INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
1560–8530/2005/07–6–979–984
http://www.ijab.org

Responses of Eucalypt Trees to the Insect Feeding (Gall-Forming Psyllid)

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ABSTRACT

Galled (diseased) and un-galled (healthy) eucalypt leaves were collected to study the different changes resulted from the biotic stress challenged with insect feeding. Insect feeding disrupted the leaf tissues (mesophyll & xylem) and elicited the formation of cavities in the xylem tissue of eucalypt leaves-particularly in the midribs and petioles of the leaves. A decrease in Ca^{2+}, Mg^{2+}, pigments levels, carbohydrates, amino acids, lignin, total soluble protein contents as well as GA_3 and zeatin were obtained in the diseased leaves compared with the un-galled healthy ones. However, an increase in K^+, proline, IAA and ABA levels were obtained after insect feeding. Insect infestation increased the lipid peroxidation product (malondialdehyde), ascorbic acid and enzymatic antioxidants such as superoxide dismutase, catalase, phenol oxidase, ascorbic acid oxidase and peroxidase activities. However, glutathione level and polyphenol peroxidase activities were reduced after insect feeding. A new polypeptide of molecular weight 47 kDa, were characterized in the diseased leaves. The findings were discussed in relation to the insect feeding and defense reactions against herbivore.

Key Words: Eucalypt; Primary metabolites; Secondary metabolites; Total soluble protein; Antioxidants

INTRODUCTION

Although *Eucalyptus* had no insect pests or diseases for almost a long time now at least four feeding guilds of insects, borers, defoliators, leaf-galling and sap-feeding insects were observed challenged with *Eucalyptus* trees (Paine & Millar, 2002). These insects occur in large areas in the middle-east, Mediterranean and Africa forming serious pest in young plantations. Moreover, heavy galling prevents further development and cause distortion of the infested *Eucalyptus* trees. For example, gall-forming psyllid kills million of eucalypts trees in many areas of Egypt.

It was reported that young leaf tissues and meristems are most susceptible to herbivory during young stages of phenological development (Orcutt & Nilsen, 2000). Chlorosis is the most obvious plant injury symptom on wheat plants after insect feeding and is indicative of chlorophyll loss (Heng-Moss *et al*., 2004; Wang *et al*., 2004). In addition, many physiological changes have been recorded in *Abies grandis* plants following insect infestation by *Adelgas piceae* such as a reduction in water permeability as well as the level of carbohydrate reserves (Putritch, 1971). Insects also altered the patterns of translocation and growth of their host plants and remove nutrients, amino acids and carbohydrates from the phloem (Miles, 1989).

Several defense reactions against herbivores have been reported in different plant species. In general, plant metabolites and macromolecules (e.g. peptides, proteins, enzymes, lignin, phenolic metabolites, cuticular waxes) can serve for defense against herbivores (Wink, 1997; Gutterman & Chauser–Volfson, 2000). Cambium of the infested shoot of white spruce produced traumatic resin canals in the xylem. These canals empty their contents into a duct and larvae galleries, which kills eggs and larvae (Alfaro, 1995). Accumulation of secondary defensive substances, such as tannins, their phenolic precursors, lignin, are considered defensive reactions against wounding and the presence of foreign organism (Rohfritch, 1981). Changes in plant protein profiles have been also reported in many plants (Jerez, 1998).

Phytohormones may play role in regulating the response of plants to herbivory by insects (Mapes & Davies, 2001). Higher and lower levels of growth inhibitors and promoters were found in the radish after infestation by *Myzus persicae* (Hussain *et al*., 1974). IAA level increased in galled than in normal tissues (Schaller, 1968). Zeatin riboside increase in rice leaves infested by insects was regarded as indicator of serious injury (Wu *et al*., 2004).

Plants have several scavenging mechanisms to limit the accumulation of harmful amounts of active oxygen species. The antioxidants such as glutathione, ascorbic acid, phenolics, carotenoids and tannins scavenge reactive oxygen radicals, and they are substrates for the antioxidant enzymes (Wise, 1995). It has been shown that insect herbivory induces oxidative responses in plants (Chaman *et al*., 2001; Ni *et al.,* 2001). Cereal aphid feeding results in increased peroxidase activity in resistant plants (Xinzh *et al.,* 2001). Similarly, chinch bug feeding increased peroxidase activity in susceptible buffalograsses (Heng–Moss *et al*., 2004). Felton *et al.* (1994) found increased peroxidase activity in response to bean leaf beetles and three-corned alfalfa leafhoopers in resistant soybean.

Chaman *et al*. (2001) showed different enzymatic responses in wheat, barley and oat to feeding by the different mechanisms of aphid resistance.

The aim of the present work was to study the alterations in different parameters of *Eucalyptus* leaves infested by xylem-feeding insect (gall- forming psyllid).

MATERIALS AND METHODS

Fully expanded healthy and damaged leaves of *Eucalyptus obliqua* trees were collected from different localities of Cairo, Egypt to analyze the damage induced by certain gall-forming insects (gall-forming psyllid). The insect forming galls was identified by the Plant Protection Institute. The damaged *Eucalyptus* leaves were infested by xylem-feeding insects and showed many bump-shaped galls on their midribs and petioles (not shown). All the following measurements were determined in both healthy and galled eucalypt leaves.

Structural changes. To examine the anatomical and cellular changes occurred in the galled leaves compared with the healthy ones, transverse leaf sectioning was made. The free-hand sections were stained using safranine and light green for microscopic examination.

Chemical determinations. Chlorophyll a, chlorophyll b and total carotenoids were measured spectrophotometrically according to Metzner *et al.* (1965) and their contents were calculated according to the formula of Lichtenthaler (1987).

Total soluble carbohydrates were extracted according to the method described by Naguib (1963) and determined using anthrone reagent (Fairbairn, 1953).

Free amino acids were extracted according to the method of Vartanain *et al*. (1992) and estimated using standard ninhydrin assay (Yemm & Cocking, 1959). Proline was determined according to Bates *et al*. (1973).

Soluble proteins were determined according to the method described by Bradford (1976) with BIO-RAD protein assay dye reagent using bovine serum albumin (BSA) as a standard. Lignin was determined by the method of Ritter *et al*. (1932).

Total tannins were determined according to Ranganna (1977). Total phenols were estimated according to Malik and Singh (1980). Phenolic acids were analysed and identified by using High Performance Liquid Chramatography (HPLC, Waters Millipore Company, USA) using authentic standards according to the method described by Cvikyova *et al.* (1988).

Antioxidant substances (i.e., ascorbic acid and glutathione were determined as follows. Total ascorbic acid was determined according the method of Kampfenkel *et al*. (1995) as modified by de Printo *et al*. (1999). Glutathione was determined according to Griffith (1985).

Hormones. Extraction of acidic hormones, indole acetic acid (IAA), gibberellic acid (GA3) and abscisic acid (ABA) were carried out according to the method described by Shindy and Smith (1975) and their identification and quantification were carried out using GLC (Varien Vesta, 6000) according to Vogel (1975). Cytokinin was determined by HPLC (Algilent, 1100 HPLC system, Germany) using zeatin as standard according to Muller and Hilgenberg (1986).

Ion content. Calcium, potassium and magnesium levels were determined using inductively coupled plasma (ICP) emission spectroscopy according to Donohue and Aho (1992) and Jones and Case (1990).

Lipid peroxidation and antioxidants. Accumulation of malondialdehyde was used as the measure of the degree of lipid peroxidation (Minotti & Aust, 1987). Antioxidant enzymes were extracted from frozen *Eucalyptus* health and diseased leaves by using a known volume of 0.2 M phosphate buffer (pH 7) containing 5 mM 2-mercaptoethanol. The pure extract was used for enzyme assays. Cu-Zn superoxide dismutase (Cu-Zn SOD) was measured by the method of Giannopolitis and Ries (1977). Phenol peroxidase (PX) activity was determined according to the method adopted by Bergmeyer (1974). Polyphenol oxidase (PPO) activity was measured by the method of Gonzalez *et al.* (1991). Catalase (CAT) activity was assayed following the method described by Chen *et al*. (2000). Ascorbate peroxidase (APX) and oxidase (AO) activities were assayed according to the method of Cao *et al.* (2004) and Maxwell and Bateman (1967), respectively.

Electrophoresis. The protein profiles were characterized and identified by using one-dimensional SDS-PAGE according to the method of Laemmli (1970). The destained gel was analysed by gel documentation system (GDS 8000, California, USA). The band pattern, molecular weights and the relative concentration of each band were analyzed.

Statistics. The significance in variation of healthy and diseased mean was assessed using paired student's t-test at $P \leq 0.05$ (Motulsky & Schouest, 1989).

RESULTS AND DISCUSSION

Gall-forming psyllid created specific pathological symptoms and thereby induced structural changes in the vascular vessels of the infested plants. Bump-shaped galls were formed on the lower sufrace of leaf midribs, petioles and stems of the young *Equalypt* branches (data not shown). Herbiovory by xylem-feeding insect (gall-forming psyllid) cause severe disruption of plant tissues or even death of young infested branches of eucalypt tree. Also, feeding of gall-forming psyllid can elicit a formation of a cavity in the area of the feeding, which results from digestion of xylem vessels in the infested part of the midrib and form a cavity to be a pest insect home (Fig. 1a & b). The cavity is not a schizogenous oil duct characterizing eucalypt leaf blade because it is not surrounded by certain kind of cells (Fig. 1c & d) Rohfritsch (1981) indicated that gall tissues appears to be defensive reactions against wounding and/or presence of a foreign organism. Chararas and Chipoulet (1983) reported that *Asutralian exylophagous* insect lay eggs in *Eucalyptus*

species and penetrate the phloem and xylem, thereby damage xylem by the formation of gallaries and causes digestion of wood. The previous finding is in agreement with our results where herbivory induces structural changes in the xylem cells (Fig. 1a, b, c & d).

Insect herbivory induced a reduction in the levels of Ca^{2+} and Mg^{2+} ion levels of *Eucalyptus* leaves (Table I). Such effect may be attributed to a decrease of ions uptake through the reduction of leaf area since herbivores were indicated to reduce leaf area or may be due to the digestion of xylem and/or the feeding of insect on xylem sap of host plant (Orcutt & Nilsen, 2000). Wu *et al.* (2004) similarly reported that *Nilaparvata lugens* infestation reduces the nutrient uptake of rice roots. Elicitors may modulate ion channels and thus the external signal converted into cellular response (Creelman & Mullet, 1997).

Chlorosis of *Eucalyptus* leaves results from insect inducing vascular disease. This may be explained by the significant reduction in chlorophyll a and b levels as well as carotenoids observed in the infested leaves as compared with the healthy ones (Table I). The decrease in the photosynthetic pigments may be attributed to the inhibition of pigments bio-synthesis, which may results from the alteration in water and minerals transport through the damaged xylem vessels or to the effect of reactive oxygen species on these pigments (Stacey & Keen, 1996). Wang *et al*. (2004) reported that the chlorotic symptoms observed on insect infested wheat may be elicited by unbalanced chlorophyll bio-synthesis and degradation.

The levels of total soluble carbohydrates, polysaccharides, free amino acids and the total soluble proteins of infested leaves were lower than those of the healthy ones (Table II). Restricted nutrient and reduced chlorophyll level may affect the metabolic activities of the leaves and thus resulted in reduced metabolites observed here. Drain of assimilates towards the insect away from the other plant parts may contribute to such metabolites reduction (Miles, 1989). Free proline content of the diseased leaves was greater than that of healthy leaves (Table II). This might be an indicator for biogenic stress experienced by *Eucalyptus* plants. Proline is reported to be a universal osmolyte accumulated in resposne to several stresses (Öncel *et al*., 1996), which may have a role in plant defense reactions (Kuznetsov & Shevyakova, 1997).

Phytohormones may play an important role in regulating the response of plants to environmental stresses, including herbivory by insects (Mapes & Davies, 2001). The level of endogenous auxins in the infested *Eucalyptus* leaves was much higher than that in the healthy ones (Table II). IAA probably involved in the growth of galls. The increase in free IAA level might be brought about by insect's feeding activity (Miles, 1989). A high concentration of ABA was obtained in the infested leaves compared with that of healthy ones (Table II). The high level of ABA in diseased leaves was evident that this tree subjected to biotic stress (Hopkin & Hüner, 2004). On the other hand, the amount of gibberellic acid and zeatin in the diseased leaves was reduced relative to the healthy ones (Table II). The reduction in both plant hormones may be related to the decrease in their bio-synthesis or increase in their breakdown or conjugation (Hopkin & Hüner, 2004). Similar results were reached by Wu *et al.* (2004) who reported that zeatin riboside content was decreased in rice leaves as a result of the infestation of rice plants.

Secondary metabolites such as tannins phenolics, and lignin has been implicated in defense against herbivore

Table I. Changes in element and pigment contents of eucalypt leaves subjected to biotic stress elicited by gall-forming psyllid. Each value is the mean of three different replicates ± SE

Element (g/100g DW)	Healthy leaves	Galled leaves
Calcium	1.4 ± 0.04	1.10±0.02
Potassium	0.87±0.03	1.05±0.06
Magnesium	1.27±0.09	1.03±0.10
Pigment (µg/g FW)		
Chlorophyll a	14.3±0.17	9.40±0.115
Chlorophyll b	14.65±0.03	7.30±0.17
Carotenoids	2.83±1.73	1.87±0.84
Chlorophyll a/b	0.976±0.7	1.288±1.15

Table II. Changes in the hormones and some metabolite contents of eucalypt leaves subjected to biotic stress elicited by gall-forming psyllid. Each value is a mean of three different replicates ± SE

Hormone (µg/100g FW)	Healthy leaves	Galled leaves
IAA	12.5±1.4	21.1±0.9
Zeatin	4.5±0.72	4.12±0.31
GA_3	617.7±3.2	498.3±2.1
ABA	158.4±1.7	178.4±0.9
Metabolite		
Total soluble carbohydrates (mg/g FW)	568.8±1.5	560.1±1.2
Polysaccharides (mg /g FW)	103.1±0.64	80.9±0.71
Amino acids (mg/g FW)	4.5 ± 0.3	3.0±0.91
Proline (µmol/g FW)	0.51 ±0.2	0.91±0.27
Total soluble protein (mg/g FW)	2.0±0.89	1.75±0.61

Table III. Changes in phenols, tannins and legnin contents of eucalypt leaves subjected to biotic stress elicited by gall-forming psyllid. Each value is the mean of three different replicates ± SE

Parameter (µg/100g FW)	Healthy leaves	Galled leaves
Quinones	87.8± 0.03	97.0± 0.24
Salicylic acid	125.5 ± 1.2	145.5 ± 0.91
Cinnamic acid	82.1 ± 0.15	53.8 ± 0.18
Caffeic acid	3.6 ± 0.03	4.6 ± 0.03
Feurulic acid	0.5 ± 0.11	0.8 ± 0.02
Vaniline	4.02 ± 0.21	4.8 ± 0.53
Total phenol (µg/100 g FW)	680.9 ± 2.9	704.0 ± 3.2
Tannins (µg/g FW)	176.4 ± 2.1	197.4 ± 1.3
Lignin (g/100 g DW)	26 ± 0.9	18 ± 1.1

Table VI. Changes in the specific activities of antioxidant enzymes, antioxidant substances and lipid peroxidation (measured as malondialdehyde) of eucalypt leaves subjected to biotic stress elicited by gall- forming psyllid. Each value is a mean of three different replicates ± SE

Enzyme activity (µmol/100g FW) and antioxidants	Healthy leaves	Galled leaves
Superoxide dismutase	19.3±2.1	21.8±1.2
Catalase	83.0±1.70	92.0±1.15
Ascorbic acid oxidase	14.5±0.29	20.6±0.35
Polyphenol oxidase	1.12±0.13	1.47±0.91
Ascorbic acid peroxidase	14.3±0.38	19.3±0.8
Polyphenol peroxidase	1.04±0.14	0.7±0.1
Ascorbic acid (mg/g FW)	7.14±1.9	13.3±0.15
Glutathione (GSH) (mg/gFW)	3155±2.9	2711±6.3
Malondialdehyde(µmol/gFW)	3.5±1.1	4.7±1.4

(Orcutt & Nilsen, 2000). Table III showed marked accumulation of the total phenols and tannins in the galled leaves. Fractionation of phenolic acids revealed that salicylic, coumaric, caffeic, feurulic acids and vaniline were accumulated in the infested leaves compared with healthy ones. However, the level of cinnamic acid decreased markedly in the infested leaves (Table III). Similar results have been obtained by Gutterman and Chauser-Volfson (2000) who reported increased phenols in Aloe leaves. Moreover, the increase in the salicylic acid might be involved in the protection of eucalypt tree from the reactive oxygen species and activate some antioxidant enzymes (Kawano & Muto, 2000; Kang, *et al*., 2003). Phenols have both allelochemical and antiherbivore action (Ocrutt & Nilsen, 2000).

Tannins scavenge oxygen free radicals and hence may minimize oxidative damage and thus they are antiherbivory agent (Ayers *et al*., 1997). Tannins also bind to membranes and serve to moderate membrane morphology and permeability (Johnson *et al*., 1986). This may be the case for increased level of tannins in the galled leaves observed in this study (Table III).

The percentage of lignin in infested eucalypt leaves was markedly decreased compared with uninfested leaves (Table III). This may be related to the decrease in the carbohydrate content (Table I), the decrease in polyphenol peroxidase activity (Table IV) and/or accumulation of phenols (Table III). Lignin synthesis has been reported to be via polyphenol peroxidase (Appel, 1993). Digestion of lignin by insect saliva during feeding process might contribute the decreased lignin.

Insect herbivory of *Eucalyptus* exerted a significant increase in lipid peroxidation, measured as malondialdehyde, compared with healthy ones (Table IV). Biotic and a biotic stresses stimulate the production of active oxygen and subsequently lipid peroxidation of the cell macromolecules (Baker & Orlandi, 1996). The increase in lipid peroxidation may be due to the un-capability of antioxidants to cupture all the active oxygen species produced by this biotic stress.

Infested eucalypt trees had high activities of superoxide dismutase, catalase, polyphenol oxidase, ascorbate oxidase and ascorbate peroxidase compared with their respective controls (Table IV). The enhanced activities of these enzymes may increase the scavenging capacity for oxygen free radicals resulted from stress injury (Elstner *et al.* 1994; Baker & Orland, 1996; Ni *et al*., 2001). However,

Fig. 1. Free hand transvere section of healthy (a) and galled (b) leaves showing the structural changes in the vascular tissues and cavities (d) formed in the midrib of eucalypt leaf.

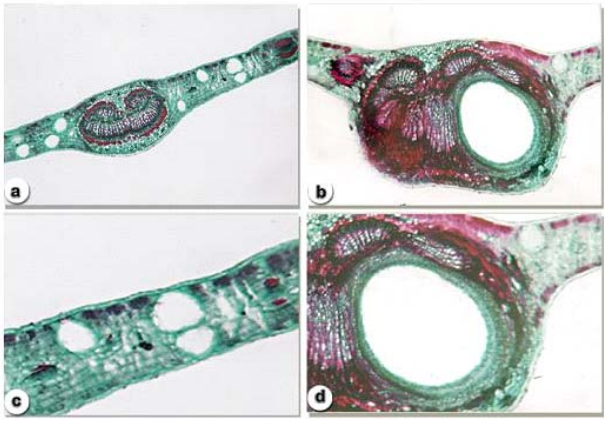
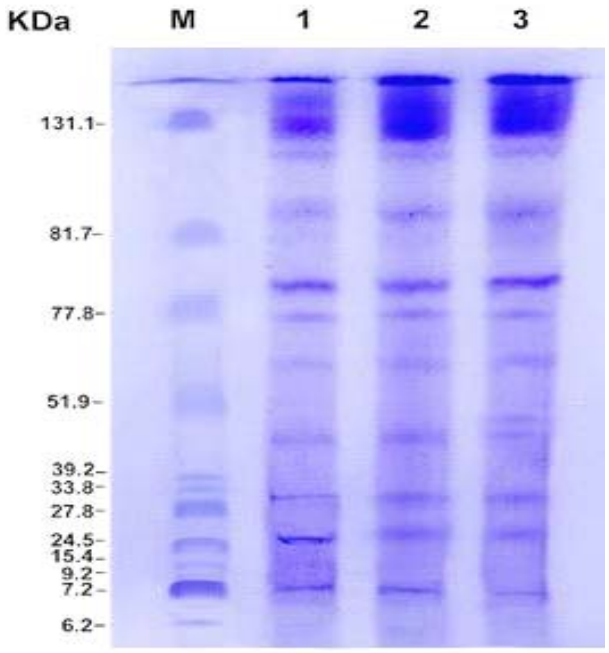


Fig. 2. Protein-banding pattern of healthy and galled eucalypt leaves. Lane M protein markers. Lane 1, ungalled leaves of healthy tree. Lane 2, ungalled leaves of diseased tree. Lane 3, galled leave of diseased tree.



no significant differences in polyphenol peroxidase activity was obtained in healthy and infested leaves (Table IV), which may point to the absence of association between this enzyme and resistant to oxidative damage. Similar result was reported by Chaman *et al*. (2001) who reported a decrease in peroxidase activity in 16-d-old barley infected by aphid.

Stressed eucalypt leaves showed a significant increase in the level of ascorbate and a decrease in that of the glutathione (Table IV). Ascorbate may, therefore, play a role in antioxidant defense (Smirnooff *et al*., 2001).

Infested eucalypt leaves exhibit decrease in the total soluble protein (Table II), which might be attributed to the decrease in protein synthesis. Singla and Grover (1994) recorded that the rate of protein synthesis declines during stress condition. Qualitative differences were observed in polypeptide patterns of galled leaves relative to healthy ones (Fig. 2). There were 12 polypeptides detected in both healthy leaves while 13 polypeptides were shown in the infested galled leaves. A new polypeptide with molecular weight 47 kDa, were detected in the galled leaves. This polypeptide may be related to dehydrine (40-45 kDa). However, dehydrines may interact with compatible solutes to serve as structural stabilizers of macromolecules under water deficit (Han *et al*., 1997). Our results were in agreement with those obtained by Rafi *et al.* (1996) and Jerez (1998) who reported changes in plant protein profiles in resistant plants after insect feeding. However, no differences in protein profiles were observed between chinch bug-infested and uninfested buffalograss (Heng–Moss *et al.*, 2004).

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(Received 20 August 2005; Accepted 09 October 2005)