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Primary Host Interaction of Root Parasite Sandalwood (*Santalum album* L) : Morphological and Biochemical Responses during Interaction with Legume Host *Cajanus* and Non-Legume Host *Alternanthera*

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ABSTRACT

Sandalwood (*Santalum album* L.), a root hemiparasite, endemic to Southern India, is a tree of great commercial value globally. Considering the growing demand and the diminishing supply of sandalwood from its natural habitat, there is a great potential for cultivating sandal trees in agricultural land, home gardens and other agro forestry systems. The cultivation of sandalwood is challenging because of the poor understanding of host parasite relationship as sandalwood is a root parasite. In this context a study was carried out to understand the relationship of sandalwood with its two primary hosts, *Cajanus* (legume) and *Alternanthera* (nonlegume). The results showed that sandalwood grown with *Cajanus* (T_3) have greater plant height (176.57 cm), stem girth (12.01 cm) chlorophyll content (3.29 mg/g FW of leaves) in contrast to sandalwood grown with *Alternanthera* (T_2) and control (T_1) without a host. The variations in activity of SOD, Peroxidase (POX) and accumulation of phenols, flavonoids, proline in sandalwood indicates differences in sandalwood response at molecular level towards the two hosts. There was a clear shift in SOD isozyme bands in sandalwood when it interacted with *Cajanus* as compared to *Alternanthera* (T_2) and control (T_1). The increase in peroxidases observed in T_3 (11.30µg/mg protein) was significantly higher compared to the T_2 and T_1. The same trend was observed for the phenols, flavonoids and prolines. The greater reduction in defense response in the host plant *Cajanus* in comparison to *Alternanthera* when planted with sandalwood signifies the deliberate vulnerability of *Cajanus* towards sandalwood parasitism, making it a superior host. Indeed, a polybag experiment to evaluate the direction of root growth shows a clear preference of sandalwood root to *Cajanus*, as compared to *Alternanthera*. Overall from the phenotypic observations and also from the biochemical estimations, the leguminous host *Cajanus* was found to be a better primary host than *Alternanthera* in the field condition. The insights gained from the present investigation can be used for sandalwood cultivation in large scale in the natural habitat as well as in other agroforestrty region.

Keywords : Sandalwood, Root hemiparasite, Host association , SOD, POX, Isoforms, Secondary metabolites

PARASITISM is a successful life strategy that represents the most extreme interaction between plants and widespread among living species (Poulin, 2011). The parasite steals water and nutrients from another plant or fungus. Parasitic plants are characterized by the ability to feed directly on other plants, invading either the roots or shoots of their hosts through parasitic structures called haustoria (Kuijt *et al.,* 1969). On the basis of the degree of host dependence, parasitic plants are classified as facultative parasites which can live autotrophically and reproduce without host contact, but will opportunistically parasitize neighboring plants when available and obligate parasites which must parasitize a host in order to complete their life cycles. Based on the status of photosynthesis, parasitic plants are of hemiparasitic (photosynthetically competent, though the efficiency of photosynthesis varies considerably between different species) and holoparasites (lack photosynthetic activity and obtain all their reduced carbon through haustorial connections with a host) (Irving and Cameron, 2009). The Sandalwood (*Santalum album* L.) belongs to the genus *Santalum* is considered as obligate root hemiparasite. The parasitic nature of sandalwood was reported by John Scott in 1871. The importance of sandal wood

parasitism was realized after Barber (1902, 1906 and 1907) reported in detail about haustorial formation, growth and development of haustoria.

Sandalwood (*Santalum album* L.) is a prized gift of the plant kingdom woven into the culture and heritage of India. India is the second largest producer with Karnataka leading in area, production and productivity of sandalwood. This widely distributed and economically important *Santalum* genus belongs to the family Santalaceae which includes 30 genera with about 400 species, many of which being completely or partially parasitic (John, 1947). The word Sandal has been derived from Chandana (Sanskrit), Chandan (Persian), Savtador (Greek) and Santal (French). It is the second most expensive wood in the world and highly valued for its aromatic heartwood which is used as finest natural materials for carving. Sandalwood oil is used in perfumes, cosmetics, aromatherapy and pharmaceuticals. Sandalwood chips are generally used for making agarbattis. Seasoned sapwood may be carved into curios, toys, carom coins and lacquerware. In medicine, it finds use as an antiseptic, antipyretic, antiscabietic, diuretic, expectorant, stimulant and for treatment of bronchitis, dysuria, gonorrhoea and urinary infections (Kumar *et al*., 2012).

The monopoly of sandalwood trade by the Governments of Karnataka, Tamil Nadu and Kerala and indiscriminate illegal felling of sandal wood from natural habitats has pushed *S. album* into the vulnerable category of the IUCN Red List. The average production of Sandalwood in Karnataka between the years 1960-1961 and 1964-1965 was 2287.8 tons which drastically reduced to 366.74 tons by the end of the last five years of the twentieth century (1995-1996 to 1999-2000). The average production further fell to a mere 61.57 tons in the first decade of this century (Kumar *et al*., 2012).

Considering the growing demand and the diminishing supply of sandalwood from its natural habitat, there is a great potential for cultivation of sandalwood tree in not only forest areas but also in private land like home gardens and other agroforestry systems. For the plants having high economic value, sustainability needs to be maintained through the efforts of regeneration. However, the cultivation of sandal tree has been challenging because of the poor understanding of host parasite relationships. Production of sandalwood can be increased by extensive plantation of this species after properly understanding the host-parasite relationship, proper production of planting materials and deeper knowledge of silviculture of this species.

Being root hemi parasite the silvicultural requirements of sandalwood are unique and there is no adequate understanding of the same. Its regeneration and establishment have been problematic because of the poor understanding of host-parasite relationships (Surendran *et al.,* 1998). Insights into *Santalum* host parasite interaction can contribute to successful establishment of Sandalwood in both natural habitat and in cultivation. Sandal can parasitise over 300 species of host plants found in nature from grasses to leguminous trees. It was also reported that sandal requires a primary host at nursery stage, secondary long term host and permanent host in the field (Annapurna *et al*., 2006). This species shows different growth patterns with different host species. Legume plants seem to act as better hosts for *Santalum* indeed, direct haustorial establishment on root nodules have been reported (Radomiljac *et al.,* 1998 and Lu *et al*., 2014). However, some contrary evidence demonstrates that host N is not always a reliable predictor of parasite performance (Jiang *et al*., 2008). Nge *et al*., 2019 studied the host specificity of *Santalum acuminatum* (quandong) and found that *Acacia saligna* was a superior host, as growth of quandongs were stronger when they were paired with *Acacia saligna* than when grown with other legumes and non-legumes, indicating Acacia to be better host. The present study was carried out to understand the effect of two primary hosts *Alternanthera* (non legume) and *Cajanus* (Legume) on sandalwood early growth under field condition. The study evaluates biochemical changes when sandalwood associates with *Cajanus*, or *Alternanthera* in comparison to sandalwood growing without association with any host plant. The study finally looks at the direction of sandal wood root growth when in proximity to both *Cajanus* and *Alternanthera.*

Material and Methods

Collection of Planting Material

The planting material - sandalwood saplings of same age (8 months old) along with the leguminous host *Cajanus cajan* and non leguminous host *Alternanthera* were collected from Tapovana Sandalwood Nursery, Bengaluru.

Performance of Sandalwood with *Cajanus* and *Alternanthera*

The sandalwood seedlings were grown in the field located at Department of Plant Biotechnology, UAS, GKVK, Bengaluru during December 2019. The experiment was designed in RCBD with three treatments and seven replications with spacing of 8 ft x 8 ft. The treatments details are listed in Table 1. The field management was followed as per the methods developed by IWST, Bangalore. The host plants were planted at one ft distance to each sandalwood plant. The plant were allowed to grow for one year before the recording of data.

Table 1

Treatment details of the field experiment

Treatment Number	Treatments	Description
T_1	Control	Sandalwood planted without any host
T_2	Sandalwood with *Alternanthera*	Sandalwood was planted with *Alternanthera*
T_3	Sandalwood with *Cajanus*	Sandalwood was planted with *Cajanus cajanus*

Phenotypic Observations

The plant height was measured as the height between base of the plant to the tip of topmost leaf in centimetre. The stem girth was measured by using measuring tape in centimetre at uniform height from the base of each individual plant.

Chlorophyll Estimation

Chlorophyll was extracted and estimated as per the method of Arnon (1994). Essentially, chlorophyll was extracted from leaf samples using 80 per cent acetone and DMSO in the ratio of 1:1. Absorbance was determined at 663 nm and 645 nm using a UV - spectrophotometer (Eppendroff Biospectrometer Basic). Using the molar absorption coefficient, the amount of chlorophyll was calculated.

Protein Extraction

Healthy mature leaves from three replications of the different treatments (T_1, T_2, T_3) were frozen in liquid nitrogen, to prevent proteolytic activity and homogenized using a mortar and pestle. The homogenate was then suspended in extraction buffer [Phosphate buffer 0.1 M, pH 7.8, 1 mM PMSF (protease inhibitor) and 0.1 per cemt of poly vinyl pyrrolidone (PVP)] and kept on ice for 15 min. The crude protein extracts were centrifuged at 14,000 rpm at 4°C for 30 min. The pellet was discarded and the supernatant containing the soluble proteins was used for further experiments. Protein concentration was determined by the method of Lowry (Lowry *et al.,* 1951) using BSA as standard. Equal quantity (µg) of protein from T_1, T_2 & T_3 was taken for the enzyme assays.

Defense Enzyme Assays

Spectrophotometric Assay of Guaiacol Peroxidase (POX) Activity

Peroxidase activity in the protein extract was measured by the method proposed by Castillo *et al.* (1984) with slight modification. Reaction mixture (3 mL of Phosphate buffer (100 mM. pH 6.1), Guaiacol (96 mM), Distilled water) was prepared and protein extract (100 µg of protein) was added to it. Absorbance due to formation of tetra-guaiacol was measured at a time interval of 30 sec up to one min at 470 nm using a UV-Vis spectrophotometer (Eppendroff Biospectrometer Basic). Peroxidase activity is assayed as increase in optical density due to the oxidation of guaiacol to tetra-guaiacol.

Native PAGE was performed as the method described by Davis (1964) for peroxidase isoenzyme activity by using 10 per cent resolving gels and five per cent stacking gel. Protein extract (25 µg) of all treatments were loaded in gel. Electrophoresis is

The Mysore Journal of Agricultural Sciences

performed initially at 60 volts and when the protein entered the resolving gel, the voltage was increased to 120. Electrophoresis was conducted at 4°C for about 3h. Later the gel was stained for peroxidase isoenzymes.

Spectrophotometric Assay of Superoxide Dismutase (SOD)

SOD activity was measured by the method described by Dhindsa *et al*. (1981) with slight modifications. SOD activity in the supernatant was assayed by its ability to inhibit photochemical reduction of nitro blue tetrazolium (NBT). 100 µg of protein was taken from each treatment and assayed and the reduction of NBT was measured by using a UV-Vis spectrophotometer at 560 nm using a UV-Vis spectrophotometer (Eppendroff Biospectrometer Basic).

Native PAGE was performed according to the method described by Davis (1964) for superoxide dismutase isoenzyme activity by using five per cent stacking gels and 10 per cent resolving gels. Protein extract (25 µg) from all treatments were loaded to gel. Electrophoresis was performed initially at 60 volts and after the protein entered the resolving gel the voltage was increased to 120. Electrophoresis was conducted for 3h at 4^0C. The gel was incubated in a staining solution containing 0.1 per cent NBT (w/v), 0.2M EDTA (w/v), 0.1M sodium phosphate buffer (pH 7.5), commercial grade TEMED and five per cent riboflavin (w/v) for 30 min until the bands appeared. The isoenzyme bands appeared white/ colourless in a dark blue background and the isoenzyme pattern was photographed.

Phenolic and Flavonoid Extraction

One gram of leaf sample was homogenized in 10 ml of 80 per cent ethanol in a pestle and mortar. The homogenate was centrifuged at 10000 rpm for 20 min, the supernatant was collected and the residue re-extracted with five times the volume of 80 per cent ethanol and re centrifuged. After this the supernatant was collected and evaporated to dryness and the residue dissolved in two ml of distilled water.

Estimation of Total Phenolics

Phenols were estimated by following the method described by Bay and Thrope 1954; Kumbar *et al.,* 2013 with slight modifications. The phenol extract of 0.5 ml was diluted with distilled water to make the volume to one ml. Folin-ciocalteau reagent (0.5 ml) was added to it and the content was mixed properly. After three minute two ml of saturated sodium carbonate was added and the mixture was kept for one minute in boiling water bath. After cooling to room temperature the absorbance was taken at 650 nm using a UV-Vis spectrophotometer (Eppendroff Biospectrometer Basic). The phenolic concentration present in the test samples were calculated using standard curve of gallic acid and the concentration is expressed as mg gallic acid equivalents (GAE).

Estimation of Flavonoids

Total flavonoid content was measured by the aluminium chloride colorimetric method following the procedures described by Woisky and Salatino, 1988. The ethanolic extract (0.5 ml) was diluted with distilled water to make the volume to one ml and then 1.5 ml 95 per cent methanol, 0.1 ml of 1 M sodium acetate and 0.1 ml 10 per cent aluminium chloride were added. The absorbance was recorded at 415 nm using UV-Vis spectrophotometer (Eppendroff Biospectrometer Basic) against the blank. The flavonoid concentration present in the test samples were calculated using standard curve of Rutin and the concentration is expressed as mg Rutin equivalants (RE).

Proline Content

Proline was extracted together with total amino acids, pigments, soluble sugars by heating plant material twice with 80 per cent ethanol and once with 50 per cent ethanol as described by Cross *et al.* (2006), which results into a 70:30 ethanol : water mixture (v/v). The resulting mixture is left overnight a 4°C and then centrifuged at 14000 g (5 min). The proline concentration was determined by following the protocol of Carillo and Gibon, 2011 in which, ninhydrin one per cent (w/v) in acetic acid 60 per cent (v/v), ethanol 20 per cent (v/v) were used as reaction mixture.

Total Soluble Sugar

Total sugar was estimated by following the methodologies of Dubious *et al*., 1956; Umashree *et al.,* 2010. One ml extract was diluted to 1.0 ml of five per cent phenol. To this mixture five ml of 96 per cent sulphuric acid was added and mixed well for 10 minutes. The mixture was incubated in a water bath at 25-30°C for 10 minutes. After cooling to room temperature, the absorbance was read at 496 nm against the reagent blank

Effect of Sandalwood Parasitism on Host Plant

The host plants *Cajanus* and *Alternanthera* were grown separately in the field without any sandal wood plant. This was used to compare with the host that were being grown along with Sandalwood in the earlier described field experiment in RCBD design. The biochemical analysis including defense enzymes, phenol, flavonoids, proline for both the host plants were carried out by following above methodologies. The *Alternanthera* without sandalwood was considered as A_1 and with sandalwood A_2, similarly for *Cajanus* C_1 and C_2 were named for the *Cajanus* without sandalwood and *Cajanus* with Sandalwood, respectively.

Evaluation of Direction of Sadalwood Root Growth when Grown with *Cajanus* and *Alternanthera*

The sandalwood plants were grown in specially designed and fabricated polybags with three interconnected compartments. The polythene wall between two compartments had a central connecting oval hole of 15 cms diameter (top to bottom) and eight cms diameter across, connecting the central compartments to the peripheral compartments, as shown in Fig. 4a. The planting was taken up in January 2021, placing sandalwood plantlet in central compartment and *Cajanus and Alternanthera* on the left and right peripheral compartments. After 10 months the sandalwood roots were uprooted and the direction of sandalwood root was observed. The total number of roots from sandalwood towards host plant and the roots from host plant towards sandalwood along with number of haustoria present in the roots of both the host plants were recorded.

Statistical Analysis

In the field experiments, the RCBD design was used with seven replicates. The mean values (seven replicates) of phenotypic and biochemical parameters are statistically compared by using the RCBD design of OPStat (Sheoran *et al*., 1998). The mean value (seven replicates) of all the biochemical parameters in host plants was compared by using student T test. For the root growth experiment in polybags, the mean values of three replicates were compared by student T test.

Results and Discussion

Sandalwood-host interactions remain an enigma. In the current study, field and polybag experiment were taken up to gain insight into the phenotypic and biochemical responses of sandalwood when grown with a legume and non-legume primary host. The observations and biochemical data are presented in the following sections.

Assessment of Morphological and Physiological Traits of Sandalwood

The morphological and physiological changes observed in sandalwood field experiments are presented in Table 1 and Fig. 1. The plants growing with *Cajanus*, were visibly more robust with higher plant biomass (Fig. 1 a, b) with greener leaves than the sandal plants growing with *Alternanthera* or without host (Fig. 1 c, d, e).

Plant height : The observation on plant height, stem girth, chlorophyll content and IRGA photoas similation rate were recorded exactly after one year of planting in December 2020. The mean plant height of T_3 (sandalwood with *Cajanus*), T_2 (sandalwood with *Alternanthera*) and T_1 (Control-sandalwood without host) were statistically significant and different from each other. The highest mean values was observed in T_3 (176.57 cm) followed by T_2 (116.14 cm), which was significantly higher in comparison to T_1 (71 cm). Plant height in T_3 was significantly higher than in T_2.

Fig. 1: Field view of sandalwood at GKVK, Bangalore. a) Sandalwood without any host and sandalwood with *Cajanus*; b) The sandalwood with *Cajanus* and Sandalwood with *Alternanthera*; c) Control, sandalwood without any host; d) Sandalwood with *Alternanthera* and e) Sandalwood with *Cajanus*

Stem girth : Stem girth is an important criterion to identify superior genotypes having higher heartwood content and the heartwood diameter is positively correlated with tree diameter (Kumar *et al*., 2011). In the current study, the observed stem girth of all three treatments was found to be statistically different. Among them T_3 showed higher stem girth (12.01 cm) followed by T_2 (6.6 cm) and T_1 (2.68 cm). The plant height and stem girth data clearly indicated better growth of sandalwood with the two hosts. In addition, among two hosts sandalwood was found to be more vigorous with *Cajanus* compared to *Alternanthera* (Table 2), thus better biomass was achieved with *Cajanus* as compared to *Alternanthera* as host. In addition, it was also observed that sandalwood with *Cajanus* had transited to reproductive stages bearing flowers and fruits much earlier than sandalwood with *Alternanthera,* and sandalwood without any host. This result proved that for enhanced heartwood

TABLE 2

Effect of two different primary host plants on sandalwood growth parameters

Treatment No.	Treatments	Plant Height(cm)	Stem Girth(cm)	Chlorophyll content (mg/g FW of leaves)	Photoassimi-lation rate (IRGA)
T_1	Sandalwood without any host	71 ^{c}	2.68 ^{c}	1.08 ^{c}	10.31 ^{b}
T_2	Sandalwood with *Alternanthera*	116.14 ^{b}	6.6 ^{b}	2.36 ^{b}	20.55 ^{a}
T_3	Sandalwood with *Cajanus*	176.57 ^{a}	12.01 ^{a}	3.29 ^{a}	18.35 ^{a}
	SE (m)	8.87	0.25	0.03	0.72
	CD @ 5 %	27.64	0.78	0.10	2.26
	CV	19.36	9.38	4.19	11.69

formation the host planting is crucial in the sandalwood plantation. Our results are also in line with Das and Tah (2017), where they conducted experiments in different soil environments of South West Bengal, both in nursery and field condition after transplantation of sandalwood saplings with different hosts singly or in combination of hosts. They found that Arhar (*Cajanus cajan*) is the best host followed by Arhar & Tulsi (*Ocimum sanctum*) combination followed by Tulsi singly. Though sandalwood plants survive without host but the girth, height and growth are much better with the hosts.

Total Chlorophyll : Chlorophyll (Chl) is a vital photosynthetic pigment in plants, largely determining photosynthetic capacity and hence plant growth (Li *et al*., 2018). This study recorded a significant higher chlorophyll content in T_3 (3.29 mg/g FW of leaves) and T_2 (2.36 mg/g of FW) as compared to control (Table 2, Fig. a, b). The significant differences in levels of chlorophyll among treatments was not surprising, as visually the intensity of greenness in leaves varied among the treatments, with T_3 exhibiting darker green leaves as compared to T_2 and T_1.

IRGA was used to assess the photoassimilation rate among the treatments. Higher photoassimilation rate was recorded through IRGA in T_2 and T_3, which were significantly higher in comparison to control (Table 2). Li *et al*., 2016 suggested photosynthetic rate is a good predictor of growth and biomass in woody species. In this context our results confirmed the necessity of a better host for enhanced growth and biomass of sandalwood. This is in corroboration with work of Rocha *et al*., 2014, who examined influence of secondary host plant, *Casuarina equisetifolia*, on carbon assimilation, water and nutrient absorption in a 6-year-old field-grown sandal tree. Sandalwood trees growing with host showed higher pre-dawn plant water potential, carbon assimilation rate, leaf nutrient contents compared to sandal trees growing without host plant. This study thus indicates that the leguminous, nitrogen fixing *Cajanus* was a superior host as compared to *Alternanthera*.

Our study surprisingly showed that the photo as similation rate measured by IRGA in sandalwood grown with *Cajanus* (T_3) was slightly lower than when when grown with *Alternanthera* (T2) (Table 2), although this difference is insignificant. It is possible that a good host such as *Cajanus* provides sufficient nutrient support to sandalwood that allows for higher biomass in T_3 as compared to T_2 (host-*Alternanthera*). In addition, the higher biomass in T_3 results in more number of leaves (canopy) and thus higher total surface area of leaf, allowing for much higher photosynthetic assimi lation in T_3 than T_2, this further adds to robust plant growth in T_3. Future studies on this aspect are warranted.

Estimation of Defense Enzymes in Sandalwood while Interacting with Host Plants

Superoxide dismutases (SOD) are ubiquitous enzymes found in nearly all aerobic organisms and they play major role in defense against oxidative stress. SODs are classified into four groups based on the metal co-factor used by them; iron SOD (FeSOD), manganese SOD (MnSOD), nickel SOD (NiSOD) and copper-zinc SOD (CuZn-SOD). The SOD activity measured through spectrophotometric assay was found to be higher in control (10.16 µg protein for 50 % inhibition) in comparison to the T_2 (29.36 µg protein for 50 % inhibition) and T_3 (20.93 µg protein for 50 % inhibition) (Table 3).

The in gel analysis of SOD showed changes in isoform pattern of SOD in the treatments (Fig. 2a), which indicates variation in molecular responses in sandalwood when it is with no host (control) or while interacting with different host plants. Although the control showed intense SOD bands as compared to T_2 and T_3 there was a clear change in banding pattern of SOD isozymes when sandalwood interacted with *Cajanus,* suggesting induction of different SOD enzymes. To our knowledge, this is the first report of variation in the parasite sandalwood's molecular response while interacting with different host species, which implicates the molecular ability of sandalwood to distinguish between host species. The reduction in SOD activity also indicates a lowering of

The Mysore Journal of Agricultural Sciences

TABLE 3

Effect of primary host plants on defense enzymes (SOD and POX) of Sandalwood

Treatments No.	Treatments	Superoxide dismutase activity (µg protein for 50 % inhibition)	Peroxidase activity (µg / mg protein)
T_1	Sandalwood without any host	10.16 [c]	3.09 [c]
T_2	Sandalwood with Alternanthera	29.36 [a]	8.92 [b]
T_3	Sandalwood with Cajanus	20.93 [b]	11.30 [a]
	SE (m)	0.27	0.12
	CD @ 5 %	0.82	0.39
	CV	3.62	4.32

guard of sandalwood plant as it interacts with the host and makes the interaction possible. Though the lowest activity of SOD was observed in sandalwood that interacts with *Alternanthera*, a significant lower SOD activity was also observed in sandalwood interacting with *Cajanus,* with a concomitant change in isozyme pattern. Based on biomass data it is clear that *Cajanus* is the better host and a lowering of defences in sandalwood may go far in helping a congenial parasitic interaction.

The peroxidases (POX) activity detected using a spectrophotometric assay was significantly higher in T_3 (11.30 rg/mg protein) followed by T_2 (8.92 µg / mg protein) and T_1 (3.09 µg/mg protein) (Table 3). The same trend was observed in the native gel for POX isozymes (Fig. 2b). This implied that sandalwood exhibited enhanced POX enzyme activity with host interaction and highest activity was observed with interaction with *Cajanus* (T_3).

Lowering of SOD activity and an enhancement of Peroxidase activity was observed when sandalwood interacts with host plants as compared to control (Table 3). Although a lower activity of SOD was detected, different isoforms of SOD appear on the activity gel suggesting the induction of some specific isoforms when sandal interacts with a host. SOD is an enzyme that gets activated during biotic and abiotic stresses. A lowering of the total SOD activity indicates a lowering of defense / stress response of a plant and thus possibly allowing a robust establishment of parasitic interaction between host and sandal plant.

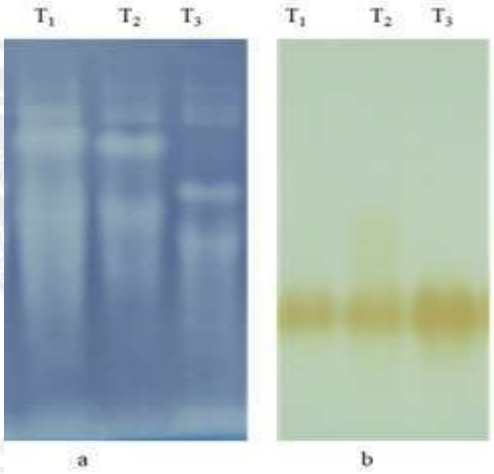

Fig. 2: a) In gel SOD (Superoxide dismutase) isoenzyme activity; b) In gel Peroxidase activity in proteins of leaf samples from Sandalwood in different treatments. T_1 – Control, T_2- Sandalwood with *Alternanthera*, T_3- Sandalwood with *Cajanus.*

Peroxidase enzyme, that acts downstream of SOD in the H_2O_2 homeostasis, is induced when sandalwood associates with host plant. The reduction of SOD and an increase in peroxidase both contribute to lowering of H_2O_2 that could result in supporting parasitic interactions

In addition, peroxidases have many other functions in the plant cell such as lignin synthesis. Plant peroxidases act as catalysts for the polymerization of phenolic compounds to form suberin and lignin in the cell wall, which act as barrier for the entry of pathogen by thickening the cell wall (Topal *et al*., 2017). In the context of host-parasite interaction, the increased POX activity in sandalwood interacting

with *Cajanus* could lead to higher tolerance to abiotic and biotic stresses.

Effect of Host Plants on the Levels of Secondary Metabolites in Sandalwood

The secondary metabolites including total proline, total phenols, flavonoids and total soluble sugars which were measured through the spectrophotometric assay by following the standard protocol mentioned in material and methods. Among the three treatments T_3 was found to be with significantly higher proline content (211.77 mg / 100 g FW) as compared to control or T_2 (Table 4). In fact, sandalwood plant growing with *Alternanthera* had lower proline in their leaves than the control (Table 4). The determination of proline is very useful to assess the physiological status and more generally to understand stress tolerance in plants. The over production of proline in T_3 suggests that growing sandalwood with *Cajanus* may impart abiotic stress tolerance to the plants and this needs to be tested in future studies.

Phenolic compounds are crucial for plant growth and development and are produced as a response to environmental factors (light, chilling, pollution, etc.) and to defend injured plants. Phenolics play important roles in plant development, particularly in lignin and pigment biosynthesis. They also provide structural integrity and scaffolding support to plants (Bhattacharya *et al*., 2010). In the present study the estimated phenol content was significantly higher in T_3 (107.86 mg / 100g FW) followed by T_2 (87.22 mg / 100g FW) in contrast to T_1 (82.80 mg / 100g FW) (Table 4). The increased phenol content in the sandalwood grown with *Cajanus* (T_3) may contribute to tolerance against any biotic and abiotic stresses and improve overall fitness of plants.

Flavonoids are structurally diverse secondary metabolites in plants, with a multitude of functions. They have functions in regulating plant development, pigmentation and UV protection, to an array of roles in defence and signalling between plants and microorganisms and with other plants. Flavonoids are incorporated into the cell wall of necrotic and adjacent cells and they play a role in tightening of

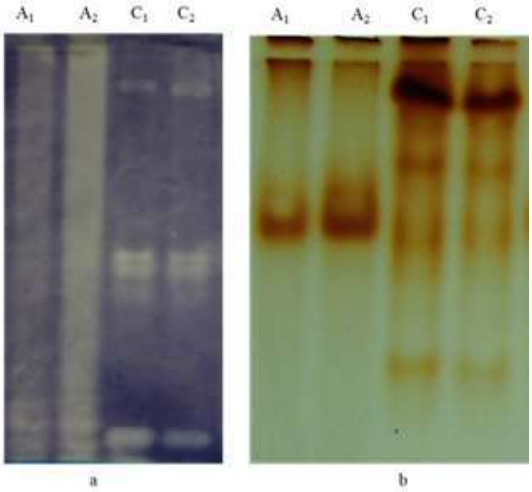

Fig. 3: a) In gel SOD (Superoxide dismutase) isoenzyme activity b) In gel POX (Peroxidase) in proteins of leaf samples from *Alternanthera* and *Cajanus*. A_1- *Alternanthera* without Sandalwood, A_2- *Alternanthera* with Sandalwood, C_1- *Cajanus* without sandalwood, C_2- *Cajanus* with sandalwood.

TABLE 4

Effect of two different primary host plants on metabolites of Sandalwood

Treatments No.	Treatments	Total proline content (mg/ 100g FW)	Phenol content (mgGAE/ 100g FW)	Flavonoids content (mgRE/ 100g FW)	Total soluble sugar (mg/ 100g DW)
T_1	Sandalwood without any host	113.05 ^{b}	82.80 ^{c}	104.59 ^{c}	10.02 ^{a}
T_2	Sandalwood with *Alternanthera*	99.99 ^{c}	87.22 ^{b}	293.81 ^{a}	9.85 ^{a}
T_3	Sandalwood with *Cajanus*	211.77 ^{a}	107.86 ^{a}	184.93 ^{b}	6.90 ^{b}
	SE (m)	2.42	0.53	1.61	0.09
	CD @ 5 %	7.56	1.59	4.84	0.28
	CV	4.10	1.52	2.20	2.68

TABLE 5

Effect of sandalwood parasitism on host plants *Alternanthera* and *Cajanus*

Parameters	*Alternanthera* without sandalwood (A1)	*Alternanthera* with sandalwood (A2)	*Cajanus* without sandalwood (C1)	*Cajanus* with sandalwood (C2)
SOD (µg protein for 50% inhibition)	81.91 ± 7.21	39.93 ± 1.6 **	14.91 ± 1.2	20.14 ± 1.27 **
POX (µg / mg protein)	3.33 ± 1.44	4.64 ± 1.91	11.54 ± 1.03	7.5 ± 3.59 *
Phenols (mgGAE/ 100g FW)	114.73 ± 7.42	85.38 ± 4.11 **	158.27 ± 7.45	125.31 ± 3.13 **
Flavonoids (mgRE/ 100g FW)	58.99 ± 6.22	41.87 ± 1.17 **	151.58 ± 9.39	89.46 ± 10.48 **
Proline (mg/ 100g FW)	182.98 ± 42.12	235.52 ± 7.76 **	36.05 ± 2.13	12.47 ± 4.68 **

(* Statistically significant at 5 % level of significance, ** Statistically significant at 1 % level of significance)

the plant structure and tissues by modulating auxin (IAA) activity which can lead to differentiation of tissues, promotion of callose and tylose formation and closure of the vascular system to prevent pathogen infection (Mathesius, 2018). The study showed the significantly enhanced flavonoid content in the leaves of T_3 (184.93 mg / 100g FW) and T_2 (293.81 mg / 100g FW) compared to control (104.59 mg / 100g FW) (Table 4). Sandalwood with *Alternanthera* accumulated relatively higher levels of flavonoids than sandalwood with *Cajanus*. The increase in biomolecules such as proline, phenolics and flavonoids in sandalwood when grown with a hosts indicates an induction of an array of secondary metabolites when associated with host plants, especially the leguminous host *Cajanus*. Whether these secondary metabolites also play a role in host selection and in successful establishment of parasitism at the root level is to be explored.

The total soluble sugars (TSS), on the other hand, was found to be lower in T_3 (6.90 mg / 100g DW) as compared to T_2 (9.85 mg / 100g DW) and T_1 (10.02 mg / 100g DW) (Table 4). T_2 and T_1 were on par for this parameter. As the TSS is an indicator for stress, the result suggests that sandalwood without any host, or an inappropriate host (*Alternanthera*), may be partially stressed. This can be overcome in the presence of its appropriate host which was found to be *Cajanus,* in this study.

Effect of Sandalwood Parasitism on Host Plants

Alternanthera : The presence of the parasitic plant - sandalwood, had profound effect on *Alternanthera,* which was evaluated by studying the SOD, POX activities and Proline, Phenol and Flavonoids (Table 4, Fig. 3 a, b) levels in leaves of *Alternanthera*. The presence of Parasite (Sandal) enhanced SOD (39.93 ± 1.6 µg protein for 50 per cent inhibition) and POX (4.64±1.91 µg / mg protein) activity along with the proline (235.52±7.76 mg/ 100g FW) in *Alternanthera* (A_2) as compared to the host grown alone (A_1), that showed lower SOD activity (81.91 ± 7.21 µg protein for 50 % inhibition), POX activity (3.33±1.44 µg / mg protein) and proline levels (182.98±42.12 mg/100g FW), respectively. The in-gel analysis of SOD and POX isozymes also confirmed the spectrophotometric increase of defense enzymes (Fig. 3 a, b). The secondary metabolites phenols and flavonoids also reduced with interaction with sandalwood. A_2 has a significantly lower flavonoids (41.87±1.17 mg / 100g FW) and phenol (85.38±4.11 mg / 100g FW) content than A_1 (58.99±6.22 mg / 100g FW, 114.73±7.42 mg / 100g FW flavonoids and phenols, respectively). But, overall there seems to be heightened defense in *Alternanthera* when associated with parasite sandalwood, and therefore this may prove detrimental to optimal establishment of parasitism and ultimately the robust growth of sandalwood.

Cajanus cajan : Similarly, the effect of sandalwood parasitism on host- *Cajanus* was studied for all the above mentioned parameters. The defense enzymes SOD and POX activity were decreased in C_2 (20.14±1.27 µg protein for 50 per cent inhibition , 7.5±3.59 µg / mg protein respectively) as opposed to the C_1 (14.91±1.2 µg protein for 50 per cent inhibition, 11.54±1.03 µg/mg protein respectively) which was confirmed by in-gel assay of SOD and POX enzymes (Fig. 3 a, b). The secondary metabolites including total phenol content, flavonoid content and proline content estimated through spectrophotometric assays, were found to be lower in *Cajanus* parasitized by sandalwood (125.31±3.13, 89.46±10.48, 12.47±4.68 mg/100g FW respectively) in comparison to *Cajanus* grown alone C_1 (158.27±7.45, 151.58±9.39, 36.05±2.13 mg/100g FW, respectively) (Table 5). Therefore, in *Cajanus*, all stress and defense

TABLE 6

Sandalwood root connection with *Alternanthera* and *Cajanus*

Parameters	*Alternanthera*	*Cajanus*
Host roots towards Sandalwood	0.67 ± 1.15	3.67 ± 1.53 *
Roots from Sandalwood	1.67 ± 0.57	3.67 ± 1.33 *
Haustoria	3.67 ± 0.58	10.33 ± 1.53 *

(* Statistically significant at 5 % level of significance)

Fig. 4: a) Design of three compartment bag; b) Uprooted Sandalwood plant with *Cajanus*, *Alternanthera;* c) Sandalwood with *Cajanus* and *Alternanthera;* d) Haustoria found in the *Cajanus* plant marked in yellow circle

The Mysore Journal of Agricultural Sciences

parameters were found to be lower in presence of parasite. This indicates a substantial lowering of guard and reduced defense in *Cajanus*, more so than *Alternanthera*, allowing a robust interaction with the parasite benefiting the parasite. Radomiljac *et al*., 1998 proposed that legume plants can act as better host for sandalwood. *Cajanus* is a legume plant and seems to show significant flexibility and modulation of its defense system when it interacts with sandalwood. This may contribute to *Cajanus* being a superior primary host than *Alternanthera*.

Assessment of Sandalwood Root Growth and Haustorial Connections with different Hosts

Polybag experiment was taken up with custom designed polybags (having interconnected three compartments) with sandal planted in central compartment and *Alternanthera* and *Cajanus* in the peripheral compartments. Alter 10 months, the plants were carefully removed from the polybags and soil gently separated from the roots to see the direction of the sandal roots. The number of sandalwood roots were found to be predominantly directed towards *Cajanus* (3.67 ± 1.33) which was significantly higher as compared to *Alternanthera* (1.67 ± 0.57) (Table 6). Also the bending (U turn) of sandalwood root towards *Cajanus* plant was observed. It was also observed that number of *Cajanus* roots growing towards sandalwood plant (3.67 ± 1.53) was higher than *Alternanthera* (Table 6). Haustorial root connection were significantly higher with *Cajanas* (10.33 ± 1.53) as compared to *Alternanthera* (3.67 ± 0.58) (Table 6) (Fig. 4). This experiment, validates the inference from the field experiment and biochemical evaluations that *Cajanus* is a better host and sandalwood prefers *Cajanus* over *Althernanthera* with more number of roots growing towards *Cajanus* with concomitant higher number of haustorial connections.

Though sandalwood plants survive without host, the girth, height and growth of sandalwood are much better with the hosts. From the current study it is clear that the leguminous *Cajanus cajan* is a better primary host for sandalwood in comparison to the nonleguminous *Alternanthera*. At nursery stage and in field conditions for the first year of sandalwood growth, *Cajanus* can thus be used as host to enhance the growth, chlorophyll content and also photoassimilation rates. The outcome of this present investigation can be used to meet the silvicultural requirements of sandalwood and also for the long term plantation. The biochemical analysis indicates a lowered defense in host *Cajanus* while interacting with sandal giving a molecular understanding of why it might be a more suitable host. While in sandalwood, an interaction with *Cajanus* changes the SOD isozymes and also lowers SOD activity. An enhancement of POX, Proline, phenols and flavonoids was observed while interacting with both *Cajanus* and *Alternanthera*. The evaluation of root directionality indicates preferred direction of sandalwood towards *Cajanus* compared to *Alternanthera*. In this regard further study is warranted to investigate more about the root connection in the course of sandalwood growth along with host plants. As the present study was restricted to only primary host, the secondary host requirement and its selection at field level is the next step towards understanding the host selectivity of sandalwood.

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