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Short communication

Localisation and isolation of fungal endophytes from healthy tissue of *Stevia rebaudiana* (Bert.)

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Abstract

Endophytes are the microorganisms living inside the plant tissue without causing any harm to the host. For past few decades, research community has keen interest in endophyte research as endophytes are able to produce secondary metabolites similar to the one produced by host plant, which can be used as potential therapeutics against several diseases. *Stevia rebaudiana* (Bert.) is known as a sweetener plant, but besides as sweetening agent it has many other therapeutic potential and can be used as antihypertensive, anti-tumour, vasodilator and neuroprotective drug. Research efforts have been made to isolate and identify fungal endophytes from *Stevia rebaudiana* (Bert.) but no efforts have been taken to study the potential of endophytes for steviol glycosides production. So the present study includes histological studies, isolation and purification of fungal endophytes from different parts of *Stevia rebaudiana* (Bert.) plant. Maximum localisation of endophytes was observed in phloem tissue of stem. Two colonies were purified, each from stem and leaf tissues showing cream and white morphology respectively. Microscopic studies revealed long, branched and septate hyphae with club shaped sporangium.

Keywords: Endophyte, *Stevia rebaudiana* (Bert.), Secondary metabolites, Lactophenol cotton blue.

Introduction

Microorganisms are ubiquitous and indispensable for every aspect of life forms. Throughout the ages every process in the biosphere has been affected by the microbes, starting from the production of oxygen, which we need to live. Microbes decompose the garbage, provide nutrients to plants, and even help to digest our food. In addition to these microbes have practical applications, making bread, wine. They can change the genetic composition of plants or animal and can be used to change any trait. Microbe are also known for the diseases they cause, but better understanding of their life cycle and mode of action enabled scientists to prevent and treat diseases by producing vaccines and antibiotics.

More recently development has been made by isolating a special class of microorganisms that internally infect the living plant tissue but does not shows any symptoms of disease and live in mutualistic association within the plant tissue for at least a part of their life cycle, and are commonly called endophytes.[1] All types of microorganisms (fungi, bacteria, and actinomycetes) have been discovered as endophytes. But the most frequently encountered endophytes are fungi [2] Plant- microbe association may exist from evolution of higher plants and mediated via plant chemical defence. [3] Later on, many studies have been done by considering the several aspects in number of endophytes from different species. This leads to its better understanding and worldwide application. It was found that endophytes have the natural potential for accumulation of various bioactive metabolites which may directly or indirectly be used as therapeutic agents against numerous maladies [4]. These natural products have many therapeutic effects such as anticancerous, anti-inflammatory, antimicrobial activity.[5,6]

Fungal endophytes posses huge diversity morphologically and biochemically. [7] Tropical and temperate rainforest are most diverse ecosystem on the earth. Number of factors influences the diversity such as host vegetation, habitat of endophyte, temperature, [8] urban and rural area. [9] And the environmental diversity leads to chemical diversity in endophytes.

Biosynthetic pathways of pharmacologically important active natural products such as Taxol, Camptothecin etc. in endophytes have been investigated. [10,11] Endophytes and plants have some common genes and some are different for biosynthesis but the accurate biosynthetic pathway in endophytes is still under study. Different ideas are given for origin of secondary metabolites in endophytes. One concept is that plants and endophytes co-evolved with pathways, another argument is that either of them produced the product and transfers it to other. Another concept is that horizontal gene transfer took place between plants and microbes. [12,13]

Stevia rebaudiana (Bert.), a sweet perennial herb contains diterpene glycosides,viz. Stevioside, rebaudioside A, Rebaudioside B, Rebaudioside C, Rebaudioside D, Rebaudioside E, Dulcoside A, Steviolbioside. These all glycosides are sweet in nature.

Stevioside is 300 times sweeter than sucrose. Diet conscious and diabetic persons with hyperglycemia can use steviosides as an alternative sweetener [14] as it regulates the blood glucose level by stimulating insulin secretion [15]. Stevioside can also be used as an antihyperglycaemic [16], antihypertensive [17], anti-tumor [18], vasodilator drug [19].

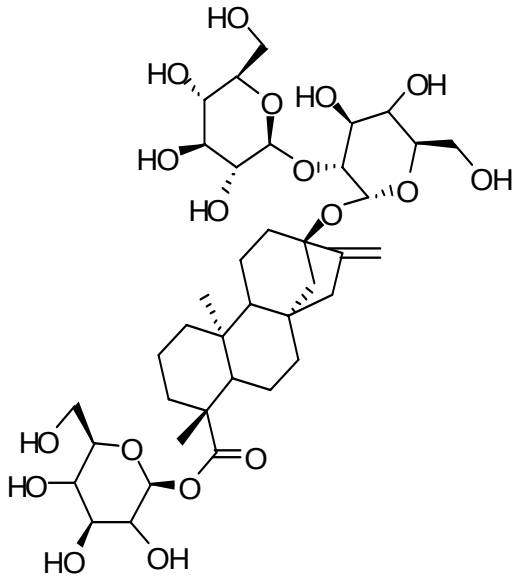


Figure1: Stevioside

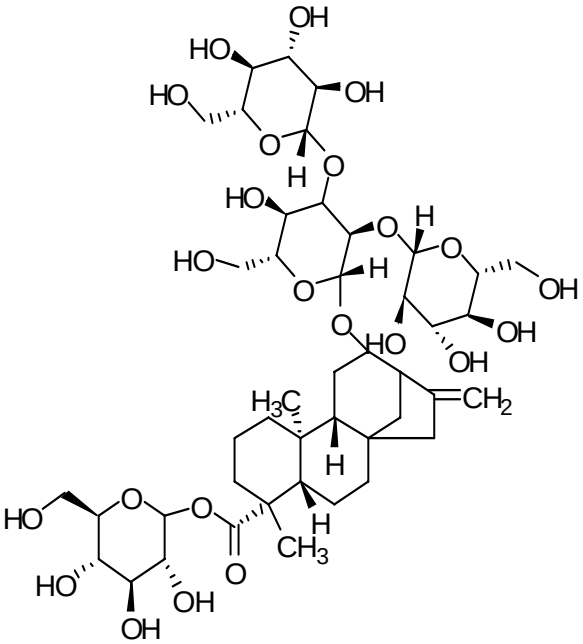


Figure 2: Rebaudioside A

Scattered reports are available on endophytes from *stevia rebaudiana*. Different fungal taxa were identified and effect of association of fungi and plant was studied [20], but no reports are available for the observation of role of endophytes on synthesis of steviol glycosides. Therefore, this study this study is conducted for isolation of endophytic fungi from *Stevia rebaudiana* (Bert.). Histological studies of *Stevia rebaudiana* (Bert.) leaf stem and root tissues were carried out to approach the location and diversity of fungal mycelium within the tissues.

Materials and Methods

Histological investigations of healthy tissues of *Stevia rebaudiana* (bert.) to localize fungal endophytes

The endophyte distribution and localization was studied with the help of microscope (Olympus CH20 Japan) under the magnification of 100 X and 1000X, attached with a digital camera (Canon 16megapixels). The fungal colonies were observed as blue after staining it with Lactophenol Cotton blue.

Plant material and collection site

Plant material:	*Stevia rebaudiana* (Bert.)
Kingdom:	Plantae
Phylum:	Magnoliphyta
Class:	Magnoliopsida
Order:	Asterales
Family:	Asteraceae
Genus:	Stevia
Species:	*Stevia rebaudiana* (Bertoni)

Figure 3: *Stevia rebaudiana* (bert.)

Explant preparation

Plantlets were collected from Indigenous Medicinal Plant Garden of Birla Institute of Technology, Mesra campus. 6-7 months old samples were randomly selected for study. The plants were uprooted from the soil and the roots were washed under tap water.

Samples were processed for their histological investigation within 6 hr of collection. Leaf, stem and root were separated by sterile blade and washed again by distilled water. Plant parts were cut into thin sections.

Slide preparation

The transverse section (T.S.) and longitudinal sectios (L.S.) were stained with Lactophenol cotton blue for 2 minutes and washed with distilled water 4-5 times to remove the extra stain. The sections were then observed under the microscope (Olympus CH20 Japan), attached with a digital camera (Canon 16 megapixels).

Isolation of endophytic fungi from *Stevia rebaudiana* (Bert.)

For isolation of endophytic fungi, matured explants were randomly selected from previously examined plantlets and were surface sterilised.

Surface sterilisation

Collected explants were washed in running tap water for 10-15 minutes and then kept in liquid soap solution (Tween 20) for 6-7 minutes. Detergent was completely washed by distilled water. Explants were then treated with antifungal agent (Bavistin 0.2%) 6-7 minutes by continuous shaking and rinsed with distilled water. Finally explants were surface sterilised with 0.1% mercuric chloride for 2-3 minutes and washed thoroughly by sterilised distilled water to remove the traces of mercuric chloride.

Inoculation on PDA media

Surface-sterilized leaves were then cut into small pieces of 1 1 cm size. Stem were also cut into length of 1.5cm. Leaf pieces and stem cutting were aseptically transferred to Petri dishes containing agar (2%) solidified Potato Dextrose Medium (PDA) and left for 5 minutes and impression of explants was taken by slight pressing. These petri dishes were considered as control plates.
After 5 minutes explants were taken out carefully under laminar hood and transferred to another petri dish with same media composition. Plates were incubated at 28ºC for 5- 7 days. After 4-5days, fungal mycelia grew on the surface of leaves and stem. Grown mycelia were picked and transferred onto PDA plates for pure culture. Plates were kept for 10- 15 days for sporulation.

Results and Discussion

Through histological investigations, localisation of endophytes within the tissue was easy and the particular tissue can be selected for isolation of endophytes in PDA media. Microscopy of stained sections of root, stem and leaf of *Stevia rebaudiana* (bert.) revealed that sections of stem harbour maximum colonies of endophytes then leaves and root. Colonies observed in higher magnification were spherical and oval in shape.

In T.S. of root, endodermis region was rich in endophytes and gradually decreased in epidermis, whereas scattered colonies were observed in ground tissue and vascular bundles.

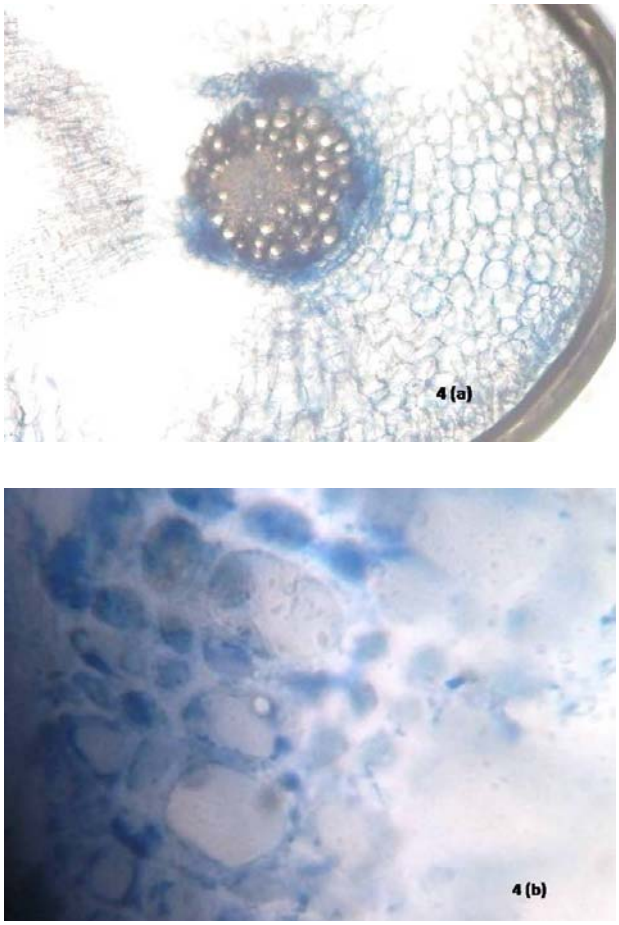
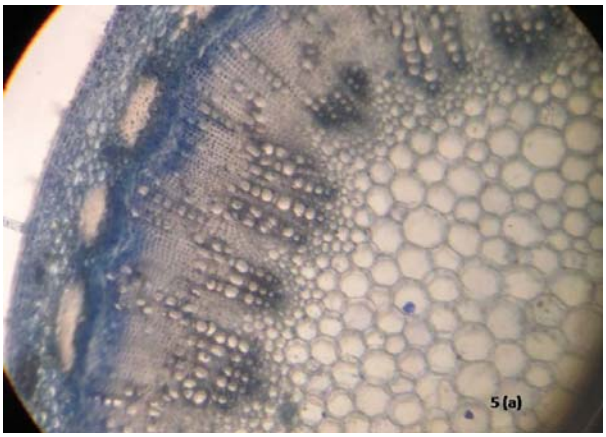


Figure 4: Root explants (a) T.S. of root at 100X magnification, dense blue fungal colonies in the endodermis region. Scattered colonies were observed in epidermis and cortex region. (b) T.S. at 1000X intracellular blue colonies.

In stem sections, both T.S. and L.S. at 100X display plenty of endophytes in phloem region and slightly lower in epidermis region. Scattered colonies were observed in xylem and cortex. At 1000X colonies were closely observed as spherical and present extracellularly and intracellularly. Leaf T.S. showed dense blue colonies in epidermis and mesophyll region and scattered colonies in vascular bundles. Both the intracellular and extracellular colonies were present.



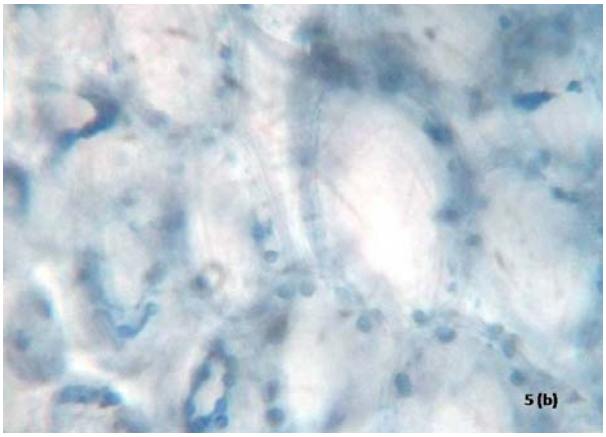
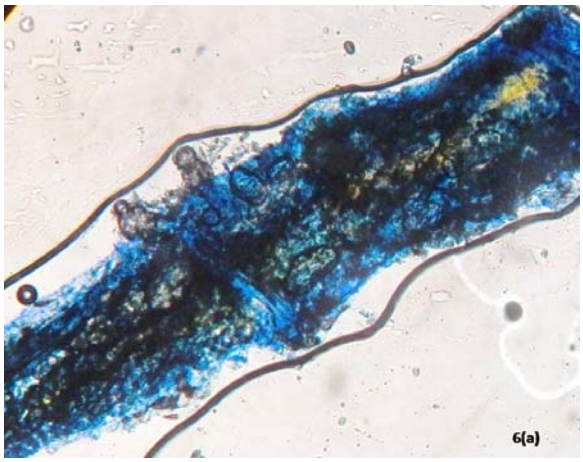


Figure 5: Stem explants (a) T.S. at100X, dense blue endophytic colonies in phloem region. Scattered colonies around the xylem and cortex of stem explants. (b) Transverse section at 1000X, spherical and oval blue fungal colonies of stem explant.



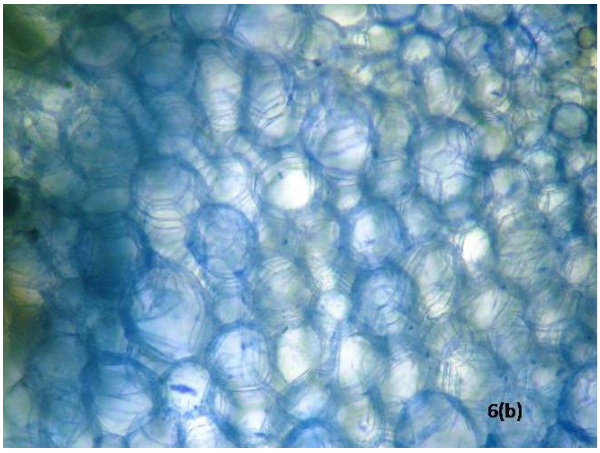


Figure 6: Leaf explants (a) fungal colonies scattered around the vascular bundle (Vas cam) of leaf section, T.S. at 100X magnification (b) Intracellular and extracellular endophytic, T.S. at 1000X.

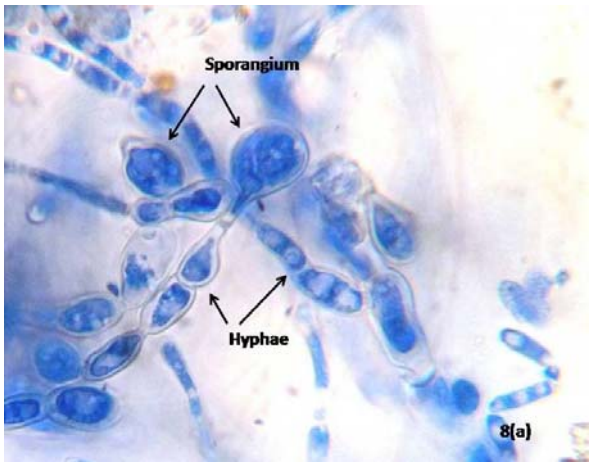
Isolation and purification of endophytic fungi

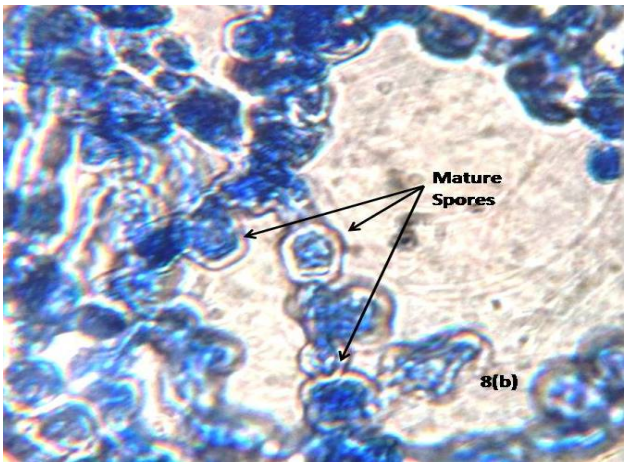
After 4-5 days of inoculation of explants fungal colonies appears on the cut end of stem and on the margins of leaf. Cream coloured dense morphology was observed on cut ends of stem whereas white furry structure appears on leaf margin. According to the morphology and growth pattern of fungal colony four different types of isolates, CK2001, CK2002, CK3001, and CK3002 from stem and leaf were purified on potato dextrose agar (PDA) media. Single colony was taken for purification so that uniform growth was observed.

Figure 7: (a) fungal colonies on the margin of leaf, (b) fungal colony in cut end of stem with different morphology, (c) Purified culture

Microscopy of isolates

Previous studies [21] reports isolation of *Alternaria, aspergillus, Monodictys,* and *curvularia* species from leaf of *Stevia rebaudiana* (Bert.). On microscopy of spores, CK2001 and CK3001 showed long, branched, septate, uninucleate hyphae with club shaped sporangium but CK3002 have vesicle structure at the base and spores were cylindrical in shape. Further characterisation is required for identification of species.





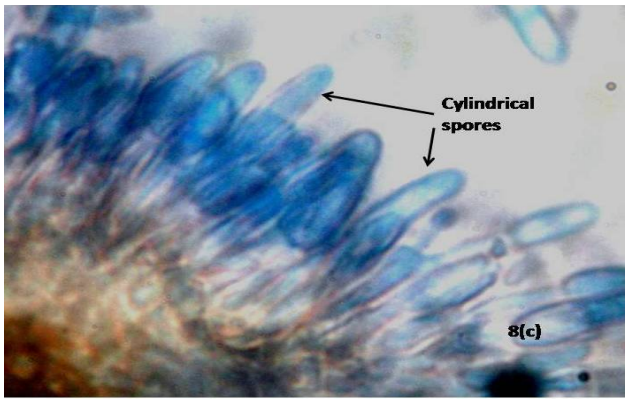


Figure 8: (a) oval sporangium and long hyphae of CK2001 and CK3001 (b) round shaped mature spores (c) vesicle structure at the base and cylindrical spores.

Conclusion

The present study revealed the gradual decrease in fungal colonies from stem to leaf and then root. Dense colonies were observed in phloem region of stem. Endophytic colonies increase from young to matured explants. In leaf tissue uniform distribution was observed whereas in root tissue colonies were concentrated on endodermis region. During isolation, in leaf explants colonies appear earlier than stem. Four isolates were purified but for identification and their prospects for glycoside production further characterisation and biochemical tests are required.

Authors Contributions

Madhumita kumari - has performed the laboratory work and written the manuscript.

Dr. Sheela Chandra - helped in designing and supervising the experiment and done critical reading and editing of manuscript.

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