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Comparative Analysis of Histopathological Alterations at Different Developmental Stages of Leaf Midrib Gall and Petiole Gall during Insect Infestation on *Eucalyptus tereticornis* Grown in the Semi-arid Region of Rajasthan, India

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ABSTRACT

The hymenoptera insect *Leptocybe invasa* causes elliptical or irregular shape galls on leaf midrib, petiole and stem of *Eucalyptus tereticornis*. The pathogen injects some elicitors into plant tissue, which alter plant metabolism and results into a tumorous outgrowth known as plant gall. In gall mainly vascular tissues are damaged which results in the formation of gall cavity due to processes of hypertrophy and hyperplasia on the outer surface of midrib and petiole. The Insect escapes through an opening on lower side of leaf, called ostiole, from which the female comes outside and starts laying eggs. Galls are elliptical or irregular in shape and pale green in colour when young but become brownish at maturity. Infestation of *Leptocybe invasa* on *Eucalyptus tereticornis* alters the anatomy of leaf midrib and petiole by disrupting the mesophyll and vascular tissues. In the present investigation studies on histopathology were carried out at young, mature and old stages of gall development on leaf midrib and petiole.

***Key words*:** *Eucalyptus tereticornis, Leptocybe invasa*, Hypertrophy, Hyperplasia, Ostiole, Histopathology

Eucalyptus tereticornis is a tall evergreen tree belongs to family Myrtaceae. It is a native plant to Australia but in the past few years it has spread to other parts of world including India. In India, gall infected plants were reported from Andhra Pradesh, Kerala, Pondicherry, Gujarat, Madhya Pradesh, Uttar Pradesh, Maharashtra, Goa and Delhi [1]. It was also found in Punjab, Haryana and semi-arid region of Rajasthan.

Galls are localized outgrowth of various host organs in which host are stimulated to excessive growth by parasite [2]. These tumorous outgrowths are arisen mostly by hypertrophy and hyperplasia under the influence of pathogenic organism. Gall tissues often act as physiological sink because they accumulate several chemical substances which are used by pathogenic organisms for feeding purpose. Plant gall shows alteration in the metabolism of affected part as reflecting in the biochemical analysis of galls and their normal counterparts [3]. The galls caused by the insect occur on the both sides of the blades of the plant [4]. The insect stimulates a perturbation in growth mechanisms and alters the differentiation processes in the host plant, modifying the plant architecture to its advantage [5].

A new pest of *Eucalyptus* plant was found in the Middle East and the Mediterranean region in 2000. Since then, it has spread to most Mediterranean countries and many of the *Eucalyptus* grown areas in northern and eastern Africa [6]. Galls on the *Eucalyptus tereticornis* is caused by a Hymenoptera insect *Leptocybe invasa* commonly called as Blue Gum Chalcid, presumed to have originated from Australia, the pest was subsequently identified as a gall wasp, *Leptocybe invasa* Fisher & La Salle (Hymenoptera: Eulophidae) [6]. Adult female measures 1.1 to 1.4 mm and lays eggs inside tender leaves and stem. After about 7 days of oviposition, the openings of the injury were blocked by a secretion from the wound [7]. Larvae after hatching out of eggs remains in a cavity formed within the plant tissues and feed on the tissues which results in the formation of galls [8].

Histopathological preparations, concern with study of diseased tissues in respect of pathogen infection. Histopathological modifications in host tissues are associated with the synthesis of various chemical substances including auxin and phenolics which results in hypertrophy and hyperplasia of plant tissues. *Eucalyptus tereticornis* is affected with Leaf midrib, petiole and stem gall. Eucalyptus oil is obtained from the leaves of the plant which has immense medicinal properties. Galls severely attack leaves and decrease yield of leaves so it is important to study histological alteration during gall development. In the present investigation histopathological studies of leaf midrib gall, petiole gall and their normal counterparts has been carried out.

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MATERIALS AND METHODS

Collection of plant material

Normal and infected plant materials viz. midrib gall and petiole gall were collected from Jaipur and nearby areas in the month of October-November. Galled material was collected at various stages of development and graded as young, mature and old for further histopathological studies. After collection plant materials (Gall and Normal) were washed with tap water for removal of dirt and debris.

Fixation of plant material

Normal and galled material were fixed in 37% F.A.A. solution (Table 1) and stored in 70% alcohol for further studies. 70% alcohol is used for the long-term preservation of plant material.

Table 1 Preparation of F.A.A. Solution

Chemicals	Concentration
50 or 70% Ethyl alcohol	90 ml
Glacial acetic acid	5 ml
Formalin (40% of Formaldehyde)	5 ml

Microtome sectioning

Plant materials were first of all washed with tap water for removal of all the traces of fixatives. Thin and fine sections of Normal and Galled plant material were cut with the help of automated Leica RM 225 Microtome for histopathological studies. Tertiary butyl alcohol method was used for dehydration and embedding [9]. Cut sections were passed from a series of TBA of different concentration in ascending order for complete dehydration of plant material. After dehydration plant material was transferred to 50% liquid paraffin wax and then to solid paraffin at 40°C for overnight so the wax can be embedded into the plant tissues. Wax embedded samples were sectioned with automated Leica RM 225 Microtome.

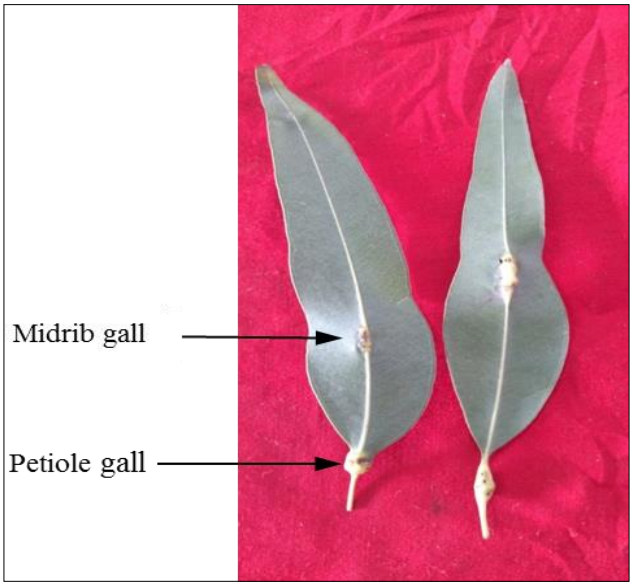

Fig 1 Leaf of *Eucalyptus tereticornis* showing midrib and petiole gall with ostiole

Staining

Microtome processed sections were stained with 1% safranin to study the anatomy of gall tissues with their normal counterparts. D.P.X. was used as mounting agent. Slides were observed under Leica DM 500 photomicroscope for anatomical studies.

Morphological studies

It was observed that plant of *Eucalyptus tereticornis* growing in the Semi-arid region of Rajasthan were infected more with leaf midrib and petiole galls than stem galls. Galls are elliptical or irregular in shape and pale green in colour at the young stage but become brownish at maturity. Generally, number of galls at midrib and petiole vary from 1-8 galls at each infested leaf and number may reaches up to 45-50 in heavily infected leaf. Sometimes complete length of petiole and midrib are covered with gall. Petiole galls are usually larger than midrib galls. It was found that young plantlets were more infected with galls than the mature ones. In case of young plantlets galls are initially pinkish in colour and diameter of gall is also more as compared to mature galls. The Insect escapes through an opening on the lower side of leaf, called ostiole (Fig 1). The length of a single unit gall was about 2.1 mm and two-unit gall or two fused gall units containing two wasps was about 3.6 mm so there were no major differences occur between the length of a single gall or of a two-unit gall [6].

Histology of healthy leaf midrib

Eucalyptus tereticornis is a dicotyledonous plant and it bears isobilateral leaves which are a feature of monocotyledonous plants as dicotyledonous plants bear dorsiventral leaves. Due to the presence of isobilateral leaves, there is no differentiation of mesophyll into palisade and spongy tissues. Equal number of stomata is found on both surfaces. Patches of sclerenchymatous tissues are present above and below the large vascular bundle. Epidermis of healthy leaves is continuous without rupturing. Vascular bundles were present in the center of the mesophyll tissues. Normal leaf midrib showed compact vascular tissues surrounded by mesophyll cells. Upper and lower epidermis is not ruptured before oviposition (Fig 2A).

Histopathology of midrib galls

Hymenoptera insect *Leptocybe invasa* cause elliptical galls on the lower side on leaf midrib of young leaves. At the stage of young gall on midrib, upper epidermis is continuous and not ruptured but the lower side is slightly ruptured due to oviposition by the adult female insect. After oviposition signals spread to nearby cells so cells start showing hypertrophy as the cell elongation takes place. After hypertrophy cells showed hyperplasia in which rapid mitotic cell division takes place so both width and length of galls increased (Fig 2B). Mesophyll cells start disrupting as the gall matures. Larvae travels it path towards vascular tissues. Fully matured gall shows an oval shaped or irregular cavity in place of vascular bundles. Larvae remain present within this cavity (Fig 2C) and mainly feed upon the vascular tissue. Old stage of gall development showed complete disruption of mesophyll cells as the mesophyll cells leaving a passage which terminates into the ostiole of the gall from which the female emerged outside and started laying eggs. At this stage gall become dry and dark brown in colour (Fig 2D).

Histology of normal petiole

Healthy petiole showed somewhat circular shaped boundary made up of uniform epidermal cells. Stomata are equally distributed on the complete boundary of petiole and intact vascular bundle is present in the center (Fig 3A).

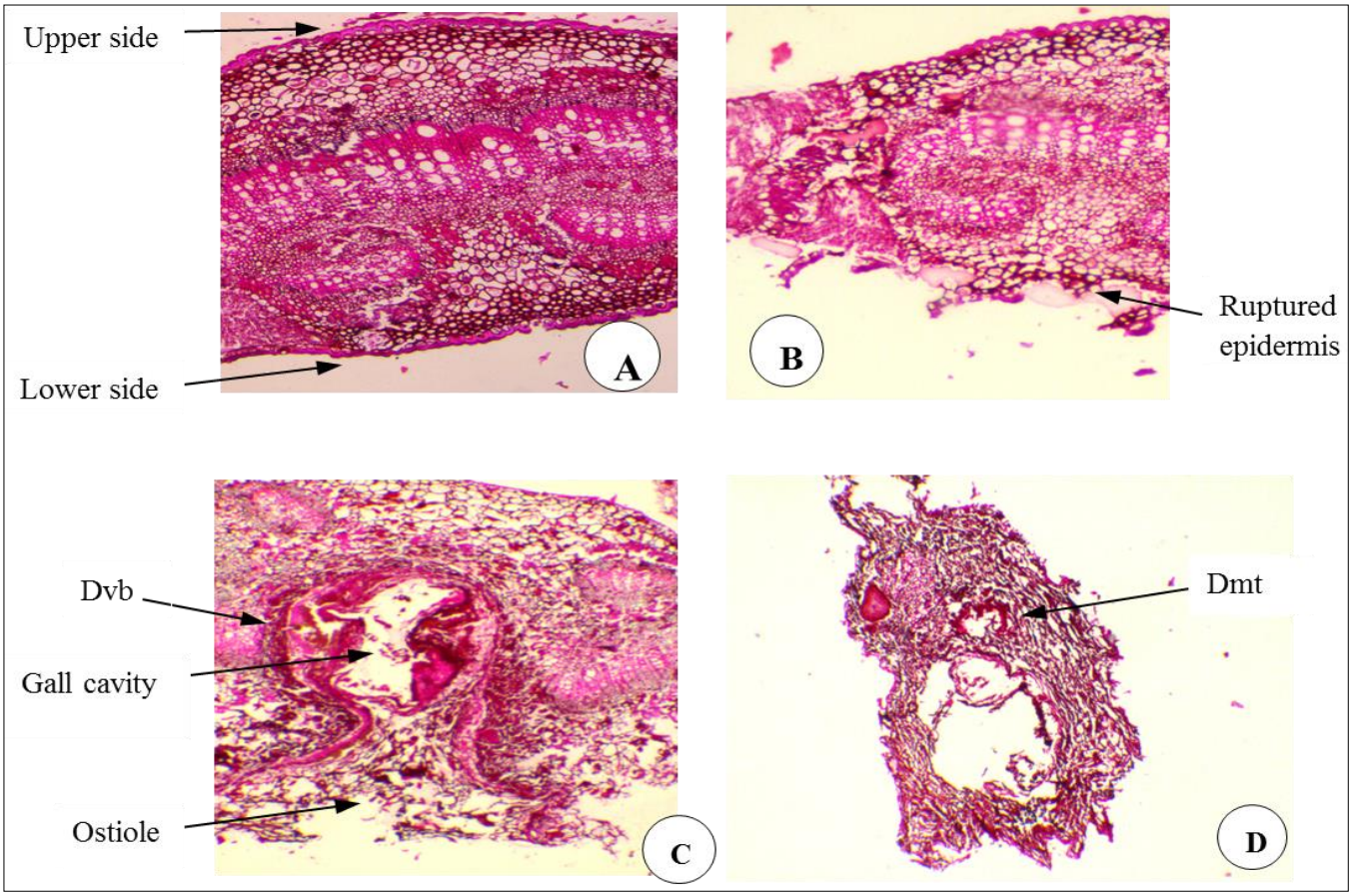

Fig 2 (**A**) Normal leaf midrib T.S. – Intact epidermis and vascular bundles. (**B**) Midrib gall (Young) – ruptured epidermis. (**C**) Midrib gall (Mature) – Completely visible gall cavity and formation of ostiole from where adult female goes outside. Disrupted vascular bundle and mesophyll tissue are also visible. (**D**) Midrib gall (Old) – Gall tissues become completely dessicated.
Abbreviations: Dvb – Disrupted Vascular Bundle, Dmt – Disrupted Mesophyll Tissue

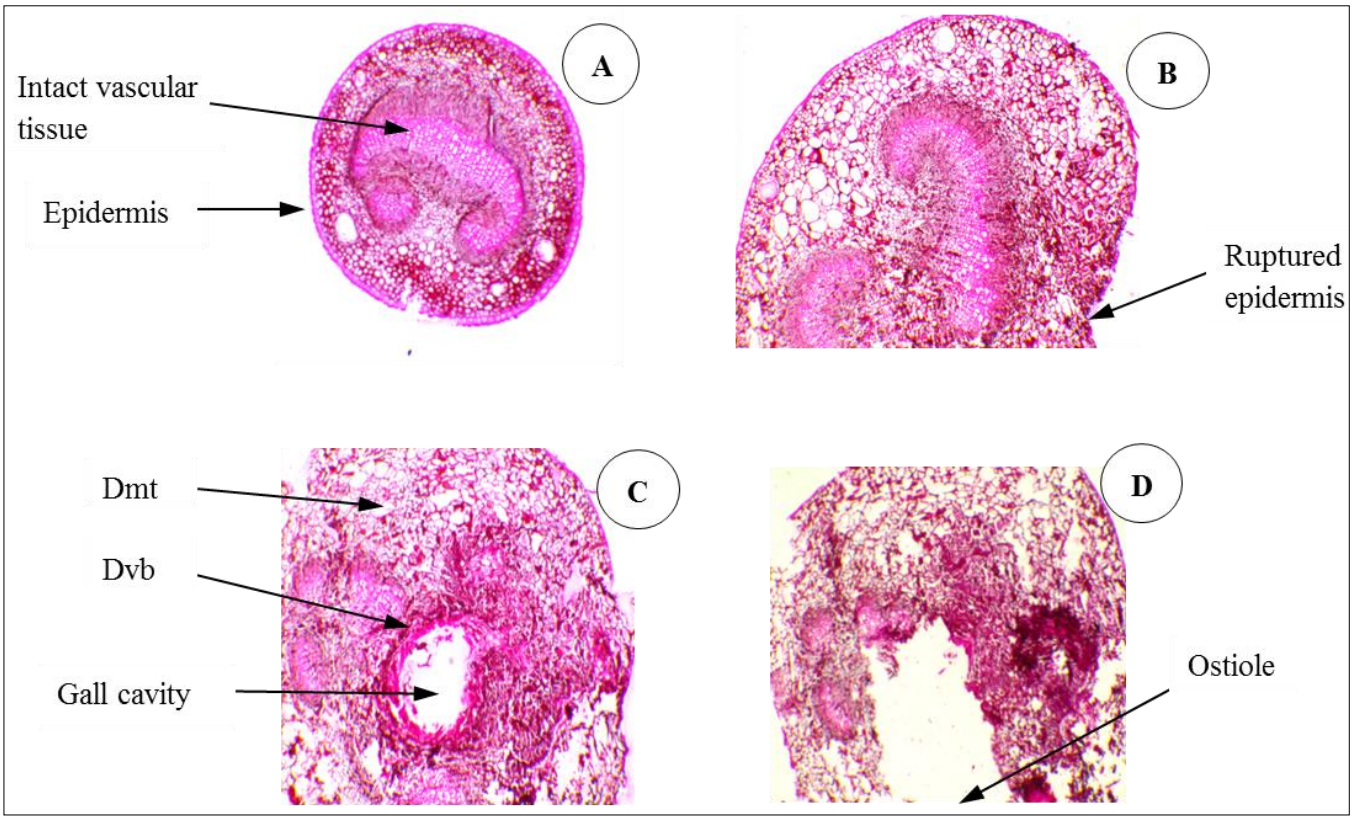

Fig 3 (**A**) Normal Petiole T.S. – Intact vascular bundle. (**B**) Petiole gall (Young) – Outline of petiole became ruptured at initial stage and vascular bundle is still intact. (**C**) Petiole gall (Mature) – Gall cavity is present in the central part which disrupt central vascular system and mesophyll tissue. (**D**) Petiole gall (Old) – Ostiole is visible and complete dessication of gall tissue and disruption of vascular bundle takes place
Abbreviations: Dvb – Disrupted Vascular Bundle. Dmt – Disrupted Mesophyll Tissue

Histopathology of petiole galls

Young stage of gall development showed ruptured epidermis where adult female laid eggs. After oviposition larvae enter into mesophyll tissue and alter the normal histological pattern by spreading stimulus into mesophyll tissue. Cells Undergo hypertrophy and hyperplasia and leads to cell elongation and rapid mitotic cell division appeared as outgrowth on petiole. Healthy petiole is somewhat circular in shape. As gall development starts the outline of petiole, become irregular (Fig 3B). Mature stage of gall development shows distinct alteration in the normal histological pattern with disruption of mesophyll tissue. The vascular bundle is disrupted because of the formation of oval shaped or sometime irregular gall cavity. Many intercellular spaces have been created because of mesophyll tissue disruption. Normally a single gall cavity was present in a single gall. Epidermis was more ruptured as compared to young stage gall. Desiccation starts as gall development reaches maturity (Fig 3C). Old stage of gall development is characterized by a passage through mesophyll cells which terminates into ostiole from which the adult female goes outside. Epidermal cells are almost completely ruptured in this stage. Mesophyll cells get completely disrupted and gall become desiccated and turns dark brown in colour. Size of old stage gall decreased due to dessication (Fig 3D).

RESULTS AND DISCUSSION

The insect galls are unique example of complex interactions and mutual adaptation between the host and the pathogen characterized by cellular hypertrophy and hyperplasia [2). As the development of larvae proceeds, larvae change its feeding site from parenchymatous tissue to vascular tissues. The histopathology of leaf gall of *Syzygium jambos* and observed that the inner side of the gall contained an oval or circular cavity which opened outside by a minute pore called ostiole formed on the abaxial surface of leaf, from which adult insect comes outside [10]. Our findings on the insect induced gall in *Eucalyptus tereticornis* revealed that gall formation alter the anatomy of normal leaf midrib and petiole by disrupting the mesophyll and vascular tissues. Histopathology of midrib is more complex as compared to petiole. Sometime individual gall may fuse to form a compound gall. Due to the outgrowth on one side of leaf, an invagination is formed on the other side which results in the distortion of leaf shape. Similar results were observed by [11] on leaf gall of *Ficus mysorensis* induced by *Psyllid*. More than one gall cavity is present in midrib gall while single gall cavity is present in petiole. Size of petiole gall is usually more than midrib galls. In both cases gall cavity was embedded into vascular tissue in which development of larvae takes place. Galling insects alters the metabolism of host plant and manipulates source-sink relationship for their own benefit. Gall tissues act as sink of energy and different nutrients for insects because they uptake nutrition from gall tissues [12-13). Histopathology of Insect and mites induced galls has also been studied by [14-18).

CONCLUSION

Anatomical changes are clearly seen in the gall tissues as compared to their normal counterparts. Oviposition by adult female on the lower side of leaf results in ruptured epidermis and formation of elliptical or sometimes irregular shaped gall. Hypertrophy and hyperplasia are observed in the gall tissues. Initially, the galls are pale green or pinkish in colour but become brownish at the time of maturity. Essential oil glands are present in the mesophyll tissue of Eucalyptus tereticornis, from which eucalyptus oil is obtained. Due to gall formation mesophyll tissue was disrupted, so it may significantly affect the yield of eucalyptus oil.

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