Impact of Nitrogen Fertilization on *Phytophthora cinnamomi* Root-related Damage in *Juglans regia* Saplings

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Abstract. Excessive nitrogen (N) use in agriculture has been associated with increased severity of the damage caused by *Phytophthora* species. In this study, we investigated the impact in vitro and in vivo of N about Phytophthora cinnamomi. The preliminary in vitro assay showed the effect of different N sources on the mycelial growth of P. cinnamomi. This assay indicated that ammonium nitrate (NH_4NO_3) and ammonium sulfate [(NH₄)₂SO₄] allowed for greater control of *P. cinnamomi* mycelia in comparison with calcium nitrate $[Ca(NO_3)_2]$ and potassium nitrate (KNO₃) when used with 1000 ppm N. The in vivo assay showed the severity of P. cinnamomi in 5-month-old Juglans regia saplings grown under greenhouse conditions. We selected NH₄NH₃ as the source for N for the greenhouse assay, considering the inhibitory effect on the ingrowth of P. cinnamomi and the intensive use of this fertilizer in agriculture. Walnut saplings were fertilized with 0, 35, 70, 140, 210, and 1050 ppm N and were inoculated with zoospores of P. cinnamomi 45 d after the application of nitrogen treatment (DAA). They were harvested at 90 DAA. We found that a 70-ppm N fertilization reduced the development of P. cinnamomi, resulting in lower root and canopy damage indices (DIs) than the unfertilized inoculated treatments and fertilized treatments greater than 140 ppm. The results of the in vitro and in vivo assay agree that increased N concentrations were associated with reduced mycelium growth of P. cinnamomi, providing further evidence that N fertilization can mitigate this disease. Greater root and canopy damage was observed in saplings fertilized with 1050 ppm N, regardless of whether they were inoculated with P. cinnamomi, as a result of N phytotoxicity (verified through foliar analysis). In contrast, inoculated and unfertilized saplings (N0) also showed high root and canopy DIs associated either with the inoculation with P. cinnamomi or the no fertilization treatment. We postulate that 70 ppm N is the best fertilization rate for J. regia saplings because the positive effects of N on growth are maximized and the damage caused by *P. cinnamomi* is mitigated.

Phytophthora species are known to cause important diseases in agriculture (Kurbetli, 2013). The factors that determine the severity of the damage caused by Phytophthora can be biotic [e.g., insects and other pathogenic microorganisms that act as antagonists or synergists (Huber, 2002)] and abiotic [e.g., pH, free water, temperature, and mineral nutrition such as N (Huber and Haneklaus, 2007; Mur et al., 2016)]. Specifically, N can have both a beneficial and harmful impact on the diseases caused by Phytophthora species (Erwin and Ribeiro, 1996). It affects the incidence of root decomposition caused by Phytophthora parasitica Dastur. in citrus, Phytophthora sojae Kaufm. & Gerd. in Glycine max L., Phytophthora cinnamomic Rands. in Persea americana Mill., and the incidence of late blight in Solanum tuberosum L. caused by Phytophthora infestans (Mont) De Bary. (Elmer and Datnoff, 2014; Erwin and Ribeiro, 1996; Huber and Graham, 1999). However, questions regarding how the N source and dose affect disease incidence remain mostly elusive. Previous studies have associated N source and dose as factors that might affect the interaction between Phytophthora species and the host (Elmer and Datnoff, 2014; Erwin and Ribeiro, 1996; Huber and Graham, 1999; Huber and Thompson, 2007; Huber and Watson, 1974). However, this effect is inconsistent and extremely dependent on the pathogenic species and the corresponding host (Huber, 2002; Huber and Haneklaus, 2007).

The high N inputs used in walnut production (Gupta et al., 2012; Simorte et al., 2001; Weinbaum and Van Kessel, 1998) and the high susceptibility to *P. cinnamomi* reported in *Juglans regia* L. (Guajardo et al., 2017, 2019) highlight the need to investigate this interaction further. The importance of assessing the severity of the disease is enhanced by the worldwide presence of this pathogen. For example, Guajardo et al. (2017) reported that 93.3% of the walnut orchards in Chile show damage caused by oomycete species. There have been similar cases in France, Italy, Hungary, and Iran, in which species of Phytophthora have been recovered from infected walnut roots (Kurbetli, 2013). In our study, we aimed to test whether N can control Phytophthora cinnamomi attack in growing J. regia saplings. First, we conducted a preliminary in vitro assay to elucidate the effect of the dose and the source of N on the mycelial growth of P. cinnamomi. Then, we studied the effect of different doses of N and inoculation with P. *cinnamomi* on the root and canopy growth of J. regia saplings grown under greenhouse conditions.

Materials and Methods

In vitro assay

An in vitro assay was conducted in the Plant Pathology Laboratory of Pontificia Universidad Católica de Valparaíso (PPL-PUCV) to observe the interaction between N source and dose on Phytophthora cinnamomi mycelial viability. This experiment served also to select the treatments (N source and dose) that were later used in the greenhouse assay described in the next section. For this experiment, the isolate PN1955 [i.e., P. cinnamomi isolated from a walnut orchard and preserved in the Fungi and Oomycetes Bank of the PPL-PUCV, and previously virulence checked by Guajardo et al. (2017)], was used. This in vitro assay used a fully randomized design with a double factorial structure and five replicates. The first factor (N source) involved four levels: NH₄NO₃ (33% N), Ca(NO₃)₂ (16% N), KNO₃ (14% N), and (NH₄)₂SO₄ (21% N). The second factor (N dose) consisted of 10 levels: 0 (control), 1, 5, 10, 20, 50, 100, 200, 500, and 1000 ppm N. The assay was conducted in a laminar flow hood and consisted of mixing CMA culture media (corn meal agar medium) with 10 mg pimaricin (Mellipore, Biochemistry CAS 7681-93-8, Darmstadt, Germany) per 1000 mL distilled water] prepared previously with the respective sources and doses for each treatment. Subsequently, each plate was inoculated with an 8-mm CMA plug obtained from the P. cinnamomi mycelial advance zone (P. cinnamomi was incubated in advance for 72 h to inoculate the assay plates). After 72 h in a growth chamber at 25 °C in the dark, the mycelial diameter was measured using a Vernier caliper. Analysis of variance and Tukey's honestly significant difference test were used to verify the effect of the N source and the N dose on the mean diameter of the growth of the P. cinnamomi colony in millimeters. Last, the effective concentration necessary to reduce the P. cinnamomi colony by 50% (EC₅₀) was calculated as described by Finney (1964). This experiment was repeated twice.

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Greenhouse experiment

This experiment was conducted at the School of Agriculture, PUCV, Quillota, Chile. The greenhouse was air-conditioned and did not have artificial light. Temperature (20 to 24 °C) and humidity (65% to 70%) were maintained day and night. Previously, a first experiment was performed under shading, which generated complications regarding the behavior of the saplings. Particularly for this article, only the second trial was completed under controlled conditions. The significant results of that first experiment are presented in Supplemental Fig. 1.

Plant material

In Nov. 2017, 5-month-old 'Vina' walnut saplings were obtained from the nursery (they came from the nursery in a 1-L plastic bag) and had been fertilized previously with Basacote® Plus 3M (2 g/L substratum) on one occasion. They were selected from 120 saplings for assay and the selection was based on the uniformity of the saplings in terms of size (plant height range, 15-20 cm). Saplings were transplanted to 7-L pots filled with natural inert peat, yellow sand, and perlite (50% to 40% to 10%, respectively). The baseline fertilization without N consisted of a mix of a modified half Hoagland solution [26 ppm phosphorus, 124 ppm potassium, 90 ppm calcium, 24 ppm magnesium, 16 ppm sulfur, 1.6 ppm iron, 0.27 ppm manganese, 0.26 ppm boron, and 0.12 ppm zinc (Saa et al., 2015)]. Irrigation was applied throughout the experiment manually using deionized water obtained through a reverse osmosis filter (Aqua-Win, RO Water System Model A-102-7, Taiwan, China). The quantity and frequency of irrigation was defined based on plant weight according to the substrate's saturation and field capacity. Calcium was applied separately and directly onto the substrate to prevent precipitation with other nutrients at a dose of 45 mg/ sapling based on the Hoagland solution mentioned earlier. The pH of the irrigation solution was adjusted for each irrigation to \approx 6.5 and 7 using potassium hydroxide with a pH meter (model YaliTector3; Yalitech, Santiago, Chile). In addition, the electrical conductivity of the irrigation solution was measured using a conductivity meter (EC-138 Conductivity Tester) to ensure it did not exceed 0.16 dS·m⁻¹. Fifteen days after transplant, N treatments started and continued through to the end of the experiment (i.e., 105 d in total). The selected N source was NH₃NO₄, which was mixed with the baseline fertilization described previously. Saplings were inoculated with zoospores from the P. cinnamomi isolate PN1955 45 DAA according to the protocol described in the next section.

Treatments

Treatments were distributed in a fully randomized block design. The structure of the experiment was factorial with 10 replications, and the first factor (N level) consisted of six levels: 0 ppm (N0), 35 ppm (N35), 70

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ppm (N70), 140 ppm (N140), 210 ppm (N210), and 1050 ppm (N1050) using NH₃NO₄ as the N source. The second factor (inoculation) consisted of two levels—noninoculated and inoculated with *P. cinnamomi*—resulting in a total of 12 treatments and 120 saplings for the assay.

Preparation of inoculum and inoculation

Saplings were inoculated 45 DAA when growth differences resulting from the different N treatments were observed between the experimental units. The inoculation of the saplings was conducted as described by Larach et al. (2009). Initially, the mycelia of P. cinnamomi (PN1955) were placed on petri dishes that contained MSP media (i.e., CMA with 10 mg pimaricin, 200 mg vancomycin, and 100 mg PCNB (Pentachloronitrobenzene; Sigma-Aldrich CAS 82-68-8, Darmstadt, Germany) in 1000 mL distilled water) and were incubated for 5 d at 25 °C. Ten 8-mm pieces were cut from the mycelium advance zone and transferred to recipients with 300 mL carrot juice (i.e., 200 g of carrot per 1 L of sterilized water) sterilized previously in an autoclave. The inocula were kept in an isolated room for 3 d, which was previously disinfected with 98% alcohol and 10% sodium hypochlorite and was equipped with artificial light and temperature (25 °C) day and night to promote mycelial growth. After these 3 d, the inocula were submerged for 30 s in 300 mL saline solution (comprised of 2.36 g Ca(NO₃)₂, 0.5 g KNO₃, and 1 g magnesium sulfate/L distilled water) and were then kept in distilled water for 3 d.

Last, the presence of mature sporangia was confirmed using an optical microscope (Nikon H55L Eclipse Ni; Nikon, Tokyo, Japan), and the inoculum preparation was resumed. The release of the zoospores was promoted by exposing the contents to a temperature of 10 °C for 30 min and 25 °C for 1 h. Subsequently, a sample was taken with a Pasteur pipette to measure the inoculum concentration in a Neubauer chamber (Hirschmann Techcolor, Eberstadt, Germany) by counting the number of existing propagules (zoospores and mycelia). From this, the existing concentration was calculated, and the mix was diluted to the inoculum concentration required (1 \times 10⁴ propagules/mL). Finally, 100 mL inoculum was applied per plant, and the substrate was saturated for 48 h to ensure the presence of free water and pathogen infection.

Measurements

Root and vegetative growth. Height, stem diameter, and leaf length were measured for each sapling. Measurements were conducted at three subsequent dates, starting at 0, 45, and 90 DAA. Total growth was calculated as the difference between 0 and 90 DAA from the data collected. To measure the leaf length, three compound leaves were measured per sapling between 0 and 90 DAA. Total dry weight was recorded by harvesting and drying the saplings at 90 DAA for 2 d at 60 °C until a constant dry weight was

Leaf N analysis. Four leaflets were selected randomly and foliar analyses were conducted for each sapling at 45 and 90 DAA. These analyses were conducted by the Soil Laboratory of the School of Agriculture, PUCV, using the Kjeldahl method for vegetable N analysis described by Sadzawka et al. (2004).

Canopy and root damage index. The saplings were evaluated at 90 DAA using adapted models for the canopy damage index (DI_C) proposed by Guajardo et al. (2017) and the root damage index (DI_R) proposed by Vettraino et al. (2003) (Table 1). These types of variables are obtained through a qualitative analysis of each plant, assigning a "score" to the severity of the damage caused by *Phytophthora* on both the canopy and the roots. From the data obtained from the DI_{R} , the most likely damage index (DIRP) was identified by constructing an ordinal logistic model (chi square) in which the DI_R was a function of the complete factorial combination (level of N × level of inoculation). After the model was constructed, the probability vectors were created for each DI_R level under each factorial combination. The DI_R^P was identified by finding the maximum probability of each DI_R vector in each factorial combination.

Statistical analysis. Data were modeled using JMP software (version 12.0.1; SAS Institute Inc., Cary, NC) as a generalized linear mixed model with normal distributions. Block effect was introduced as a random effect in the model; the N and inoculation factors were included as fixed effects for all response variables.

Results

In vitro assay. In vitro growth of P. cinnamomi mycelia was affected significantly by the different N sources and correlated negatively with increasing N concentration in the media (Fig. 1). For instance, ammoniacal sources, such as NH₄NO₃ and (NH₄)₂SO₄ at 1000 ppm inhibited mycelial growth by 37% and 59% vs. the control (0 ppm), respectively. Nitric sources, such as Ca(NO₃)₂ and KNO₃, inhibited mycelia growth by 28.3% and 10.1% vs. the control, respectively. On the other hand, KNO₃ did not have a significant effect on mycelial growth vs. the control, regardless of the N concentration used. The greatest in vitro inhibition was produced with 1000 ppm N in the form of $(NH_4)_2SO_4$ in comparison with all treatments. In general, the ammonia sources showed a greater inhibitory effect compared with the other sources. Specifically, mycelial growth decreased from 50.5 mm to 32 mm under NH₄NO₃ and from 48.8 mm to 20.5 mm when exposed to $(NH_4)_2SO_4$, when the doses changed from 0 ppm to 1000 ppm, respectively. The inhibitory Ca(NO₃)₂ effect on mycelial growth was observed at higher doses than with NH₄NO₃ and (NH₄)₂SO₄. For instance, the inhibition of the in vitro growth of

Table 1. Scale of damage index for leaves and roots used to evaluate Juglans regia saplings

Scale	Canopy	Roots			
0	Healthy plant, no evidence of leaf damage	Roots without damage, presence of abundant			
	(chlorosis, necrosis, and decay)	roots and rootlets in active growth			
1	Saplings with light chlorosis and wilting	Roots with damage: a loss of less than 25% of			
		roots and slight rotting of roots or rootlets			
2	Saplings with chlorosis, wilting,	Roots with damage: 26% to 50% root loss, vascular			
	and leaf necrosis	damage at the level of the collar and root rot			
3	Wilted sapling, with loss of leaves and presence of canker at neck level	Roots with damage: 50% to 75% root loss and evident root rot, vascular damage at collar level			
4	Dead sapling	Roots with damage: 76% to 100% root loss and evident rot, canker visible at collar level (optional)			

Source: Adapted from Vettraino et al. (2013) and Guajardo et al. (2017).



Fig. 1. In vitro growth of *Phytophthora cinnamomi* mycelia (average diameter in millimeters) in Corn Meal Agar (CMA) medium combined with different nitrogen sources (ammonium nitrate, calcium nitrate, potassium nitrate, and ammonium sulfate) and concentrations (0, 1, 5, 10, 20, 50, 100, 200, 500, and 1000 ppm) 72 h postinoculation in petri dishes. The significant differences of means were analyzed using Tukey's honestly significant difference test ($\alpha = 0.95$). Bars represent SEM (n = 5).

P. cinnamomi with 500 ppm N as $Ca(NO_3)_2$ was not significantly different from the inhibition with 200 ppm N as NH_4NO_3 and 20 ppm N as $(NH_4)_2SO_4$.

In addition, the effective concentration values that inhibited 50% of the pathogen's mycelium (EC₅₀) coincided with the mycelial growth results (Supplemental Fig. 2). The EC₅₀ values for NH₄NO₃, (NH₄)₂SO₄, and Ca(NO₃)₂ were 4×10^3 , 2×10^3 , and 1×10^4 ppm, respectively. The greatest EC₅₀ value was 2×10^{12} ppm in the KNO₃ treatment.

Vegetative and root growth of walnut. An overall decrease in root biomass was observed as the level of N increased in both noninoculated and inoculated plants. Inoculated saplings showed the lowest canopy biomass at 0 ppm and 1050 ppm N, whereas noninoculated saplings showed less canopy biomass as the N level increased (Figs. 2 and 3).

The greatest root biomass was observed at 0 ppm N applied whereas the least root biomass was observed at 1050 ppm N. At 70 ppm N, canopy and root growth were maximized (Supplemental Table 1; Fig. 3). Stem diameter, height, and leaf length were not significantly different among any treatment (Supplemental Table 1).

Growth and damage variables. The DI_R was affected significantly by the inoculation

and had a significant relationship with N level (r = 0.54) and percentage of foliar N (r =0.48; Supplemental Fig. 3). Unfertilized saplings (N0) showed a DI_R value of 2.2 compared with intermediate doses N35 and N70, which had a DI_R value of 1.6. The N140 and N210 doses had DI_R values of 2.3 and 2.8, respectively, which are not significantly different than the value of N0. The N1050 treatment displayed the greatest DI_R value of 3.7. Overall, ID_R values of noninoculated saplings were less compared with inoculated plants. For instance, inoculated unfertilized (N0) saplings showed a DI_{R} of 2.2, which was significantly greater than the DI_R of 0.5 recorded in noninoculated N0 plants. A direct relationship between the DI_R and the DI_C (r = 0.6), as well as an indirect relationship (r = -0.39 and r = -0.57, respectively)between canopy and root dry weight was observed (Supplemental Fig. 3).

The canopy-to-root ratio (C/R) increased as the N level increased. The lowest C/R occurred in unfertilized saplings (N0), with values close to 0.3. These values did not differ significantly from those of the "intermediate" treatments (N35, N70, N140, and N210), which ranged between 0.39 and 0.55. The N1050 treatment had a C/R of 0.9, which was the greatest among all treatments. The C/R was not significantly different between noninoculated and inoculated saplings (Fig. 3B).

Root dry weight was affected significantly by N levels (Fig. 3C). In addition, root dry weight correlated directly to canopy dry weight (r = 0.42) and indirectly to the DI_R (r = -0.57) (Supplemental Fig. 3). The NO treatment had the greatest dry root weight (24.15 g) among all treatments. Nonetheless, the N35 and N70 treatments did not differ significantly from N0, averaging root dry weight values of 21.8 to 20.0 g, respectively. In contrast, the N1050 treatment had the lowest root dry weight (7.61 g), which was significantly less than the unfertilized treatment (N0). The root dry weight of the N140 and N210 treatments was not significantly different between N35 and N70, with overall mean values of 17.13 and 16.29 g, respectively. Overall, no significant differences between inoculated and noninoculated saplings were observed across the treatments for root dry weight.

The DI_C was affected directly by the N level and, to a lesser extent, by the level of inoculation (Fig. 3D). In addition, the DI_C



Fig. 2. Saplings of *Juglans regia* noninoculated (left) and inoculated (right) with *P. cinnamomi* fertilized with 0, 35, 70, 140, 210, and 1050 ppm nitrogen (from top to bottom). Photograph taken of canopy and roots during harvest at 90 d after the application of nitrogen treatments from a representative repetition of each treatment.

showed a positive correlation with the DI_R (r = 0.6) and leaf N concentration (r = 0.23), as well as a negative correlation with the dry weight of the canopy (r = -0.37) and roots (r = -0.31) (Supplemental Fig. 3). In noninoculated plants, the overall DI_C increased with N application. However, N70 saplings showed a DI_C of 2.1, which was the lowest value recorded in the experiment and was not significantly different from N0 plants. Treatments N140, N210, and N1050 showed the greatest DI_C in comparison with the rest of the treatments, which had DI_C values of 3.3, 2.9, and 3.2, respectively. Saplings inoculated with the N0 and N1050 treatments showed the greatest DI_C values (3.2 and 3.3, respectively), without significant differences between them. The rest of the inoculated treatments (N35, N70, N140, and N210) did not show significant differences, with DI_C values ranging between 2.7 and 2.8.

Leaf N concentration at 90 DAA correlated directly to N levels (Fig. 3E, Supplemental Fig. 4). In addition, there was an interaction between N level and inoculation at high doses (1050 ppm), with values of 3.95% and 3.37% for noninoculated and inoculated plants, respectively. Treatments N0 and N35 were not significantly different, averaging leaf N concentrations of 1.81% and 1.96%, respectively. The intermediate treatments N70, N140, and N210 were not significantly different either, varying from 2.17% to 2.73%. Finally, the treatment with N1050 showed the greatest leaf N concentration, with values of 3.66%, and showed significant differences with respect to the rest of the treatments.

Canopy dry weight was affected significantly by N level (Fig. 3F), correlated positively to root dry weight (r = 0.42), and correlated negatively to leaf N concentration (r = -0.13) (Supplemental Fig. 3). Levels N0 and N1050 showed the lowest canopy weight and did not differ from each other, with values between 6.95 and 5.74 g in noninoculated saplings, and between 6.78 and 5.70 g in inoculated saplings for treatments N0 and N0150, respectively. The N35, N70, N140, and N210 treatments showed no significant dry weight differences among them.

The DI_R^P was affected significantly by the level of N and the level of inoculation, with a lower DI_R^P observed in noninoculated saplings compared with saplings inoculated with P. cinnamomi (Fig. 4). In noninoculated plants, N levels of N0, N35, and N70 had a DI_{R}^{P} value of 0 (i.e., without visible symptoms in the roots). However, N140 and N1050 had DI_R^P values of 2 and 4, respectively. Saplings inoculated with P. cinna*momi* showed low DI_R^P values when they were not fertilized with nitrogen (0 ppm), averaging a DI_{R}^{P} value of 2, which remained constant for N0, N35, N70, and N140. However, for the N210 and greater N treatments, the DI_R^P value was 4 (i.e., severely affected plant). Saplings with N levels of 140 and 1050 ppm, either noninoculated or inoculated with *P. cinnamomi*, had similar DI_{R}^{P} values at each level.

Discussion

This study suggests that specific N fertilization rates (using NH₄NO₃ as the N source) can minimize P. cinnamomi damage in walnut saplings. We found that 70-ppm N fertilization reduced disease severity in roots and canopy while maximizing the positive effect of N in walnut sapling growth. The greatest root and canopy damage observed in saplings fertilized with 1050 ppm N, regardless of whether they were inoculated with P. cinnamomi, was likely a result of N phytotoxicity. In contrast, inoculated and unfertilized saplings (N0) showed high DI_R and DI_C values associated with the inoculation of P. cinnamomi. The results presented here might have a direct impact at the field level by improving the agricultural management of this disease in walnut orchards.

The results of the preliminary assay suggest that N sources NH_4NO_3 and $(NH_4)_2SO_4$ allowed greater control of *P. cinnamomi* mycelia in comparison with $Ca(NO_3)_2$ and KNO_3 . These results corroborate previous findings by Utkhede (1984) and Scarlett et al. (2013), suggesting that *P. cinnamomi* growth decreased with N doses greater than 40 ppm NH_4NO_3 and 30 ppm $(NH_4)_2SO_4$. The $Ca(NO_3)_2$ results of this study are aligned with the results obtained by Utkhede (1984), suggesting that although the growth of the *P. cactorum* mycelia was inhibited, this





Fig. 3. Effect of nitrogen level (measured in parts per million) and inoculation on (**A**) root damage index (ID_R), (**B**) canopy-to-root ratio, (**C**) root dry biomass, (**D**) canopy damage index (ID_C), (**E**) leaf nitrogen percentage, and (**F**), canopy dry biomass. Nitrogen concentrations were 0 ppm, 35 ppm, 70 ppm, 140 ppm, 210 ppm, and 1050 ppm. The levels of inoculation were noninoculated plants (black shading) and plants inoculated with *P. cinnamomi* (gray shading). Data were obtained at 90 d after the application of nitrogen treatments. Significant differences of means in continuous variables (dry biomass, canopy-to-root ratio, and leaf nitrogen percentage) were analyzed using Tukey's honestly significant difference test ($\alpha = 0.95$). Significant differences in categorical variables (damage index) were analyzed with a nonparametric Kruskal Wallis test. The bars represent the SEM (n = 120).

inhibition occurred only with more than 60 ppm N. The N source selected for our greenhouse assay was NH_4NO_3 , considering the inhibitory effect on the in vitro growth of *P. cinnamomi* and the intensive use of

this fertilizer in agriculture compared with $(NH_4)_2SO_4$ (International Fertilizer Association, 2017).

Canopy and root growth had a differentiated behavior between noninoculated and inoculated plants. Noninoculated saplings were affected mainly by the excess of N, with the lowest canopy and root growth in saplings fertilized with 1050 ppm N. Excessive N has been associated with physiological





and morphological disorders, such as restricted growth of structures exposed to areas with greater concentrations of N (Mills and Jones, 1979). Noninoculated and inoculated saplings from the unfertilized treatment (N0) probably used the N storage and carbohydrate accumulation in the canopy and root system during from the nursery to support sapling growth (Boussadia et al., 2010). Regardless of inoculation, canopy and root growth in saplings fertilized with 1050 ppm N displayed limited root growth because root exploration to supply N demand was not necessary (Garnett et al., 2009; Lynch et al., 2012). On the other hand, we found that the canopy and roots grew more when the doses provided were balanced and sufficient for the sapling (i.e., N70 treatment), with roots possibly exploring the substrate to take up resources (i.e., water and nutrients) to deliver them up to the rest of the plant.

Following the categories described by Romheld (2012) and Goodman et al. (2013), three nutritional stages could be identified in this study. Treatments N0 and N35 corresponded to the "deficiency" stage, when saplings show symptoms of N deficiency (Romheld, 2012) and the percentage of foliar N is less than 2.1% [considered to be deficient by Beutel et al. (1976)]. At N70, the "sufficiency" stage begins with an increase in growth, when the percentage of N is $\approx 2.2\%$ [considered adequate by Beutel et al. (1976) and Goodman et al. (2013)]. A 3.2% leaf N concentration associated with the N140 to N210 treatments can be identified as "luxury

consumption." Last, the N1050 treatment can be associated with the "toxicity" stage, as leaf N concentrations exceeded 3.3% N, which has been reported as a toxic level for J. regia in the past, according to Simorte et al. (2001). Previous studies on excess N and its interaction with Phytophthora have suggested that root diseases caused by this pathogen are exacerbated by high doses of N (i.e., diseases caused by P. cinnamomi in Eucalyptus macranthera, Angophora costata, and Corymbla gummifera; P. palmivora in Durio zibethinus (Murr.) and Carica papaya L.; as well as P. cactorum in Malus pumila (Mill.) (Elmer and Datnoff, 2014; Scarlett et al., 2013; Utkhede and Smith, 1995). The results obtained here highlight that, in inoculated J. regia saplings, both a deficit and excess of N increased the DIR and DI_C. The unfertilized saplings were affected directly by P. cinnamomi, causing a decrease in the quantity and growth of leaves and roots, chlorosis and leaf necrosis, root decomposition, and canker in the collar (visual observations), which is similar to the results reported by Vettraino et al. (2013) and Guajardo et al. (2017). On the other hand, saplings fertilized in excess (1050 ppm), both inoculated and noninoculated, had high DI_R and DI_C values associated with the phytotoxicity of N when applied under high concentrations. The lowest DI was associated with 70 ppm N, where found NH₄NO₃ reduced the mycelium growth of P. cinnamomi, and the rate of 70 ppm N enhanced plant growth.

The DI_{R}^{P} can assist with N fertilization management in horticulture. These likelihoods suggest that saplings not inoculated and fertilized with less than 70 ppm N grow less as a result of an N deficiency. In other words, we postulate that 70 ppm N is the best fertilization rate for J. regia less than 1 year old because the positive effects of N on growth are maximized, and the damages caused by P. cinnamomi are mitigated. At 140 ppm N, the beneficial effect of N in canopy and root size seems to be countered by the negative effect of P. cinnamomi. When the N rate exceeds 210 ppm, there seems to be an additive effect between N and P. cinnamomi, which causes a greater probability of death. In contrast, saplings not inoculated and fertilized with 210 ppm show less damage associated with excess N, exhibiting no growth gain when compared with noninoculated N70 saplings. This finding suggests that 210 ppm N is an excessive application of N. This situation is even more apparent for the case of saplings fertilized with 1050 ppm N, which showed an even greater probability of death.

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Supplemental Table 1. Vegetative growth of Juglans regia samplings with regard to stem growth (diameter, height, and length of leaves) and dry biomass of the canopy, roots, and total in fertilized samplings with different levels of nitrogen dose.

	Stem growth (cm)			Dry biomass (g)			
Level of N dose (ppm)	Diam	Ht	Length of leaves	Canopy	Roots	Total	
N0	2.22 ± 0.19 ab	1.98 ± 0.33 b	5.95 ± 0.49 ab	6.87 ± 0.39 ab	24.14 ± 1.85 a	31.06 ± 2.03 a	
N35	2.93 ± 0.25 a	2.89 ± 0.61 ab	7.26 ± 0.69 ab	8.15 ± 0.59 a	21.79 ± 1.52 ab	29.94 ± 1.86 a	
N70	2.57 ± 0.20 ab	$2.18 \pm 0.27 \text{ ab}$	5.72 ± 0.53 ab	7.96 ± 0.45 a	20.00 ± 1.69 ab	27.95 ± 1.96 a	
N140	2.80 ± 0.23 a	4.17 ± 0.84 a	6.04 ± 0.68 ab	8.21 ± 0.41 a	17.13 ± 1.23 b	25.03 ± 1.39 a	
N210	2.87 ± 0.22 a	3.54 ± 0.43 ab	7.34 ± 0.64 a	7.90 ± 0.47 a	16.29 ± 1.07 b	24.50 ± 1.31 a	
N1050	$1.88\pm0.13\ b$	3.90 ± 0.50 ab	$4.92\pm0.48~b$	$5.70\pm0.27~b$	$7.6\pm0.88~c$	13.30 ± 1.01 b	

Nitrogen (N) dose levels: N0 = 0 ppm, N35 = 35 ppm, N70 = 70 ppm, N140 = 140 ppm, N210 = 210 ppm, and N1050 = 1050 ppm. Values are means \pm sE (n = 20). Different letters in the same column symbolize significant differences (Tukey's honestly significant difference, α = 0.95). Results of diameter, height, and length of leaves were calculated based on the difference between 0 and 90 d after the application of N treatments (DAA). The measurement of dry biomass was made at 90 DAA after harvesting the samples.



Supplemental Fig. 1. Effect of nitrogen level (measured in parts per million) and inoculation on root damage index (ID_R) and canopy damage index (ID_C). Nitrogen levels were nitrogen concentrations at 0 ppm, 35 ppm, 70 ppm, 140 ppm, and 210 ppm. The levels of inoculation were noninoculated plants (black shading) and plants inoculated with *P. cinnamomi* (gray shading). Data were obtained at 90 d after the application of nitrogen treatments. The significant differences were analyzed with a nonparametric Kruskal Wallis test. The bars represent the sE (n = 100).



Supplemental Fig. 2. Linear regression of probit inhibition and logarithm of the concentration of four sources of nitrogen: (A) potassium nitrate, (B) calcium nitrate, (C) ammonium nitrate, and (D) ammonium sulfate. The minimum inhibitory concentration value of 50% of the pathogen (EC₅₀) is presented in conjunction with the equation of the line.

	ID _R	Dry biomass roots	Canopy/root ratio	Dry biomass canopy	Leaf nitrogen percentage	ID _C
Nitrogen level	0,54	-0,61	-0,09	-0,34	0,82	0,23
	1,44 x 10 ⁻¹⁰	1,65 x 10 ⁻¹³	0,35	1,22 x 10 ⁻⁴	1,61 x 10 ⁻³⁰	0,01
ID _R		-0,57	-0,12	-0,39	0,48	0,60
		8,26 x 10 ⁻¹²	0,18	1,26 x 10 ⁻⁵	3,58 x 10 ⁻⁸	7,51 x 10 ⁻¹³
Dry biomass roots			0,05	0,42	-0,57	-0,31
			0,56	1,63 x 10 ⁻⁶	1,38 x 10 ⁻¹¹	5,73 x 10 ⁻⁴
Canopy/roots ratio				0,13	4,5 x 10 ⁻³	-0,03
				0,17	0,96	0,77
Dry biomass canopy					-0,20	-0,37
					0,03	2,56 x 10 ⁻⁵
Leaf nitrogen						0,23
percentage						0,01
ID _C						

Supplemental Fig. 3. Pearson correlation coefficient between variables such as radicular damage index (ID_R), dry root biomass, canopy-to-root ratio, dry canopy biomass, leaf nitrogen percentage, and canopy damage index (ID_C). The greater values correspond to the correlation coefficient and the lesser value corresponds to the *P* value. The pairs of variables with positive correlation coefficients and *P* values less than 0.05 tend to increase together (no shading). For the pairs with negative correlation coefficients and *P* values less than 0.05, one variable tends to decrease while the other increases (light gray). For pairs with *P* values greater than 0.05, there is no significant relationship between the two variables (dark gray); n = 120.



Supplemental Fig. 4. Leaf nitrogen percentage in *Juglans regia* at 45 and 90 d after the application of nitrogen treatments (DAA) in (**A**) noninoculated plants and (**B**) plants inoculated with *Phytophthora cinnamomi* at different nitrogen levels: 0 ppm, 35 ppm, 70 ppm, 140 ppm, 210 ppm, and 1050 ppm nitrogen. 45 DAA = the moment of preinoculation (black bars); 90 DAA = the postinoculation moment (hatched bars). Significant differences were analyzed with Tukey's honestly significant difference test, $\alpha = 0.95$. The bars represent the sE (n = 120).