2015,
Accepted on 10 Aug 2015

***Correspondence**
For Author
Dr. Shweta Sao
Associate Prof. & Head
Dept of Life Sciences , Dr. C. V. Raman University
Kota, Bilaspur,
Chhattisgarh.

ABSTRACT

Fungal endophytes have been studied from three ethno medicinal plants such as *Terminalia arjuna* (Arjun), *T. chebula* (Harra) and *Shorea robusta* (Sal) of Achanakmar-Amarkantak Biosphere Reserve Mungeli, Chhattisgarh. All the plants were found colonized with endophytic fungi. Endophytes are microorganisms that form symptomless infections within plants. They have widespread existence in various plants. Although the mechanism is unclear, an endophytic fungus actually plays an important role in local ecology. Physiological and nutritional studies on some important endophytic fungi i.e., *Botrytis* sp., *Curvularia* sp., *Monodyctis* sp., *Scytalidium* sp. and *Verticillium* sp. were performed viz. requirement of suitable media, carbon and nitrogen requirement, temperature study, pH study etc. which revealed some important characteristic of those fungi and their role in the environment.

KEYWORDS: Endophytic fungi, Physiological studies, Achanakmar-Amarkantak Biosphere Reserve, Chhattisgarh.

INTRODUCTION

Endophytic fungi are microorganisms that colonize and cause asymptomatic infections in healthy plant tissues (Wilson, 1995). The term endophyte has been introduced by de Bary (1866) and was initially applied to any organism found within a plant. Carroll (1986) defined endophytes as organism that causes asymptomatic infections within plants. The endophytic

mycobiota are distributed based on the ecological and physiological factors in plants (Khan et al., 2010) such as geographical location (Fisher et al., 1994; Collado et al., 1999), age and specificity of host tissue (Khan et al., 2010; Sahashi et al., 2000; Bills and Polishook, 1991). Endophytic fungi forms a major role for plant physiology and it absorb nutrients from the host, but at the same time host plant were also benefited by symbiotic relationship with endophytes (Clay and Schardl, 2002; Thrower and Lewis, 1973). Zhang et al., (2006) reported that endophytic fungi harboured in medicinal plants makes the host to adapt at extreme climatic condition. Krishnamurthy et al., (2008) stated that fungal community lives inside the healthy tissue of medicinal plants increases absorption of soil nutrients inducing changes in nutrient cycle. However, endophytic fungi associated with leaves of woody angiosperms, especially in tropical forests, are poorly known. Studies were, therefore, carried out to assess the physiological requirements of endophytic fungi.

Nutritional supplementation is required to overcome low thermal tolerance in some organisms while in others growth at elevated temperatures is accomplished by an increased nutritional sufficiency (Campbell and Williams, 1953; Langridge, 1963). Although each species has a very specific nutritional requirement for its optimum growth and activity, the necessity to identify a unique nutrient media having a capacity to support a wide range of endophytic fungal flora is required so as to maintain the cultures and to conduct further studies on enzymatic systems, degradation and recycling of complex debris in nature and other related studies.

Chhattisgarh, which was carved out from erstwhile Madhya Pradesh in November 2000, is rich in forest and mineral resources, laying between lat $17^0$47’ and $24^0$06’N and long $80^0$15’ and $84^0$24’E. The geographic area of the state is 13.52 million which constitutes 4.1% of the land area of the country. The state shares its boundaries with the 6 Indian states i.e. Madhya Pradesh on the northwest, Uttar Pradesh on the north, Jharkhand on the north-east, Orissa on the south-east, Andhra Pradesh on the south and Maharashtra on the south-west. Agro-climatically the state can be divided into three zones, viz. the Chhattisgarh plains, the northern hills of Chhattisgarh, and the Bastar plateau.

Chhattisgarh state is one of the best representatives of the Deccan Peninsular bio-geographic zone in India which possesses rich biodiversity in its tropical semi evergreen Sal forests covering about 44% total geographical area of the state. The state forests are known to provide shelter for great varieties of flora and fauna of ethno-botanical importance.

MATERIALS AND METHODS

Study site

Study site Achanakmar-Amarkantak Biosphere Reserve (ABR) located in the Mungeli district of Chhattisgarh and it is a 14th Biosphere Reserve of India, which recently selected for Tiger project in central India. Few medicinally important tropical forest tree species were selected for the isolation of endophytic fungi. Plants should be selected mainly on the basis of their unique environmental setting, ethno botanical history, endemism, unusual longevity, and large areas of biodiversity. The ABR has an area of 552 sq. km. lies between lat. 22 ^{o} 15' to 22 ^{o} 58' N and 81 ^{o} 25' to 82 ^{o} 5' E, partly falling in Madhya Pradesh and partly in Chhattisgarh state. The tree species were selected on the basis of their highest abundance and usefulness in this area. Sampling of plant tissues were made in the morning randomly in the forest.

Endophyte isolations

For all surveys of endophytic infection in the present study, healthy leaves were harvested from Arjun, Harra and Sal trees. Samples were washed in running tap water and processed within 4 hrs of collection. Healthy leaves were defined as leaves undamaged by herbivores and free of overt symptoms of disease. Segments of 0.5 to 1.0 cm were cut from the midrib portion of each leaf. Leaf segments were surface sterilized by sequential washes in 70% ethanol (2 min) and 0.5% NaOCl (2 min), rinsed with sterile water and allowed to surface dry under sterile conditions (Arnold et al., 2000). This method of surface sterilization eliminates epiphyllous microorganisms present at the leaf surface (Arnold et al., 2000; Schulz et al., 1993). Ten leaf segments were plated in each Petri dish containing potato dextrose agar (PDA) medium amended with chloramphenicol (150mg^{-1}) to control the growth of bacteria. The Petri dishes were then sealed with parafilm and incubated under cool daylight fluorescent lamps (12 hrs of light and 12 hrs dark interval) at 25 ±1ºC. Fungi that grew out from the segments were periodically isolated and identified. The identified pure cultures of endophytes were maintained on PDA slants.

Identification of endophytic fungi

The technique of James and Natalie (2001) was adopted for identification of the unknown isolated fungi using cotton blue in lactophenol stain. The identification was achieved by placing a drop of the stain on clean slide with the aid of a mounting needle, where a small portion of the mycelium from the fungal cultures was removed and placed in a drop of

lactophenol. The mycelium was spread very well on the slide with the aid of the needle. A cover slip was gently applied with little pressure to eliminate air bubbles. The slide was then mounted and observed with the microscope and microphotographs of different endophytic fungi were taken (Fig.1). The species encountered were identified in accordance with Cheesbrough (2000).

The most important sets of characteristics to be observed are the conidia and the process involved in their formation. Additionally, the pigmentation and shape of hyphae, the presence or absence of septa, the occurrence of sclerotia, chlamydospores, conidia, conidiomata or any other particular hyphal element, considered helpful to assist in classification of both anamorph (asexual state) or telomorph (sexual state) phases (Bononi and Grandi, 1998). Yet, this method requires a taxonomic expertise taking into consideration its complexity.

Physiological studies

The physiological studies were conducted in accordance with the standard procedures laid down by Lilly and Barnett (1951) and Tuite (1969) with some modifications. Four solid media namely, Potato dextrose agar (PDA), Malt extract agar (MEA), Czapek's Dox, Yeast peptone agar (YPA) were used. These different medium were then inoculated with 5 mm mycelia discs of pure culture of test fungi i.e., *Botrytis* sp., *Curvularia* sp., *Monodictys* sp., *Scytalidium* sp. and *Verticillium* sp. under investigation raised on potato dextrose agar medium and cut with the help of sterilized cork borer under aseptic conditions. After that they were incubated at 25 ±1ºC for 14 days.

Effect of carbon source

Carbon compounds tested in the study were dextrose, sucrose, maltose, starch and cellulose. A medium free of any carbon source served as a control containing only potato and agar (PA) and was used as the basic medium. Carbon sources were added to the basal medium PA at 9g of carbon per litre of medium. Each Petri plate containing different carbon sources was inoculated with 5 mm mycelial disc of 7 days old fungal culture and incubated at 25 ±1ºC for 14 days.

Effect of nitrogen source

Four different nitrogen sources used in this study were peptone, ammonium nitrate (NH_4NO_3), sodium nitrate ($NaNO_3$) and urea (NH_2CONH_2). The nitrogen sources were omitted from the control treatments. Test organisms were inoculated with 5 mm mycelial disc of 7 days old fungal culture under aseptic condition and incubated at 25 ±1ºC for 14 days.

Effect of pH

Effect of pH on the growth of the test fungi were performed in the laboratory using solid cultures containing different pH levels (viz. 6.0, 6.5, 7.0, 7.5 and 8.0). PDA medium was used to study the effect of pH on the growth of test fungi. The pH of the medium was adjusted to the desired pH by adding 0.1N NaOH or 0.1N HCl (Naik et al., 1988) using pH meter. The medium was buffered with Disodium hydrogen phosphate citric acid buffer according to the schedule of Vogel (Vogel, 1951). Petri dishes were inoculated with each isolate using 5 mm diameter mycelial disc under sterile conditions. Inoculated Petri dishes were incubated at 25±1 ºC for 14 days and mycelial growths were measured.

Effect of temperature

Endophytes were subjected to different temperature conditions to study the best suited temperature level for the growth of the fungus. Each Petri plate was inoculated with each isolate using 5 mm diameter mycelial disc under sterile conditions on PDA medium. Inoculated Petri plates were incubated at 20 ºC, 25 ºC, 30ºC and 35 ºC for 14 days. The diameter of each culture defined as the average of five measurements taken randomly across each of the three plates was measured every two days for ten days.

RESULTS AND DISCUSSION

Effect of different media on the growth of endophytic fungi

The average mycelial growth of tested strains on different solid media is given in Table 1. Most of the endophytes utilize dextrose as a carbon source and peptone as a nitrogen source for their growth and development. The data obtained from detailed study of *Botrytis* sp., *Curvularia* sp., *Monodictys* sp., *Scytalidium* sp. and *Verticillium* sp. in different media revealed that PDA supported maximum mycelial growth followed by YPA and MEA (Plate 1). The medium Czapek's Dox was less preferred by these organisms. The result was similar to the work of Winder (1999) on *Fusarium avenaceum* who reported that PDA rich in starch and glucose was a much better growth substrate for this fungus. The fungus *Botrytis* sp. and *Curvularia* sp. was found to grow best in the PDA medium showing colony diameter of 85 and 82 mm in 7 days respectively followed by *Verticillium* sp. with the colony diameter of 78 mm. Least growth was obtained in the Czapek's Dox medium in which *Monodictys* sp. shows minimum mycelial growth of 35mm.

Table1. Media study of different fungal endophyte. Colony diameter (mm) measured in 7 days at 25^0C.

S.No.	Endophytes	Culture medium			
		PDA	YPA	Czapek's Dox	Malt extract
1	*Botrytis* sp.	85	84	55	70
2	*Curvularia* sp.	82	75	60	64
3	*Monodictys* sp.	65	50	35	60
4	*Scytalidium* sp.	75	70	65	60
5	*Verticillium* sp.	78	67	70	68

Size in millimetre

Effect of carbon sources on the growth of endophytic fungi

Among the different carbon sources used, dextrose supported the maximum mycelial growth followed by sucrose (Table 2). The minimum growth was obtained on cellulose. Glucose, maltose and sucrose have been reported to be best carbon source for maximum mycelial growth (Asiegbu, 2000; Dong and Yao, 2005). The fungus *Botrytis* sp. and *Curvularia* sp. were found to grow best in the dextrose medium showing colony diameter of 82 mm. and 75 mm. in 7 days respectively followed by *Verticillium* sp. with the colony diameter of 72 mm. Least growth was obtained in the cellulose medium in which *Scytalidium* sp. shows minimum mycelial growth of 37 mm.

Table 2. Growth of endophytic fungi against different carbon source. Colony diameter (mm) measured in 7 days at 25^0C.

S.No.	Endophytes	Carbon source				
		Dextrose	Sucrose	Starch	Maltose	Cellulose
1	*Botrytis* sp.	82	65	64	60	40
2	*Curvularia* sp.	75	70	65	75	40
3	*Monodictys* sp.	65	55	50	55	40
4	*Scytalidium* sp.	70	65	62	60	37
5	*Verticillium* sp.	72	80	65	60	60

Size in millimetre

Effect of nitrogen sources on the growth of endophytic fungi

Most of the test fungi showed common preferences for peptone as a nitrogen source. Other nitrogen source such as ammonia, ammonium salts, amino acids and complex organic nitrogen are equally important for the growth and development of fungi (Garraway and Evans, 1984). In the study it was found that peptone supported maximum mycelial growth followed by ammonium nitrate and sodium nitrate (Table 3). Bilgrami and Verma (1978) also reported that ammonium nitrate is extensively utilized by the fungi as a source of nitrogen. The fungus *Botrytis* sp. was found to grow best in the ammonium nitrate medium showing colony diameter of 78 mm. followed by *Scytalidium* sp. and *Verticillium* sp. with the colony

diameter of 72 mm. Least growth was obtained in the urea medium in which *Curvularia* sp. shows minimum mycelial growth of 20 mm.

Table 3. Growth of endophytic fungi against different nitrogen source. Colony diameter (mm) measured in 7 days at 25^{0}C.

S.No.	Endophytes	Nitrogen source			
		Urea	Ammonium nitrate	Sodium nitrate	Peptone
1	*Botrytis* sp.	60	78	75	76
2	*Curvularia* sp.	20	60	55	75
3	*Monodictys* sp.	25	40	50	57
4	*Scytalidium* sp.	70	72	72	72
5	*Verticillium* sp.	37	72	68	70

Size in millimetre

Effect of temperature on the growth of endophytic fungi

A perusal of the data in Table 4 showed that endophytic fungi grew well at 25^{0}C followed by 30^{0}C. Limited growth was observed at the temperature of 35^{0}C, gradual growth retardation has been observed at higher temperature. The temperature and fructification optima occurred around 25^{0}C. Lu et al. (2000) also reported that most preferable temperature range of most of the fungi is between 25^{0}C- 30^{0}C. The fungus *Botrytis* sp. was found to grow best at the temperature of 25^{0}C, showing colony diameter of 82 mm followed by *Curvularia* sp. and *Verticillium* sp. with the diameter of 74 mm, least growth was obtained at 35^{0}C in which *Monodictys* sp. shows minimum mycelial growth of 26 mm.

Table 4. Growth of endophytic fungi in different temperature: Colony diameter measured in 7 days at 25^{0}C.

S.No.	Endophytes	Temperature			
		20^{0}C	25^{0}C	30^{0}C	35^{0}C
1	*Botrytis* sp.	72	82	77	36
2	*Curvularia* sp.	55	74	67	35
3	*Monodictys* sp.	50	64	54	26
4	*Scytalidium* sp.	65	72	70	32
5	*Verticillium* sp.	58	74	60	34

Size in millimetre

Effect of pH on the growth of endophytic fungi

In the present study endophytes were grown on a wide range of pH. The result obtained revealed that pH 7 supported maximum mycelial growth, followed by pH 6.5 (Table 5). Fungi mostly have optimal growth rates between pH 4 and pH 7, rarely at basic pH (Garraway and Evans, 1984). Lilly and Barnett (1951) reported that a medium having pH values between 5 and 6 at the time of inoculation was suitable for most fungi. According to them, fungi generally tolerate more acid than alkali. The pH optimum for growth of all tested endophytic fungi was pH 7. The fungus *Botrytis* sp. and *Verticillium* sp. was found to grow best in the pH 7, showing colony diameter of 82 mm, followed by *Curvularia* sp. with the colony diameter of 70 mm, least growth was obtained in the pH 8.0 in which *Monodictys* sp. and *Scytalidium* sp. shows minimum mycelial growth of 40 mm.

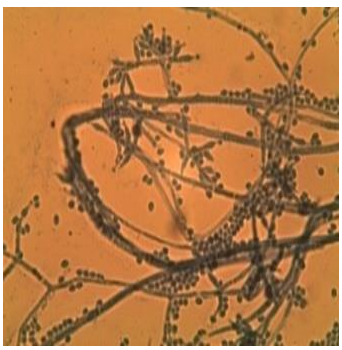
Table5. Growth of endophytic fungi in different pH: Colony diameter measured in 7 days at 25^{0}C.

S.No.	Endophytes	pH range				
		6.0	**6.5**	**7.0**	**7.5**	**8.0**
1	*Botrytis* sp.	75	80	82	68	55
2	*Curvularia* sp.	65	77	70	60	45
3	*Monodictys* sp.	55	60	57	55	40
4	*Scytalidium* sp.	70	77	68	55	40
5	*Verticillium* sp.	75	80	82	73	45

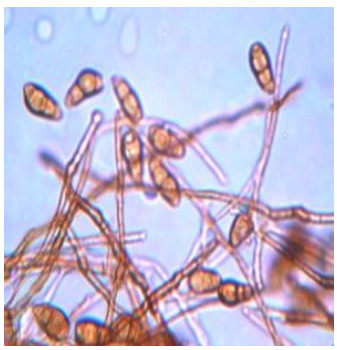
Size in millimetre

CONCLUSION

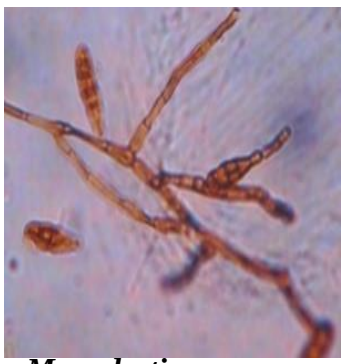
Isolates of endophytic fungi under study varied in their ability to grow in different temperature and pH levels and to utilize different carbon and nitrogen sources. Dextrose was found to be the best source of carbon. Among the nitrogen sources tested, Ammonium nitrate supported the maximum growth of the fungi. In most of the isolates preferred temperature range was between 20-30^{0}C for their growth and development. The entire test organism grew well at pH of 6.5 to 7.0 while pH 8.0 was not preferred by the endophytes.



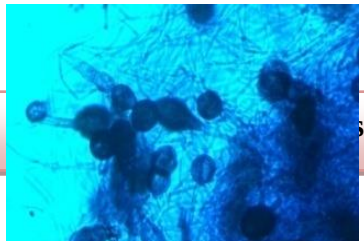
a- *Botrytis* sp.



b- *Curvularia* sp.,

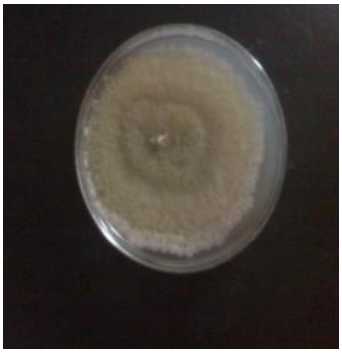


c-*Monodyctis* sp.



d- *Scytalidium* sp., **e- *Verticillium* sp.**

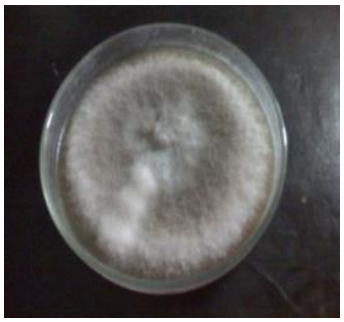
Fig no – 1 Microphotographs of endophytes V



***a. Botrytis* sp.**

b- *Curvularia* sp

c- *Monodictys* sp



d- *Scytalidium* sp

e- *Verticillium* sp.,

Fig no 2 - Colonies of endophytes

REFERENCES

1. Arnold, A. E., Maynard, Z., Gilbert, G. S., Coley, P. D. and Kursar, T. A. Are tropical fungal endophytes hyperdiverse? *Ecology Letters., 2000;* 3: 267–274.
2. Asiegbu, F. O. Effects of carbohydrates, ethanol and selected cell wall phenolics on *in-vitro* growth of necrotrophic fungi *Heterobasidion annosum* and *Fusarium avenaceum*. *J. Basic Microbiol*., 2000; 40: 139-148.
3. Bilgrami, K. S. and Verma, R. N. (1978). *Physiology of Fungi*, Vikas Publishing House Private Ltd., New Delhi, 493p.
4. Bills, G.F., Polishook, J.D., 'Microfungi from Carpinus caroliniana'. Can. J. Bot., 1991; 69; 1477-1482.

5. Bononi, V. L. R. and Grandi, R. A. P. Eds. (1998). Zigomicetos, Basidiomicetos, Deuteromicetos: Noções Básicas de Taxonomia e Aplicações Biotecnológicas. Instituto de Botânica, Secretaria de Estado do Meio Ambiente, São Paulo, pp. 184.
6. Campbell, L. L. Jr. and Williams, O. B. The effect of temperature on the nutritional requirements of facultative and obligate thermophilic bacteria. J. Bact., 1953; 65: 141-145.
7. Carroll, G. C. Fungal endophytes in stems and leaves: from latent pathogen to mutualistic symbionts. *Ecology*. 1986; 69: 2-9.
8. Cheesbrough, M. District Laboratory Practice in Tropical Countries Part 2, Cambridge University Press, Cambridge. 2000; 47-54.
9. Clay, K., Schardl, C. Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. *The American Naturalist., 2002;* 160S: 99-127.
10. Collado, J., Platas, G., Conzalez, I., Pelaez, F., 'Geographical and seasonal influences on the distribution of fungal endophytes in Quercus ilex'. New Phytologist., 1999; 144: 525–532
11. de Barry (1866). Morphologie and physiologie der pilze. Flechten and Myxomyceten Vol ll *Hofmeister"s Handbook of physiological botany*, *Leipzig* 316.
12. Dong, C. H. and Yao, Y. J. Nutritional requirements of mycelial growth of *Cordyceps sinensis* in submerged culture. *J. Appl. Microbiol*., 2005; 99: 483- 492.
13. Fisher, P. J., Petrini, O., Petrini, L. E., Sutton, B. C., 'Fungal endophytes from the leaves and twigs of *Quercus ilex* L. From England, Majorea and Switzerland'. New Phytol., 1994; 27: 133-137.
14. Garraway, M. O. and Evans, R. C. (1984). Fungal nutrition and physiology. Wiley, New York, 8.
15. James, G. C. and Natalie, S. (2001). Microbiology. A laboratory Manual (ed.). 211-223.
16. Khan, R., Shahzad, S., Choudhary, M. I., Khan, S. A., Ahmad, A., 'Communities of Endophytic Fungi in Medicinal Plant *Withania somnifera*'. Pak. J. Bot., 2010; 42(2); 1281-1287.
17. Krishnamurthy, Y. L., Naik, S. B., and Jayaram, S., 'Fungal Communities in herbaceous medicinal plants from the Malnad Region', Southern India. Microbes Environ., 2008; 23: 24-28.
18. Langridge, J. Biochemical aspects of temperature response. A. *Rev. Pl. Physiol., 1963;* 14: 441-462.

19. Lilly, V. G. and Barnett, H. L. 1951). Physiology of the fungi. McGraw-Hill Book Company, lnc. NewYork, 464.
20. Lu, H, Zou, W. X., Meng, J. C., Hu, J., Tan, R. X. New bioactive metabolites produced by *Colletotrichum* sp., an endophytic fungus in *Artemisia annua*. *Plant Sci*., 2000; 151: 67–73.
21. Naik, M. K., Hiremath, P. C. and Hegde, R. K. Physiological and nutritional studies on *Colletotrichum gloeosporioides*, a causal agent of anthracnose of beetlevine, *Mysore J. Agric. Sci., 1988;* 22: 471-474.
22. Sahashi, N. Y., Miyasawa, T., Kubano, S., Ito, T. 'Colonization of beech leaves by two endophytic fungi in northern Japan'. Forest Pathology., 2000; 30: 77-86.
23. Schulz, B., Wanke, U., Draeger, S., Aust, H. J. Endophytes from herbaceous plants and shrubs—effectiveness of surface sterilization methods. *Mycol.* Res., 1993; 97: 1447–1450.
24. Thrower, L. B., Lewis, D. H. 'Uptake of sugars by Epichloe typhina (Pers. Ex Fr.) Tul. In culture and from its host, Agrostis stolonifera L'. New Phytologist., 1973; 72: 501-508.
25. Tuite, J. (1969). Plant Pathological Methods in Fungi and Bacteria. Burgess Publ. Comp. Minn., p.239.
26. Vogel, A. I. (1951). Inorganic quantitative analysis, Long-mans Green and Co., Toronto, 872p.
27. Wilson, D. Endophyte-the evolution of a term, and clarification of its use and definition. *Oikos., 1995;* 73: 274–276.
28. Winder, R. S. (1999). Evaluation of *Colletotrichum* sp. and *Fusarium* spp. as potential biological control agents for marsh reed grass (*Calamagrostis canadensis*). *Can. J. Plant Pathol*., 1999; 21: 8-15.
29. Zhang, H. W., Song, Y. C. and Tan, R. X. (2006). Biology and chemistry of endophytes. Natural Product Reports., 2006; 23: 753-771.