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Ultrastructural and biochemical studies on insect induced leaf galls in *Alstonia scholaris* L.

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ABSTRACT

Morphological, histopathological, histochemical and biochemical changes in the galled leaves of *Alstonia scholaris* R. Br. (Apocynaceae) infested by the insect *Pauropsylla tuberculata* Crawf. (Order: Homoptera) were recorded. The differentiation of mesophyll in palisade and spongy parenchyma was lost The cells adjoining the insect cavity undergo hypertrophy and hyperplasia and produce semi-globose, irregular in shape and dark and dry conical galls. The light (LM) and scanning electron microscopic (SEM) observations showed that in mature galls are enclosed by a thick periderm. The differentiation of mesophyll into palisade and spongy parenchyma was completely lost and number of slightly enlarged chloroplasts increased. The gall cavity opens on the lower surface through an ostiole. The vascular bundles were reduced in size. A sclerenchymatous layer develops around mature gall cavity. Transmission electron microscopic (TEM) observations indicated that cellulosic microfibrillar architecture of cell walls was lost in mature galls. The number and size of organelles, chloroplast in particular in infested galled leaves was reduced. The differentiation of grana and stroma was lost and the thylakoid membranes were thin and loosely arranged. The number of mitochondria and golgi complex along with rough endoplasmic reticulum and micro-bodies in the undifferentiated mesophyll cells was reduced. The histochemical localization of total carbohydrates of insoluble polysaccharide made by PAS reaction indicated the presence of suberin on the upper surface of mature galls. The undifferentiated mesophyll showed moderate or low intensity of the reaction. Mesophyll cells in mature galls showed marked reduction in total carbohydrate of insoluble polysaccharides (TCIP). The vascular tissues of galled leaves also showed reduction in the intensity of PAS reaction. The quantity of chlorophyll, chlorophyll a and b was significantly reduced in severely infested leaves. The quantity of total proteins in infested leaves was reduced. There was a spectrum of 9 amino acids (cystine, lysine, histidine, glycine, threonine, proline, tyrosine, tryptophan and phenylalanine) in the healthy and infested leaves. However, the quantity of cystine and lysine in galled leaves increased, while the quantity of proline, histidine and phenylalanine was significantly reduced and quantity of glycine, threonine, proline, tyrocine, and tryptophan was also low but it was insignificantly. The quantity of total phenolics in the infested leaves increased gradually with the increase in the intensity of infestation. The quantity of total and non-reducing sugars was higher in infested leaves at all the stages of infestation and total biomass was lower in the infested leaves.

Keywords : *Pauropsylla tuberculata,* thylakoids, mitochondria, PAS reaction, proteins, chlorophylls, proline, phenolics, sugars.

Plant galls have attracted the attention of naturalists from very early times. They present intriguing problems of morphogenesis and complex interrelations of associated organisms. In recent years, the study of galls has gained considerable new significance. Mani (1973) has so far described over seven hundred and fifty different insect galls from the Indian flora; out of them 55% of the galls in our country are leaf galls. Raman

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(2001) has reviewed the work of Mani (2000) on Plant Galls of India.

A gall is a cumulative expression of a suite of adaptations achieved by the host plant for accommodating -the inducing insect. In principle, a gall provides nutrition and shelter to the inducing insect and, in a few taxa, to its progeny as well. The insect activates a perturbation in growth mechanisms and alters the differentiation processes in the host plant, modifying the plant's architecture to its advantage (Raman 2003). Since insects derive their nutrition from gall tissue, the gall becomes a sink for different nutrients and energy that will be vital for the insect's growth (Raman 2003). A majority of gall-inducing insects stimulate host-plant tissue to develop into galls by their feeding action, whereas species of Hymenoptera trigger gall development via ovipositor. Even the vascular tissues can be modified by gall induction; so that they supply nutrients and water sub serving the needs of the inducing insect (Raman 2003). Raman (2007) has discussed some of the unresolved questions on insect-induced plant galls of India.

Alstonia scholaris (Family: Apocynaceae) is an elegant, beautiful ever green ornamental tree with grayish rough bark. Leaves are arranged in a whorl of 4-7, leathery, dark green above, and covered with a whitish bloom beneath. It is planted in the gardens and avenues for its dense crown of dark green leaves and several branches of creamish-white flowers with sweet fragrance. The beauty of this highly ornamental tree is distorted by the infestation of an insect- *Pauropsylla tuberculata* which not only induces gall formation on leaves alone but also on flowers and fruits of *Alstonia scholaris*.

Present investigation has been undertaken to study the morphological, histopathological (by LM, SEM & TEM) and biochemical changes in the leaves of *Alstonia scholaris* infested by the insect *Pauropsylla tuberculata*.

MATERIAL & METHODS

Present investigation was carried out on fresh, fixed and dried healthy and galled leaves of *Alstonia scholaris* collected from School of Life Sciences, Dr. B.R. Ambedkar University Khandari Campus; Shastripura; Vishwakarma Enclave, Agra; Bharti Nagar, Lodhi Estate, New Delhi and All India Institute of Medical Sciences, New Delhi.

A. Morphological studies: Morphological changes in *Pauropsylla tuberculata* infested leaves of *Alistonia scholaris* were recorded with the initiation of galls up to severe infestation.

B. Histopathological studies:

a. Light Microscopy (LM) : Pieces of healthy and galled leaves at were fixed in F.A.A (Formalin Acetic Acid). These were dehydrated in ethyl alcohol and n-Butyl alcohol. Infiltration was done by making 3 changes in wax and n-butyl alcohol. Sections were cut at 7-12µm and stained with Delafield haemotoxylin for 30 minutes. They were dipped 10-12 times in acidic and alkaline 70% ethyl alcohol. Dehydrated in tertiary butyl alcohol (TBA) and cleared in xylene and mounted.

b. Scanning Electron Microscopy (SEM): Samples were fixed in 3% glutaraldehyde in 0.1M phosphate buffer (pH-6.8) for 8-20h at room temperature, washed in the same buffer. Post fixation was done in 1% osmic acid in the same buffer for 4 hrs at 4ºC, washed in 75% ethyl alcohol and passed through the graded series of ethyl alcohol for 1 hr at room temperature. Transferred to a mixture of 1:1 100% ethyl alcohol and isoamyl acetate (3-methyl butyl acetate) and kept at room temperature for 30 minutes and transfered to pure isoamyl acetate making 2 changes. The samples were dried with liquid CO_2 in a HCP-2 Hitachi critical point dryer at 1000 lb per inch, mounted on brass stubs and coated with gold (20 mm coating) in a SCD 0.2 sputter coating unit (Polorn Equipment Ltd.; Walford, England). Photographs were taken in LEO-EM-SEM at All India Institute of Medical Science, New Delhi.

c. Transmission electron Microscopy (TEM): Fresh healthy and galled leaves were cut into small pieces and fixed in 3% glutaraldehyde in 0.1 M phosphate buffer at 6.8 pH for 8 h at room temperature. Post fixation was done in 1% osmic acid in the same buffer for 4 hours at 4ºC. Dehydrated and transferred to propylene oxide for 30 minutes at 4ºC with two changes. Pieces were embedded in Epon (Spurr's low-viscosity embedding media) in rubber embedding trays and kept in the oven at 40-45ºC for 2 days and there after the temperature were raised to 70ºC. Ultra-thin sections (0.75–1.5 µm) were cut in an automatic ultra-microtome (ultra-cut-E-F (4) and placed on the grids. Sections were stained with uranyl acetate and lead citrate. And viewed and photographed in a JEM 100s electron microscope at

80 KV at All India Institute of Medical Science, New Delhi.

C. Histochemical Studies : Total carbohydrates of insoluble polysaccharides (PAS reaction) were localized in the healthy and galled fresh leaves by the methods after Jensen (1962). Pieces of both healthy and galled leaves were fixed in formalin-acetic-alcohol for 24 hours, dehydrated in alcohol-xylol series, embedded in paraffin-wax and sections were cut at 7-12 µ m. Haupt's adhesive was used to affix the section on to the slide. Slides were placed in aqueous solution of 0.5% periodic acid for 5-30 minutes at room temperature. Washed in distilled water for 10 minutes and placed in Schiff's reagent for 10-15 minutes. Destained in 2% potassium sulphite for 1-2 minutes. Passed through graded up series of alcohol-xylol and mounted with DPX. The polysaccharides imparted purplish red stain and starch reacted strongly.

D. Biochemical studies : The following biochemical estimations were carried out in galled and healthy leaves.

1. Total chlorophylls : Chlorophylls were estimated in the healthy as well as galled fresh leaves following Arnon's (1949) method and the quantity of chlorophyll a and b was determined by measuring the optical density on UV-VIS spectrophotometer at 663 nm and 646 nm. Acetone and alcohol mixture was used as blank. The quantity of chlorophyll was estimated by using following standard formulae. Readings from 10 replicates were taken and the quantity was expressed as average values.

mgchla/g tissue = [12.7 (OD 663) - 2.69 (OD 645] X _V_X wt.

mg chl. b/g tissue = [22.9 (O D 645) - 4.68 (OD 663] X VX wt

mg total chl. /g tissue = [20.2 (OD 645) - 8.02 (OD 663] X VX wt

mg total chl /g tissue = OD 653 x 1000 X V

2. **Total proteins :** Quantity of total proteins was estimated in fresh healthy as well as galled fresh leaves by method after Lowery *et al.* (1951) using diluted Folin Ciocaltue reagent (BDH) with rapid mixing. For the development of colour, it was allowed to stand for 30 minutes. The optical density was recorded at 750 nm on a spectrophotometer. Protein values were estimated using a standard curve prepared from Bovine serum albumin.

3. Free proline : Quantitative estimation of free proline in the healthy as well as galled fresh leaves was made by the method of Bates *et al.* (1973). Optical density (OD) was read at 520 nm. Standard curve was prepared by using pure proline (BDH).

4. Amino acid spectrum :1 g fresh healthy and galled leaf tissue was boiled in 20 ml of 80% alcohol. The samples were homogenized and kept for 4-5 days and filtered (filtrate no. 1). The residue was washed with 80% ethyl alcohol three times with 5-8 ml each time and filtered (filtrate no.2). Filtrate 1 and 2 were combined and concentrated in vacuum.

Two dimensional paper chromatography : The loaded chromatograms were run in first direction as is done for mono-dimensional chromatography mentioned above. Air dried chromatograms were run in second direction in the other solvent (125 ml water was added to a 500 g bottle of phenol, kept overnight and before use a few drops of 0.88 ammonia were added and mixed thoroughly. Chromatograms were dried in the oven and developed with 0.2% ninhydrin in acetone. Chromatograms were dipped in Cu $(NO_3)_2$ in HNO_3 as described above. Amino acids were identified using standards (BDH).

Quantitative estimation of amino acids : After calculating the Rf values, the spots were cut and eluted in 4 ml 75% ethanol containing 0.05% $CuSO_4$ 5 H_2O and were read at 540 nm in a spectrophotometer. The concentration of different amino acids was expressed as glycine equivalents.

5. **Total phenol:** Total phenols were estimated in the healthy as well as galled fresh leaves after the method of Bhatia *et al.* (1972) using Folin Dennis reagent and reading taken at 725 nm. The total phenol were calculated using a standard curve using tannic acid.

6. Total carbohydrates: Quantity of total carbohydrates in healthy and infested leaves was estimated after the method of Somogyi (1952) and Nelson (1954) using Somogyi reagent and OD was read at 510 nm.

STATISTICAL ANALYSIS : Data obtained for various biochemical analyses were subjected to analysis of variance (ANOVA) after Snedecor & Cochrom (1967).

RESULTS & DISCUSSION

A. Healthy Plant: *Alstonia scholaris R. Br*. is an ever green medium to large tree, of about 8-15 m height with corky grayish rough bark. Branches whorled very much like the ribs of an umbrella. Leaves 4-7 in a whorl,

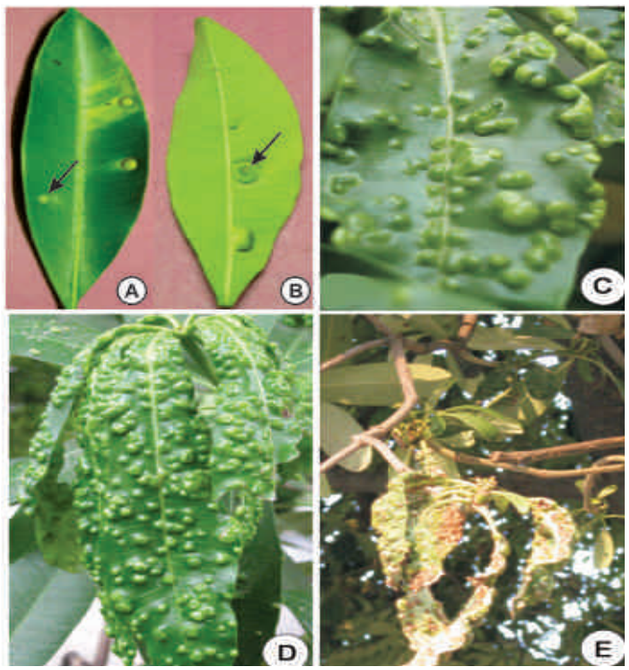

Fig. 1—A-E. Stages of leaf gall development on *A. scholaris*. A. Young galls on upper surface and B. Gall initiation on lower surface of leaf at stage a. C. Galls developed at stage b, D. Fully infested leaf at stage c and E. Mature dark brown galls at stage d.

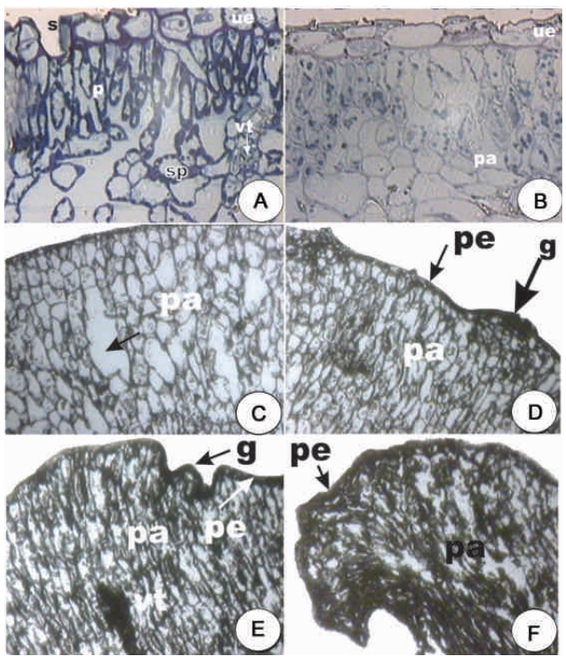

Fig. 2— A-F. V.S. of leaves healthy and infested leaves. A. Healthy leaf showing differentiated palisade and spongy parenchyma, stomata on upper surface and developed vascular tissue, B-F. Infested leaves. B. Leaf with undifferentiated mesophyll. C. Mesophyll with loosely arranged parenchyma, D. Formation of periderm and young gall on upper surface, E. Gall formation at stage b covered with periderm and F. Mature gall covered with thick periderm. (g: gall; pa: parenchyma; pe: periderm). 480X

leathery, in shape rather like those of the mango tree; dark green on the ventral surface and the dorsal surface is yellowish green. Flowers are sweetly scented, white or greenish-white in little clusters on slender long stalks at the ends of the final branches. Fruit is long follicle 30-50 cm long, 3-4 mm thick; pendulous in pairs, produced in such large numbers as to change the general aspect of the tree.

B. Infested Plant : The galls on *Alstonia scholaris* leaves and flowers are induced by the insect *Pauropsylla tuberculata.* The formation of galls on leaves takes place in four successive stages (a-d).The insect enters the leaf from the dorsal surface by making a hole in the epidermal cells (Fig. 1 B). At stage a, very small swellings of light greenish colour appear on the ventral surface (Fig. 1A). At stage b, the galls enlarge in dimensions and assume irregular shape of light greenish colour (Fig. 1C). The dark green surface of the surrounding area of the gall turns yellow. In the third developmental stage (c), the galls further enlarge and assume highly irregular shape of dark green colour spreading on the entire ventral as well as dorsal surface of the leaf (Fig. 1D). The galls are semi-globose, conical and about 2-5 mm in diameter at the tip and 3-6 mm at the base, glabrous, hard, and scattered irregularly in large numbers on the leaf surface. Sometimes they are present on the petioles also. The galls in the fourth developmental stage (d) or dehiscence phase become more irregular in shape and turn brown and dry with a large depression in the center and cover the entire leaf surface and the infestation is so severe that leaves become dry (Fig. 1E) and the entire tree gives a weathered look.

II. HISTOPATHOLOGICAL STUDIES :

A. Light (LM) & Scanning Electron Microscopy (SEM):

a. Healthy Leaf : The V.S. of healthy leaf shows the structure of typically dorsiventral leaf with the differentiation of palisade and spongy parenchyma (Figs.2A, 3A). The cells in the epidermis are isodiameteric and compactly arranged. Stomata are present on both the surfaces with higher frequency on the dorsal surface. There are two layers of compactly arranged palisade cells with large number of chloroplasts (Fig. 2A). The spongy parenchymatous cells are loosely arranged with large number of intercellular spaces between them (Figs. 2A, 3A). The vascular tissue in the mid-rib is well differentiated into xylem enclosed by phloem and is surrounded on both sides by the sclerenchymatous hypodermis.

b. Infested Leaf : The tangentially elongated epidermal cells in the infested leaves at stage b are narrow and covered with thin cuticle (Fig.2B). Formation of a thin layer of periderm starts which grows in thickness and at stage d; the galls show a thick layer of periderm enclosing the gall tissue. At stage c, the epidermal cells get disorganized at places to give the entry to the gall inducer (Fig. 2D) and bulge due to initiation of gall formation (Figs. 2D, E). There is no differentiation of mesophyll into palisade and spongy parenchyma (Figs.2B, C, D, E & 3B). At stage b and c, the entire mesophyll is represented by thin walled compactly arranged enlarged parenchymatous cells (Figs.2 A, B, 3B). These cells possess a limited number of chloroplasts reduced in size. The palisade cells and spongy parenchyma cells become hypertrophied. In between parenchyma cells a pear shaped cavity with an opening on the lower surface of leaf develops for the insect to live(Fig.3D).In mature galls at stage d, a sclerenchymatous layer develops surrounding the pear shaped gall cavity which opens through an orifice on the dorsal surface of the leaf (Fig. 3D). At stage d, the vascular bundles in the galled tissue are much disorganized and xylem and phloem is reduced (Fig. 3D). The mature galls show only a thick layer of periderm enclosing an undifferentiated mesophyll consisting of only degenerated parnechymatous tissue (Figs. 3B, D). The mature galls with large opening are irregular in shape and become agglomerated (Fig.3E). Each opening or orifice is surrounded by galled tissue with much enlarged and elongated cells (Fig.3E). At the openings of the galls well developed fissures and several large sized crystals or raphides of calcium oxalate develop (Fig.3F).

Present histopathological observations are supported by those of several others. Rohfritsch (1999) recorded the process of a so called “rudimentary gall” induced by the gall midge *Physemococcis hartigi* on leaves of *Tilia intermedia*. The small gall cavity is induced by cell wall maceration between the vascular bundle sheath cells and the phloem. These cells were

structurally modified into nutritive tissue which are hypertrophied and turgid, and have a centrally located enlarged nucleus and small vacuoles; the hydrated cytoplasm contains numerous concentric layers of endoplasmic reticulum, as well as many dictyosomes and autophagic structures. Kraus *et al*. (2002) have observed the anatomy and ontogeny of hymenopteran leaf galls of *Struthanthus vulgaris.* The vascular parenchyma shows hyperplasia. Fissures appear on the gall epidermis and the neoformed parenchyma is conspicuous, with a cortical and a medullar region. The senescent galls show the orifices of the exit channel made by the adult gall-makers**.** Harper *et al.* (2004) have achieved cynipid gall formation by an insect-plant interaction whereby cynipid gall wasps redirect host-plant development to form novel structures to protect and nourish the developing larvae.

Arduin *et al.* (2005) studied histopathology of leaf galls induced by *Baccharopelma dracunculifoliae* (Hemiptera: Psyllidae) on *Baccharis dracunculifolia* (Asteraceae) respectively. According to them, the galls pass through five developmental stages. Moura *et al.* (2008) recorded the species-specific changes in tissue morphogenesis induced by *Aceria lantanae* in *Lantana camara* leaves. At senescent stage, nutritive tissue suberization occurs, indicating the end of the inducers feeding activity. The suberization of nutritive tissue indicates the end of cell cycles, an event that may be related to the death of the deutogyne female, or to the limits imposed by the age of *L. camara* host leaf.

Marmit *et al.* (2008) have made a study to understand the definite alteration of metabolic activity at cellular level in *Magifera indica* leaf galls induced by *Amardiplosis allahabadensis.* A marked difference in the anatomy was observed between galls and normal tissue. Moura *et al.* (2008) have recorded the changes in tissue morphogenesis induced by an arthropod (*Acerialantanae*) in *Lantana camara* leaves. Gall induction causes hyperplasia of epidermis and ground system. At senescent stage, nutritive tissue suberization occurs, indicating the end of the inducers feeding activity. The suberization of nutritive tissue indicates the end of cell cycles, an event that may be related to the death of the deutogyne female, or to the limits imposed by the age of *L. camara* host leaf.

Susy *et al.* (2011) described morphological, anatomical and biochemical alterations in foliar galls of *Alstonia scholaris* induced by the insect *Pauropsylla tuberculata*. Galls occur isolated or agglomerated on the abaxial surface of the leaf. According to them, the insect *Pauropsylla tuberculata* deposits the egg along with some physiologic fluid which acts as a stimulant for the induction of the gall. This stimulus brings about hypertrophy followed by hyperplasia of cells.

B. Transmission Electron Microscopy (TEM):

a. Healthy Leaf : TEM observations indicate well organized cellulosic microfibrillar architecture of the cell wall (Figs. 4A, B). The intercellular spaces between the spongy parenchymatous cells are few in numbers as well as smaller in size. The cytoplasm is dense and consists of large number of rough as well smooth endoplasmic reticulum near to the nucleus, elongated chloroplasts, mitochondria and Golgi complex (Figs. 4 A, B). The cytoplasm in the mesophyll cells of the older leaves is highly vacuolated and lies on the periphery or accumulates in the center of the cells (Figs. 4 A, B). The cells in palisade layer consist of well-organized chloroplasts showing clear distinction of stroma and grana (Figs. 4 C, D). The thylakoid membranes are thick and compactly arranged (Fig. 4 C). The mitochondria in the mesophyll cells of the healthy leaves are well organized and they are of variable in number as well as in size (Figs. 4C & D). The mesophyll cells contain numerous invaginations of the plasmalemma bordering the chloroplasts and evaginations of the outer membrane of the opposing chloroplast envelope. In places, these membranes appear continuous with each other. A limited number of the mitochondria are exceptionally large, while others are much broad in outline. Several starch grains are seen in the chloroplasts (Fig. 4B. The minor veins consist of tracheary elements, xylem parenchyma cells, sieve-tube members, companion and phloem parenchyma cells, and other cells simply designated vascular parenchyma cells. The companion and phloem parenchyma cells are typically larger than the sieve-tube members with the companion cells containing a much denser cytoplasm that the phloem parenchyma.

b. Infested Leaf : The cell walls of the cells in the galled tissue at stage a, retain their cellulosic microfibrillar architecture as is seen in the healthy leaf which is lost in subsequent stages of gall development (Figs. 5 A-E). The cytoplasmic contents are much

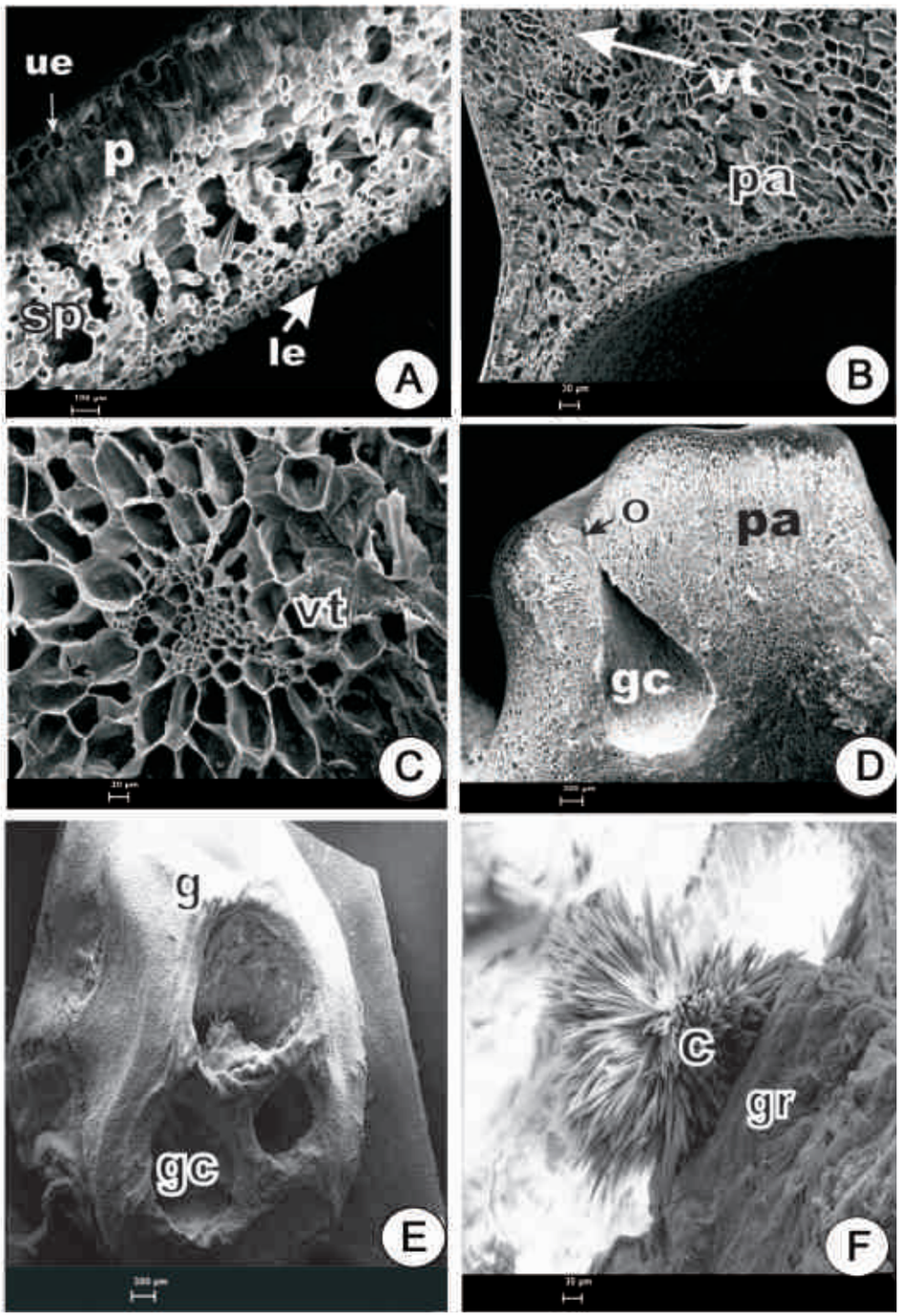

Fig.3— Scanning electron microphotographs (SEM) of V.S. leaves. A. Healthy leaf showing differentiation of palisade and spongy parenchyma. Bar= 100 µm. B. Infested leaf without mesophyll differentiation showing poorly developed vascular tissue. Bar=30 µm. C. Magnified view of poorly vascular tissue in infested leaf. Bar=20 µm. D. Leaf with gall (g) at stage b showing cavity surrounded by parenchyma, an ostiole. Bar=300. E. Mature galls at stage d with deep gall cavity (gc) surrounded by gall rim (gr). Bar=300 µm. E. Surface of mature galls covered with raphides (c). Bar=30 µm. (le: lower epidermis; pa: parenchyma; o; ostiole; sp: spongy parenchyma; ue: upper epidermis; vt: vascular tissue).

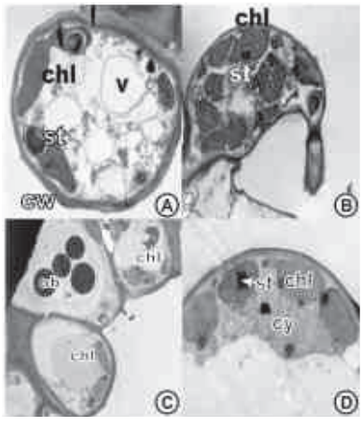

Fig. 4— A-B. Transmission electron microphotographs (TEM) of mesophyll cells of healthy leaves showing cellulosic microfibribillar architecture of cell wall, several large and small vacuoles; some osmiophilic bodies and chloroplasts with starch Figs. 4 C & D TEM of palisade cells of healthy leaf showing the structure of chloroplasts (chl). Note the presence of well organized grana, thylakoid membranes stroma in chloroplasts. (Figs. A, B: 3500; C: 4500X and D: 5500X). (chl: chloroplast; cy: cytoplasm; cw: cell wall; g: grana; ob: osmiophilic bodies; st: starch; str: stroma; thy: thylakoid; v: vacuole).

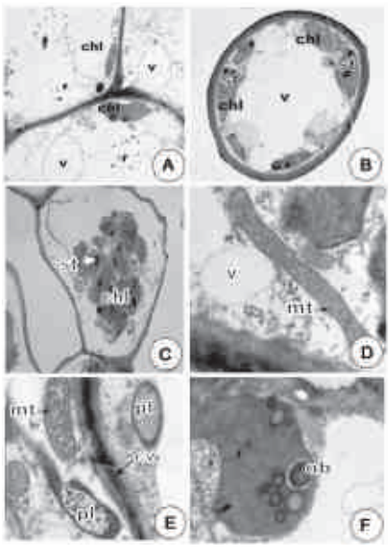

Fig. 5— A-F.TEM of palisade cells of infested leaves showing the structure of chloroplasts (chl) and mitochondria (mt). Note the presence of poorly developed cell wall (cw), plastids (pl), degenerated grana and stroma (st) in chloroplasts (chl), mitochondria (mt), and large vacuoles (v) and osmophilic bodies (ob). (Figs. A, B & C: 4500X and D, E & F: 5500X).

reduced and the number and size of these organelles is reduced (Figs. 5 A-E). The cytoplasm is highly vacuolated (Figs. 5 B, C & D). The size and the number of the chloroplasts in the infested galled leaves is considerably reduced (Figs.5 A-D) and the reduction is directly proportional to the intensity of the infestation. There is a gradual loss of differentiation of grana and stroma in the chloroplasts and at stage A, the thylakoid membranes are thin and loosely arranged and there is a further loss in the differentiation of grana and stroma at stage b (Figs. 5C). At stage, the mesophyll cells also show further reduction in the cytoplasmic contents and their organelles loose organized structure (Figs.5 A-D). In the galled leaves at stage c, the differentiation between the grana and stroma is completely lost and the thylakoid membranes are thin, loosely arranged (Figs.5A-C). The number and size of mitochondria in the undifferentiated mesophyll cells also decreases and they are much elongated in size in infested leaves at stage d (Figs. 5 D). The mesophyll parenchyma in the galled tissue at stage a have a centrally located enlarged nucleus and small vacuoles; the hydrated cytoplasm contains numerous concentric layers of endoplasmic reticulum, as well as many dictyosomes and autophagic structures (Figs.5 E & F). However, in subsequent stages, particularly in mature galled tissue (stage D), the number of golgi complexes also decreased along with rough endoplasmic reticulum and micro-bodies. However, in the infested leaves at stage D (severely infested), the mitochondria and other cell organelles including golgi bodies are seen in completely degenerated form (Fig. F). The protoplast in the cells of the galled tissue is much reduced and lies as a thin layer at the periphery of the cell enclosing a large central vacuole. The nuclei are also in much degenerated form. Intensity of degeneration of cell organelles including mitochondria is directly proportional to the intensity of the infestation (Figs. A, B, C & D).

Orion & Wergin (1982) have recorded chloroplast differentiation in tomato root galls induced by the root-knot nematode *Meloidogyne incognita.* Ultra-structural observation of the parenchyma tissue in galls from inoculated cultures indicated that starch containing plastids or amyloplasts, which are usually present and remain undifferentiated in these root cells, developed into chloroplasts.

III. HISTOCHEMICAL STUDIES:

A. Localization of total carbohydrates of insoluble polysaccharides (PAS reaction): PAS reaction has been used to evaluate the intensity of total carbohydrates of insoluble polysaccharides in the normal and galled leaves. This has been done largely to evaluate the changes in the cell wall constituents and photosynthetic ability of the mesophyll cells in galled tissue as compared to that in normal leaves.

Histochemical evaluation of total carbohydrates of insoluble polysaccharides (TCIP) made by periodic acid Schiff's (PAS) reaction in the healthy as well insect infested leaves at different stages of infestation showing low (stage a), moderate (stage b), intense (stage c) and severe infestation (stage d) is arbitrarily divided in to four parts, sight or low (+), moderate (++), intense or high (+++) and most intense or highest (++++). Similarly, the presence of PAS positive bodies in various parts of the healthy and infested leaves, particularly in the galled tissue is marked by low (*), high (**) and highest (***). Table 1 shows evaluation of PAS reaction in various parts of the healthy and infested leaves at different stages of infestation.

a. Healthy leaves : The cells in the upper epidermis exhibit moderate reaction, while the cuticle covering them shows intense reaction, but the cells in the lower epidermis and the guard cells of stotmatal openings exhibit intense PAS reaction (Fig. 6A). The palisade and mesophyll parenchya cells exhibit moderate reaction and contain several highly intense PAS positive bodies (Figs. 6A & B). The laminar vascular bundle exhibits most intense PAS reaction (Fig. 19 A). The xylem elements exhibit more intense PAS reaction while the phloem cells are less intensely stained.

b. Infested Leaf :

i. Stage a: The intensity of PAS reaction in various parts of infested leaf at stage a, is more or less similar to that exhibited by healthy leaves with a few exceptions. The cells in the upper epidermis show intense reaction (Figs. 6B, C, D). The hypodermal cells show highly intense PAS positive reaction indicating the presence of suberin (Figs. 6C, D). However, the ruptured epidermal cells show reduction in the intensity of the reaction and are moderately PAS positive (Figs. 6C, D). The

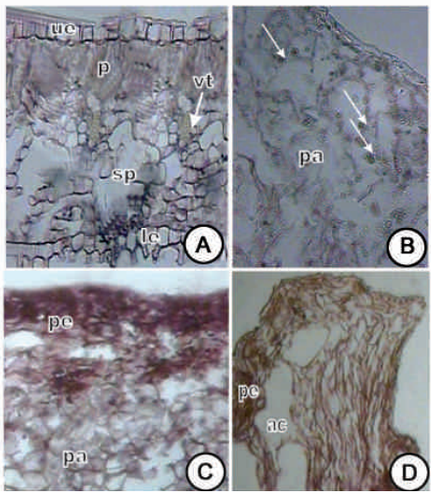

Fig. 6— A-D. V. S. of healthy and infested leaves showing PAS reaction. A. Healthy leaf. The cells in upper and lower epidermis cuticle on upper epidermis, mesophyll cells showing intense reaction. B. Infested leaf showing poor PAS reaction. C. Deposition of suberin on the epidermal cells at stage b and poorly stained parenchyma of the gall. D. Ruptured epidermis on the gall cavity with suberized cells. Gall tissue at different stages (a & c) showing intense PAS reaction in the periderm cells of the gall. (ac: air chambers; pa: parenchyma; le: lower epidermis; p: palisade; pc: periderm; sp: spongy parenchyma; ue: upper epidermis). 480X

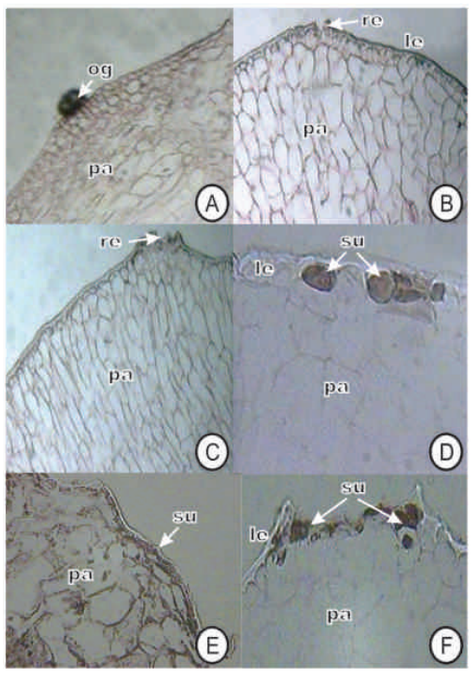

Fig. 7— A-F.V.S. of infested leaves showing PAS reaction. A. Initiation of infestation. Note the presence of PAS positive body as an outgrowth (og) on the epidermal surface. B. Ruptured (re) lower epidermis (le). C. Ruptures epidermis (re) and formation of lenticels and poor PAS reaction in parenchyma (pa); D. Ruptures lower epidermis (le) and lenticels showing deposition of suberin (su) and poor PAS positive parenchyma. E. Infested leaf showing intense PAS positive epidermal cells and suberized (su) hypodermis and thin walled parenchyma in the mesophyll and F. Ruptured lower epidermis (le) and deposition of suberin (su) in the lenticles. 480X

epidermal cells in the ruptured area show the accumulation of suberised substance (Figs.7A, B, C, F). The mesophyll cells which fail to show differentiation of palisade and spongy parenchyma cells show moderate or low intensity of the reaction at all the stages of gall development (Figs.7 B, D). At stage a, the mesophyll cells show the presence of total carbohydrate of insoluble polysaccharide (TCIP) bodies (Figs.7B, D, E). The vascular tissues in the galled leaves at this stage a, also show reduction in the intensity of PAS reaction (Fig.7C).

ii. Stage b: The intensity of the reaction decreases and is either low or moderate in different parts of the leaf. However, the cells in the upper epidermis show intense reaction (Figs.7 A, B, C & D).

iii. Stage c: The cuticle, the cells in the upper and lower epidermis, guard cells, spongy parenchyma cells, xylem, phloem and cells in the bundle sheath show moderate reaction while rest of the parts show only low PAS reaction (Fig. 7B).

iv. Stage d: Interestingly**,** the intensity of PAS reaction increases in all the parts of severely infested leaf and it is intense in the cells of the upper epidermis and in the cells below the epidermis forming the periderm (hypodermal cells) are highly intense and undifferentiated mesophyll cells also show intense PAS reaction (Figs. 7A, C & D). These cells in mature galls are much distorted in shape and size but show moderate PAS reaction. There are several large inter-cellular spaces between them (Fig. 6D). However, the PAS-positive bodies indicating the presence of TCIP are absent from these mesophyll cells (Table 1). The upper three to four layers of cells in the mature galls showing most intense PAS reaction indicating the presence of suberized periderm, which protects the galled tissue (Figs.6C, D, 7E). The vascular tissue in the bundles scattered in the galled tissue show only low or moderate PAS reaction.

Several investigators have also undertaken histochemical studies localizing not only cell wall constituents, but also various other metabolities e.g. tannins, starch, proteins, lipids, lignin and some enxymes e.g. acid phosphatase, peroxidase and poluphenol oxidase (Kant 2000, Arora & Patni 2001, Debnath *et al.* 2002, Singh *et al.* 2005, Raghav *et al.* 2007, Marmit *et al.* 2008).

Singh *et al.* (2005) have observed some changes in the metabolites and enzymes in leaf gall of *Ficus racemosa* induced by *Pauropsylla tuberculata*. Raghav *et al.* (2007) have studied histochemical changes in the leaf galls of *Alstonia scholaris* induced by *Pauropsylla tuberculata*. They have recorded relatively higher amount of metabolites and enzymes (starch, cellulose, lignin, carbohydrates, proteins, lipids, tannins, polyphenol oxidase, peroxidase and acid phosphatase) in galled tissues.

IV. BIOCHEMICAL STUDIES:

Biochemical changes in various metabolites in the galled leaves are recorded at four different stages of gall formation, namely, stage a : leaves showing initiation of

Table 1—Histochemical evaluation of PAS reaction in various parts of the Healthy and infested leaves.

H/I Leaf	Cut	Upper Epid	Palsiade Cells	Spongy . Paren	Mid rib		Bundle Sheath	Lower Epi	Sto.
					Xy.	Phl.			
H	++++	+++	+++***	++ **	++	+++	++	+++	+++
I (stage a)	+++	++	+++***	++**	++	++	++	++	+++
I (stage b)	+++	++	++**	++*	++	+	++	++	++
I (stage c)	++	++	+**	++*	++	+	++	+	++
I (stage d)	+++	++	+++*	+++*	++	++	+++	++	+++

I : Infested; Cut.: Cuticle; Epid.: Epidermis; Paren.: Parenchyma; Xy.: Xylem; Phl.: Phloem; Sto.: Stomata. Intensity of the reaction: sight or low (+), moderate (++), intense or high (+++) and most intense or highest (++++). Intensity of the PAS positive bodies: low (*), high (**) and highest (***).

gall formation; stage b: moderately infested leaves with 7-12 galls/leaf; stage c: intensely infested leaves and stage d: severely infested leaves. The quantity of various metabolites estimated is compared with that in healthy leaves.

A. Total chlorophylls : The quantity of chlorophyll a; b; their ratio and total chlorophylls in the healthy and infested leaves is shown in Table 2.

It is evident from the data in Table 2 that healthy leaves contain maximum chlorophyll a and b and total chlorophyll. On the other hand, the quantity of both chlorophyll a and b declines with the intensity of infestation of the aphid and gall formation. In leaves showing mild or initiation of infestation (stage a) with minimum number of small sized galls, the quantity of both chlorophyll a and b is slightly lower than that in healthy leaves. However, with the increase in the intensity as well number and size of the galls, the quantity of chlorophyll a and b and total chlorophylls declines and in moderately infested leaves (stage b). The chlorophylls a and b and total chlorophyll in such leaves are 0.95±0.5; 0.75±0.5 and 1.75±0.5 mg/g respectively. In intensely infested leaves (stage c), the quantity is further reduced significantly (0.75±0.5; 0.35±0.5 and 1.0±0.5 mg/g respectively) and in severely infested leaves (stage d), which are showing more or less complete withering, the quantity of chlorophylls is significantly reduced and is more or less negligible (0.25±0.025; 0.15±0.025 and 0.40±0.025 mg/g respectively).

B. Total proteins : The quantity of total proteins in the healthy and infested leaves is shown in Table 3.

Table 3—Quantity of total proteins in the healthy and galled leaves.

Type of leaves	*Total proteins (mg/g).
Healthy	6. 25±0.5
Infested (Stage a)	4.75±0.45
Infested (Stage b)	**3.45±0.5
Infested (Stage c)	**1.75±0.6
Infested (Stage d)	**0.85±0.25

*Mean value of 10 replicates, **Significant at 5%, Significant at 1% level; ±Standard deviation

The quantity of total proteins in the healthy leaves is highest (6.25±0.5 mg/g). On the other hand, the quantity gradually declines in the infested leaves and the maximum reduction being the most significant in severely infested leaves (0.85±0.25 % mg/g). However, there is a slight reduction in the quantity of total proteins (4.75±0.45) in infested leaves showing the initiation of infestation with a few small galls on the leaves (Stage a) and there is further significant reduction in leaves showing moderate (stage b) (3.45±0.5 mg/g) and intense infestation i.e. stage c (1.75±0.6 mg/g).

C. Amino acids: The spectrum of amino acids in the healthy and infested leaves is shown in Table 4.

It is evident from the data in Table 4 that there is a spectrum of 9 amino acids present in healthy as well as infested leaves. The amino acids present are: cystine, lysine, histidine, glycine, threonine, proline, tyrosine, tryptophan and phenylalanine. However, the quantity of these amino acids is different in infested leaves as compared with the healthy leaves. The amino acids

Table 2— Quantity of chlorophyll a & b and total chlorophylls and their ratio in healthy and infested leaves.

*Chlorophylls (mg/g tissue)	Healthy leaf	INFESTED LEAF			
		Stage a	Stage b	Stage c	Stage d
Chl. A	1.78±0.5	1.25±0.45	**0.95±0.5	**0.75±0.5	#0.25±0.025
Chl. B	0.95±0.5	0.85±0.5	**0.75±0.5	**0.35±0.5	#0.15±0.025
Total chl.	2.73±0.5	2.10±0.5	**1.70±0.5	# 1.10±0.5	#0.40±0.025

*Mean value of 10 replicates; ** Significant at 5%; # Significant at 1% level; ±Standard deviation

Table 4— Quantiy of amino acids in the healthy and galled leaves.

S. No.	Amino acids	*Quantity of amino acids in healthy and infested leaves (µg/100 mg dry weight)				
		Healthy	INFESTED			
			Stage a	Stage b	Stage c	Stage d
1.	Cystine	265 ±26	287 ±30	318 ±53	**379 ±58	**407 ±61
2	Lysine	285 ±29	302 ±34	**368 ±34	**412 ±49	**467 ±54
3	Histidine	277 ±15	287 ±28	255 ±25	232 ±55	228 ±40
4	Glycine	415 ±21	410 ±40	366 ±28	320 ±29	**287 ±41
5	Threonine	345 ±22	341 ±38	302 ±30	277 ±30	**241 ±20
6	Proline	625 ±27	595 ±45	524 ±48	415 ±40	**302 ±26
7	Tyrosine	432 ±29	415 ±40	367 ±36	335 ±35	**288 ±28
8	Tryptophan	655 ±31	618 ±50	551 ±51	**423 ±33	**316 ±22
9	Phenylalanine	324 ±12	335 ±31	289 ±25	275 ±37	269 ±31

*Mean value of 10 replicates, **Significant at 5%, ±Standard deviation

showing significant increase in their quantity in intensely (stage c) and severely infested (stages d) leaves are cystine and lysine showing 53.58 % and 63.85% increase respectively. On the other hand, the quantity of histidine and phenylalanine is reduced in infested leaves, but the reduction is not significant even at stage D (17.68% and 20.44% respectively). However, the quantity of glysine, threonine, proline, tyrosine, and tryptophan in the infested leaves at stage d is significantly decreased and the reduction is 30.84, 25.06, 51.68, 33.33, and 51.75% respectively. A comparative view of the quantity of different amino acids in both healthy as well as infested leaves at various stages of infestation is shown with the help of histograms. The percentage of increase and reduction in the quantity of various amino acids in infested leaves as compared to healthy leaves is also been shown by histograms.

D. Free proline: The quantity free proline in the healthy and infested leaves is shown in Table 5.

Table 5 shows that the quantity of free proline is highest in the healthy leaves as compared to that in leaves showing infestation of various intensities. In the healthy leaves, the quantity of free proline is 3. 45±0.25 mg/g is significantly higher to that in infested leaves. Leaves showing mild infestation (stage a) contain 2.55±0.25 mg/g free proline and the difference in the quantity of free proline with the healthy leaves is not significant, while moderately (stage b) and intensely infested leaves (stage c) show significant reduction in the quantity of free proline and the quantity is 2.05±0.20 and 1.75±0.20 mg/g respectively. Severely infested leaves (stage d) show further and significant reduction as they contain only a very small quantity of free proline (0.95±0.20 mg/g). It is interesting to note that the quantity of free proline decreases with the increase in the moisture content in the galled leaves.

Table 5—Quantity of free proline in the healthy and galled leaves.

S. No.	Healthy/Galled leaf	*Free proline (mg/g)
1.	Healthy	3. 45±0.25
2.	Infested (Stage a)	2.55±0.25
3.	Infested (Stage b)	**2.05±0.20
4.	Infested (Stage c)	**1.75±0.20
5.	Infested (Stage d)	# 0.95±0.20

*Mean value of 10 replicates, **Significant at 5%, # significant at 1% level; ±Standard deviation

E. Total phenolics : The quantity of total phenolics in the healthy and infested leaves is shown in Table 6.

It is evident from the data in Table 6 that the quantity of total phenolics is low in the healthy leaves (28.5±2.5 mg/g dry wt.) as compared to that in infested leaves. On the other hand, the quantity of total phenolics in the infested leaves increases gradually with the increase in the intensity of infestation and the reduction is inversely proportional to the intensity of infestation. In severely infested leaves (stage c and d), the quantity of total phenolics was significantly high (40.0 and 44.0±2.5 mg/g dry wt. respectively). However, in the infested leaves with the initiation of infestation (stage a) and in moderately infested leaves (stage b) the quantity of phenolics is also low but not significantly different from the healthy leaves.

Table 6—Quantity of total phenolics in the healthy and infested leaves.

S.No.	Healthy/galled leaves	*Total phenolics mg/g dry wt.
1.	Healthy	28.5 ±2.5
2.	Infested (Stage a)	34.7 ±3.0
3.	Infested (Stage b)	35.5 ±3.0
4.	Infested (Stage c)	**40.0 ±2.5
5.	Infested (Stage d)	**44.0 ±2.5

*Mean value of 10 replicates, ** Significant at 5%, ± Standard deviation

F. Total sugars—The quantity of and total sugars in the healthy and infested leaves is shown in Table 7.

The quantity of total and non-reducing sugars is increased in infested leaves at all the stages of infestation as compared to that in healthy leaves. However, significant increase is recorded only in the quantity of non-reducing sugars (2.35±0.25 % dry wt.) in the infested leaves at stage d. On the other hand, the quantity of reducing sugars is reduced in the infested leaves at all the stages of infestation as compared to that in healthy leaves. The maximum reduction (1.60±0.12 % dry wt.) is recorded in the severely infested leaves (stage d).

From the foregoing biochemical observations on it is evident that the leaves infested with the insect *Pauropsylla tuberculata* show marked reduction in the quantity of chlorophyll, total proteins, total sugars and some amino acids, proline in particular. However, there is an increase in the quantity of some amino acids and total phenolics.

Chandraguru *et al*. (1987) recorded increased sugar contents in leaf galls in *Calycopteris floribunddus* and *Mimus opselengi* induced by the thrips *Austrothrips cochinensis* Karny and *Arrhenothrips ramakrishnae* respectively.

The loss in chlorophyll contents in various diseased plants has also been reported earlier by several workers (Chauhan *et al.* 1984a, b, Chandraguru *et al.* 1987, Blanchfield *et al.* 2006).

Present findings have indicated that the amount of free amino acids in infested leaves get disturbed by the preferential utilization by the pathogen or a reaction of the host. The loss in the total proteins also gives an indication about their breakage into simpler units. On the other hand, the loss in certain amino acids may be due to their utilization by the gall-producer. The loss in the concentration of tryptophan may be due to its utilization in the synthesis of IAA as also reported by Miles (1968) and Balasubramanium & Purshothaman (1974).

Table 7—Quantity of total sugars in healthy and infested leaves.

S.No.	Healthy/ Galled leaves	*SUGARS (% dry wt.)		
		Total	Reducing	Non-reducing
1.	Healthy	3.25±0.25	2.00±0.15	1.25±0.15
2.	Infested (Stage a)	3.35±0.16	1.85±0.12	1.75±0.15
3.	Infested (Stage b)	3.45±0.15	1.70±0.13	2.05±0.25
4.	Infested (Stage c)	375±0.14	1.65±0.17	2.15±0.25
5.	Infested (Stage d)	3.95±0.15	1.60±0.12	**2.35±0.25

*Mean value of 10 replicates, **significant at 5%, ± Standard deviation

According to Andres (1957, 1958) amino acids provided the stimulus for the gall formation and found 4 to 14 amino acids in the saliva while IAA was absent. They have successfully induced galls on the roots of rape seedlings by growing them in solutions of amino acids (especially tryptophan, histidine and glutamic acid) either singly or in combinations that he claimed were present in the saliva of the grape *phylloxera*. Chauhan *et al.* (1982) have studied the changes in amino acid spectrum in insect induced leaf galls of some species of *Ficus* and *Quercus.*

Yoshitaka *et al.* (2004) have observed that aphid galls accumulate higher concentrations of amino acids: a support for the nutritive hypothesis for gall formation. The nutritive hypothesis for the adaptive significance of insect gall formation postulates that galls accumulate higher concentrations of nutritive compounds than uninfected plant tissue.

The loss in proteins as result of aphid infestation and gall formation is well known since early days in large number of plants and it has reported that the presence of enzyme protease in the salivary glands of the insects which is released in the galled tissue and this in turn hydrolyzes the proteins into amino acids. Chauhan *et al.* (1982, 1984a, b) have recorded a significant reduction in the quantity of total proteins in insect induced leaf galls in some spcies of *Ficus* and *Quercu* and *Boerhaavia diffusa* infested by the midge *Punaravomyia boerhaaviaefolie.*

On the other hand, Chandraguru *et al.* (1987) recorded an enhanced level of proteins at all stages in the galls induced by *Austrothrips cochinensis* and *Arrhenothrips ramakrishnae* respectively in *Calcopteris floribundus* and *Mimusops elengi*as compared to the normal leaves. El-Akkad (2004) compared the protein patterns of healthy and galled leaf extracts of *Populus nigra* infested with the aphids *Pemphigous populi*. There was an expression of specific proteins (molecular weights were, 65, 45, 37 and 25 kDa) in the protein pattern of the leaves with galls, which were not found in the healthy leaves. The presence of these specific polypeptides induced by galling in galled tissues suggests that, some of these proteins may be stress proteins.

Yang *et al.* (2007) have observed life time deficiency of photosynthetic pigment-protein complexes CP1, A1, AB1 and AB2 in two cecidomyiid galls derived from *Machilus thunbergii* leaves. Susy *et al.* (2011) observed that with an increase in the growth of the galls on *Alistonia* leaves, chlorophyll content in the gall tissue decreases. A steady increase of sugar content is noticed. On the contrary of the results of present investigation, Susy *et al.* (2011) recorded almost two fold increases in the protein content in immature galled tissue and a very high increase in the proline content in the mature galled tissue compared to the immature galled tissue indicating a stressed condition of the galled tissue. Saini & Sarin (2012) using electrophoretic protein analysis technique revealed that amount of total protein increased in the young galls but falls down in older stages.

Dhingra *et al.* (1982) and Chauhan *et al.* (1984b) have shown a reduction in the quantity of free proline under pathological stress. Chauhan *et al*. (1984b) have recorded a reduction in the quantity of free proline in the galled leaves of *Boerhaavia diffusa.* Proline usually accumulates in the plant tissue under stress conditions (Levitt 1972). The deficiency of free proline in galled leaves of *Alstonia scholaris* is associated with the higher quantity of water as compared to the healthy leaves (Kumar Unpublished). More over proline acts as a reservoir and all the major amino acids are pooled into it and when ever required, the proline is utilized in the formation of the amino acids (Chauhan 1997). It is quite possible that the deficiency of free proline in galled leaves may be due to its conversion into other amino acids, some which accumulate while others have been utilized in gall making.

Several investigators have also made studies about the role of phenolic compounds in the gall and these compounds have been assigned the role of resistance against the infection. Chauhan *et al.* (1982) have studied the changes in phenolics in insect induced leaf galls of some species of *Ficus* and *Quercus.* They have recorded an appreciable increase in the amount of phenolics in the galled leaves and according to Chauhan *et al.* (1982) this is a reaction in defense. According to Hartley (1999), insect-induced galls are similar to any microbe-induced abnormal growth (e.g. tumours induced by *A. tumefaciens*), in that both systems accumulate large quantities of phenolics compounds.

Pascual-Alvarado *et al*. (2008) studied the interactions between galling insects and leaf-feeding insects for evaluating the role of plant phenolics compounds and their possible interference with herbivores in several plants of tropical dry forest in Western Mexico. Four plant species had significantly greater total phenol concentrations in galled than healthy leaves. In three plant species associated with galls, host total phenol concentrations were significantly greater in short than in tall plants. Biswas *et al*. (2014) found antioxidant enzymes and phenolics activity was positively correlated with different stages of gall leaves whereas chlorophyll content exhibited strong negative correlation in *Alstonia scholaris*, elegant evergreen tree foliar galls caused by *Pauropsylla tuberculata*. Marmit & Sharma (2008) have analyzed total sugar, reducing sugar, a-amylase activity and invertase activity in galled leaves of *Mangifera indica* as compared to normal leaves. Infested leaves showed significantly higher quantity of total sugars, a-amylase and lower amount of reducing sugar. Recently, Huang *et al.* (2015) have recorded structural, biochemical and physiological characterization of photosynthesis in leaf-derived cup-shaped galls on *Litsea acuminate* induced by *Bruggm anniella* sp. (Diptera: Cecidomyiidae).

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