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Biochemical Changes Induced in *Populus nigra* Leaves by Galling Aphids *Pemphigous populi*

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ABSTRACT

The insect, *Pemphigous populi* infects *Populus nigra* producing large galls at the bases of leaf blades. The protein patterns of ungalled and galled leaf extracts were compared. There was an expression of specific proteins (molecular weights were, 65, 45, 37 & 25 kDa) in the protein pattern of the leaves with galls, which were not found in the ungalled leaves. The presence of these specific polypeptides induced by galling in galled tissues suggests that, some of these proteins may be stress proteins. The amount of auxins and gibberellins was substantially higher in gall tissue (twice that of normal and galled leaves). The mean zeatin, kinetin and benzyladenin levels in the ungalled *populus* leaf tissue were low. However, in marked contrast to this, the mean zeatin level in the *populus* gall tissue was much higher than in the ungalled leaf tissue and in galled leaves.

Key Words: *Populus;* Insect gall; Protein pattern; Phytohormone; HPLC; Enzyme activities

INTRODUCTION

A variety of organisms can programme plant development, producing novel and abnormal structures such as galls. The most fascinating and elaborate galls are those produced by insects. The mechanisms of gall formation by plants in response to the insect attack remain largely unknown. In addition, the stimuli, which trigger gall formation, are also unknown.

Gall insects modifying minute areas of host plants by soliciting gene expression from adjacent cells in such a way that new developmental events occur (Ananthakrishnan, 1998). New morphological potentialities of the host plant are brought into play, enabling differentiation of new cell types that are unknown in the normal plant organ. Additionally, organising new differentiation centres ultimately results in the expression of a gall system with well defined nutritional as well as defensive systems. Gall inducers have the ability to control and manipulate the growth of a plant; the degree of such manipulation varies widely from simple cell proliferation to the production of complex structures not normally produced by the plant (Rohfritsch & Anthony, 1992). Gall inducers may utilise a wide range of mechanisms including behavioural, mechanical, chemical or genetic manipulation of the host plant to produce galls (Hori, 1992). It would appear that the host plant and its parasites are engaged in a struggle.

Among gall inducing insects, the aphids are unique. It has been established that along with saliva, aphids inject chemicals into the leaf such as amino acids and plant growth substance (Schaller, 1968; Hori, 1976) and the plant produces secondary metabolites as a defense mechanism. The inducing molecules are thought to be released by plant cells when insects attack their cell walls (Broadway & Duffey, 1986).

Some sort of phylogenetic signals emanating from the insects must be responsible for this complex plant response. There is a wealth of literatures describing the anatomy of galls, little is known of the nature of these signals, which the author believes are chemical in nature and possibly proteins.

Plant growth hormones, especially auxins, have been implicated in gall formation because abnormal growths induced by these materials are similar to cells and tissues in natural galls (Overbeek, 1966). Much of the evidence indicate that the plant growth hormones are responsible for gall formation comes from work on galls produced in response to nematode, fungal or bacterial invasion (Bouillenne & Gasi'Ak, 1970). Relatively little work has been done on changes in hormone levels in insect-induced galls (Galston & Davies, 1969).

Populus nigra (Family: Salicaceae) is a plant cultivated in Egypt and propagated by cuttings (El-Hadidi & Boulos, 1988). In St. Katherine (Sinai, Egypt), the gall-forming aphid *Pemphigous populi* attacks the leaves of most *Populus nigra* trees in late April indicating large galls at the base of leaf blades (El- Akkad & Zalat, 2000).

The interactive nature of the systems and the fact that the galls themselves are static and many are readily visible in *Populus nigra* makes this system practical as a model for study. Our knowledge of insect galls is still so poor but in addition to the studies of a vast body of professional workers there are still opportunities for amateur naturalists to make valuable scientific contributions. Generally, what chemical signals are involved in gall formation and how such growth is controlled remains undetermined. Little has been written about the physiology of *Populus* insect galls grown in St. Katherine and the stress that they inflect on their host plants.

The objectives of this study were to evaluate insect/plant interactions when attacked by *P. populi* and the nature of the signals that result in galls in induction of galls in *Populus nigra.*

MATERIALS AND METHODS

In *Populus nigra* trees, between late April and early May aphids select some leaves for infestation and form characteristic galls at the base of leaf blades (galled leaves) and other leaves that do not have infestations are without galls (ungalled leaves). In the present work the analyses were done on: ungalled leaves, galled leaves and gall tissues (associated with the galled leaves).

The plant samples were collected from Wadi El-Arbain, St. Katherin, and Sinai, Egypt. Different biochemical measurements were done as follows:

Quantitative estimation of protein. Plant tissue samples of 0.5 g (air dry tissue), were hydrolysed in 5 mL saline solution (NaCl) and then centrifuged at 6000 xg for 10 min. The supernatants contained the proteins extracted, protein contents were measured (in mg/g dry weight of galled, ungalled leaves & gall tissue), using Folin-Cicaltew reagent according to the procedure described by Daughaday *et al.* (1952).

Protein Electrophoresis

Protein extraction. Three mL tris-HCl extraction buffer at pH 8.5 were added per gram fresh weight of *Populus* leaves, ground together and the slurry was then kept in the refrigerator over night before centrifugation the next day, 20 minutes at 5000 xg.

The supernatants were decanted to fresh tubes and 0.7 mL of absolute ethyl alcohol was added per 1ml supernatant to precipitate the proteins. These extracts were then kept over night again in the refrigerator before centrifugation for 20 min at 5000 xg, the supernatants discarded, the pellets washed in 70% acetone, centrifugation and dissolving the dried pellets in a minimum volume of extraction buffer.

Electrophoresis. Electrophoresis using 12% polyacrylamide gel was carried out following the method of Laemmli (1970). Standard proteins of known molecular weights (7.1, 20.6, 28.9, 34.8, 49.1, 80.0, 124.0 & 209 kDa) were run on corresponding gels and used to determine the molecular weights of *Populus* polypeptides.

The staining of protein was done overnight in a mixture of 0.25 g Comassie blue, 90 mL water: methanol (1:1 v/v) and 10 mL of glacial acetic acid. Then transferred to a mixture of glacial acetic acid: methanol : water (3:17:20 v/v/v) for staining.

Estimation of phytohormone concentration by HPLC. The effects of galling on the endogenous auxins, gibberellins and abscisic acid were investigated within the plant. These different growth regulators were extracted in pure forms and quantified by HPLC (high performance liquid chromatography). The tissues used in this analysis were both galled and ungalled leaves. Gall tissues were also analysed. The extraction of indole acetic acid (IAA), gibberellic acid (GA_3), abscisic acid (ABA) was achieved by using the method adopted by Guinn *et al.* (1986). Extraction, methylation and estimation of cytokinin (zeatin, benzyladinine (BA) and kinetin were done according to the method adopted by Muller and Hilgenberg (1986).

Accurate concentrations of the plant growth regulators were obtained by comparing their respective peak areas in the plant extracts with their corresponding areas obtained with the authentic control samples. The peak areas and plant growth regulator concentrations were measured automatically by the HPLC software.

Estimation of proline. Proline content was extracted with sulphosalycilic acid and estimated spectrophotometrically in fresh shoot systems according to Sadasivam and Manickam (1991).

Enzyme Activities

Extraction. For assaying the activities of Indole acetic acid (IAA) oxidase and polyphenol oxidase (PPO), the plant materials were extracted following the method of Kar and Mishra (1976) with slight modifications. Two grams of fresh plant tissue samples were homogenized in cold phosphate buffer (0.05 M at pH 6.5). The homogenates were centrifuged at 10 x 10^3 xg for 10 min. The pigments were removed from the supernatant by adsorbing with activated charcoal and filtered. The enzyme contents of the filtrates were compared to known volumes of enzyme standards.

Indole acitic acid oxidase (IAA oxidase). The activity of this enzyme was assayed following the method of Gordon and Weber (1951).

Poly phenole oxidase (PPO). Polyphenol oxidase was assayed following the method described by Kar and Mishra (1976).

RESULTS AND DISCUSSION

Protein content and protein electrophoresis. Samples were collected from galled, ungalled leaves and gall tissue. The total soluble protein were extracted and quantified (Table I). The results indicated that protein content was different in the three samples with higher levels in the gall tissues of *Populus*. These results were in line with Arora and Patni (2001). Their results indicated a greater localisation of proteins in gall tissues of rachis as compared to the normal tissue. Also galled tissues of *Viola odorata* of all ages showed elevated total amino acid values compared to normal leaf tissues (Birch, 1974). A comparative histochemical study of the normal as well as gall tissues of *Mimusops elongi* Linn. Also revealed a higher incidence of protein in gall tissues (Gopinathan, 1987).

The obvious increase in protein content in gall tissue of the present work, together with the disorganisation and malformation which appears as a reduction in most of the morphological criteria in *Populus* leaves as result of galling (El- Akkad & Zalat, 2000). This suggested that the increase

Table I. Total protein content and proline content of the ungalled leaves, galled leaves and gall tissue of *Populus nigra*

Sample	Ungalled leaves	Galled leaves	Galled tissues
Total protein (mg/ g)	164	162	175
Proline U mol/ g tissue	0.589	1.183	0.689

Table II. Data matrix of the protein patterns of total protein extracts of ungalled leaves and gall tissue (two samples from each taken from two different branches) of *Populus nigra* tree

Molecular Weight	Ungalled leaves	Galled tissue	Ungalled leaves	Galled tissue
124.0	++	+	++	+
80.0	+	++	+	++
75.0	++	+	++	+
65.0	0	++	0	++
57.0	+	++	+	++
45.0	0	+	0	+
41.0	+	+	+	+
37.0	0	+	0	+
34.8	+	+	+	+
30.0	+	+	+	+
25.0	0	+	0	+
23.6	+	++	+	++
13.0	+	+	+	+

of the protein content is for the benefits of the insects and at the expense of the other metabolites in the leaves.

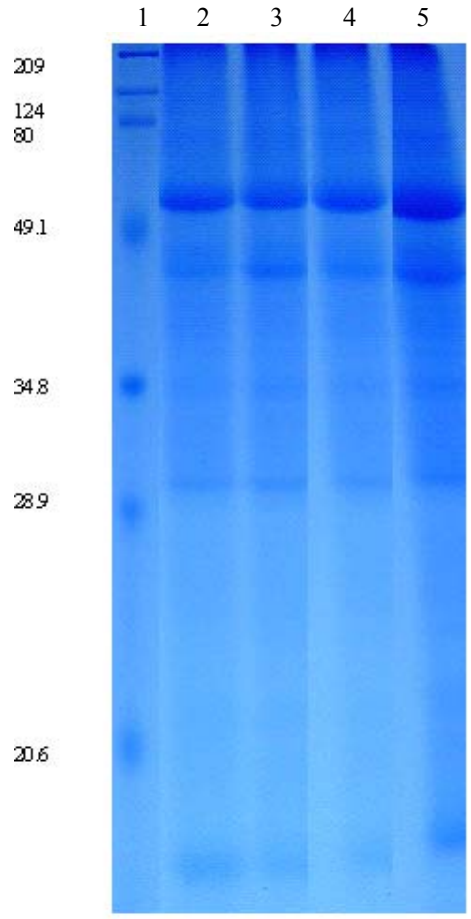
The protein patterns of total protein extracts of ungalled and galled leaves (two samples each taken from two different branches) are represented in Fig. 1 and the qualitative representation of the bands developed are shown in Table II.

Considerable differences in the protein patterns were observed between ungalled and galled leaves. Eleven polymorphic bands were detected in both kinds of tissues with molecular weights ranging from 124 to 13 kDa.

As shown in data matrix (Table II), there is an obvious variation in the number and intensity of bands between the gall tissue tissues and ungalled *Populus* leaves. As we see from the data there was an expression of specific proteins in the protein pattern of the galled leaves protein extracts, which are not found in the ungalled leaves, their molecular weights were 65, 45, 37 and 25 kDa. The induction of certain protein during the galling process was also recorded by Garango *et al.* (1988). In their work, the protein produced by galled and ungalled tissues of *Solidago altissima*, produced by the larva of the tephritid fly *Eurota solidaginis* were examined, and the hyperinduction of a 58 kDa protein was observed in galled tissues. The presence of this protein suggests that a substance secreted by the larva may function as Trans-acting gene regulator.

Several proteins that accumulate to high levels in normal leaf tissues show reduced levels in the galled leaves. It is also clear from the results that the bands of molecular

Fig. 1. Electrophoretic pattern of *Populus nigra* proteins. Lane 1 represent relative molecular weights (MW) of protein standards. Lanes 2 and 4 represent ungalled leaves protein. Lanes 3 and 5 represent galled leaves protein



weights 80, 57, 23 kDa were present in much lower concentrations in non-galled leaves compared to their concentration in galled leaves. On the other hand, the intensity of the band with molecular weight 124, 75 kDa indicated that protein of this weight was present in higher concentrations in ungalled compared with the galled leaf.

Two of the protein bands (65 & 37 kDa) recorded, which were induced in galled tissue were very close to the molecular weights of the bands recorded by Schonrogge *et al.* (2000). These investigators showed that *cynipid* gall formation might involve the ectopic expression of specific proteins and the total protein signatures of galled tissues were different from those of non-galled plant tissue. There were two abundant proteins from the galled tissue their molecular weights were 62 and 34 kDa.

McDermott *et al*. (1996) examined the proteins that accumulate in the leaf (hackberry) and the gall (nipple gall) and have identified several proteins that accumulate specifically in the gall. They found that even adjacent non-

galled leaf tissues do not show the expression of the gall-specific proteins. Furthermore, several proteins that accumulate to high levels in the normal leaf tissues show reduced levels in the gall tissues. They found that 37 kDa proteins (in galled tissues) apparently arise from a 59 kDa protein (in leaf tissue) implying that gall formation induces specific degredation of the 59 kDa proteins.

The detection of unique proteins in galled leaves could lead to the method of characterisation of the cause of gall formation. That characteristic protein could be compared with other galling plants and this might be used as a marker for plants susceptible to the infestation by gall producing insects.

The protein expression within the galled tissue has been shown to vary for some proteins between species, but also contain common proteins found in several gall species (Schonrogge *et al.,* 2000). These proteins, expressed in several gall tissues but are not normally expressed in leaf or stem tissue, may provide vital clues as to how the larva are reprogramming plant development to achieve gall formation (Harper *et al*., 2004).

Also the induction of specific proteins in galled tissues may give an indication that this protein is one of the stress proteins. Such plant stress protein are produces as a defence mechanism to protect from invaders (biotic stress) or stresses factors (abiotic stresses) and are believed to be adaptive response to the altered conditions. Syntheses of diverse plant proteins are believed to be of importance in defence were suggested by Ananthakrishnan (2001). So it might be concluded that when plants are attacked by insects the plants they generate signals and one of these signals is the initiator of expression of certain polypeptides that may be useful in providing the basis for new crop protection strategies.

Synthesis of diverse plant proteins believed to be of importance in defence is also known (Reinbothe *et al.,* 1994). Defensive proteins that block the action of proteolytic enzymes from herbivores are found in legumes, tomatoes and other plants. These proteins, known as proteinase inhibitors, rapidly accumulate throughout plants that are being fed upon by insects and even accumulate in undamaged areas of plants that are far from the initial feeding site (Ananthakrishnan, 2001).

Changes in phytohormone levels. The concentration of endogenous plant growth regulator of ungalled and galled leaves as well as the gall tissue of *Populus nigra* (Table III) showed big differences. The amount of auxin was substantially higher in gall tissue than in normal and galled leaves, suggesting that some types of auxin was responsible for gall formation.

Extracts of insect larvae of midge *Jantiella* sp. causing galls at the base of young needles of *Pinus* did not contain auxins at detectable levels. This suggested that at the time of the gall formation, protein expression in the plant to produces abnormally high auxin levels and thus causes gall formation directly (Byers *et al.,* 1976).

Higher levels of gibberellin-like materials were found in gall tissue than in normal and galled leaves (Table III). This was in concomitant with the results of Byers *et al.* (1976). However, high levels of exogenous gibberellin are known to increase auxin levels in plants, possibly by increasing the orthophenols that inhibit peroxidase (Kogl & Elema, 1960). Thus the gall- forming larvae might secrete or induce the production of gibberellins which, would in turn increase auxin levels or perhaps act synergistically with IAA to cause the abnormal growths.

A reverse situation was shown in the present work, regarding the changes in ABA. The ABA concentration in the galled leaves was higher than that of the gall tissue and ungalled leaves. It is well known hat ABA affects stomatal closure during stress and some of ABA's activity seems related to its effect on protein synthesis (Saigo & Saigo, 1983). ABA is sometimes called the stress hormone, for it forms when a plant is subjected to unfavourable conditions and initiates specific defensive responses.

The structure of the *Populus* gall indicates that cell proliferation is likely to be involved in its development. Cytokinins are known to be involved in the control of plant division. In addition, they have also been shown to stimulate the production of certain mRNAs and proteins (Flores & Tobin, 1988; Lu *et al.,* 1990). We therefore thought to determine the levels of cytokinins (zeatin, kinetin & benzyladenins) present in the normal, galled leaves and the gall tissues. The mean zeatin, kinitine and benzyladenin levels in the ungalled *Populus* leaf tissue were low. However, in marked contrast to this, the mean zeatin level in the *Populus* gall tissue was much higher than the ungalled leaf tissue and it is also higher than in galled leaves (leaf blade associated with the gall) (Table III). Previously, the concentrations of zeatin, zeatin riboside, isopentenyladinine, and isopentenyladenosine were found to be higher in golden rod ball gall tissues than in normal goldenrod stems (Mapes & Davies, 2001).

Other workers have implicated cytokinins involvement in insect gall development (Engelbrecht, 1971; Abou-Mandour, 1980). Abou-Mandour (1980) has suggested that the elevated levels of cytokinins might be responsible for redirecting nutrients required for gall development.

The data in Table III also show that the auxin level was lower than that of cytokinin in gall tissue. Skoog and Armstrong (1970) found that if a high cytokinins- to- auxin ratio is maintained, meristimatic cells produce a callus. The increase in auxin content in galled leaves, and an obvious increase in cytokinins agree with the idea of Salisbury and Ross (1992) that cytokinins and auxins are important in controlling formation and development of tumors (galls).

Table III. The concentrations of endogenous Indole acitic acid (IAA), Gibberellic acid (GA_3), Abscisic acid (ABA) and Cytokinin (Zeatin, Kientin and Benzyl adenine) of the ungalled leaves, galled leaves and gall tissue of *Populus nigra*

Hormone	Ungalled leaves	Galled leaves	Galled tissues
IAA (µg/100 g)	2.087	2.384	5.940
GA3 (µg/100 g)	57.759	157.297	205.128
ABA (µ/100 g)	1.439	5.017	3.783
Zeatin (mg/100 g)	989.494	363.978	1975.11
Kinetin (mg/100 g)	6824.330	1964.785	8322.506
Benzyl adenine (mg/100 g)	861.358	1040.869	1234.006

Table IV. Total protein content, prolin content and the activity of polyphenol oxidase indole acetic acid oxidase activities of the ungalled leaves, galled leaves and gall tissue of *Populus nigra*

Sample	Ungalled leaves	Galled leaves	Galled tissues
Polyphenol oxidase (PPO) optical density/g/h	0.515	0.536	0.600
Indole acitic acid (IAA) oxidase enzyme activity/g fresh tissue/ h	1.50	1.30	1.10

IAA oxidase and PPO activity. As shown in Table IV, there is a variation in IAA oxidase activity between the ungalled, galled leaves and gall tissue. An obvious decrease in the activity of that enzyme was detected in the gall tissue in comparison with that of ungalled and galled leaves. This may be one of the reasons for the increased level of auxin concentration in the same sample (Table III).

It was evident from the previous work of El-Akkad and Zalat (2000) on the same plant that within the same tree, the total phenol concentration was much higher in galls than in leaves (ungalled and galled). This was explained by that phenols may be involved in protection of the gall-maker from pathogens such as fungi or other natural enemies. There was another explanation for the increase in total phenol content in gall tissue is that o-and p-dihydroxy phenols and polyphenols act as IAA-oxidase inhibitors (Shekhawat *et al*., 1978). Shekhawat *et al*. (1978) also concluded that there is a direct correlation between increased phenols and decreased IAA-oxidase activities. This inhibition of IAA-oxidase activity by protectors leads to hyperauxinity in the tissues, resulting in gall formation. The results obtained in the present study show a decreased level of IAA oxidase activity accompanied with an elevated level of PPO activity in gall tissue. It was evident that injuries to plants, whatever their cause, are liable to induce an increase in the content of toxic phenolic compound (Peries, 1994). This transformation is under the influence of polyphenol oxidase. This enzyme could be produced either by the plant or by invading organism (Miles, 1968). A comparative histochemical study of the normal as well as gall tissues of *Mimusops elongi* Linn. Revealed a higher incidence of polyphenol oxidase in gall tissues (Gopinathan, 1987). A localisation of polyphenol oxidase was also seen in rachis gall tissues as compared to the normal tissues (Arora & Panti, 2001). Of the chemical changes that take place during induced damage, the increase of the oxidative enzyme PPO appear to be most critical to the induction of resistance (Stout *et al.,* 1996).

Changes in proline content. The results obtained (Table I) showed an increase in proline content in the galled leaves of *Populus* compared with ungalled leaves and gall tissue. The increased level of proline in galled leaves might confirm the idea that galling has stressful effect on the infested leaves of *Populus*. Proline accumulation in response to stress is widely reported, and may play a role in stress adaptation within the cell, which is of great interest to those studying stresses in plants (Gibon *et al*., 2000)

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