Synergic Effect of Mucuna pruriens var. Utilis (Fabaceae) and Pontoscolex corethrurus (Oligochaeta, Glossoscolecidae) on the Growth of Quercus insignis (Fagaceae) Seedlings, a Native Species of the Mexican Cloud Forest

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Introduction

Cloud forest hosts around 6790 plant species (Villaseñor, 2010). Regrettably, this diversity is in decline due to several factors of disturbance, most of which are anthropogenic (Ramírez-Marcial et al., 2001; Lamb et al., 2005). This situation affects the population dynamics of plant species that are endangered, threatened or susceptible to forest fragmentation (Saunders et al., 1991; Cayuela et al., 2006). This is the case with Quercus insignis, a species native to this ecosystem. Despite a broad geographic range that spreads from eastern-central Mexico to Costa Rica, this species is a narrow habitat-specialist and is highly susceptible to disturbance (Valencia, 2004). It was included in the Red List of Oaks under the endangered category (Oldfield & Eastwood, 2007). More recently, and due to the accelerated destruction of its habitat, this species was re-classified to the Critically Endangered category in the Red List of Mexican cloud forest trees (González-Espinosa et al., 2011).

A potential mechanism to reverse this situation and restore degraded areas is the establishment of forest plantations, or the reintroduction of locally extinct species (Vázquez-Yanes & Cervantes, 1993; Meli, 2003; Pedraza & Williams-Linena, 2003; Lamb et al., 2005). This process must be complemented by the production of native species in nurseries (Benítez et al., 2002). At present, there is nil to very low availability of native species in local nurseries in Mexico, and the few plants that are produced are lacking in both quality and size (Arriaga et al., 1994; Meza-Sánchez et al., 2009). Moreover, the use of black plastic bags with repeated watering causes the soil compaction and poor root development in the seedlings. Novel propagation techniques are therefore required in order to improve seedling quality in nurseries (Benítez et al., 2002), as well as complementary strategies with which to facilitate the future field establishment in the field.

In nurseries that are devoted to the massive production of native forest species, common use is made of organic remains,
sand, fertilizers and pesticides. However, the use of green manure could become an important source of nutrients (mainly nitrogen) to increase soil fertility (Smyth et al., 1991; Blanchart et al., 2006). The legume *Mucuna pruriens* var. *utilis* has been used as a cover crop for these purposes (Hulugalle et al., 1986; Smyth et al., 1991; Buckles, 1995; Ortiz-Ceballos et al., 2012) and its use has also enabled the biological control of weeds, pests and diseases, while it acts to influence the composition and activity of the soil biota, particularly the earthworms. All of these effects may indirectly favor plant growth (Ortiz-Ceballos & Fragoso, 2004; Blanchart et al., 2006).

The soil biota is known to regulate the availability of nutrients necessary for plant growth and development (Wardle et al., 2002; Bhadauria, 2010) and enhancement of plant growth (Lee, 1985; Haimi & Einbork, 1992; Pashanasi et al., 1992; Edwards & Bohlen, 1996; Doube et al., 1997; Scheu, 2003; Ortiz-Ceballos et al., 2007). However, most information published to date on plant-earthworm interactions is derived from studies based on domesticated plants (Edwards & Bater, 1992; Brown et al., 1999; Scheu, 2003; Ortiz-Ceballos & Fragoso, 2004). It is therefore important to examine the influence of earthworms on plants from natural environments (Scheu, 2003), such as cloud forest. The earthworm *Pontoscolex corethrurus* (Müller, 1857) is a peregrine species of the Glossoscolecidae family and native to the Neotropics. It is an endogeic earthworm with a broad environmental tolerance that occurs in various habitats and soil types (Lavelle et al., 1987; Lee, 1985; Fragoso et al., 1999; Buch et al., 2011), including cloud forest (Fragoso et al., 1999). This species plays a crucial role in organic matter decomposition as well as the mineralization of nitrogen and phosphorous (Barois et al., 1999; Bhadauria & Saxena, 2010) and has been successfully used in bio-fertilization and bio-stimulation techniques, producing significant positive effects on crop yields (Lavelle & Pashanasi, 1989; López-Hernández et al., 1993; Senapati et al., 1999).

Obtaining information on the interaction between *P. corethrurus* and native cloud forest plant species in general and *Q. insignis* in particular may be of great relevance, as this can allow us to develop new practices aimed at supplementing and reinforcing the management and propagation of native plant species within conservation and restoration programs for this mountain ecosystem. The goal of this study was to evaluate integrated soil fertility management, *i.e.*, how the combination of biomass (*Mucuna pruriens* var. *utilis*), inorganic fertilizer, and earthworms could impact the soil fertility as well as the growth and leaf nitrogen content of *Quercus insignis* seedlings under nursery conditions.

**Methods**

**Study Site and Soil**

The study was conducted in the greenhouse and laboratory of the Instituto de Biotecnología y Ecología Aplicada (INBIOTECA) in the center of Veracruz State, a region of Mexico that features cloud forest. A total of 200 kg of soil was collected to a depth of 40 cm from the Plan de San Antonio cloud forest (19°26′N, 96°59′W; 1430 m elevation), located 20 km from the town of Coatepec city. Soil samples were sealed in plastic bags and transported to the INBIOTECA greenhouse where they were air-dried at ambient temperature. The physical and chemical soil properties of the collected soils were: pH 5.7, organic matter content 16.6%, total nitrogen 0.68%, 0.32 cmol-kg$^{-1}$ K, 0.4 cmol-kg$^{-1}$ Ca, 1.7 cmol-kg$^{-1}$ Mg, 26.9 cmol-kg$^{-1}$ cation exchange capacity, 54% moisture content (soil collected), 17% clay, 32% silt, 49% sand.

**Earthworms, Green Manure and Oak Seedlings**

Juvenile individuals of *P. corethrurus* used in this study corresponded to the first generation obtained under laboratory conditions from earthworms collected in a secondary cloud forest. *Pontoscolex corethrurus* is an exotic species that has been reported in Mexican cloud forests and is also commonly found in areas that have been transformed from cloud forest to pastureland or crops, such as maize or beans, among others (Fragoso et al., 1999; Fragoso, 2001).

Juvenile *P. corethrurus* earthworms were raised following the protocol of Ortiz-Ceballos et al. (2005): (1) two adult earthworms, each with a conspicuous clitellum, were placed in plastic boxes ($12 \times 12 \times 8$ cm) filled with 300 g of soil, mixed with 3% *Mucuna* and wetted to field capacity (42%). The boxes were incubated at 26°C ± 2°C, and the soil replaced and cocoons collected fortnightly. These cocoons were incubated in Petri dishes at 27°C. In the greenhouse, plants of *M. pruriens* were grown for a period of three months (September - November 2009). The foliage was then collected, dried (63°C ± 3°C, 48 h), ground (to 2 mm), and placed in paper bags; total nitrogen content of the *M. pruriens* was determined (2.23%) using the Kjeldahl digestion method following the Mexican Official Norm NOM-021 (SEMARNAT, 2002). Seeds of the studied oak species, *Quercus insignis* were collected in November 2009 from cloud forest fragments located at 19°12′N, 96°59′W, at an elevation of 1460 m. This species bears large acorns (5 cm diameter) and its seeds may be classed as recalcitrant. Seeds were placed in nursery beds (1 × 8 m), where they germinated between 15 - 20 days after sowing, presenting a germination rate > 60%.

**Experimental Setup**

The experiment was conducted in the greenhouse of INBIOTECA from May to November 2010. We used eight treatments: 1) *Pontoscolex* (P); 2) *Pontoscolex-Mucuna* (PM); 3) *Pontoscolex*-fertilizer (PF); 4) *Pontoscolex-Mucuna*-fertilizer (PMF); 5) *Mucuna* (M); 6) fertilizer (F); 7) *Mucuna*-fertilizer (MF); 8) Control (C) [only soil]. There were 20 replicates (15 × 25 cm plastic containers) by treatment. The containers were filled with 540 g of dry soil that was then wetted to field capacity (43%), and placed on a metallic table. One day later, an 8 - 12 day old *Q. insignis* seedling of approximate height 15 cm was planted in each container. One week later, two juvenile *P. corethrurus* earthworms (106 ± 12 mg) were added to the appropriate treatments, and *Mucuna* foliage was added to the soil surface (8 g; 2.23% N) (nitrogen content of *Mucuna* is equivalent to that of the applied inorganic fertilizer), as well as the slow-release fertilizer (ammonium nitrate [0.7 g; 26 N 13 S%]) according to the requirements of each treatment. Seedlings were grown for a period of 180 days and watered weekly to maintain the soil moisture at field capacity (43%). Mean temperature in the greenhouse was 25.6°C ± 4.3°C, and weeds were manually removed from the soil. During the experiment, aphids
were found on the back of some leaves, these were controlled manually and no pesticide was applied.

We recorded seedling height and basal diameter monthly. A destructive harvest of all plants from all treatments was carried out 180 days after the start of the experiment. For each plant, the biomass weight was calculated (dried 65°C ± 3°C; 72 h) in roots, stem and leaves. To determine leaf nitrogen content a sample of the leaves was taken for each treatment and nitrogen content was determined with the Kjeldahl digestion method, following the Mexican Official Norm NOM-021 (SEMARNAT, 2002). At the end of the study, the number of earthworms and cocoons in each treatment was recorded.

Statistical Analysis

Analysis of variance (ANOVA) was performed to compare between treatments for each of the following variables: a) growth rate in height, b) growth rate in basal diameter, c) total and component-specific dry biomass, and d) total foliar nitrogen content. When the ANOVA yielded a significant result, means were compared with a Tukey multiple-comparison test. Prior to the ANOVA, numeric values were transformed to natural logarithm, the normality and homogeneity of variance were fulfilled. Growth rate ($T_C$) was estimated with the following equation:

$$T_C = (\ln C_2 - \ln C_1)/t$$

where $C_2$ is final seedling height or diameter, $C_1$ is initial seedling height (cm) or diameter (mm), and $t$ is elapsed time (months). In addition, biomass allocation to leaves ($L$) was assessed relative to root biomass ($R$), and expressed as the $LR$ ratio between treatments. Percentage values of total nitrogen content in the leaves were also compared between treatments with a Tukey test (GLM Proc, SAS 9.2; SAS Institute Inc., 2009).

Results

Growth of Quercus insignis Seedlings

Basal diameter growth rate of the $Q$. insignis was significantly higher in the treatment PMF ($F = 5.33; P < 0.0001$) than in all the other treatments (Figure 1).

Regarding plant height, significant differences were found ($F = 2.84; P < 0.0087$) among the treatments PMF, fertilizer (F) and the control treatment (C) (Figure 2). However this trait did not differ significantly between the treatments $P$. corethrurus-M. pruriens (PMF), $P$. corethrurus-fertilizer (PF), $M$. pruriens-fertilizer (MF), $P$. corethrurus-M. pruriens-fertilizer (PMF), $M$. pruriens (M), fertilizer (F), $M$. pruriens-fertilizer (MF), Control (C). Mean values of seedling basal diameter are shown. Vertical lines represent standard errors. Different letters indicate significant differences between treatments.

Total Leaf Nitrogen

Total foliar nitrogen content in $Q$. insignis seedlings was significantly different between treatments ($F = 2.54; P < 0.01742$). Values of this trait were significantly higher in the treatment PF compared to the other treatments. It is noteworthy that the $Q$. insignis plants that grew in the control treatment (soil only) presented the lowest values of foliar nitrogen, as is shown in Figure 3.

Biomass in $Q$. insignis Seedlings

Significant differences between treatments ($F = 2.32; P < 0.0290$) were recorded for leaf biomass values in $Q$. insignis. This was particularly noteworthy in the contrast between the $P$.
corethrurus-fertilizer (PF) treatment and the control. However the root and shoot biomass Q. insignis seedlings did not differ significantly between treatments (see Table 1).

Total biomass was similar between treatments, and significant differences ($F = 3.71; P < 0.0011$) were only found among the treatments PF, F and the control (Table 1). The leaf/root biomass ratio was $>1$ in all treatments, indicating a larger resource allocation to photosynthetic tissues in Q. insignis seedlings, regardless of treatment. No significant differences were observed between treatments in this trait.

**Discussion**

It is widely accepted that the presence of earthworms is generally beneficial to plant growth (Edwards & Bohlen, 1996; Bohlen et al., 2002, 2004; Scheu, 2003; Eisenhauer et al., 2009). Similarly, the addition of organic matter to the soil, mediated by leguminous plants such as M. pruriens, is known to provide nutrients such as nitrogen, and to improve soil fertility and thus increase plant productivity (Becker et al., 1995; Konboon et al., 2000). In our study, the treatments with P. corethrurus, M. pruriens, inorganic fertilizer and the interaction between these, presented similar values in terms of growth variables. However, significantly higher values in all plant growth variables were found between P. corethrurus-M. pruriens-fertilizer (PMF) treatment versus the control treatment. Brown et al. (2004) and Scheu (2003) reported increases in the growth of several cultivated plant species, mostly cereals and grasses, in the presence of earthworms. Likewise, other studies have led to the conclusion that the use of Mucuna pruriens favors the growth and productivity of Zea mays (Ile et al., 1996; Buckles & Triomphe, 1999; Eilitta et al., 2003).

Our results indicate that the combination of P. corethrurus-M. pruriens-fertilizer (PMF) positively affects the growth in height of Q. insignis plants. However, the combination P. corethrurus-M. pruriens (PM) and P. corethrurus-fertilizer (PF) can be equally effective in producing increased height, an aspect which is of vital importance to tree species that are propagated in greenhouses. The use of inorganic fertilizers may potentially induce variability in the effects of earthworms on plants, which may largely depend on soil nutrient status (Laossi et al., 2010a).

According to our findings, inorganic fertilizer appears to interact synergistically with P. corethrurus and M. pruriens, or with M. pruriens only, promoting the growth of Q. insignis during early stages of its life cycle. This condition is likely to be the result of the relatively rapid liberation of nitrogen provided by the synthetic fertilizer. If true, this would partly explain the improved plant growth in the treatments with P. corethrurus, M. pruriens and synthetic fertilizer, and in combinations of these. The fact that the inorganic fertilizer alone cannot produce a growth increase in these seedlings must not be overlooked.

Increases in stem and root biomass in plants that have been exposed to earthworm activity have been repeatedly documented (Wurst & Jones, 2003); however, other studies have reported contrasting results (Scheu, 2003). Ortiz-Ceballos et al., (2007) observed increased root biomass in plants of Zea mays exposed to the combined effects of the activity of the earthworm Balanotrichus pearsei with the leguminous M. pruriens. Conversely, the presence of P. corethrurus, and its combination with M. pruriens, did not significantly affect root and stem biomass in Q. insignis seedlings. It must be stressed that most studies reporting increases in root and stem biomass have focused on species with short life cycles (annual or biennial) that are often herbaceous, while the effect of this interaction with long-lived trees is less well understood. Moreover, the effect of earthworms on plant growth may also vary depending on the functional traits of the different plant and earthworm species (Brown et al., 2004; Eisenhauer et al., 2009; Laossi et al., 2010b). As a long-lived tree species, Q. insignis could respond differently in terms of resource allocation to different tissues during a very early phase of its juvenile development. Ultimately, this possibility could obscure the synergistic effect of P. corethrurus and M. pruriens on root and shoot biomass allocation in Q. insignis seedlings at the early stages of their development in a nursery.

Leaf biomass is seldom assessed as a response in studies of plant-earthworm interactions. In our analysis, the comparison of leaf biomass of Q. insignis revealed a homogeneous increase between the various treatments, with the exception of the control. The uniform increase of leaf biomass among treatments

**Table 1.** Effects of treatments with P. corethrurus (P), M. pruriens (M) and fertilizer (F), and combinations of these, on the biomass of aboveground and undergound components and leaf/root ratio of Q. insignis seedlings.

<table>
<thead>
<tr>
<th>Treatments (n)</th>
<th>Root biomass (g dry weight)</th>
<th>Stem biomass</th>
<th>Leaf biomass</th>
<th>Total biomass</th>
<th>Leaf/root ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>P (17)</td>
<td>2.2 ± 0.9a</td>
<td>2.3 ± 1.2a</td>
<td>3.7 ± 1.7ab</td>
<td>8.3 ± 2.2ab</td>
<td>2.1 ± 1.2a</td>
</tr>
<tr>
<td>PM (16)</td>
<td>2.1 ± 0.8a</td>
<td>1.9 ± 0.7a</td>
<td>3.2 ± 1.3ab</td>
<td>7.1 ± 1.5ab</td>
<td>2 ± 1.1a</td>
</tr>
<tr>
<td>PF (18)</td>
<td>2 ± 0.9a</td>
<td>2.6 ± 1a</td>
<td><strong>4.1 ± 1.3a</strong></td>
<td><strong>8.8 ± 2.3a</strong></td>
<td><strong>2.4 ± 1.6a</strong></td>
</tr>
<tr>
<td>PMF (16)</td>
<td>1.9 ± 0.9a</td>
<td>1.8 ± 0.8a</td>
<td>3.4 ± 0.7ab</td>
<td>7.0 ± 2.1ab</td>
<td>2.2 ± 1.3a</td>
</tr>
<tr>
<td>M (16)</td>
<td>1.8 ± 0.8a</td>
<td>1.8 ± 1a</td>
<td>2.9 ± 1.5ab</td>
<td>6.5 ± 2.2ab</td>
<td>2 ± 1.2a</td>
</tr>
<tr>
<td>F (17)</td>
<td>1.9 ± 0.6a</td>
<td>1.7 ± 0.9a</td>
<td>2.7 ± 1.4ab</td>
<td>6.3 ± 2.1b</td>
<td>1.6 ± 0.9a</td>
</tr>
<tr>
<td>MF (17)</td>
<td>2 ± 0.6a</td>
<td>1.9 ± 0.8a</td>
<td>2.9 ± 1.2ab</td>
<td>6.7 ± 2.5ab</td>
<td>1.7 ± 1.1a</td>
</tr>
<tr>
<td>C (17)</td>
<td>1.7 ± 0.4a</td>
<td>1.8 ± 1a</td>
<td>2.6 ± 1.4b</td>
<td>6.1 ± 1.6b</td>
<td>1.6 ± 1a</td>
</tr>
</tbody>
</table>

Note: Values are means ± SE. Values in parentheses (n) indicate the number of plants per treatment at the end of the experiment. Different letters within the same column indicate significant differences between treatments. p < 0.0290; **p < 0.0011**.
of native species aimed at conserving and restoring fragmented forest ecosystems.

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