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Original Research Article

Histological and biochemical evaluation of *Santalum album* L. seedlings cocultivated with different hosts

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Abstract: *S. album* establishes haustorial connections with the roots of specific hosts to fulfill its nutritional requirements during the primary stages of growth. Haustoria are the specialized structures having meristematic cells establishing an inverted flask shaped vascular connection with host stele for the transport of nutrients. In the present study we examined the morphological and anatomical features along with biochemical parameters of sandal haustoria developed with the roots of five host species from leguminous (*M. pudica* L., *C. ternatea* L., *C. pubescens* Benth. and *A. glabrata* Benth.) and non-leguminous (*V. zizanioides* (Linn.) Nash) plants. Sandal plants also showed auto-parasitism or intraspecific parasitism. The plants attached with the roots of *M. pudica* showed better growth (total chlorophyll, protein and carbohydrate content) compared to sandal plants attached with other hosts. Number and size of haustoria were more on the roots of sandal attached with *Mimosa* roots, but it was less in sandal attached with *Vetiveria* roots. Histologically sandal haustorium has a hyaline body on the periphery of host roots and a penetration peg which penetrates into the host tissue and formed a laterally flattened ellipsoidal disc at the cortex of host root. A thick mass of parenchyma was present at the interface of haustorium and host xylem. Red secretions were observed at the interface region where a thick mass of haustorial parenchyma attached with xylem tissues of the host. Haustorial xylem to host xylem connections at the interface region were prominent in sandal attached with *Mimosa*, but haustorial parenchyma to host xylem connections at the interface region were prominent when sandal attached with other hosts. Irregularly shaped penetration peg was present in *V. zizanioides* which intruded into the host root xylem pith. In the present study we focused on the impact leguminous and non-leguminous primary hosts on sandal plants and their influences on growth parameters, which give important information for further studies.

Key words: *Auto-parasitism, haustorium, hyaline body, intraspecific parasitism, primary host, total chlorophyll content.*

Introduction

Santalum album L. (Indian sandal wood) is a tropical evergreen hemi-parasitic tree belonging to the family Santalaceae. The family includes 44 genera and more than 400 species in which *S. album* is the most economically important species due to its essential oil and medicinal values. The plant is distributed in India, Australia, Indonesia, New Zealand and other countries. Sandal seedlings derive nutrients from its seed reserves in the initial stages of development and later they attach with proper hosts for further mineral uptake due to their reduced root system. Chemical signal released from host roots has no role in haustorial induction. But the haustorial initiation is triggered by contacts with the inert objects or sometimes with the roots of same plant. *Santalum spicatum*(R.Br.) A.DC. forms haustoria on many inert objects in the absence of potential hosts.

Barrett and Fox (1997) emphasized that the fully fertilized seedlings developed haustoria within three months and seedlings without minerals had fewer haustoria. Nagaveni and Srimathi (1985) observed 70% of seedlings initiated haustoria within 30 days of germination in the presence of potential hosts and 90% within one year.

Haustoria are the bell shaped structures penetrating in to the vasculature of host roots for absorbing mineral nutrients. A typical haustorium consists of clasping folds, collapsed layers, inverted conical flask shaped vascular tissue, a central hyaline body and endophyte tissue through which the exchange

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of essential mineral nutrition between host and parasite occurs (Zhang *et al.,* 2012). Different hosts provide different minerals to sandal. Mainly hosts are the source of N, P and K. The unattached sandal seedling growing in medium with deficiencies of N, P, K, S and Ca showed specific morphological symptoms, chlorophyll levels, haustorial induction and accumulation of different minerals in different parts of seedlings (Barrett and Fox, 1997). A detailed anatomy and penetration mechanism of haustoria in *S. album* attached with *Tithonia diversifolia* (Hemsl.). A. Gray was discussed by Tennakoon and Cameron (2006). Haustorial vascular tissues connected only with xylem of host roots but not with phloem elements. Haustorial and host root xylem elements are connected through their pits for membrane transport of nutrients and water. Micronutrient level in sandal is not influenced by host attachment, but macronutrient (N, P and K) level was highly influenced in sandal by the association of suitable hosts. Host plants are also maintaining the water levels and reduction of water stress in sandal plants. Removal of hosts from sandal-host association will lead to wilting and leaf shedding in sandal (Rocha *et al*., 2014). The phytohormone levels are increased in sandal haustoria after pre and post attachment with suitable host plant *Kuhnia rosmarnifolia* Vent. Compared to hormone levels in sandal and host roots before attachment (Zhang *et al*., 2012). Tennakoon *et al*. (1997) suggested that proline is the important aminoacid synthesized and transported through haustoria in *S. acuminatum* attached with *Acacia rostellifera* Benth. and also nitrate reductase activity was induced in haustoria during nitrate uptake from host plant. The presence of endophytic fungi in the cortical cells of sandal and its host plant *K. rosmarinifolia* roots were reported by Sun *et al*. (2014). 25 taxa of endophytic fungi were isolated from sandal-*Kuhnia* interactions in which Penicillium sp 1 and Fusarium sp 1 were predominant.

Many studies on sandal-host relationship were conducted by using different host species and explained their influence on morphological and physiological attributes of sandal, haustorial establishment with host roots, anatomy of sandal haustoria attached on different hosts, fungal symbiosis in the roots of sandal and hosts *etc*., but the most suitable annual primary host for proper growth and development at the primary stage in sandal silviculture is still unknown. In the present study we selected both legumes and non-legumes as annual primary host plants to identify the better host plant and provided both biochemical and histological data to clarify the results.

Materials and Methods

Sandal seeds for the study were procured from KFRI, Peechi, Thrissur, Kerala. The seeds were pretreated with 98% (v/v) H_2SO_4 and sowed in a tray filled with soil and sand in 1:1 ratio. Seventy percentages of seeds were germinated after 3 weeks. The seedlings were transplanted in to polythene bags (.16 X .10 m) contained potting mixture (soil: sand: cowdung in 1:2:1 ratio) and kept for one month in the polyhouse. These sandal plants were taken out from the polybags and planted in the field and assigned to six treatments with eight replications. One treatment maintained as control without host. Each plant was planted at 0.5 m gap with two hosts each in the field. Leguminous (*Mimosa pudica* L.*, Clitoria ternatea* L.*, Centrosema pubescens* Benth. and *Arachis glabrata* Benth.) and non-leguminous (*Vetiveria zizanioides* (Linn.) Nash*)* host seedlings were planted along with sandal seedlings about .02 - .03 m apart. Morphological analysis (stem length, stem girth, number of leaves, leaf length, leaf breadth, leaf area, number of nodes and number of branches), biochemical analysis (total protein, sugar and chlorophyll estimations) and histological analysis (observation of transverse sections of haustoria-host root interface under phase contrast microscope) of treated plants were carried out after one year. Leaf samples from each treatment were collected and cleaned thoroughly and analyzed for total proteins by Lowry *et al*. (1951) protocol, total chlorophyll content by Arnons (1949) protocol and total sugar by anthrone method (Hedge and Hofreitre, 1962). Optical density of all samples was measured by using UV-VIS spectrophotometer, shimadzu at specific wavelengths.

Roots of sandal plants along with host roots from each treatment were collected from the field and washed well to observe the shape, diameter and distribution of sandal haustoria developed on host roots. Sledge microtome was used to prepare thin sections of haustoria-host root interface from fresh samples. The sections were stained with safranine and mounted in glycerin. Starch grains were detected with Iodine Pottasium Iodide stain (Lugol's iodine). Histological observations were done by Lab. A1. ZEISS Axiolab Phase Contrast Microscope (Germany, Caul ZEISS).

Results

Morphological analysis

Sandal plants grown along with leguminous hosts (*M. pudica, C. ternatea, C. pubescens* and *A. glabrata)* showed better morphological growth parameters compared to sandal grown along with non-leguminous host (*V. zizanioides)* and control plants (Fig. 1). Among the leguminous hosts *M. pudica* was the best host which improved total biomass production and metabolism in sandal. Sandal attached with the non-leguminous host, *V. zizanioides*, also showed higher growth rate than control plants. Control plants showed diminutive growth and high mortality because of the lack of mineral uptake from host plants.

Figure 1: Morphological analysis of *S. album* associated with leguminous and non-leguminous hosts (Values are mean ± standard error, n=10)

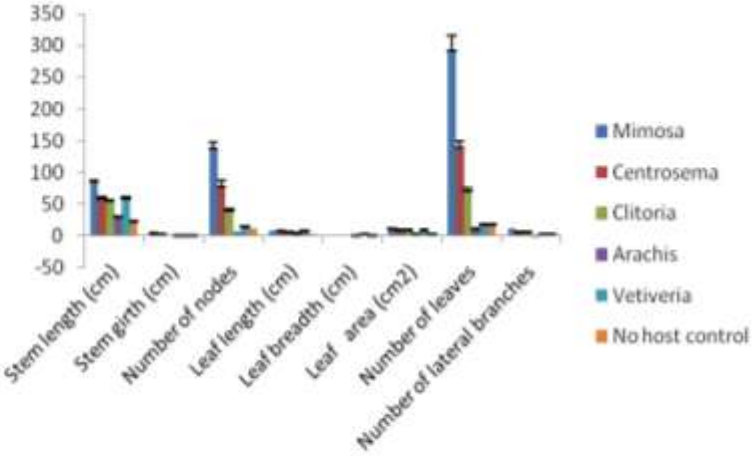

Table 1: Biochemical analysis in *S. album* associated with leguminous and non- leguminous hosts (Values are mean ± standard error, n=3).

Sandal+Host	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Total chlorophyll (mg/g)	Total protein (mg/g)	Soluble sugar (mg/g)	Total sugar(mg/g)
Sandal+*M. pudica*	$4.2x10^{-3}\pm7.8x10^{-5}$	$41\ x10^{-3}\pm\ 1.5x10^{-4}$	$6.7x10^{-3}\pm6.7x10^{-5}$	$0.033\pm1\ x10^{-3}$	$0.84\pm7x10^{-2}$	$0.042\pm4\ x10^{-3}$
Sandal+ *C. ternatea*	$4.3x10^{-3}\pm1.8x10^{-4}$	$39\ x10^{-3}\ \pm6.7x10^{-5}$	$6.6x10^{-3}\pm1.1x10^{-4}$	$0.031\ \pm\ 2\ x10^{-3}$	$0.36\pm4x10^{-4}$	$0.022\pm\ 3\ x10^{-3}$
Sandal+ *C. pubescens*	$4x10^{-3}\pm7.4x10^{-5}$	$29\ x10^{-3}\pm\ 1x10^{-3}$	$5.5x10^{-3}\pm4.7x10^{-5}$	$0.029\ \pm\ .7x10^{-3}$	$0.48\pm4x10^{-3}$	$0.025\pm\ .7x10^{-3}$
Sandal+ *A. glabrata*	$4.2x10^{-3}\pm2.4x10^{-5}$	$35\ x10^{-3}\pm\ 2.9x10^{-4}$	$6.2x10^{-3}\pm1.2x10^{-5}$	$0.029\ \pm\ .2x10^{-3}$	$0.56\pm2x10^{-2}$	$0.034\pm\ 1\ x10^{-3}$
Sandal+ *V. zizanioides*	$3.4x10^{-3}\pm2.04x10^{-6}$	$27\ x10^{-3}\pm\ 4.6x10^{-5}$	$4.8x10^{-3}\pm5.8x10^{-6}$	$0.029\pm\ .4x10^{-3}$	$0.32\pm6x10^{-3}$	$0.033\ \pm3\ x10^{-3}$
Control without host	$2x10^{-3}\pm1.6x10^{-4}$	$12\ x10^{-3}\pm4.2x10^{-4}$	$2.5x10^{-3}\pm1.2x10^{-4}$	$0.027\pm\ .5x10^{-3}$	$0.11\pm4x10^{-4}$	$0.029\pm\ 1\ x10^{-3}$

Estimation of chlorophyll, sugars and proteins

Quantity of Chlorophyll a, chlorophyll b, total chlorophyll, soluble sugar, total sugar and total protein contents were higher in sandal grown along with leguminous hosts compared to sandal grown along with *V. zizanioides* and no host control plants. Among the treatments sandal grown along with *M. pudica* showed better results (Table 1).

Morphology of haustoria

Morphology of haustoria attached on sandal roots varied in each host-parasitic interaction (Fig. 2). Number, size, shape and distribution of haustoria were different in sandal attached with different hosts. There were no haustoria on roots of control plants but sandal produced large number of haustoria on root surface of *M. pudica*. Haustoria attached with *M. pudica* roots were large in size (0.002-.01 m) compared to haustoria produced on the roots of *C. ternatea* (0.004-0.007 m), *C. pubescens* (0.003-0.004 m), *A. glabrata* (0.003-0.005 m) and *V. zizanioides* (0.002-0.003 m). In the study sandal haustoria attached on *V. zizanioides* were smaller in size compared to haustoria on other host roots. All the haustoria were globular in shape and some of the haustoria on *M. pudica* roots were bell or cup shaped. Clustered appearance of two or three haustoria was present on *C. pubescens* roots, but haustoria on roots of all other hosts were solitary in distribution. In *C. ternatea* and *C. pubescens,* haustoria were attached mainly on elongated primary roots. Haustoria-rhizome interactions were prominent in *A. glabrata*. There was no correlation between number of haustoria and sandal root length because root length was almost same in all treatments.

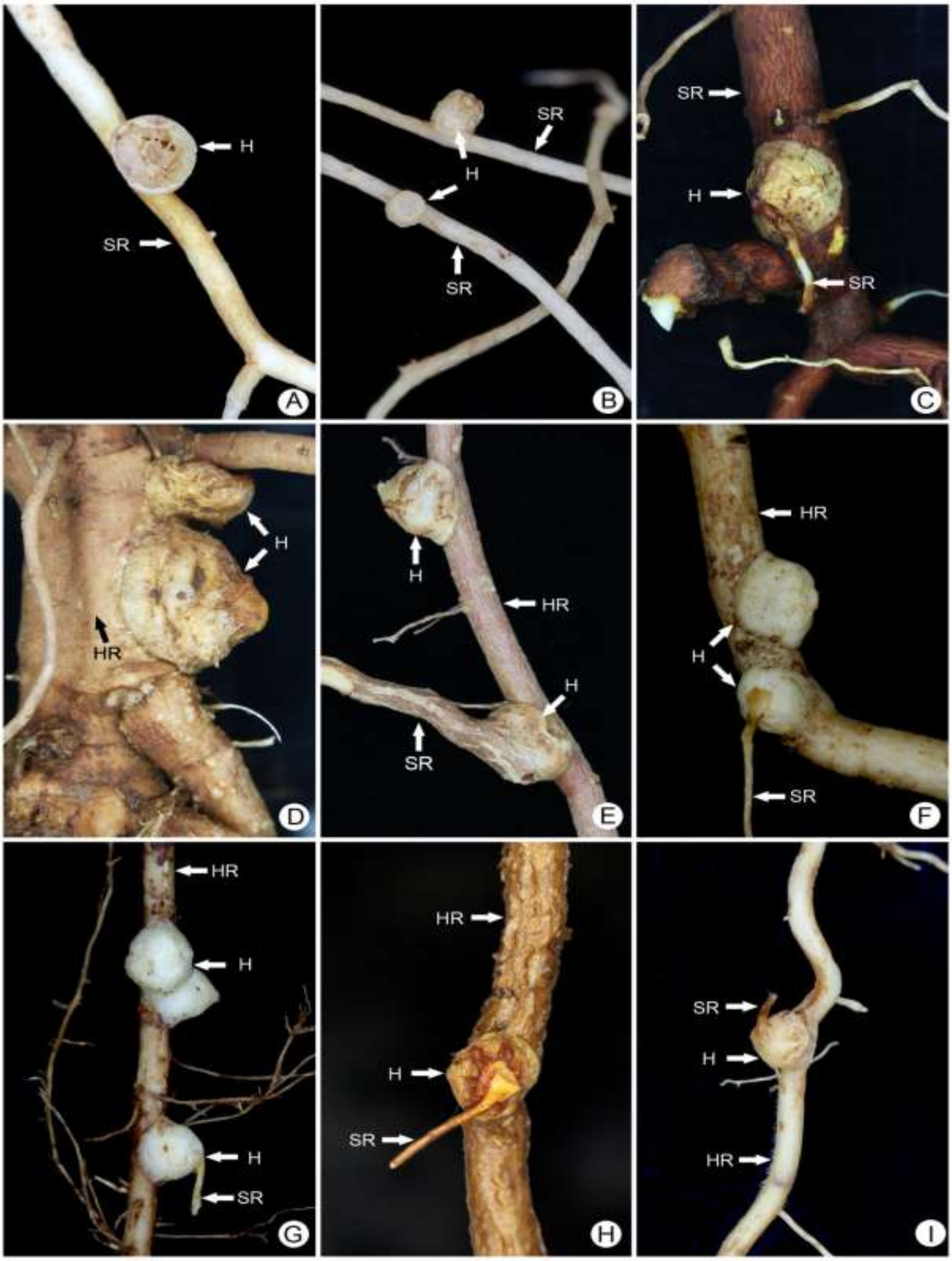

Fig. 2 (**A**) Sandal haustorium (H) without host root connections. (**B**) Intraspecific haustoria (H) on sandal roots (SR) at the younger stage. (**C**) Matured intraspecific haustorium (H) on sandal root (SR). (**D**) & (**E**) Sandal haustoria (H) attached with the root of *M. pudica* (HR). (**F**) Sandal haustoria (H) attached with the root of *C. ternatea* (HR). (**G**) Sandal haustoria (H) attached with the root of *C. pubescens* (HR). (**H**) Sandal haustorium (H) attached with the root of *A. glabrata* (HR). (**I**) Sandal haustorium (H) attached with the root of *V. zizanioides* (HR).

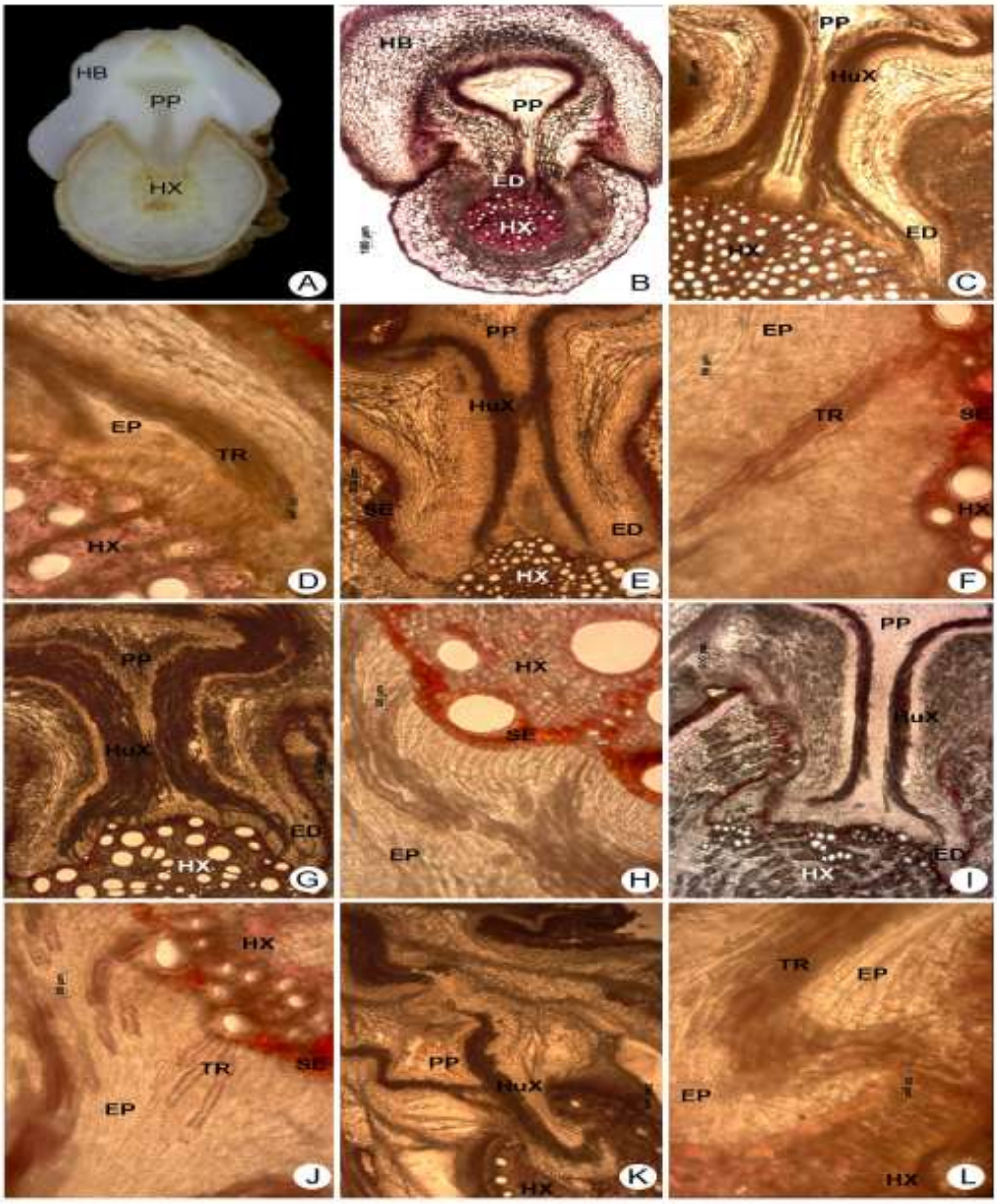

Fig. 3 (**A**) Transverse section of haustorium-host root interaction showing cup shaped hyaline body (HB) attached on host root surface and invasion of penetration peg (PP) in to the xylem of host root. (**B**) Transverse section of intraspecific haustorium showing attachment of hyaline body (HB) on sandal root and invasion of penetration peg (PP) in to the xylem of sandal root. (**C**) Anatomical view of penetration peg (PP) and ellipsoidal disc (ED) attachment with xylem elements of *M. pudica.* (**D**) Enlarged view of haustorial interface of sandal with *M. pudica* roots showing haustorial elongated parenchyma (EP) and trachied (TR) connections with host xylem elements. (**E**) Anatomical view of sandal haustorial interface with root xylem of *C. ternatea* showing blunt ended ellipsoidal disc (ED). (**F**) Enlarged view of sandal haustorial interface with xylem elements of *C. ternatea* showing reddish secreation (SE) to host xylem (HX). Trachieds (TR) and elongated parenchyma (EP) show attachment with host xylem. (**G**) Transverse section showing sandal haustorium connection with root xylem of *C. pubescens*. (**H**) Enlarged view of sandal haustorium interface with root xylem of *C. pubescens* showing reddish secreation (SE) to host xylem (HX). Some of the haustorial xylem elements and elongated parenchyma (EP) show attachment with host xylem (HX) elements. (**I**) Transverse section shows penetration peg (PP) with ellipsoidal discs (ED) connection with root xylem of *A. glabrata*. (**J**) Anatomical view of penetration peg-root xylem attachment in *A. glabrata*. Reddish secreation (SE) presents in the host root xylem. A dense mass of elongated parenchyma (EP) and some of the trachieds (TR) attached to host xylem (HX). (**K**) Anatomical view shows irregular shaped haustorial penetration peg (PP) invasion in to the root xylem pith of *V. zizanioides*. (**L**) Enlarged view of haustorial trachied (TR) and elongated parenchyma (EP) connections with xylem elements of *V. zizanioides* roots through pith tissues.

Anatomy of haustoria

Intraspecific (haustoria of sandal roots attached on the roots of nearest sandal plant) haustorial induction and auto parasitism (haustoria of sandal root attached on the roots of same plant) were observed in sandal roots when the seedlings were grown in a tray contained potting mixture for one month. Histologically intraspecific haustoria were similar to other haustoria attached on leguminous and non-leguminous host roots (Fig. 3). Sandal haustoria had two parts, the outer dome shaped hyaline body (HB) and the inner inverted conical flask shaped penetration peg (PP). The hyaline body was made up of 14-16 layers of parenchyma cells with scattered starch grains which helped to attach haustoria with the periphery of host root. Penetration peg intruded through epidermis, hypodermis, cortex and cambium and finally attached with xylem elements of host root which firmly adhere the haustoria and host root. Elongated parenchymatous tissues with numerous starch grains (EP) were present at the centre and on both sides of vascular duct which was extended up to the host xylem. The tip of penetration peg was bifurcated into two lateral ellipsoidal discs (ED) within the host cortex (Tennakoon and Cameron, 2006). In the present study the shape of the lateral disc was different in haustoria attached with different host species. In *M. pudica*, *A. glabrata* and *C. pubescens* roots the shape of haustorial lateral disc was elliptical, but in *C. ternatea* roots only two projections were present at the tip of penetration peg. Irregular hyaline body and penetration peg were present in haustoria attached with *V. zizanioides* root and the penetration peg was directly entered in to the pith of vascular cylinder of host root for intake of nutrients. A bright yellowish-red secretion (SE) was present at the interface of haustorium and host roots. A dense mass of elongated parenchyma (EP) tissue was present at the host-parasite interface which was connected with host xylem elements. A few tracheary elements present in haustoria showed pit to pit connections with host root xylem elements for transport of nutirents. Macerated or any damaged tissues were absent at the interface region due to the pressure created by penetration peg in the cortex of host root.

Discussion

Sandal without the attachment of a host normally showed slow growth and mortality may be due to the lack of enough nutrients in the plant. Root nodules of leguminous plants are the centre of nitrogen fixation due to the presence of nitrogen fixing bacteria. Sandal haustoria can uptake more nitrogen compounds into sandal from leguminous hosts which improve growth and metabolite productions. So we selected leguminous hosts in the present study. Organic nitrogenous compounds in the form of aminoacids were transported from host through haustorial connections and haustoria were the major site for synthesis of proline in *S. accuminatum* (Tennakoon *et al*., 1997). *M. pudica* developed a much branched root system with numerous root nodules establishing more haustorial connections and better mineral transport between *M. pudica* roots and sandal haustoria. In *C. ternatea* and *C. pubescens* root system, number of attached sandal haustoria was less and distributed mainly on primary roots to absorb maxium minerals and water. Root system of *V. zizanioides* was fibrous type with many root hairs having nitrogen fixing microbes, azotobacter (Zhao *et al*., 2009). Attachment with *V. zizanioides* reduced sandal growth due to less haustorial connections and less solute transport through host-parasite interactions compared to other leguminous host plants.

Without host interaction sandal could not survive as a perrenial plant because root system of sandal is less branched. So the plant could not get enough water and minerals through root system. To overcome the mineral and water deficiency plants develop vascular connections with suitable hosts. Hemiparasitic species can efficiently assimilate organic carbon from their hosts (Tesitel *et al*., 2010). Increase in photosynthetic pigments, sugar and protein contents in sandal was observed due to the leguminous host interactions. These envisages transport of carbon and nitrogenous compounds through haustorial connections to sandal.

The type and quantity of minerals, nitrogenous compounds and organic carbon compounds transported through haustoria dependent upon the nature of hosts attached with sandal. From the study it was concluded that number and size of haustoria were dependant on the availability of nutrients from the host plants. *S. album* produced large number of haustoria with *M. pudica* roots because of the availability of vast amount of nitrogen compounds. The prominent haustorial xylem to host xylem connections in sandal-*Mimosa* interactions also gives a histological evidence to higher mineral and water transport between them (Fig. 3 C & D). Mineral transport through haustorial xylem to host xylem connections are very fast compared to haustorial parenchyma to host xylem transport because of the presence of cell membranes which acts as barriers. In all sandal-host interactions haustorial parenchyma to host xylem connections were prominent. From the histological study it is clear that a good percentage of mineral transport is occurring through membrane transporters present on cell membranes at the haustoria-host interface in addition to pit transports. The presence of red secretion at the interface region of haustorium and host root helps to firm the adhesion (Tennakoon and Cameron, 2006). In the present histological work, the secretion was present in all haustorial-host interfaces.

Conclusion

The evidences from higher biomass productions, metabolite synthesis and haustorial connections in *S. album* support the selection of leguminous plants as better hosts for sandal. Among leguminous host plants the influence of *M. pudica* was better on sandal growth, development and haustorial inductions. So we can conclude that sandal plants can intake excess nitrogenous compounds from leguminous hosts through haustoria and used for further metabolism. Histological studies also support the membrane transport and xylem pit to pit transport of nitrogenous compounds at the haustorial-host interface. In the study *M. pudica* was selected as better host for sandal growth in the primary stage which has short life span and less biomass production compared to other perennial hosts.

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