Histological localization of fungal endophytes in healthy tissues of *Adhatoda vasica* Nees

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A research protocol has been developed to locate endophytic fungi present inside the healthy leaves and stems of *Adhatoda vasica* using the microtome technique. The surface-sterilized explants after staining with lactophenol cotton blue showed the presence of endophytic fungi in intercellular spaces of ground and dermal tissues. In transverse sections of leaf at 100× magnification, dense blue colonies of endophytic fungi in epidermis and mesophyll region were recorded, while sprinkled colonies were seen in vascular bundles. In transverse sections of stem at 100× magnification, dense colonies of endophytic fungi in phloem and slightly lesser in epidermal region were observed. Scattered colonies were also recorded in xylem and cortex tissues. The fungal colonies were also present in intracellular and extracellular regions of both explants. Further studies will be required to identify these fungal isolates through pure culture on PDA plates to explore their biosynthetic pathway.

Keywords: *Adhatoda vasica*, endophytic fungi, histological localization, microtome.

ENDOPHYTIC fungi are microorganisms occurring asymptomatically within plant tissues[1]. Occurrence of endophytes enhances the defence mechanism of the host plants. They led to the hypothesis that plants might have accommodated endophytes to improve their fitness in the environment under certain stressful conditions and co-evolve with plant hosts and possess species-specific interactions[2]. Medicinal plants are the precious source of wealth for a healthy society. All plants in the natural ecosystem appear to be symbiotic with fungal endophytes[3]. These plants contain numerous biologically active compounds which are helpful in improving the quality of the life and in the treatment of diseases[4]. In the present scenario, a variety of medicines have been produced using different plant species, viz. *Catharanthus roseus* (L.) G.Don for the production of vinblastin and vincristine and *Stevia rebaudiana* Bertoni for stevioside and rebaudioside[5–8]. This is triggered by the presence of dense fungal colonies and hyphae in the xylem, phloem and pith region of leaf and stem tissues. Endophytic fungi have been isolated from healthy tissues of many plant species, viz. *Rauwolfia serpentina* (L.) Benth. ex Kurz, *Achyranthes bidentata* Blume. and *Myricaria laxiflora* Franch[9–11].

Taxonomy – Kingdom: Plantae; Subkingdom: Tracheobionta; Division: Magnoliophyta; Class: Magnoliopsida; Subclass: Asteridae; Order: Lamiales Family: Acanthaceae; Genus: *Adhatoda*; Species: *Adhatoda vasica.*

Adhatoda vasica Nees belonging to the family Acanthaceae, is commonly known as adusa. It is found in many regions of India and throughout the world, and is used in Ayurvedic and Unani systems of medicine. The leaves have been used for centuries with much success to treat asthma, chronic bronchitis and other respiratory conditions due to the presence of a variety of natural products such as vasicine, vasicinone, vasakin, vasicinolone, adhatonine and glycodin[12,13]. A poultice of the leaves of adusa may be applied to wounds for their antibacterial and anti-inflammatory properties; it also exhibits antispasmodic, expectorant and blood-purifying qualities[14]. It is believed to have abortifacient properties and used in some parts of India to stimulate uterine contractions, thus speeding up childbirth[15]. The present study analyses the localization, distribution and significant presence of fungal endophytes in different tissues of *A. vasica*.

The plant under study was selected from the premises of Manipal University, Jaipur campus located at 26.8430°N, 75.5650°E. The leaf and stem explants were washed under tap water before packaging them in sterile paper bags for transportation to the laboratory for processing. Samples were processed for their histological study within 5 h of collection; the time and date of collection was 10 : 30 a.m. and 6 June 2015 respectively.

The leaf and stem explants were surface-sterilized under running tap water to remove soil particles, oil remnants and surface debris. Subsequently, explants were subjected to surface sterilization in laminar air flow by successively rinsing in 70% ethanol (1 min) and 2% sodium hypochlorite (2–3 min) followed by washing with double-distilled water[16].

The explants (leaf and stem) from *A. vasica* were sectioned into smaller pieces (10 μm) and immersed in the fixing agent (FAA – formalin, acetic acid and alcohol). Later, tissues were dehydrated using Randolph's series of ethanol and *n*-butyl alcohol and substituted by liquid paraffin. The liquid paraffin was then substituted with paraffin wax (in an oven), and infiltrated specimens were poured into moulds and affixed to microtome chucks using a microtome (Rotary Erma Microtome, Japan). The top and bottom faces of the square were trimmed and oriented such that when sectioned a ribbon was formed; further the tissues were sectioned and rehydrated carefully[17,18]. A previous study has shown the presence of endophytic fungi using a microtome and sections were stained with KOH–aniline blue[19]. After staining the sections using safranin, they were washed with water (until the water became colourless). Later, sections were passed

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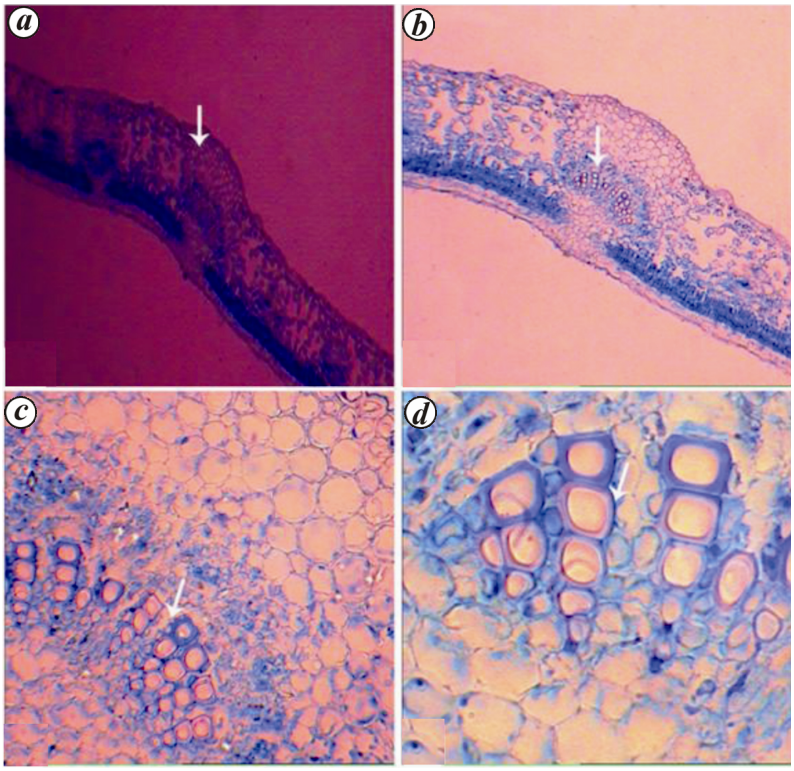

Figure 1. Vertical transverse section of *Adathoda vasica* leaf showing the presence of endophytic fungi (↑). ***a***, Intercellular mycelium (4×). ***b***, Blue colonies in epidermis region (10×). ***c***, Dense blue colonies in epidermis and mesophyll region (40×). ***d***, Sprinkled colonies of fungal endophytes in vascular bundles around stomatal subsidiary cells (100×).

through a graded series of alcohol for dehydration (30%, 50% and 70%), and lactophenol cotton blue was used as counter stain. Sections retained the stain quickly. Further, destaining was done with 90% alcohol followed by clearing with xylene and mounting was done in Canada balsam. The distribution of fungal endophyte and localization was studied with the help of a microscope (Magnus Microscope, inclined binocular microscope model MLX-B with LED-45 degree inclined standard set complete with LED light source). The dense fungal colonies were observed as blue colour after staining with lactophenol cotton blue in vascular bundles and cortex region of leaves and stem. Photographs were taken under different magnifications (4, 10, 40 and 100×).

Thin microtome sections stained with lactophenol cotton blue showed the presence of endophytic fungi in the intercellular spaces of ground and dermal tissues (Figure 1 *a* and *b*). Transverse section of leaf at lower magnification showed dense blue colonies in epidermis and mesophyll region (Figure 1 *c*) and at high magnification sprinkled colonies proximal to vascular bundles were observed (Figure 1 *d*). Similar results have been reported to favour localization and abundance of endophytic fungi in the tissues of *C. roseus*[8]. Transverse section of stem at lower magnification showed fungi in intercellular mycelium and lesser amount in epidermal region (Figure 2 *a*); at higher magnification there was a large amount of endophytic fungi in phloem region and slightly lesser in epidermis (Figure 2 *b* and *c*). However, scattered colonies were observed in phloem region with spores (Figure 2 *d*). Endophytes within healthy tissues of *Azadirachta indica* (A. Juss.) have been well documented by Verma *et al.*[19]. Similarly, *Metarhizium* and *Beauveria* showed preferential localization within plant tissues of field and tissue culture-raised plants[20]. Pianesse IIIB stain is a differential technique that facilitates detection of fungal mycelium within the plant tissues[21].

Findings reported by Wang *et al.*[22] in *Pinus tubulaeformis* were also in consonance to the outcome of the present results.

Further, leaves show dense colonies of endophytic fungi compared to the stem region. However, season and type of explants also affect the colonization of endophytic fungi within plant tissues. During the present study, leaf explants showed more fungal endophytic colonization in summer season and less in winter season on potato dextrose agar medium (Figure 3). However, Mishra *et al.*[23] reported contrasting results in *Tinospora cordifolia* (Willd.) Miers ex Hook F. & Thomas, while Kharwar *et al.*[24] reported that frequency of colonization (CF) varied more strongly with tissue type and season than location. CF was maximal during monsoon followed by winter and minimum during summer. *Guignardia* and *Acremonium* were isolated from the leaves followed by

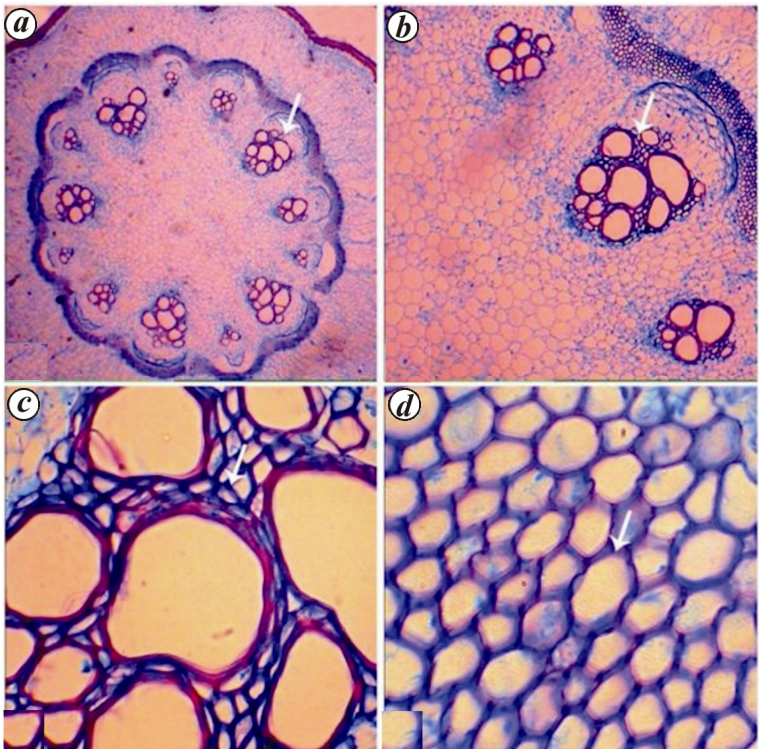

Figure 2. Transverse section of *Adathoda vasica* stem showing the presence of endophytic fungi (↑). ***a***, Endophytic fungi in epidermal region (4×). ***b***, Endophytic fungi in intercellular mycelium and lesser in epidermal region (10×). ***c***, Dense colonies of endophytic fungi in phloem (40×). ***d***, Dense colonies of endophytic fungi in phloem with spores (100×).

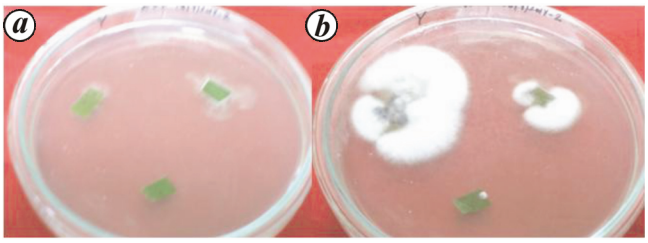

Figure 3. Colonization of endophytes in leaves of *A. vasica* on potato dextrose agar during winter and summer seasons. ***a***, ***b***, Emergence of endophytic fungi (five days) in (***a***) winter season and (***b***) summer season.

Colletotrichum spp. (11.8%), *Cladosporium* spp. (8.9%), *Chaetomium globosum* (8.1%) and *Curvularia* spp. (7.6%).

Direct approach to culture explants for identification of fungal endophytes on suitable media is time-consuming. Hence, histological localization of endophytic fungi within plant tissues would be beneficial in a short duration of time. Further, pure culture of endophytic fungi would be helpful to accelerate the growth of plants under natural/laboratory conditions, when used as biological elicitors. It may be possible to develop a complete catalogue of fungal endophytes within host tissues, as well as augment the level of understanding about the interaction of endophytes.

Conflict of interest: The authors declare that there is no conflict of interests regarding the publication of this paper.

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ACKNOWLEDGEMENT. We thank Prof. Sandeep Sancheti, President Manipal University, Jaipur for providing the necessary laboratory facilities.

Received 9 November 2015; revised accepted 23 November 2016

doi: 10.18520/cs/v112/i10/2112-2115

Seasonal variation in nearshore wave characteristics off Cuddalore, Southeast coast of Tamil Nadu, India

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Wave data collected using wave rider buoy between January 2010 and January 2011 off Cuddalore coast, Tamil Nadu, India, have been analysed season-wise in this study. Wave steepness method was used for the separation of sea and swell wave parameters. Also parameters such as significant wave height of total wave, sea and swell (H_s, H_{sw} and H_{ss}), zero crossing periods (T_z, T_{sw} and T_{ss}) and mean wave directions (θ, θ_{sw} and θ_{ss}) have been studied. The study shows a distinct shift in sea wave direction of about 90° between June and October as well as November and February. Throughout the year, the predominant swell direction remained around 135°. The contribution in total H_s by H_{sw} was 76% and the remaining 24% by H_{ss} in the yearly cycle. The sea wave height was dominant by more than 90% during November to May. Regression analysis showed good positive Pearson's correlation of 0.94 between H_s and H_{sw}; however, it was 0.65 between H_s and H_{ss}. The maximum and significant wave heights of 5.7 and 2.7 m were recorded during cyclone *Jal* on 7 November 2010.

Keywords: Regression analysis, seasonal variation, spectral energy density, wave characteristics.

WAVE characteristics, viz. wave height, period, direction, energy of sea and swell play a crucial role in nearshore processes, planning and design of coastal structures, navigation and forecasting[1]. The wave and wave-dominated processes are the predominant factors for alteration of coastal geomorphology. Sea waves are generated by wind and as they propagate away from the generating area, they are called swell waves. Swell waves are known to travel long distances across the globe. Wind waves are generated locally and are strongly coupled to the local wind field, surpassing the contribution of tides, tsunamis and coastal surges[2]. Identification and separation of wave coagulations of wind sea and swell provide a more realistic depiction of the sea state and are of great importance to oceanography and engineering applications[3].

The wave climate of the seas around India varies from southwest monsoon (June–September) to northeast monsoon (October–January) and fair weather (February–May) period[4]. Seasonally, the wave climate can be subdivided

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