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Qualitative and Quantitative Study of Cytochrome Oxidase Activities in the Haustoria of *Cassytha filiformisn* L. *Cuscuta reflexa Roxb.* (Stem Parasites) and *Santalum album* L. (Root Parasite)

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Abstract

In *Cassytha filiformis* L. cytochrome oxidase activity was more pronounced at the tip and margin of the young haustoria while in mature haustorium, the activity is more at the vascular core. In *Cuscuta reflexa*, during the initial stage of haustorial penetration the enzyme activity appears only at the tip of the haustorium and very scanty, but in matured haustorium the activity is more in the region of vascular core. In the root parasite *Santalum album,* the enzyme activity appears to be present both in host and parasite. But in young haustorium the concentration of enzyme is very less when compared to matured haustorium. Formation of gland increases the enzyme activity and the entire gland is filled with the enzyme.

***Key words*:** Haustorium, Endophyte, Cytochrome oxidase, Vascular core

Angiosperms comprises of about 90% of the plant kingdom. Parasitic plants are a small group of dicotyledonous plants which exhibit a great wealth of structural diversity and are quite distinct from other weeds in forming intimate attachments to their host crops either to roots or to the stem. Based on the nutritional requirements, parasitic plants are grouped into hemiparasites and holoparasites. The hemiparasites contain chlorophyll and synthesize their organic needs using carbon. However, some species have a reduced photosynthetic activity compared to their host [1-2]. On the other hand, holoparasites do not contain chlorophyll; they are more specialized and depend entirely on the host for their nutritive needs, since they are unable to synthesize organic nutrients by using atmospheric carbon. The holoparasites are necessarily obligate parasite, while hemiparasites may sometimes be facultative. The term haustorium was first coined by De Candolle [3] with reference to *Cuscuta*. It was originally defined by Koch [4] as part of the parasite that develops within the host tissue. It is a part of parasite which penetrates the host tissue for the absorption of nutrients. The origin of haustoium is not uniform in all parasitic angiosperms.

In a living cell many chemical reactions are controlled by some specialized substances called enzymes. The term enzyme was coined by Kuhne [5]. Enzyme localization in cells and tissues is a challenging goal in the field of biochemistry. Enzyme reactions are also activated in the cells when the tissue is sectioned. The end product of enzyme reaction is also detected. Raciborski [6-7] identified the localization of peroxidase in the phloem, guardcells, and lenticels, gap cells of endodermis and absorption zones of root surface. Further he found that α-naphthol and hydrogen peroxide are the better reagents than guaiacol or dimethyl paraphenylenediamine.

The development, structure and variation in the haustoria of many species of Santalaceae were described by Rao [8]. Glick developed a method for the use of buffered nadi reagent on fresh tissue sections. He mixed the reagent with a buffer in the pH range 3.5 to 5.5. This condition is recommended to be suitable for plant tissue [9]. Seligman and Rutenburg (1951) first proposed the histochemical localization of Succinic dehydrogenase by tetrazolium method, in which tetrazolium salt functions as hydrogen acceptor in the reaction catalyzed. The tetrazolium salt reduced to insoluble colored substance called formazon, because of its important function during respiration. Succinic dehydrogenase activity may reasonably be expected in all living cells [10]. Cytochemical localization of acid phosphatase in endophyte cells of semi parasitic angiosperm *Camandra umbellata* (Santalaceae) was studied by Toth and Kuijt [11]. The authors also reviewed the ultrastructure of the young haustorial gland in *Comandra*. Histochemical localization of Succinic dehydrogenase activity in the haustorium of *Cassytha filiformis* L and *Cuscuta reflexa* Roxb. was investigated by Rajanna and Suresha [12]. During the present investigation, qualitative and quantitative studies of cytochrome oxidase activity in the haustoria of two stem parasites and one root parasite was performed because of their importance in cellular reactions.

MATERIALS AND METHODS

Qualitative analysis

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Test for cytochrome oxidase activity: (Nadi reaction method) [13].

Fresh and freehand sections of the haustorium all the three plants were taken and incubated for about 30 seconds in a reaction mixture at lab temperature.

The reaction mixture consisting of 25 ml of 0.05M phosphate buffer at pH 7.6, {It is prepared by dissolving A) 3.15 g of sodium phosphate dibasic in 1000 ml of distilled water and B) 3.026 g of potassium phosphate monobasic dissolved in 1000 ml of distilled water. 172 ml of solution **A** and 27 ml of solution **B** were mixed}, 1% α-naphthol solution (It is prepared by dissolving 1gram of α-naphthol in 40% alcohol) and 1ml of 1% solution of dimethyl paraphenylene diaminehydrochloride (1 g of reagent dissolved in 100 ml of distilled water). Then the sections were rinsed in water and mounted it in glycerin. In control heat killed sections were placed in a reaction mixture.

Enzyme assay

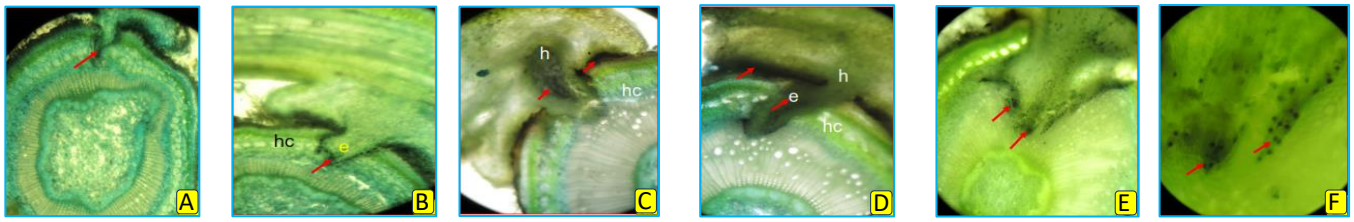
Extraction of cytochrome oxidase

200 mg of each plant material was taken and ground in a pestle and mortar. The grounded tissue is suspended in 1.2 ml of extract buffer, [It is prepared by mixing 0.1 M KH_2PO_4, pH 7.5 and 0.1% (w/v) Triton X]. The extract was than centrifuged at 13000 g for 5 min. The supernatant was decanted without disturbing pellet and used for the spectroscopic assay. The cytochrome C stock was prepared in KH_2PO_4 buffer. 2 ml of cytochrome C was taken in cuvette and was noted its reading as blank at 550 nm. 20 ml of enzyme extract was then added to cytochrome C solution and incubated for 10 minutes. Readings were observed after 10 min.

RESULTS AND DISCUSSION

Cytochrome oxidase enzyme activity appears to be present both in the host and parasite. Its reaction is indicated by bluish green color. In the early stage of haustorial development, the cytochrome oxidase activity is confined to the epidermal layer of the host. As the development proceeds, the concentration of the enzyme gradually appears to be more at the tip and margin of the endophyte at the region of penetration (Fig 1).

In the early stages of development, the enzyme activity is not noticed in the haustorium (Fig 1 B-C). As the haustorium matures, the concentration of the enzyme gradually increases, (Fig 1 A-B), the endophyte penetrates the host cortex, in this condition the enzyme concentration appears to be more in the region of vascular core and upper portion of the haustorium. Cytochrome oxidase activity was clearly observed even at the region of cell division and growing part of the endophyte (Fig 1 C-D).



A and B, V/s of the young haustorium showing cytochrome oxidase activity (arrow)
Note: the endophyte (e) of the haustorium penetrated the host cortex (hc)
C and D, V/s of haustorium penetrated the host cortex (hc) shows cytochrome oxidase activity
Note: the haustorium (h) is completely filled with the enzyme (arrows)
E and F: Cross section of mature haustorium enlarged to show the dividing cells possess enzyme activity (arrows)
Fig 1 *Cassytha filliformis* L.

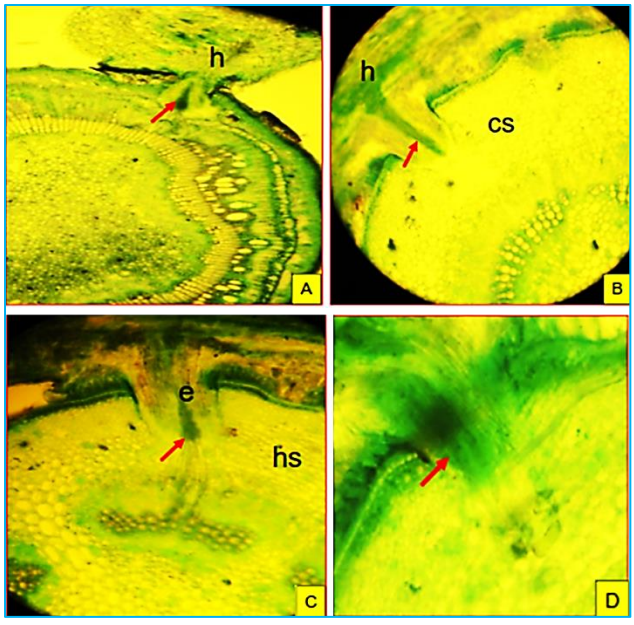


Fig 2 *Cuscuta reflexa*

A: cross section of young haustorium (h) tested for cytochrome oxidase
B: V/s of matured haustorium which penetrate the host cortex (hc)
C: V/s of matured haustorium the endophyte penetrates the host stele (hs)
D: Cross section of haustorium tested for cytochrome oxidase activity (arrows)

Cytochrome oxidase activity: in Cuscuta

Cytochrome oxidase activity appears bluish green in colour, further its activity noticed both in the host and parasite. In the initial stage of haustorial penetration the enzyme activity appears in the region of vascular core of the endophyte at the point of contact with the host tissue, and the enzyme concentration is very poor in the region of haustorium (Fig 2A). When the growth of haustorium precedes the endophyte penetrates the host cortex and in this condition the enzyme activity appears both in the haustorium and vascular core of the endophyte (Fig 2B). Gradually the endophyte pushed inside the host stele and established the firm contact with the host vascular tissue (Fig 2C). As the haustorium matures the enzyme secreted more in the region of vascular core of the endophyte and also in the upper region of the haustorium (Fig 2 A-D).

Cytochrome oxidase activity: in Santalum

The sites of this enzyme activity appear bluish green in colour. The activity of this enzyme appears both in host and parasite. When haustorium is young the activity is less when compare to mature haustorium. During the early stages of development of the haustorium, cytochrome oxidase activity is noticed between the epidermal cells of the host and the parasite (Fig 3A), but after the formation of gland the enzyme activity gradually increases, the entire gland is surrounded by the accumulation of Cytochrome oxidase activity. It is assumed that

the Cytochrome oxidase enzyme is synthesized and released from the gland (Fig 3B-C). This enzyme activity appears to be present both in the parasite haustorium and the host with which it establishes contact. The mature haustorium of *Santalum album* is richly filled with cytochrome oxidase at the region of vascular core and the tip of the endophyte (Fig 3B-C). As the development of the haustorium still continues the terminal region of the endophyte loses its activity and accumulated more at the region of clasping folds and even in the cortex region of the host tissue (Fig 3 B-C).

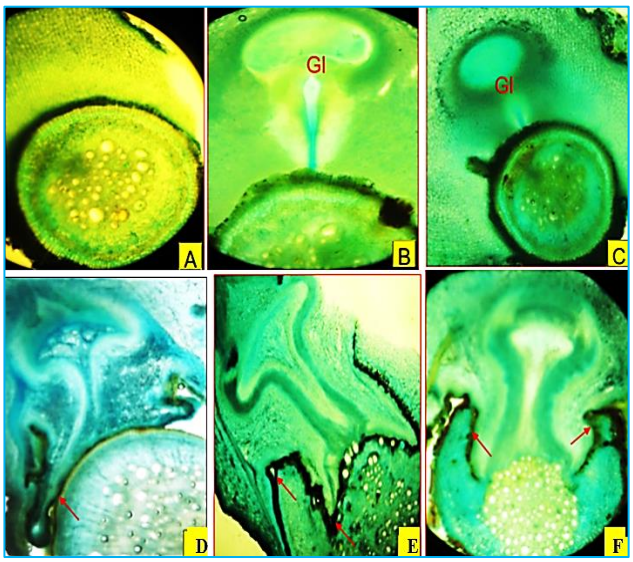


Fig 3 *Santalum album* L.

A: Vertical section of haustorium with the host root cut transversely showing the initiation of haustorium.
B and C: Vertical section of haustorium with gland (GI) shows cytochrome oxidase activity
D, E and F. V/s of mature haustorium showing cytochrome oxidase activity (arrows)

The quantitative assay of enzyme activity was studied and the graph is plotted parasites verses enzyme concentration, in all the three parasites it is revealed that the enzyme cytochrome oxidase concentration is very much appeared in *Santalum album* when compared to other two stem parasites. In *Cassytha filiformis* the enzyme concentration is little more when compared to *Cuscuta reflexa.*

All the three taxa were found to develop haustorial connections widely on different dicotyledonous and monocotyledonous host species and exhibit great variation in the extent of parasitism. In some cases, the occurrence of hyper parasitism has been reported, this clearly demonstrates that the angiosperm parasites do not have any host specificity [12]. Self-parasitism was common in both, *Cassytha filiformis* and *Cusscuta reflexa.*

Haustorium is one of the most important specialized organs among parasitic angiosperms. Normally, two kinds of haustoria are recognized on the basis of their origin [14]. If the root apical meristem of the embryo gets transformed into a haustorium, it is referred to as "primary haustorium". This is observed among the members of Orobanchaceae, Loranthaceae, Viscaceae and *Striga orobanchoides* of Scrophulariaceae. On the other hand, haustoria developing from regions other than the radicular apex of embryo are called "Secondary haustoria". All three parasitic taxa selected for the present study developed only secondary haustorial contacts with their hosts. A peculiar kind of haustoria is the leaf haustoria formed directly from the scaly leaves in *Hyobanche* of Scrophulariaceae [15].

The size of the haustoria of root parasitic angiosperms normally very small organs, measuring in mm or at the most few cms in diameter. The contact between the haustorium and host organ is very delicate and the two get separated easily even with slight pressure so is the nature of contact between haustorium and its host stem in case of *Cuscuta reflexa.* But in case of *Santalum album* and *Cassytha filiformis* the haustoria are firmly anchored to the host plant.

Chain like haustoria formation between parallely lying parasite and host organ has been observed in all three parasites selected for the present study. They were capable of forming haustorial connections simultaneously on different hosts.

In *Santalum* and *Cassytha* the haustoria are initiated just behind the apex of growing roots. Such observations have been made by Fineran (1965) [16] in *Exocarpus bidwillii*, Tooth and Kuijt [11] in *Comandra umbellata*, Musselman and Dickinson (1975) [17] in some Scrophulariaceae. In *Cassytha filiformis* haustoria are formed by narrower haustorial shoots which surrounds the host branches.

Fig 4 Assay of cytochrome oxidase activity

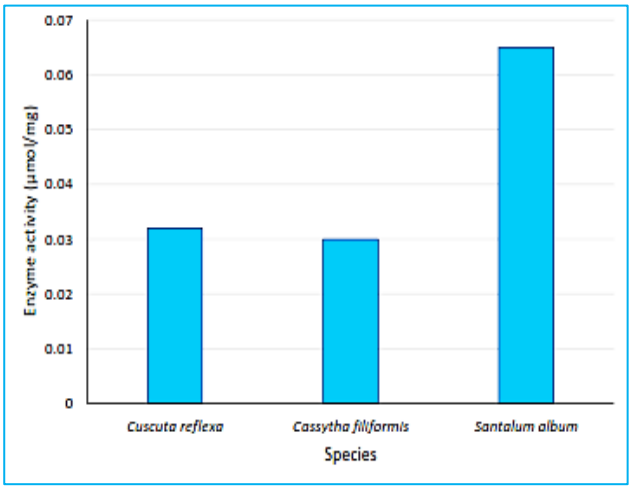


Fig 5 Graphical representation of cytochrome oxidase activity

In all the three taxa of present investigation, the haustorial formation initiated by the meristematic activities of cortical cells of the root *in Santalum album*, in case of *Cassytha filiformis*, prior to the initiation of haustorial primordium an adhesive disk from the epithelial cells developed into a secretary epithelium of unicellular trichomes, which latter produces finger like projections from their tips. Similar type of haustorial formation has also been reported in *Cuscuta* [18].

Exogenous origin of haustoria has been reported in many taxa belonging to Santalaceae [8], [16], [19-20]. Similarly, the exogenious origin of haustoria in some members of Scrophulariaceae was studied by Chaung and Heckard [21] Musselman and Dickison [17] and in members of Lauraceae by Kuijt [22-23].

The function of root is augmented by the haustorium involved in absorption of water and nutrients from the host root, its exogenous origin has been a matter of interesting discussion [22] and it was concluded by Kuijt [23] that haustorium represents a root in form and evolutionary in origin.

A well-marked gland is organized within the developing haustorium just before the penetration of the host root in *Santalum album*. While in both *Cassytha filiformis* and *Cuscuta reflexa* gland formation was not observed. This unique feature is reported in the developing haustorium of only *Santalum* members so far. A structure similar to gland organization and function has not been described in any other parasitic angiosperms outside the santalales. The gland is reported to be absent in the aerial members of Loranthaceae [23]. In contrast to this work, Rao [8] reported that an internal gland is formed in the developing haustorium only if a hard and woody root has to be penetrated.

The haustoria of various *Cuscuta species* have been thoroughly invrestigated by early workers [24-26], nevertheless even today new findings demonstrate the new complexity of this organ. Recently, results in particularly increasing highlight the close relationships between the structural nature of the haustorium and its specialized function. There are several reasons why the haustorium of *Cuscuta odorata*, is ideally suited to study such a correlation. It is a holoparasitic plant and develops cellular system for water and assimilate uptake. *Cuscuta* is an extremely quick growing plant, so this uptake system must be highly efficient. *Cuscuta* constantly establishes new contacts to the host plant so that the development of the haustorium from endogenously arising meristamatic tissue [27] to a highly differentiated organ occurs rapidly and repeatedly within a few days. In the meantime, within the middle of the haustorium cells develops into xylem and phloem elements. These haustorial conducting elements do not derive from procambial strands, but from apparently normal haustorial cells, which divide either into a sieve tube and companion cell or into a xylem element. In this way rather unusual short conducting elements are formed.

Early reports about the penetration process, based on low resolution light microscopy, claimed that cell contents of the host cortical parenchyma are by means of a secretion from the intrusive cells and that the parasite is able to feed on dissolved substances. Now a days, deposit a great gap in knowledge we know that parasitic *Orobanchae* do not dissolve the cells found in their way, even though some parasites (*Agalinis aphylla*) may break through host cells [17]. Histological observations clearly showed that only a combination of mechanical and enzymatic mechanisms exerted by a parasite to separate host cells, allows penetration [28-29]. The intrusive cells of *Orobanche* species penetrate by pushing their way between host cells and the concomitant mechanical pressure by the penetrating cells pushes portions of host cell walls aside so that the shape of the host cells changes and the space between them is occupied by the intrusive cells [28].

A few works presented *in vitro* evidence about pectolytic, cellulolytic and proteolytic enzymes being secreted by seedlings of *Phelipanche aegyptica* before penetration [30-32]. The endodermis with its cutinized or subarised casparian strips is another obstacle which the haustorium needs to cross its way to host conductive tissues. Indeed, a combined anatomical and immunocytochemical study revealed that penetration of the *Phelipanche aegyptica* haustorium takes place between host and epidermal cells by the dissolution of cutin of the casparian strips [28]. Cell wall degrading enzymes were also found in Striga during the penetration of Sorghum roots by *Striga hermonthica* involved alterations of host cell walls at the infection point [33], and softening and dissolution of middle lamella was observed with *Striga gesnerioides* attacking cow pea [34].

High enzyme activity was observed in the parasitic tissues, especially in the parts of the haustorium in contact with newly formed xylem tissues of the host and the parts of the haustorium close to the site of entry. The reaction was generally so intense as to make the recognition of individual cells difficult at times. It was found also, that the enzyme activity is tended to be greatest (As judged by intensity of staining) near the site of entry of haustorium in the younger stages. In older stages especially after the establishment of vascular contact, the activity is concentrated near the vascular contact surfaces. Enzyme activity was also generally high near the advancing front of the haustorium. There was usually lower activity in the host tissue, the activity being chiefly associated with the epidermis, cortex and phloem. The fact that the enzyme is also found in considerable amount near the site of entry and at the advancing front of the haustorium may implicate it in the enzymatic action that has been postulated as one of the mechanisms of host penetration by the parasite haustoria [35-37]. However, the enzyme may be mostly associating in the absorption of host materials derived from cells damaged by mechanical invasion of host tissues.

Therefore, we conclude that any enzymatic weakening of the host is a primary function of the endophyte and not a secondary result or passive condition resulting from the death of passive tissue The endophyte then moves through the host tissue by mechanically crushing host cells with the proposed enzymatic weakening being necessary preconditioning for endophyte penetration. Similar kind of enzyme localization tests were conducted in angiosperms apart from the parasites. Kumar and Mathur [38] have observed very high activity of peroxidise in the cortex, vascular region and pith cells of normal stem in *Terminalia arjuna*. Occurrence of enzymatic studies on the haustorium of parasitic phanerogams are infrequent and fragmentary, only few reports on the histochemical localization of few enzymes were made on only few parasitic angiosperms.

CONCLUSION

So far, no enzymatic study was carried out in the haustorium of parasitic flowering plants. During the present study Cytochrome oxidase activity was recorded for the first time in the haustorium of *Cassytha filiformis*, *Cuscuta reflexa* and *Santalum album*. This enzyme is very essential for the growth, development and penetration of the haustorium in to the host tissue.

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