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Histological Investigation of Fungal Endophytes in Healthy Tissues of *Azadirachta indica* A. Juss.

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ABSTRACT

The presence of endophytic fungal mycelia within healthy tissue is most commonly detected by long incubation of small pieces of surface-sterilized healthy tissue on agar plates and the emergence of their mycelia or spores. Thus, to understand their biology and distribution within the host, it is mandatory to use histological tools to locate the endophytic mycelia as well as spores *in vivo* within healthy tissue. Thin microtome sections were stained with KOH-aniline blue procedures. The prominent fungal structures visualized were the fungal mycelia and occasionally spores. Mostly, the intercellular compact mycelia were observed with a few haustoria and clamp-like structures, while at several locations, the position was ambiguous. A few spore-like structures were also visible which were suspected to be the spores of *Humicola* or *Nigrospora,* since these genera have been reported as frequent endophytes from the same host (*Azadirachta indica* A. Juss.) by isolation methods. Some smaller hyphae were found under the cuticle and within the epidermal cells; however the larger hyphae were observed within all tissues including mesophylls, palisade and spongy parenchyma and vascular tissue. The study emphasized the localization, distribution and significant presence of fungal endophytes of *Azadirachta indica* within healthy tissues, which may eliminate the suspicion with which some scientists view the presence of endophytes. The study provided further insight in the biology of the host-endophyte relationship as well as encouraging future investigations pertaining to host-endophyte interactions.

Keywords: fungal endophytes, *Azadirachta indica*, histological study, KOH-aniline blue stain.

INTRODUCTION

Contrary to an earlier view that the internal tissue of healthy plants was supposed to be sterile (Foley, 1959), there have been isolations from such tissue of bacteria and actinomycetes (Ryan *et al.,* 2007; Verma *et al.,* 2009a), flagellates (Holms 1925) and fungi—including yeast—(Marcus, 1942; Foley, 1959; Verma *et al.,* 2007, 2011a,b). Asymptomatic microbial infections are known to occur in the twigs and leaves of several vascular plants (Verma *et al.,* 2007, 2011a,b). Such mutualistic infections are also termed as latent infection, where a latent pathogen is involved. Microbial endophytes including fungi and bacteria have been reported in almost every plant studied

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so far. These microbes have potential to influence the plant health in terms of combating stresses and improving fitness for survival (Verma *et al.,* 2009b). Fungi are among the most diversified microbes reported to be present in extremely adverse conditions, such as in the internal living tissue of higher plants, where they usually reside in the intercellular (apoplast) and intracellular (symplast) spaces (Belanger, 1996). Endophytic microbial diversity has not yet been explored extensively and possibly a large amount of untold microbial diversity may reside as endophytes. Among microbial endophytes, the 'fungi' are widely studied in many higher plant hosts. Often these fungi are isolated from healthy and symptomless tissues, which have been surface sterilized with sodium hypochlorite/hydrogen peroxide, or mercuric chloride (Tervet and Hollis, 1948; Norse, 1972) as a surfactant. In the majority of cases, the 'isolation and culture method' is adopted to isolate and document the endophytic fungi, which are assumed to have originated from internal infection (Norse 1972; Verma *et al*., 2007). However, this technique provides no information about the location and distribution of endophytes within the host tissue (Bernstein and Carroll, 1977). Only fragmentary reports are available about the direct visualization of endophytic microbes within healthy living tissue using histological methods (Foley, 1959; Herr 1971; Welty *et al.,* 1986; Saha *et al.,* 1988; Wilson 1991; Johnson *et al.,* 2006). The culture method does not eliminate the possibility that fungi growing from surface-sterilized tissue may actually represent dormant appressoria or subcuticular hyphae that have survived the surface sterilization. Therefore, to determine the location and distribution of fungal endophytes within host tissue, the histological method may be an appropriate tool.

The current study selected a medicinally important host, *Azadirachta indica* A. Juss., traditionally known as 'Indian Lilac' or neem for histological study of its fungal endophytes, their location and distribution among stem and leaf tissues based on a previous study on the endophytic fungal diversity of this host by the isolation and culture method (Verma *et al.,* 2007; 2009, 2011a). Staining the fungal endophytic mycelia/spores in the asymptomatic tissues of the host plant using a suitable stain is the most utilized method for studying plant-microbe interactions. In the case of neem, a few investigations for fungal endophytes have been made through the 'isolation and culture method' but no report is available about their histological investigation to date. Therefore, this study represents the maiden report on the histology of endophytic fungi in *Azadirachta indica*. Histological studies of neem leaf and stem tissue were carried out 1) to report on the histological observation of fungal mycelia and spores with maximum possible specialized structures and 2) to assess the location and diversity of fungal mycelia and spores within the tissues.

MATERIALS AND METHODS

Plant material and collection site

Azadirachta indica A. Juss., plants growing within and around the campus premises of Banaras Hindu University, Varanasi (25.5º N 82.9º E, elevation 85 m) were selected for study. Four plants 5–8 years old were sampled randomly for selected foliage and stems/twigs at different canopy levels. Materials were placed loosely in sterile paper bags for transport to the laboratory and stored at 4 ºC before processing. Samples were processed for their histological investigation within 48 hr of collection.

Stain preparation

The staining procedure was adapted from Hood and Shew (1996). The samples were autoclaved for 15 min at 121 ºC in 50 mL of 1M KOH solution followed by rinsing three times in deionized water. Each specimen was mounted on a glass slide in several drops of stain solution

(KOH- aniline blue) that had been prepared at least 2 hr prior to use as 0.05 % aniline blue dye in 0.067 M K_2HPO_4 at pH 9. The specimens were then analyzed under a research microscope (E 600 Eclipse; Nikon Corp., Tokyo), attached with a digital camera (Coolpix 3.34 mega pixel; Nikon Corp., Tokyo).

Histological processing of samples

For microscopic investigations of endophytic mycelia, the tissues were cleaned, stained and mounted. Stem tissues were cut into large blocks 3–5 cm length and washed thoroughly with running tap water. Thereafter, the tissue was surface treated with 5% NaOCl (4% available chlorine) for 5 min and again rinsed in 70% ethanol for 5 s followed by triple rinsing in deionized water. The tissues were then sectioned with a microtome (Weswox MT- 1090A.; Haryana, India) in all possible orientations. The speed of the microtome blade was set at four. The tissue sections were about 3µm thick and after removing them from the water bath of the microtome, they were transferred into a test tube containing aniline blue stain. The tube was heated until boiling for about 1 min and then washed several times with distilled water to remove excess staining. The sections were then rinsed and cleaned several times in xylene, followed by mounting in Canada balsam and the leaf sections were cut into small discs. A solution of 2.5% KOH at 40–60 ºC were used to obtain clear leaf tissue that became straw colored after several days. The KOH solution was changed daily 2–3 times for about 5–10 d. The cleared leaf tissue was then rinsed three times in distilled water and bleached for 3 min in 5% NaOCl and then rinsed in distilled water twice. A stack of five discs was inserted into a carrot hinge that was placed in the microtome holder, with the open opposite hinge side towards the blade.

RESULTS

The habitat occupied by endophytes and their association with their host was examined in transverse cross sections of typical endophyte-infected tissues of *Azadirachta indica* (Figure 1). The whole mount and the transverse sections of the leaf and stem tissues clearly showed endophytic fungal mycelia to be intercellular and sometimes intracellular as a few hyphae showed penetration points within the mesophyll tissue of the leaf blade and the longitudinal section of stem tissue (Figures 1a and 1b); most such hyphae were thinly dispersed and appeared as single strands within the tissue (Figures 1b and 1e). However, care is necessary in extrapolating these results to plants growing in different ecological conditions as some results were notably variable from what has been documented in other hosts (Norse 1972; Pearce 1984; Johnson *et al.,* 2006). The majority of mycelial structures were observed to be intercellular (Figure 2); as a consequence, the hyphae were oriented parallel to the longitudinal axis (Figures 2 a–d). The robustness and presence of prominent scars on the apical portion of the mycelium (Figure 2b) indicated conidiophores. Since these features of conidiophores are prominent in *Cladosporium* sp., it was considered that these mycelia might be of *Cladosporium* spp. Additionally, this strain was recovered from this host through an isolation method (Verma *et al*., 2007). The hyphae were in close contact with the cells and appeared to be firmly attached (Figure 2a). The plant cells typically appeared completely unaffected by the attachment of the hyphae except for a few haustoria like-structures and penetration points were evident from where the mycelia had entered the host cell (Figures 2c and 2d) which made them intracellular. The hyphae were compact and lacking vacuoles, in contrast to most plant cells which contain large vacuoles. Typically, no endophytic hyphae were observed in the vascular bundles. Examination of leaves revealed that at

all distances from the leaf base, the hyphae were dense and lacking vacuoles. In some cases, hyphae were present in longitudinal cross sections of stem tissue and were observed to be intercellular only; however, at several locations, the position was ambiguous and could not be classified (Figures 1c and 1d). The key point that emerged from the microscopy was that the growth of the endophytes was synchronized with that of the host tissue; the hyphal growth kept pace with that of the tissue and in particular it grew better in soft tissue like leaves (Figure 1a) followed by the bark and stem, respectively. However, once the hyphae had entered mature and hard tissue, the hyphal

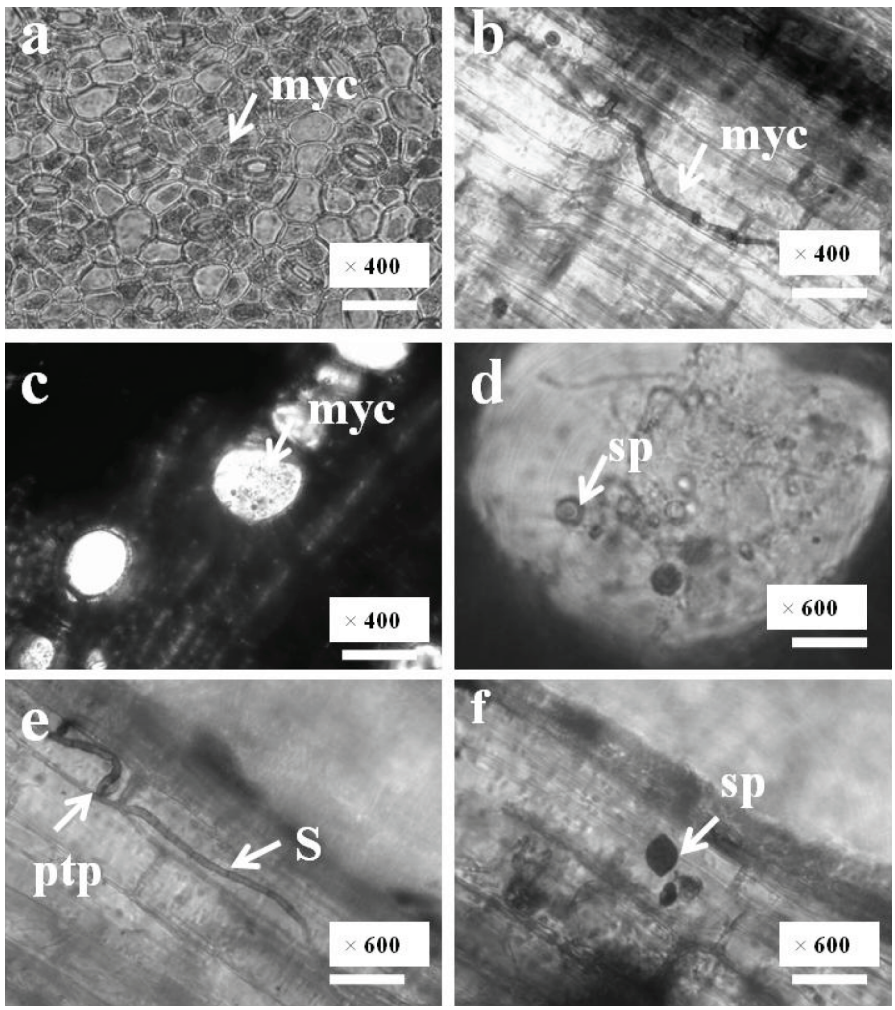

Figure 1 Leaf sections (a–b) showing stained intercellular mycelium (myc) within and around the stomatal guard cells, and subsidiary cells; (c) transverse section of stem showing interwoven mycelia within air spaces (vessels) of secondary tissue; (d) enlarged view of spaces showing some spore-like structures within them suspected to be spores of *Nigrospora* or *Humicola.* (e–f) intercellular mycelia with some locations showing protrusions as secondary mycelia/ clamp-like formations (ptp) and septation (S) with some swollen structures together with spores (sp).

growth often became slow and ultimately ceased and did not form further branches, though they did stay metabolically active resulting in much wider and darker mycelia (Figure 1e, Figures 2a and 2b). Some fungal infection has been reported to extensively invade the leaf vascular bundle (White and Margan-Jones, 1996), but this was not observed in the current study, which corroborated the earlier assumption made in the current study that the endophytic mycelia maintain a balance between mutualism and antagonism (Bacon and Siegel, 1988). Compared to the isolation methods where several strains were obtained, in the histological study it was difficult to identify any strain, as most of the fungal mycelia had only grown vegetatively. Thus, it was only possible to study the importance of their distribution within the host and their interactive association.

DISCUSSION

In addition to the isolation technique, direct observation of fungal hyphae within living tissue is critical to obtain a better insight and understanding of the biological interaction between an endophyte and its host. However, few attempts have been made to directly localize the internal fungal hyphae within healthy plant tissue. The current study described the KOH-aniline blue staining method (Hood and Shew, 1996) that provided rapid, simple, cost-effective and proper visualization and documentation of the endophyte-host interaction. Interest in detecting the interaction of endophytes with herbivores and in forage grasses with their host and insects has spurred experimentation with techniques for the visualization of endophytic mycelia in vegetative and reproductive tissue (Clark *et al.,* 1983; Welty *et al.,* 1986; Saha *et al.,* 1988; Wilson *et al.,* 1991; Hignight *et al.,* 1993) The pathogen-host interactions are the most important aspect among the various facets of pathogenic establishment. Among the more popular methods to study such interactions is staining of the fungal mycelia

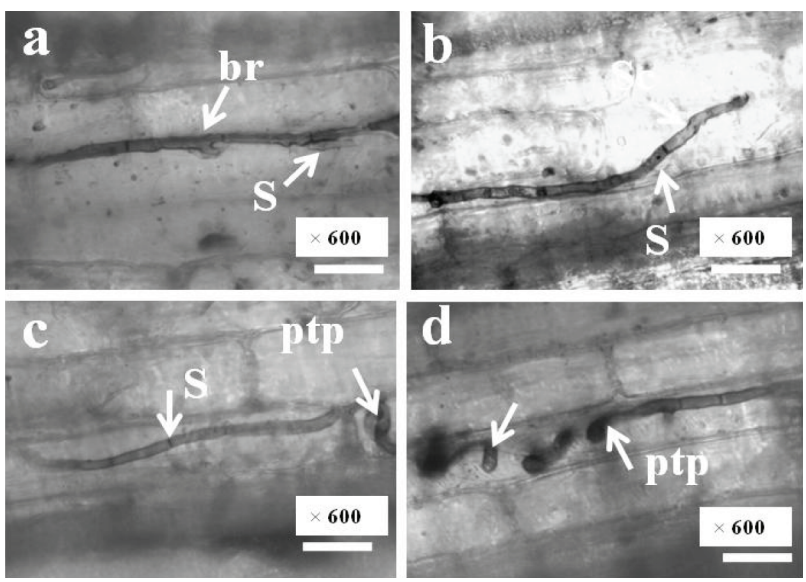

Figure 2 (a–b) robust mycelium shows septation (S), branching (br) and prominent scars (Sc) at its tip region; (c) at some locations, curling and twisting of mycelia was observed with some penetration points (ptp); (c–d) peculiarity of these structures is important because these types of modifications are not observed in mycelia isolated in plates.

within the tissue to assess the *in-vivo* position and distribution of fungal hyphae using histological tools. In many cases, endophytic fungi can be visualized easily in cleared-tissue whole mounts with a light microscope (Pearce, 1984; Stone 1987) and in a few reports by using fluorescence probe labeling (Johnston *et al.,* 2006). Chemical clearing of plant tissues has been widely used for microscopic observation of their morphology (Herr, 1971; Yadegari *et al.,* 1994), but it has not been well applied to plant tissue infected by endophytes. However, staining techniques are generally not categorized by their appropriateness for this type of application, because the staining characteristics of fungi and different plant tissue can vary substantially with the particular interaction. The histological investigations undertaken allowed the study of the interaction of various pathogenic fungi with their host under *in- vivo* conditions. Endophytic fungi have long been known as an unexplored entity for microbial diversity data, however, a lot of work has been done to explore the endophytic community from various hosts including *Terminalia* (Tejesvi *et al.,* 2005), *Spondias* (Rodrigues and Samuels, 1999), *Eucalyptus* (Bettucci and Saravay, 1993), *Aazadirachta* (Rajagopal and Suryanarayanan 2000; Mahesh *et al.,* 2005; Verma *et al.,* 2007, 2011a), *Musa acuninata* (Pereira *et al.,* 1999), *Aegle marmelos* (Gond *et al.,* 2007), *Piper longum* (Verma *et al.,* 2011b) and many other palms, orchids and thallophytes, among others. However, all these studies were similar in that screenings of endophytes were made through the simple isolation method followed by surface sterilization. However, this simple isolation procedure cannot explain the following fundamental queries. 1) What positions do the fungal mycelia and spores have within the tissue—is it inter- or intracellular? 2) At what sites are the fungal mycelia located—are they restricted to one type of tissue or evenly/unevenly distributed in all tissues? c) What is the probable percentage ratio of infection load of endophytic fungal spores and mycelia in most tissue? These questions can only be answered by adopting a technique that enables the localization of the fungal mycelia within the tissue. Staining fungal hyphae can allow these questions to be resolved. Some workers have successfully adopted the same methodology to assess the localization of endophytic fungi in *Nicotiana* sp. (Spurr and Welty, 1975), *Krunzea ericoides* (Johnston *et al.,* 2006). The current study has assessed the fungal endophytes of the neem plant to localize the endophytic mycelia within the tissue. However, various workers have already studied the endophytic mycoflora of this host using only the isolation technique (Rajagopal and Suryanarayana, 2000; Verma *et al.,* 2007; Verma *et al.,* 2011a).

Most endophytes do not produce symptoms in the host, and accordingly, isolation of such fungi into culture from vegetative tissue has usually targeted leaves or the pith of culms for excision and plating to media or for diagnostic examination (Bacon *et al.,* 1977; Bacon and Siegel, 1988). Generally, endophytes grow intercellularly in their host, and the site and extent of invasion by endophytic fungi reflects the mechanisms of nutrient transfer to the fungal symbionts (Bacon and Siegel, 1988; White and Margan-Jones, 1996). An examination of all sections in the current experiment, indicated that the number of spores appeared more limited than mycelial formation, which may have been due to a lack of favorable conditions/food supplements by mycelia to support sporulation. Alternatively, it may also happen that the required food was not being released by the host to the endophytes in order to save more energy for the host itself under stress conditions. Maximum mycelial formation could occur during periods with sufficient precipitation and humidity. Thus, their growth could occur only when the host tissue was developing and so the concentration of hyphae remained at a level that was not a substantial burden on the host plant. The association between fungal endophytes

and their host plant is the result of possibly unique adaptations including the development of a signaling system by which hyphal growth is regulated. Through this adaptation, the endophytes are able to synchronize their growth with that of their host.

CONCLUSION

A better understanding of the basic biology of endophyte-host symbiosis, along with the application of recent molecular techniques and chemical analytical procedures, will help improve the precision of knowledge about the processes involved in this unique symbiosis between fungal endophytes and a host, which have remained unclear for a long time. Through this knowledge, it may be possible to develop a complete inventory of fungal endophytes and their hosts, as well as enhance the level of understanding about the interaction of endophytes with their host.

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