

# Isolation and pathogenicity of fungi associated to ambrosia borer (*Euplatypus segnis*) found injuring pecan (*Carya illinoensis*) wood

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## ABSTRACT

*Euplatypus segnis* is an insect pest of economic importance in pecan (*Carya illinoensis*) trees grown at Parras, General Cepeda and Torreón Coahuila, Mexico. The objectives in this study were to identify the fungal strains associated to ambrosia borer body and diseased pecan wood and determine their pathogenicity. The results showed that the associated fungi to *Euplatypus segnis* and damaging the pecan wood were identified as: *Helminthosporium sp.*, *Aspergillus sp.*, *Penicillium sp.*, *Phoma sp.*, *Ascochyta sp.*, *Phaeocylomices sp.*, *Umbeliopsis sp.*, *Torula sp.*, *Fusarium solani*, *Alternaria alternata*, *Fusarium oxysporum*, and *Lasiodiplodia theobromae*. The pathogenicity tests on healthy 3 year old pecan trees cv. western using *Fusarium oxysporum*, *Fusarium solani*, *Alternaria alternata* and *Lasiodiplodia theobromae* suspension conidia shown die back tree branches after 84 days inoculation. The insect in combination with the fungal invasion eventually cause the death of trees. Additionally, the insect contributes to the spread of fungi in pecan nut orchards.

**Keywords:** Pathogenicity; Pecan Nut; *Euplatypus segnis*; Ambrosia Borer; *Carya illinoensis*

## 1. INTRODUCTION

In Mexico, *Euplatypus segnis* also known as trunk and branch borer is distributed in the states of Coahuila, Durango, San Luis Potosi, Jalisco and Chihuahua [1-3]. In the Coahuila Southwest region there are approximately 525 thousand pecan (*Carya illinoensis*) trees on produc-

tion, and annually are harvested about 19,345 tons of nuts. However, in more that 20 percent of the tree plantations the presence of ambrosia borer *Euplatypus segnis* has been reported causing yield losses of up to 4% (773 tons of nuts) [4]. In addition, *E. segnis* (Coleoptera: Platypodinae), attacks wild trees such as: *Bursera copallifera*, *Delonix regia*, *Ficus sp.* *Ficus lyrata*, *Ficus microcarpa*, *Ficus elastica* and *Ficus cotinifolia* [5], fruit trees such as avocado, apple, peach, pomegranate, apricot, and mango, and some forestry trees like silver poplar, blackberry and tropical ash [6]. There are reports on symbiotic associations between ambrosia insects and fungus, this relation help both of them to survive, per example *Xyleborus ferrugineus* and *Lasiodiplodia theobromae* in avocado (*Persea americana* Mill) [7], *Xyleborus ferrugineus* and *Fusarium solani*, *Cephalosporium sp.* and *Graphium sp.* [8], *Ips typographus* and *Ophiostoma polonicum* on *Picea abies* [9], *Hypotenemus hampei* and *Fusarium solani* on Coffee genus trees [10]. *Platypus quercivorus* and *Raffaelea quercivora* on *Quercus crispula* and *Q. serrata* [11], *Hypothenemus hampei* and *Penicillium brocae* on *Coffea arabica* [12], *Platypus mutatus* associated with a symbiotic fungus in commercial plantations of poplar [13]. In this study, fungi associated to ambrosia borer isolated from diseased pecan wood were morphologically and molecularly identified and incidence of diseases induced by these fungal species was determined using pathogenicity tests.

## 2. MATERIALS AND METHODS

### 2.1. Study Area

The sample collections of *E. segnis* specimens and pecan damaged tissues were obtained from Parras de la Fuente (25°22'N 102°11'W 1520 m), General Cepeda

(25°22'N 101°28'W 1470 m) and Torreon (25°42'N 103°27'W 1120 m) all of these counties located in Coahuila, Mexico. Three orchards were sampled by locality and at each orchard were selected three 20-years old damaged trees cv. Western, the damaged trees were in the phase 3 (50% damaged leaf area, 25 to 50 holes entries and presence of sawdust at the stem base) [14]. Wood and insects samples were placed in plastic bags and were properly coded and carried to the entomology and phytopathology laboratories for examination.

## 2.2. Fungal Isolates

The fungi were obtained from xylem, phloem and wood contained entry points and borer insects. The borer insects were sectioning into head, thorax and abdomen. Each section was disinfected used sodium hypochlorite (1.5%) by immersion for 3 min, after those insect sections were washed with sterile distilled water for 1 min and dried on sterile paper towels. Wood and insect sections were placed on Petri dishes containing potato dextrose agar (PDA: 20 g potato, 20 g of dextrose, 18 g agar and 1000 mL distilled water) as a culture medium, placing 4 pieces per plate. The Petri dishes were incubated and a continuous black light lamp 40 W to 25°C ± 1°C was using during the day. The isolates were purified by monospore cultures on water-agar (AA: 18 g agar in 1000 mL distilled water), and increased on PDA. The strains were spread on liquid medium (Pontecorbo) in order to obtain spores and mycelium production for pathogenicity test, and freeze drying and preservation.

## 2.3. Identification of Pathogenic Fungi

### 2.3.1. Fungal Morphological Characterization

After 7 - 10 days, the isolated and purified fungi from wood and insects were observed under a composed microscope and identified by morphology using taxonomic keys for ascomycetes and imperfect fungi, in this case characterization was based on characteristics of isolate reproductive structures using Barnnet and Hunter [15], Hanlin [16], Boot [17], Rotem [18], Neergaard [19], Sutton [20] keys for genus and Wei *et al.* [21] key for species. To identify *Lasiodiplodia theobromae* were used Punithalingam [22], Sutton [20] and Burgess *et al.* [23] keys.

### 2.3.2. Molecular Identification of Isolated Fungi

To confirm the morphological identification of those fungi colonies that showed the highest pathogenicity on pecan trees under field conditions. DNA was isolated from *Fusarium oxysporum*, *Fusarium solani*, *Alternaria alternata* and *Lasiodiplodia theobromae* fungi at the Molecular Biology laboratory, CIIDIR-IPN Oaxaca. The DNA was isolated using the methodology proposed by Ahrens and Seemüller [24]. Amplification of internal

transcribed spaces of ribosomal genes (rRNA) was performed by PCR using the primers ITS4 (TCC TCC GCT TAT TGA TAT GC) and ITS5 (GGA AGT AAA AGT CGT AAC AAG G) according to the methodology proposed by Ahrens and Seemüller [24] with minor modifications, PCR reaction was composed of sterile ultrapure water (13.22 µL), 10X TBE buffer (2.5 µL), MgCl<sub>2</sub> at 2.5 mM (2.08 µL), dNTPs at 0.2 mM (2 µL), primers ITS4 and ITS5 to 20 µmol (2 µL of each), DNA polymerase (biogenic<sup>®</sup>) 1 U (0.2 µL) and 80 ng of DNA sample (1 µL). The amplified product was purified with Wizard kit (Promega<sup>®</sup>) and sequenced with the Genetic Analyzer<sup>®</sup> Model 3100 (Applied Biosystems). The data base sequences with the highest value of similarity in were considered for comparison with the sequences obtained in this study. The ITS sequences obtained were analyzed using the software Lasergene<sup>®</sup> 2001, V5 (DNASTAR<sup>®</sup>, Inc.) and were aligned in the database of the National Gene Bank Center for Biotechnology Information (NCBI), by the BLAST program (Basic Local Alignment Search Tool) (<http://www.ncbi.nlm.nih.gov/BLAST/>).

## 2.4. Pathogenicity Tests

Inoculum was incremented in PDA culture medium which was autoclaved three times continues with an interval of 24 hours among sterilizations. Then were inoculated each of the twelve fungi isolated from pecan damaged wood and borer insect body: *Phoma*, *Fusarium oxysporum*, *Ascochyta*, *Phaeocylomices sp Lasiodiplodia theobromae*, *Alternaria alternata*, *Umbeliopsis sp*, *Torula sp*, *Fusarium solani*, *Helminthosporium sp*, *Aspergillus sp* and *Penicillium sp*. The isolates were multiplied in the culture media described above, then were kept in an incubator at 27°C with a photoperiod of 12:12 and a relative humidity of 50% ± 10%. Once sporulation was observed, the conidia from each isolate were harvested adding to each Petri dish 50 mL of a solution of Tween 80 (0.05%) and sterile distilled water, and removing the mold with a spatula. Subsequently, the mold solution was liquefied for 60 sec and placed in a beaker of 250 mL (stock suspension). Then, were made three successive dilutions of 100 µL of the stock suspension in 900 µL of sterile distilled water and were labeled 10<sup>-1</sup>, 10<sup>-2</sup> and 10<sup>-3</sup>. Finally, spore concentration was determined using a Neubauer's camera and microscope, the dilution samples were observed in 40× to calculate spore concentration of the stock solution which was adjusted to a final concentration of 10<sup>9</sup> conidia/mL.

Pathogenicity tests were performed *in vivo* in twenty eight three years-old healthy pecan trees cv western (height (150 cm) and stem diameter (4 - 5 cm)) established in a high technology greenhouse at a temperature of 24°C ± 2°C, at the Universidad Autonoma Agraria

Antonio Narro located in Saltillo, Coahuila. The inoculation technique used in this step was that proposed by Kuroda [11], briefly; trees bark at a height of 100 cm was sterilize with sodium hypochlorite (1.5%), then washed with sterile distilled water and dried on using sterile absorbent paper. To each tree, two holes were made on the stem until xylem with a sterile 3-mm drill diameter. Twenty four trees were inoculated with 1 mL of the conidial suspension at  $1 \times 10^9$  conidia·mL<sup>-1</sup> of each one of the twelve identified fungi and two trees were inoculated with a mixed suspension of all fungi spores and two trees with sterile distilled water (control). The inoculations were added using a manual sterile pipette, each wound was covered with parafilm to prevent rapid drying and possible contamination by other microorganisms. According to this technique was favored the low density against mass inoculations in the inoculated tree to prevent wilting and rapid reactions to characterize tissues in a cytological way in response to the inoculated fungi activities. The evaluations were done every 7 days, watching symptoms and disease progression for 84 days after inoculation. Later, from the infected tissues of inoculated stems was isolated the fungus in pure culture and compared morphologically with the fungus inoculated to confirm Koch's postulates.

## 2.5. Statistical Analysis

The response variable was the presence of the fungal specie each case, which was evaluated in a quality way (presence and absence). For results analysis a categorical data analysis was performed. In this case Sx2 tables were used, where row and columns were nominally classified. Statistical analysis was performed using SAS (8.1 versions).

## 3. RESULTS

### 3.1. Disease Symptoms in Pecan Plantations under Field Conditions

In 20 years-old pecan trees were observed the characteristic symptoms of regressive dead and insect presence mainly in weakened and/or stressed (because age, water deficiency, injuries, fires, intense cold, snow or prolonged drought) trees. In trunk and branches were detected 2 mm-diameter entry holes and sawdust at the trees base, crystal reddish exudates that eventually became dark brown and pungent, after, taking off the bark, necrotic spots were observed with the typical form of diamond. On the other hand one cans heard a sound in trunks and branches; in addition one can see the sap out of the branches when phloem and xylem are piercing by the borer. Also the presence of adults, especially during morning and evening is detected, chlorosis is observed in

the leaf area, wilting, premature abscission of stems and leaves, partial death of branches and downward total death of trees.

### 3.2. Morphological and Molecular Identification of Plant Pathogenic Fungi Associated with Plant Damage

The morphological characterization based on reproductive structures of the fungal isolates was performed using the taxonomic keys proposed by Barnnet and Hunter [15], Hanlin [16], Boot [17], Rotem [18], Neergaard [19], and Sutton [20] for genus and Wei *et al.* [21] keys were used for the specie identification. *Lasioidiplodia theobromae* was identified using the Punithalingam [22], Sutton [20] and Burgess *et al.* [23] keys. In **Table 1** are shown the fungal species associated to die-back in pecan damaged wood.

The identification to genera and specie level by sequencing of the regions ITS1 and ITS2 of ribosomal genes (rRNA) was performed only to confirm identity of those fungal more common as pathogens. *L. theobromae*

**Table 1.** Genus and species identified in samples of pecan with dieback symptoms and presence of ambrosia insects.

Name	Morphology
<i>Lasioidiplodia theobromae</i>	Hyphae septate, dark 1.5 to 2.8 microns in diameter. Dark-colored pycnidia $493.04 \times 622.82$ microns, with ostiole at the apex, formed both in artificial medium and in plant tissue. Hyaline septate conidia in immature, dark and septate with longitudinal striations in mature state $11.35 \times 26.54$ microns.
<i>Fusarium oxysporum</i>	Microscopically <i>Fusarium oxysporum</i> isolates showed abundant macroconidios, hyaline, oval, elliptical and occasionally kidney-shaped, with one or two septa, which measured $4 - 5 \times 2 - 4$ um, formed in false heads. The thin-walled hyaline macroconidia are just curved (canoe) or nearly straight, pointed at both ends, in the form of basal cell foot. Presented 3 - 5 septa and measure $20 - 59.5 \times 2 - 6$ microns. It has also marked by abundant spherical chlamydo spores, large, hyaline, with smooth or rough walls. Can be seen individually or in pairs at intervals along the hyphae or branched short lateral sections.
<i>Fusarium solani</i> (Mart.) Sacc.	<i>Fusarium solani</i> (Mart.) Sacc. Abundant microconidia hyaline, ovoid, with 0 - 1 septum and occasional 2. Born in monophialides long with collar, a false head, measuring $8 - 16 \times 3 - 5$ microns. Macroconidia slightly curved, with ventral and dorsal plane parallel in most of its length, with 5 - 6 septa, $23 - 67 \times 4 - 6$ um in, slightly curved apical cell and basal cell pedicellate, cream-colored sporodochia at the beginning, later turning blue or blue.
<i>Alternaria alternata</i> (fr.) Keissler	<i>Alternaria alternata</i> (fr.) Keissler presents colony dark green to olive, conidia (dictyosporae) in chains on short conidiophores and branching. The strings appear in dense cultures and isolated as bundles of 1 to 9 transverse septa and several longitudinal or oblique.

genome sequence was compared with the genetic sequences of *L. theobromae* deposited in the gene bank (NCBI) with accession numbers EF622073 (542 bp) and EU564805 (540 bp) in 98% and AY662402.1 in 99%; the *Fusarium solani* sequences was lineated with *Fusarium solani* sequences deposited in gene bank (NCBI) with accession numbers AF440567.1 (480 bp) in 94%, and AY310442.1 (554 bp) in 100%. The *Fusarium oxysporum* was aligned with *F. oxysporum* accession number EF495235.1 with a similarity index of 99%, *Alternaria alternata* gene sequence was compared and showed to be homologous to *A. alternata* with access numbers AF397236.1 with a similarity index of 100%, all these fungal species were confirmed as causal agents of dieback in pecan grown in Coahuila Southeast Region.

### 3.3. Fungi Associated to Insects and Damaged Pecan Trees in the Coahuila Southwest Region

There were isolated 459 fungal strains from insects and diseased pecan wood on artificial culture medium (PDA), distributed as follows: In Parras county were isolated 165 strains, in General Cepeda county 161 strains, and in Torreón county 131 strains. The genera isolated were described according to their morphological structures based on microscopic observations and literature guides.

In the **Table 2** are displayed the statistics for fungi by incidence. The Mantel-Haenszel Chi-Square (Qcs), is valid for these data since fungi is ordinaly scaled, and the response is dichotomous; it indicates that there is a strong association between fungi and incidence (Qcs =

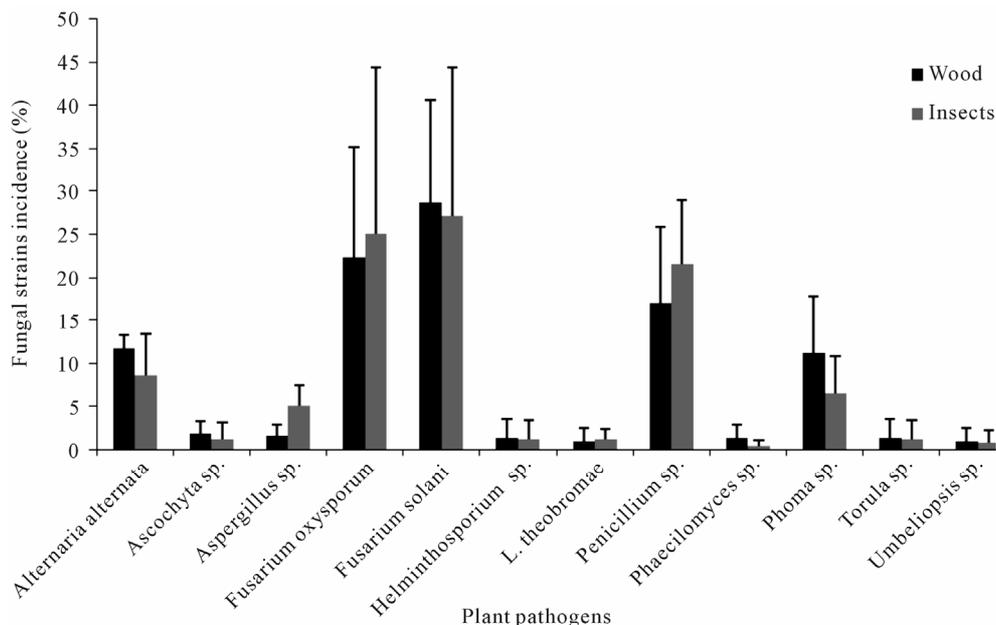
73.53) which is highly significant. By looking at the percentages in the **Figure 1**, we can see that fungi *F. solani* and *F. oxysporum* had the highest frequency of incidence.

In the **Table 3** are displays the Mantel-Haenszel results for the stratified analysis where the strata are all combinations of county and tissue used (insect o wood) is  $Q_{csMH} = 73.4498$ , which is strongly significant, in this case some fungal species were observed only in some counties and a specific tissue (wood or insect) but no in others (**Figures 2** and **3**). The association of fungi and incidence controlling for counties is  $Q_{csMH} = 73.4878$ , which is highly significant. In this case some fungal species appeared only in samples taken in specific counties (**Figures 3** and **4**). On the other hand, the Mantel-Haenszel results for the stratified analysis where the strata are all combinations of tissue used (insect o wood) is  $Q_{csMH} = 73.5103$ , respectively, which is highly significant. In

**Table 2.** Statistics for fungi by incidence.

Statistic	DF	Value	Prob
Chi-Square	11	820.44	<0.0001
Likelihood Ratio Chi-Square	11	770.72	<0.0001
Mantel-Haenzel Chi-Square	1	73.53	<0.0001
Phi Coefficient		0.33	
Contingency Coefficient		0.31	
Cramer's V		0.33	

Sample size = 7202.

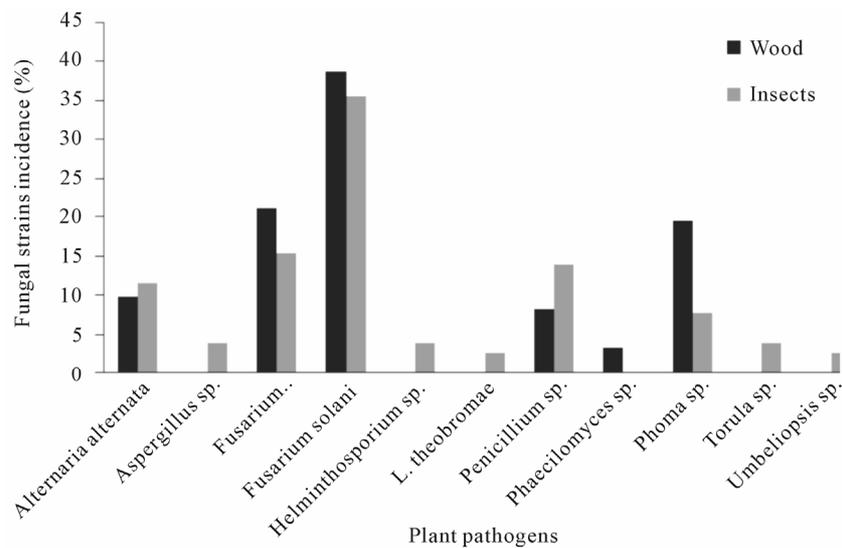


**Figure 1.** Fungi species isolated from the *Euplatypus segnis* collected attacking pecan branches and trunks at three Coahuila counties.

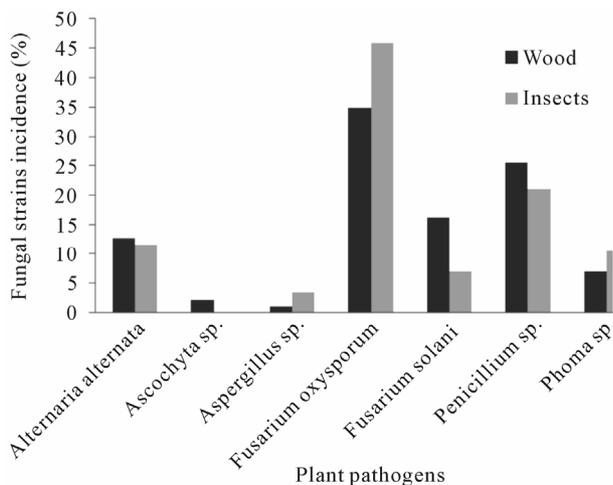
**Table 3.** Statistics for stratified by county and tissue used, stratified by county and stratified by tissue used.

Alternative Hypothesis	DF	Value			Probability
		County and tissue	County	Tissue	
Nonzero Correlation	1	73.4498	73.4878	73.5103	<0.0001
Row mean Scores Differ	11	819.7609	820.1022	820.216	<0.0001
General Associations	11	819.7609	820.1022	820.216	<0.0001

Cochran—Mantel-Haenszel Statistics (Modified Redit Scores).



**Figure 2.** Fungal species found associated with *Euplatypus segnis* and diseased pecan (*Carya illinoensis*) wood collected at Parras Coahuila, Mexico.



**Figure 3.** Fungal species found associated with *Euplatypus segnis* and diseased pecan (*Carya illinoensis*) wood collected at General Cepeda, Coahuila, Mexico.

this case some fungal species appeared only in samples taken in a specific tissue but not in the others (Figure 2). The significant association between incidence and fungal

specie remains evident.  $Q_{GMH}$  and  $Q_{SH}$  are significant too in all the analyses.

Eighty nine fungal strains were isolated from borers insect body (*Euplatypus segnis*) collected in pecan branches and trunks at Parras county, 35% of the isolates belongs to *Fusarium solani*, 15.2% to *Fusarium oxysporum*, 13.9% to *Penicillium*, 11.4% to *Alternaria alternata*, 7.6% to *Phoma sp.*, 3.8% to *Helminthosporium sp.*, *Torula sp.*, *Aspergillus sp.*, and 2.5% to *Lasiodiplodia theobromae* and *Umbelopsis sp.*, (Figure 2), also 76 fungal strains were isolated from diseased pecan wood, 38.7% belongs to *Fusarium solani*, 21% to *Fusarium oxysporum*, 19.4% to *Phoma sp.*, 9.7% to *Alternaria alternata*, 8.1% to *Penicillium sp.*, and 3.2% to *Phaeoacremonium sp.* (Figure 2).

Eighty five fungal strains were isolated from borer insect (*E. segnis*) body collected in pecan branches and trunks at General Cepeda county, these strains were distributed as follow: 45.9% belonging to *Fusarium oxysporum*, 21.2% to *Penicillium*, 11.4% to *Alternaria alternata*, 10.6% to *Phoma sp.*, 7.1% to *Fusarium solani* and 3.5% to *Aspergillus sp.* (Figure 3). On the other

hand 76 fungal strains were isolated from pecan (*C. illinoensis*) diseased wood, distributed as follow 38.7% belongs to *Fusarium oxysporum*, 21.2% to *Penicillium sp.*, 16.3% to *Fusarium solani*, 11.8% to *Alternaria alternata*, 7% to *Phoma sp.*, 2.3% to *Ascochyta sp.* and 1.2% to *Aspergillus sp.* (Figure 3).

From the samples collected in the Torreon county, sixty nine fungal stains were isolated from borer insect (*E. segnis*) body collected attacking pecan branches and trunks. These strain were distributed as follow: 38.5% belongs to *Fusarium solani*, 28.6% to *Penicillium sp.*, 10.3% to *Fusarium oxysporum*, 7.7% to *Aspergillus sp.*, 4.0% to *Helminthosporium sp.* And *Alternaria alternata*, 3.3% to *Ascochyta sp.*, 2.2% to *Phoma sp.* and 1.1% to *Phaeocylomices sp.* and *Lasiodiplodia theobromae* (Figure 4), also were isolated 62 fungal strains from diseased pecan (*C. illinoensis*) wood distributed as follow 34.7% belongs to *Fusarium solani*, 14.7% to *Penicillium sp.*, 9.3% to *Fusarium oxysporum*, 7.7% to *Aspergillus sp.*, 12.0% to *Alternaria*, 9.3% to *Phoma sp.*, 4.0% to *Torula* and *Helminthosporium sp.*, and 2.7% to *Lasiodiplodia theobromae*, *Umbelliopsis sp.*, *Ascochyta sp.* and 1.3% to *Aspergillus sp.* and *Phaeocylomices sp.* (Figure 4).

### 3.4. Pathogenicity of Fungi Strains Isolated from Pecan Trees with Dieback

At 14 days after inoculation were observed the first signs of disease in some trees (Table 4). In trees inoculated with *Fusarium solani* had discharge in the drilling at 21 and 28 days, chlorosis at 42 and 49 days, wilting at 63 and 70 days and dieback after 77 days after inoculation, the inoculated trees with *Fusarium oxysporum*-shown exudates at the inoculation point in the two treatments at 14 and 21 days, and brown leaves at 35 and 42

days, chlorosis at 45 and 56 days, at 63 and 70 days caused wilting and dieback at 77 and 84 days after inoculation. The inoculated trees by *Alternaria alternata* showed chlorosis at 63 days, wilting to 70 days and dieback to 84 days after inoculation. The trees infected with *Lasiodiplodia theobromae* presented leaves chlorosis at 56 days, premature abscission at 70 days, defoliation at 77 days and dieback at 84 days, the other tree shoots inoculated only presented reddish tip at 77 and chlorosis at 84 days; *Torula sp.* Showed brown leaves at 77 days and defoliation at 84 days, the second treatment presented only brown leaves at 84 days, the two treatments behaved similarly. *Phoma sp.*, caused wilting only 70 days to complete the evaluation at 84 days, the trees inoculated with *Phaeocylomices sp.* presented chlorotic symptoms at the end of the evaluation, *Ascochyta sp.*, caused brown leaves at 63 days, wilting from 77 to 84 days, in the second treatment was observed brown leaves at 70 days and defoliation at 84 days; *Helminthosporium sp.*, caused chlorosis at 77 days and wilting at 84 days, the other treatment only showed a slight chlorosis; The treatments that received a mixture of spores showed the most damaged where trees were killed at 77 and 84 days in both treatments, *Penicillium sp.* and *Aspergillus sp.* *Umbelliopsis sp.* and the control treatment did not cause any outward symptoms.

## 4. DISCUSSION

### 4.1. Insect-Fungi Association

*Euplatypus segnis* is is an ambrosia specie with monogenic reproductive behavior [25], polyphagous in its degree of specificity with respect to the host and xylomictophagus by feeding habit, consuming the conidia producing by

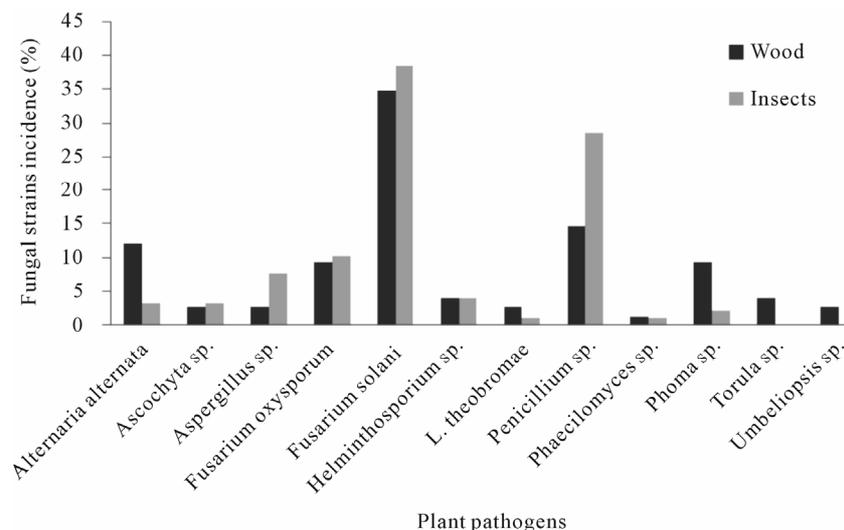


Figure 4. Fungi species found associated with *Euplatypus segnis* and diseased pecan (*Carya illinoensis*) wood collected at Torreon Coahuila, Mexico.

**Table 4.** Development of symptoms and signs in pecan trees inoculated with fungal strains isolated from *E. segnis* and damaged pecan wood.

Inoculated fungi	Symptoms after different days of inoculation												
	0	7	14	21	28	35	42	49	56	63	70	77	84
<i>Helminthosporium sp.</i>	S											C	C, M
<i>Botryodiplodia sp.</i>	S								C		AP	M, Ar	MR
<i>Torula sp.</i>	S											Hc	D
<i>Umbeliopsis sp.</i>	S												NS
<i>Ascochyta sp.</i>	S									Hc	Hc	M	D, M
<i>Phaeocylomices sp.</i>	S												C
<i>Fusarium oxysporum</i>	S		E	E	E	Ar	Ar	C	C	M	M	MR	MR
<i>Fusarium solani</i>	S			E	E			C	C	M	M		MR
<i>Phoma sp.</i>	S										M		M
<i>Alternaria alternata</i>	S									C	M		MR
<i>Penicillium sp.</i>	S												NS
<i>Aspergillus sp.</i>	S												NS
Spore mixture	S							C	C	M	M	MR	MR
Control (Sterile water)	S												NS

S: Health, NS: No symptoms, E: trunk exudates, Ar: reddish tip, Hc: Brown Leaves, C: Chlorosis, M: wilt, AP: Premature Abscission, D: Defoliation. MR: dieback.

the fungi inoculated to the trees [26]. Ulloa [27] mentions that borers have the habit of feeding on fungi introduced by them in the gallery system built into the wood. These fungi appear to be relatively non-specific and need basically moistened wood for their development. Insect species that grow fungus in galleries are called beetles or ambrosia borers and they are dominant in tropical areas [28]. Most of the buildings in the tunnel systems of ragweed borer (galleries) are in the vigorous host tissues, weakened or recently dead, though some species are specialize in colonize the bone marrow, large seeds, fruits and petiole leaves [26,29]. The term ambrosia refers to the fungi that are cultivated by beetles in the gallery walls, which are feed exclusively for insects. Beetles are necessarily dependent on fungi, from which acquired vitamins, amino acids, and essential sterols [30, 31]. The fungi are the main source of food for larvae and adults and are essential to complete insect life cycle [10]. In some insect tribes, only females perform tasks inside the culture, while males are short lived and little flight [32]. After mating, females disperse to new host substrate, carrying fungi in specialized pockets called mycangia. Once inside, the founder females planted the fungi on the walls of tunnels, lay eggs, and serve the growing brood [32]. In a way not understood, the female beetles can control fungal growth and degree of multi-species composition [30,33-35]. If the female dies, the culture is quickly contaminated by other fungi and bacte-

ria, which ultimately leads to the brood death [32,36]. The ambrosia fungi cultured by borers are not pure monocultures, also which consist of a mixture of fungi mycelium, yeasts, and bacteria [37,38]. Norris [39] calls these complex multi-species mixtures. However, recent work has shown that a fungus always dominates in primary cultures of the insect [37,40-42]. In addition, beetles carry only the primary fungus in the mycangia (although sometimes secondary fungi are isolated from the mycangia), females tend to favor the primary fungal culture, which gives them a nutritional benefit [32,41,43, 44], some auxiliary fungi also support beetle development [32]. These observations imply the primary fungus as the primary crop, while fungi, yeasts and bacteria may be secondary "weeds" or with additional auxiliary roles to play in the cultures. The fungus growing transmission among generations of farmer's beetles is transmitted vertically from parent to offspring generations [38,43,45]. Reproductive females acquire original inoculum from their crops, they take with them in specialized pockets during dispersal flights and use this as seed inoculum for their new crops. Ambrosia borers are only associated with a particular kind of primary cultures within a specific geographic region [37,41]. However, although most of the beetles are associated with a specific species of fungus in their primary area, ambrosia beetle species distantly related are sometimes associated with cultivating the same fungus, involving the exchange, direct or

indirectly. The fungal exchange between and within species of beetles can occur when several females colonize the same tree and fungal associations contaminate adjacent galleries [41]). The primary fungi are primarily asexual [46,47], while less specific auxiliary fungi are often sexual [43].

#### 4.2. Fungi Associated with Pecan in the Coahuila Southwestern Region

The identification of different fungal isolates from both wood and insect body, one may infer that dieback may be associated with *Fusarium* since it is the fungal specie most frequently isolated from pecan tissue and insect body (**Figure 1**).

This association of *Fusarium solani* and *Fusarium oxysporum* as a primary agent on pecan tree is due to exudates or gummosis presented at 14 days, later inoculated trees showed marked chlorosis and red apices, finally trees declined to dead. Inside of stalks were initially observed brown areas and when these areas were cut transversely, staining was observed in the vascular system in ring forms, this situation was observed in leaf petiole. When the fungal pathogen completely invaded the conducting vessels caused widespread of wilt and tree death. From all the isolated fungal species, *Fusarium solani* and *Fusarium oxysporum* are highlighted in this study because they have a recognized importance as ambrosia fungus [30]. *Fusarium solani* had the highest percentage of occurrence at Parras county, 35% in insects and 38.7% in diseased wood and in Torreon county, 38.5% in insects and 34.9% in diseased wood, by this reason it is considered as the primary ambrosia fungus, in contrast *Fusarium oxysporum* was the most frequent fungal specie at General Cepeda county, 45.9% in insects and 38.7% in diseased wood. As genera *Fusarium* are a ubiquitous necrotic parasite [48], and may tolerates pH changes [49], and host susceptibility to this fungus is directly influenced by temperature and osmotic pressure [50], *Fusarium* is widely distributed in soils and organic substrates, and it has been isolated from permafrost in the Arctic to Sahara sands, its presence is abundant in cultivated soils from temperate and tropical areas and it is the most frequently isolated fungi from plants by pathologists [17]. *Fusarium* species are commonly associated with some group of insects like Scolytidae and Platypodidae families habit in wood [51]. Regarding the association of *Fusarium* species and order Coleoptera insects in woods, Norris [32] mention *Fusarium solani* as a primary ambrosia fungus associated with species from *Xyleborus* genus, this report is consistent with the results obtained in this study, since 27.1% of our samples from the three different locations showed damage associated with insects of the Coleoptera order. Gil *et al.*, [52] identified

*Corthylus sp.* (Coleoptera: Scolytidae), causing damage to alder (*Alnus acuminata*) trees, which showed wood rot and death, in this case the insect was associated to some ambrosia fungi like *Fusarium solani*, *Fusarium sp.*, *Ceratocystis sp.*, *Verticillium sp.* and a yeast identified as *Pichia sp.* Bonello *et al.* [53] found that the ambrosia beetle *Xylosandrus germanus* associated with *Fusarium lateritium* and *F. oxysporum*, is related with vascular stained, cancers, wilting and dieback in black walnut (*Juglans nigra*). Tisserat *et al.* [54] found that the mortality of black walnut (*Juglans nigra*) in Colorado, USA, is the result of association of *Pityophthorus juglandis* (Coleoptera: Scolytidae) with *Fusarium solani* and *Geosmithia sp.* this fungus was latter isolated from trunk marginal cancer in the final stages of decline. *F. solani* has been mentioned as a primary symbiotic with the coffee berry borer (*Hypothenemus hampei*) [55,56]. The immature stages of *X. ferrugineus* are unable to complete the development in absence of *F. solani* [34,57-59]. Ergosterol is essential for development and reproduction of *X. ferrugineus* [60,61]. This sterol is absent in wood, but *F. solani* offer it in sufficient quantities [31]. This fungus also provides to *X. ferrugineus* essential fatty acids and phospholipids [62]. Norris [32] found that *X. ferrugineus* offspring was highly associated to the primary ambrosia fungus *Fusarium solani*, but with *Cephalosporium sp.* association was reduced by 50% and with *Graphium sp.* up to 70%. *Pityophthorus juglandis* is associated with *F. solani* in black walnut (*Juglans nigra*) trees in North America and South Africa; it is believed that trees are predisposed to cancer formation by stress factors such as optimum site conditions, improper pruning and adverse weather conditions [54], such as abandoned orchards and poor management. In our study, *F. solani* and *F. oxysporum* were consistently isolated from bone, xylem, phloem, cambium and galleries, as well as all *Euplatypus segnis* body parts. This observation and pathogenicity studies suggest an important role of *F. oxysporum* and *F. solani* in the pecan tree mortality of all ages in our region.

In the pathogenicity tests, *Lasiodiplodia theobromae* induced necrotic lesions approximately 10 cm long and 1.0 cm wide, that caused necrosis in the cortical tissue and deepened to the bone in the site of inoculation, at 56 days showed a slight chlorosis followed by early abscission, leaves with red tips, wilting and dieback tree at 84 days, the slow progression of the disease coincides with Milholl and [62] who mentioned that this type of fungus grow slowly and progress in living tissue host due to its saprophytic condition. Optionally, *L. theobromae* is a parasite that usually infects its host plants by penetrating through wounds and decaying tissue. Occurrence of this fungus is common in tropical and subtropical regions and in different ecological areas, where it has been identified

as the cause of disease in approximately 280 species of vascular plants, including avocado, apple, mango, grapes, pine, rose, rubber, cotton, cocoa, coffee, sugar cane, peanuts, tobacco, etc. [63]. Umezurike [64] mentions that *Botryodiplodia theobromae* has cellulolytic activity in *Bombax buonopozense* wood. The fungus attacks plant cells in a similar manner to soft rot fungi, use starch and other saccharides present in the initial wood substrates before degradation of cellulose and hemicellulose, but not degrade lignin. Rajput *et al.* [65] mention that decline and death of mango (*Mangifera indica* L.) trees in India, are related to the presence of *Botryodiplodia theobromae*, *Alternaria alternata*, *Fusarium moniliforme*, *Cephalosporium sp.*, *Chaetomium sp.*, *Aspergillus ellipticus*, *A. niger*, *Penicillium sp.*, *Curvularia lunata*, *Gloeosporium mangiferae*, *C. globosum*, *Daldinia sp* associated to the attack of ambrosia beetles, termites, mechanical damage to trunk, branches and pruning. Rondon [7] established the relationship between the wood borer *Xyleborus ferrugineus* F. (Coleoptera: Scolytidae) and *Botryodiplodia theobromae* Pat attacking avocado trees in Venezuela, this author found that *B. theobromae* is the most frequently isolated fungi from the body of this wood borer insect. Flores *et al.* [66] detected the association of *Scolytus sp.* and *Botryodiplodia theobromae* fungus, which causes circular lesions to elongated oval up to 15 cm long, dark color that can be covered by large masses of spores in dry periods, causing the death of tips, and branches of *Tectona grandis* trees in Ecuador. Masood *et al.* [67] identified the association of *Hypocryphalus mangiferae* (Coleoptera: Scolytidae) with *Lasiodiplodia theobromae*, *Phomopsis fimbriata* and *Ceratocystis sp.* in the sudden death syndrome in Pakistan associated with symptoms of gummosis, dry rot and vascular discoloration. Tress inoculated with *Alternaria alternata* showed at 63 days marked chlorosis, wilting leaf area at 70 days and dieback at 84 days as external symptoms and necrotic areas, browning of tissues and a deep dehydration of inoculated area. The genus *Alternaria* contains cosmopolitan species found in a wide range of materials and products, can damage food and feed, producing biologically active compounds such as mycotoxins. As pathogens reduce crop yields or affect stored plants therefore requires a precise identification of the species because each has specific characteristics (preferences for growth, pathogenicity, and production of secondary metabolites) that predict the behavior of this fungus [68]. Armengol *et al.* [69] mentioned that *A. alternata* is normally a saprophytic fungus, but in Spain some strains have been described as pathogenic on different citrus cultivars like Fortune, Nova, and Mineola. Negron *et al.* [70] described *Alternaria* as parasites of annual and bi-annual plants or saprophytes on organic substrates, however found that *Alternaria alternata* associated with *Scolytus*

*schevyrewi* (Coleoptera: Scolytidae) and other fungi affect some species of elms (*Ulmus* spp) in Colorado and Utah, which showed necrotic areas, browning of xylem tissue, wilting of foliage and general death of elms, symptoms consistent with the disease progression in the pecan trees of this study inoculated with *A. alternata* in our pathogenicity tests. Reyes [71] found that 8% of fungal strains isolated from wood samples and insect remains of adult weevils (Coleoptera: Platypodidae) belong to *Alternaria alternata* and 7% to *Phoma sp.*

The fungus *Paecilomyces sp.* is a pathogen of wide host range and wide geographical distribution, which has been isolated from soil and insects of various orders such as Coleoptera, Homoptera and Collembola, Lepidoptera, Diptera, Homoptera, Hymenoptera and spiders [72]. *Paecilomyces farinosus* may inhibit some strains of pathogenic fungi [73]. Finally, *Aspergillus*, *Paecilomyces*, and *Penicillium*, have been isolated from insect gut *Triatoma sp.* [74], which has been found sporadically in pecan orchards at the Coahuila Southeast region, Mexico.

## 5. CONCLUSIONS

Twelve genera of fungi were isolated from the body of *E. segnis* and diseased pecan wood. These fungi were identified as *Phoma sp.*, *Fusarium sp.*, *Ascochyta sp.*, *Phaeocylomices sp.*, *Lasiodiplodia sp.*, *Alternaria sp.*, *Umbeliopsis sp.*, *Torula sp.*, *Helminthosporium sp.*, *Aspergillus sp.*, and *Penicillium sp.*, and inoculated into healthy pecan trees, only *Fusarium oxysporum*, *Fusarium solani*, *Alternaria alternata* and *Lasiodiplodia theobromae* were highly pathogenic causing dieback at 87 days of inoculation, they were characterized morphologically and molecularly to the specie level.

In Mexico, this is the first study that determines to *Fusarium solani*, *Fusarium oxysporum*, *Alternaria alternata* and *Lasiodiplodia theobromae*, as the causal agent of pecan (*Carya illinoensis*) dieback in association with *Euplatypus segnis* ambrosia borer.

*Fusarium solani* and *F. oxysporum* were the most prevalent fungi and isolated from insects and diseased wood at all studied locations. It has been suggested that some species of fungi associated with ambrosia borer are in a symbiotic relationship with the insect. Although, there may be a symbiotic relationship between fungi and insects, there is no conclusive evidence to show that this relationship is sufficient, due to the great diversity of fungi found in association with *E. segnis*.

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