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Secondary Metabolite Changes in Pecan (*Carya illinoensis*) Tissue Damaged by *Euplatypus segnis* Chapuis and Associated Fungi

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Abstract: Problem statement: The borer insect *Euplatypus segnis* Chapuis is associated to *Fusarium solani*, *Fusarium oxysporum*, *Alternariaalternata* and *Botryodiplodia theobromae* fungi which produce regressive death in pecan (*Carya illinoensis*) trees. In the Mexico northern have been reported loses estimatesof more than 20% in production by the combination of insects and phyto-pathogen organisms. It also has been observed that some trees can survive with or without chemical treatment. **Approach:** The aim of present study was to determine the biochemical changes (contained of nitrogen, crude protein, terpen, condensed and hydrolyzable tannins, cellulose, lignin and silicium) in tissue of pecan trees cv. Western colonized by Ambrosia Borer (*Euplatypus segnis* Chapuis) and associated fungi complex. Three damaged trees and three healthy trees were sampled in three different plantations in each one of three Coahuila State localities. The responses variables were analyzed under a nested design, Tukey s test ($p<0.05$) was used to compare mean treatment differences. **Results:** Dataindicated that content of: terpenes, hydrolyzables tannins, cellulose, lignin and silicium increased significantly in the damaged trees in comparison to healthy trees. **Conclusion:** This results allowed inferring that these components increased as a chemical defense answer to insect invasion and to the enzymatic action of the associated phyto-pathogenic fungi.

Key words: Characterization, identification, borer insect, *Carya illinoensis*

INTRODUCTION

The Coahuila State Southern area (Parras, General Cepeda and Torreoncounties), are harvested 19,345 tons of pecan nut per year in a population of 525.060 trees. In the 20% of the pecan trees planting area has been reported the attack of ambrosia borer *Euplatypus segnis*, which causes a loss in production of 773.82 tons. This insect is associated to *F. solani, F. oxysporum*, *A. alternata* and *B. theobromae*, which are plant pathogenic fungi to pecan trees cv. Western variety under greenhouse conditions. The observed symptoms in colonized field trees are; sawdust in entry holes, brilliant reddish to brown dark and pungentliquid excretions. Removing the tree cortexare distinguished 2 mm-diameter holes and necrotic spots with different colors with a diamond tip form. In addition, other signs have been reported like turgidityloss, yellowing, leaf drop, partial and total death of branches and trees in relatively short periods (three months to a year); however, it is important to mention that some of them may survive without any treatment. This suggests that some biochemical changes are occurring as treedefense mechanisms and these changes may be structural or constitutive and are induced by the presence of pathogens (Vivanco *et al*., 2005). This kind of biochemical mechanisms of defense against insect attack and pathogen infection has been reported insome conifers (Berryman, 1972; Christiansen and Fjone, 1993). Although, terpenes are most commonly associated with conifer species, have also been detected in other plants, including

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angiosperms (Zwenger and Basu, 2008). Eyles *et al.* (2003) reported an increase of polyphenolic compounds including hydrolysable tannins, proanthocyanidins (condensed tannins), flavonoids, glycosides in *Eucalyptus globulus* (Labill) and *Eucalyptus nitens* (Maiden) xylem after artificially injury and indicated that the diverse range of secondary metabolites found in the wound wood is a response to repairthe damaged tissue. The fungal attack on the walls cell cellulose reduces flexibility and endurance, while lignin degradation affects wood compressive strength, promoting a significant loss of this resistance and occurs even before the wood rot is detected (Murace *et al.* 2006). Moreover, there are not reports that cellulose has a connection with plant defense against pathogens, but the data suggesteda relationship. Wainhouse *et al*. (1998) confirmed the importance of lignin as a tree defense mechanical barrier, because when the borer insect *Dendroctonus micans*attacked Norway spruces, it was observed a negative relationship between lignin amount and adult gallery size. Also, some biomolecules such as aldehydes, terpenes, esters monoterpenoids, cyanohydrin esters, cyanohydrins, sesquiterpenes, essential oils, furans, alkaloids and phenolic compounds (tannins) are present naturally in plants and have shown insecticidal activity or phytotoxicity during pest control (Vazquez-Luna *et al*., 2007). The aim of this study was to determine the biochemical changes occurring in Pecan tree tissue damaged by the ambrosia borer *Euplatypus segnis* and the associated fungi.

MATERIALS AND METHODS

Diseased and healthy pecan tree tissues were collected in three different Coahuila state locations, Parras de la Fuente (25° 22’N 102°11’W 1520 m), General Cepeda (25° 22’N 101° 28 ‘W 1470 m) and Torreón (25° 42 ‘N 103° 27’W 1120 m). Three orchards were sampled by location and in each orchard, three healthy trees and three wounded trees were sampled. 20 years old-wounded trees were chosen if they were in the of phase three of damage (50% damaged leaf area, from 25-50 inlet and presence of sawdust at the stem base). Healthy and damaged wood samples were taken for the chemical analysis of each pecan tree, in this case the samples were obtained from a wood portion cut to 0.30 and 2.60 m from soil in accordance to the TAPPI T 257-I 76 Norm. After each portion was cut in 2 cm size slices. Wood was obtained from the center and extreme slice terminals (lower and upper) to perform the biochemical determinations, including nitrogen, crude protein, terpenes and hydrolysable tannin, cellulose, lignin and silicium. The samples were transported in black polyethylene bags of 20 kilograms of capacity in order to avoid dehydration and the dispersion of insects (larvae and adults). Subsequently, the slices are peeled and dried in a forced circulation oven at 60°C. Slices are splintered and grounding in a Wiley type miller and passed through a No. 40 mesh (0.42 mm) for 48 h, immediately, the powder was stored at room temperature in opaque containers to avoid direct exposure to light (Salazar and Perez, 1998). Extraction of polyphenols. In Erlenmeyer flasks (1000 mL^{-1}) were added 20 mL^{-1} of acetone 70% (ratio 1:4) and 5 g of powdered samples of each healthy and diseased wood. The flasks were covered with aluminum foil to avoid light exposure and passing a reflux system at a temperature of 60°C for 12 h. After the reflux, the solution was filtered in tulle fabric, the recovered extract was centrifuged at 3500 rpm for 10 min. The solvent was removed with a rotary evaporator (Yamato res540) at a temperature of 60°C, avoiding exposure to light.

Quantification of hydrolysable tannins. A standard curve was determinated using a solution of gallic acid at concentration 0.4 gL^{-1}. Five test tubes were labeled 0 to 4 and subsequently 0, 0.1, 0.2, 0.3 and 0.4 mL^{-1} of standard solution and 0.4, 0.3, 0.2, 0.1 and 0 mL^{-1} distilled water were added to each tube respectively. Next, in others tubes was added 400 uL of the extract samples recovered from healthy and diseased wood diluted to 1:100. The tubes with gallic acid and sample were added with 400 microliters of Folin Ciocalteu commercial reagent, so was stirred and left stand for 5 min. Then, were added 400 uL of sodium carbonate (0.01 m), each tube was stirred and let stand for 5 min. Subsequently, were added 2 mL^{-1} of distilled water and the tubes were immediately read in anUV/Visible spectrophotometer (Thermo Spectronic, Biomate3) at 725 nm to determine phenols. The readings obtained from the samples were calculated using the follow equation $Y = 5.235\times-0.0256$, obtained from the calibration curve (R^2 =9991). The assay was performed in triplicate.

Quantification of condensed tannins.Astandard curve was determinate using a standard solution of catechin at a concentration 1 g L^{-1}. Five test tubes were labeled 0 to 4, then were deposited at each tube 0, 0.25, 0.50, 0.75 and 1 mL^{-1} of standard solution, respectively and 1.0, 0.75, 0.50, 0.25 and 0 mL^{-1} of distilled water. On the other hand, 500 uL of each diluted 1:100 sample was added in a test tube (16×150). All tubes contented catechin and samples was added 3 mL^{-1} of HC1-butanol 10% and 100 uL of ferric reagent. These tubes were sealed to prevent evaporation of the HC1-butanol.

After that,the tubes were placed in a boiling water bath at 100°C for 1 h, subsequently the tubes were let to cool andreadings were done in anUV/Visible spectrophotometer (THERMO SPECTRONIC, Biomate3) at 460 nm absorbance. The sample data were calculated using the follow equation Y = 1.6102×-0.329, obtained from the calibration curve (R^2 = 9967). This test was performed in triplicate.

Quantification of nitrogen and crude proteinwere determined using the Kjeldhal method. Digestion was performed with one 5 g sample. In a Kjeldhal flask was placed each sample, next were added 5 glass beads, one selenium mixture tablespoon as catalyst and 30 mL^{-1} concentrated H_2SO_4. Each flask was placed in a digester until change in tone from dark brown to light green was observed. The obtained digested solution was distillated, in a flask (500 mL^{-1}) were placed the digested solution and added 300 mL^{-1} distilled H_20, 5 zinc granules, 110 mL^{-1} NaOH 45%, 50 mL^{-1} of H_3BO_3-4% and plus 5 drops mixed indicator, it was distilled to recover 300 mL^{-1}. The degree took place at burette with H_2SO_4 at 0.11173N. The quantification of nitrogen was obtained from the wet organic matter digestion and ammonia produced from nitrogenwas quantified. Whereas, total nitrogen from protein was estimated in percentage. To quantify the crude protein was multiplied N_2 percentage times the 6.25 protein factor.

Quantification of Terpenes by the Soxhlet Method with Hexane. We used 2 g of healthy or diseased wood per sample. Each sample was placed in a ground-necked flasks pre-weighed on electric blankets adding hexane. The extraction was performed for 12 h and afterthe solvent was removed in a crucible furnace and bell-shaped drying. Each flask was weighed and by difference between the initial and final weights the hexane extract percentage was calculated, corresponding to the content of terpenes per sample (Munoz-Concha *et al*., 2004). Quantification of lignin, cellulose and silicium by the permanganate method. From each sample was weight 1 g of each healthy and damaged wood ground sample, in a 600 mL^{-1} Berzelius glass were added 200 mL^{-1} of acid detergent solution and 2 mL^{-1} of Decahydronaphthalene and then heated to boiling over grills for 1 h. The solution was filtered through a crucible vacuum on tare porous layer. The solid residue was washed twice with hot distilled water, immediately washed again with 50 mL^{-1} of acetone until colorless, removes the residual solvent and then was placed in a drying oven at 105°C for 12 h, then samples were placed in a drying hood for 30 min to obtain the Acid Detergent Fiber (ADF), which was placed on a petri dish with cold water andthen was added 25 mL^{-1} of potassium permanganate solution at least five times for 90 min. permanganate solution was removed after filtration using a vacuum funnel. The crucibles were placed on Petri dishes and demineralizer solution was added to half the volume of the pot, letting the solution sit for 5 min; The process was repeated twice, until the whitish residue, it quickly washed twice with 70% alcohol and dried crucibles at 105°C, leaving to cool in a desiccator to obtain the weight of lignin. The samples for the determination of lignin was burned in a furnace at 570°C, leaving to cool in a desiccator to obtain the weight of cellulose, which in turn was added to 48% boric acid and washed with acetone, were burned in muffle at 570°C, give yourself cool in letting marker to obtain the weight of silicium (Van Soest and Wine, 1968).

Statistical analysis the data were analyzed under a nested completely randomized design, where the orchards are nested in localities, the repetitions were nesting in localities by orchand, the physiological state by localities and physiological state in orchand nestled in localities. ANOVA was performed and means comparison was done using Tukey's test $p<0.05$ (SAS V8).

RESULTS

Chemical analysis from healthy and damaged pecan wood among localities indicated non significant difference for nitrogen, crude protein, terpenes, condensed tannins and silicium content (Table 1). Meanwhile there are significant differences for Hydrolysable Tannin (TH) concentration, cellulose and lignin.

This differences in TH content was higher in Torreon (1.02me/cat), followed by General Cepeda and Parras localities respectively. Cellulose content was higher in Torreon and Parras in relation to General Cepeda. The lignin content (Table 1) was statistically higher in the General Cepeda locality (15.49%) than in Parras locality (13.64%).

Table 1: Biochemical composition of Pecan trees sampled in three different Coahuila state locations

Localities	N (%)	Pc (%)	T (%)	TC (me/ac. gal)	TH (me/cat)	C (%)	L (%)	S (%)
Torreón	0.55484a	3.4678a	3.8928a	0.81619a	1.01965a	62.142a	14.8311ab	1.14714a
Parras	0.52610a	3.2881a	4.9817a	0.82553a	0.87363c	61.639a	13.6483b	1.17007a
G. Cepeda	0.55537a	3.4711a	4.2272a	0.79769a	0.97114b	57.102b	15.4994a	1.17108a

Means with different letters in the same column are different ($p<0.05$) N = Nitrogen, Pc = Crude Protein, T = Terpenes, CT = Condensed Tannins, TH = Hydrolysable Tannins, C = Cellulose, L =Lignin and S = Silicium

Table 2: Biochemical composition of diseased and healthy walnut Pecan trees sampled in three different localities in the state of Coahuila

Physiological state	N (%)	Pc (%)	T (%)	TC (me/ac. gal)	TH me/cat)	C (%)	L (%)	S (%)
Healthy	0.5414 a	3.384 a	2.512 b	0.898 a	0.847 b	58.808 b	10.124 b	0.784 b
Damaged	0.5494 a	3.434 a	6.222 a	0.727 b	1.062 a	61.781 a	19.194 a	1.540 a

Means with different letters in the same column are different ($p<0.05$). N = nitrogen and PC = Crude Protein

In Table 2 is shown the resultdifferences on chemical composition between diseased and healthy trees, where it is observed significant differences for tannins concentration, cellulose, lignin and silicium content. The content of chemical compounds was higher in damaged wood by the complex Ambrosia Borer (*Euplatypus segnis* Chapuis) and associated fungi, except for condensed tannins, which was higher in healthy trees.

DISCUSSION

The null differences between ochands from different localities for nitrogen, crude protein, terpenes, condensed tannins and silicium content, may be due to the same agro-ecological management: such as improving soil quality by incorporating organic matter and fertilization management.

Chemical composition of diseased and healthy trees indicated that Nitrogen (N) concentration was not significant, this results suggests a similar fertility level in the sampled orchands. In this sense, fertilization practices can have indirect effects on plant resistance or susceptibility to insect and diseases, changing the treenutrients composition. On the other hand, the total nitrogen has been considered a critical nutritional factor that modifies the abundance and behavior of insects (Mattson, 1980). The crude Protein Content (PC) in healthy trees (3.38%) and damaged trees (3.43%) was statistically similar. Torres *et al*. (2005) mentioned that different rainfall periods increases significantly the content and production of protein in the available biomass (% PC /kg h^{-1}) and not available; The protein-based chemical defenses in trees include enzymes such as chitinases and glucanases that can degrade components of invading organisms. The enzyme as inhibitors interfere with the microrganism ability to use as food resources the invaded tissue. Other inducible enzymes such as peroxidases and laccases can induce harder cell walls by promoting lignification, which directly affect the invader microorganism. Protein-based defenses can be highly specific to a particular organism, but only a small subset of them can be regulated during the attack of a specific pathogenic fungus (Hietala *et al*., 2004; Nagy *et al*., 2004).

Nitrogen and crude protein content may be havenot an associated response to plant defense to pest attack, also, there are association between the reporter chemical compounds with effect in plant defense such terpenes, tannins, lignin, cellulose and silicium. Terpenes content increased significantly in damaged pecan trees attacked by the borer-fungal complex as occurring in species of conifers (Zwenger and Basu, 2008). Trapp and Croteau (2001) found that terpene content increases as a host response to insects when they drilled the trees. Terpenes have also been reported that can inhibit development of pathogens (Klepzig *et al*., 1996). Although, there is an induced response to insects invasion, the biosynthesis of terpenes takes time and occurs after the borers have been transported to the wood, fungal hyphae and the pathogen is established, the distribution of terpenes can vary within a species (Semiz *et al*., 2007). Thompson *et al*. (2006) found in tree samples, high terpenes concentrations in the xylem and low in the phloem, as moderate levels in the sapwood. Kessler and Baldwin (2001) demonstrated that herbivorous insects can induce the emission of plant terpenes and also make the plant emits signals to attract predatory species. These experiments provide not only evidence of the powerful role of terpenes forplant defense, but also provide a model of co-evolution between plants, mites and insects.

In this study,condensed tanninsconcentration was lower in diseased than healthy trees. Alonso *et al*. (2001) reports that in *Calluna vulgaris* plants nitrogen concentrations increase, promote a reduction in total phenolics, condensed tannin levels and a increase of hydrolysable tannins in diseased wood in relation to healthy wood, these changes may be a response of plants to insect-pathogen complex attack, since tannic compounds play a significant role in the resistance mechanisms to pathogenic organisms due to its antifungal properties (Singh and Kim, 1997; Morita *et al*., 2001). Eyles *et al*. (2003) analyzed anatomically and chemically the wood produce by xylem artificially injured *Eucalyptus globulus* (Labill) and *Eucalyptus nitens* (Maiden) trees, theses authors found an increase in polyphenolic compounds including hydrolysable tannins, tannins condensate, glycosides and flavonoids and mentioned that the diverse range of secondary metabolites found in the wound wood is a repair response to pest attack. Tannins have been reported to have an important role in the plant defense mechanisms against insects, decay fungi or allelopathic agent; tanins react quickly with other biomolecules forming complex products with proteins (structural and catalytic), starch, pectin substances and cellulose. By this way, the attack by fungi or bacteria metabolism can be inactivated or substantially decreased (Laredo, 1996). Some of these

bioactive compounds such as tannins and terpenes play an important role in natural defense mechanisms of fruits and vegetables (Cowan, 1999; Howard *et al*., 2000; Lombardi-Boccia *et al*., 2004).

Cellulose content increasedin a significant way in diseased wood in comparison to healthy wood; the cellulose polysaccharide is in greater proportion in the wood and is the most important substance produced by trees and the main cell wall component (Godoy *et al*., 2007). Cellulose has different physical and mechanical properties, giving plant strength and toughness (Chowa *et al*., 2008). Fungal attack tocell wall cellulose reduces tree flexibility, whereas lignin degradation affects compressive strength of wood, a significant loss of this resistance occurs before the rot is detected in the wood (Murace *et al*., 2006), there are no reports that cellulose has a relationship with plant defense against pathogens, but the data suggest an association.

Lignin concentration was higher in damaged pecan trees by insect attack. Wainhouse *et al*. (1990) mentioned that the increase of lignin content has an negative effect on wood boring insects, affecting larval survival, insect weight andgrowth,there are a reduction of tunnels diameter and they are deformed, adults oviposit less on trees with high lignin content. Lignification may be a constitutive feature in some species, but may also occur as a process of strengthening the tissues when are subjected to physical damage and manifested during plant defense as local accumulation of large quantities of lignin in affected tissues. Lignin is produced by the enzymatic binding of phenylpropanoid units forming long polymers that confer impermeability and mechanical strength; in addition, lignin is resistant to degradation by many pathogens (Nicholson and Hammerschmidt, 1992). Wainhouse *et al*. (1998) confirm the importance of lignin as a mechanical barrier developed by trees, because when the borer *Dendroctonus micans* was established in Norway spruce, there had a negative relationship between the amount of lignin and gallery size, the galleries werebigger in trees with a low lignin concentration. When *Ascocalyx abietina* infected naturally Norway spruce wood, phenolic compounds and lignin were accumulated in the cell wall Cvikrova *et al*. (2006). When *Pinus banksiana* was attacked by the fungus *Gremmeniella abietina*, the stem bark, phloem cells and vascular cambiumwere invaded, in response, the treeaffected tissues produced ligno-suberized tissues confined the pathogen within the affected area; this is the first demonstration about anatomical mechanism of defense in conifer (Simard *et al*., 2001). Genetically engineered trees with low lignin increases the destruction of forests and ways of life and are more susceptible not only to storm damage, but also to attacks by insects, fungi and bacteria (Frankenhuyzen and Beardmore, 2004).

In this study was found that silicium increases significantly in diseased wood compared to healthy wood. Silicium forms insoluble (phytoliths) and soluble aggregates (orthosilicic acid polymers), intertwined with cellulose and cell wall components, making them strong and flexible, thus protecting plant tissue against water, air and microorganisms action (Rodrigues and Datnoff, 2005). Vegetable physiologists consider silicium as an essential element for plants, however, has been reported that the presence of silicium benefit crops, by induction of resistance and protection against various biotic and abiotic factors attributed in part to the accumulation and polymerization in the cell walls, which constitutes a mechanical barrier against attack by pathogens and insects (Epstein, 1999). Silicium activates a series of defensive genes in rice, wheat and maize, it is confirmed that some insect predators (Coleoptera: Coccinellidae) feed more on plants with higher levels of silicium, which means that silicium-supplemented plants release volatile compounds that attract natural enemies of pests (Bettiol, 2006). This leads, consequently, a decrease in the preference of insects for plants (Gomes *et al*., 2005). There are other reports that found that some biomolecules such as aldehydes, terpenes, esters monoterpenoids, cyanohydrin esters, cyanohydrins, sesquiterpenes, essential oils, furans, alkaloids and phenolic compounds (tannins) occurring naturally in plants and tested in different bioassays had showed phytotoxic insecticidal activity (Vazquez-Luna *et al*., 2007).

CONCLUSION

Terpenes, hydrolysable tannins, cellulose, lignin and siliciumconcentration significantly increased in diseased trees, it can be inferred that the increase of these components is a biochemical defense response of pecan to insect invasion and to the enzymatic action of the fungal pathogens associated. The results confirm that activation of plant defense has a complex base that depends on the coordinated expression of a set of defense mechanisms. These mechanisms respond to the expression or repression of genes where the products participate in different metabolic pathways involved in plant defense.

These genes, called defense genes, form the basis of horizontal or polygenic resistance known by plant pathologists and plant breeders. The defense mechanisms of horizontal resistance, involves the production of phytoalexins, secondary metabolites, PR

proteins, lignin deposition, hypersensitive reaction or SAR, which are responsible for acting against pathogen. In other words, plants defense is largely polygenic. On the other hand, the R genes form the basis of vertical resistance or monogenic or oligogenic. Despite what its name suggests, the R genes are not directly responsible for resistance, but also act as signal receivers (Avr proteins) that originate from the pathogen. This leads to the activation of different signaling cascades that ultimately initiate the expression of genes responsible for the defense mechanisms.

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