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Morpho-physiological Alterations in *Capsicum annuum* L. by the False Root-knot Nematode *Nacobbus aberrans*

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ABSTRACT

Nacobbus aberrans is a phytoparasitic nematode responsible for significant losses in numerous horticultural crops. For this reason, it is considered a major economic pest in several countries. This work focuses on the morphological, biochemical, and physiological changes in pepper plants due to *N. aberrans* parasitism. The experiment was conducted under controlled conditions. A significant reduction in growth was observed in the inoculated plants, showing less accumulation of dry matter in aerial parts and roots. The leaves of the inoculated plants showed lower chlorophyll and soluble protein contents than the non-inoculated plants. Net photosynthesis and transpiration decreased, thus reducing water use efficiency in the inoculated plants. Stomatal conductance in the inoculated plants was also lower. The penetration of mobile forms of *N. aberrans* in the roots damaged cell membranes, as evidenced by a more profuse release of electrolytes that, in turn, increased relative conductivity. Malondialdehyde content was higher in the roots and leaves of inoculated plants than in plants without nematodes. An increase in the catalase activity and peroxidase enzymes was observed in plants infected by *N. aberrans*. The loss of functionality in inoculated roots caused drought stress and culminated in reduced plant growth. This was also confirmed by a greater accumulation of proline and sugars, metabolites used as osmoregulators in water-deficit situations.

Introduction

Root-knot nematodes (*Meloidogyne spp.* and *Nacobbus spp*) affect many economically important crops. They are phytoparasites that usually induce gall formation on roots, which limits the absorption of water and nutrients, therefore generating losses in vegetable production (Jones et al., 2013). *Nacobbus aberrans* (Thorne and Allen, 1944) is a nematode species native to South America. It is recognized as a quarantine pest in Europe and the US (Manzanilla-Lopéz et al., 2002). It has a wide range of hosts, comprising 84 known plant species distributed in 18 families, while it can remain dormant under unfavorable conditions when they inhibit its development (Manzanilla-López, 2010; van den Akker et al., 2014). *N. aberrans* is a sedentary endoparasite that generates galls in the roots of many horticultural crops such as pepper, tomato, beet, eggplant, and potato (Castillo and Marban Mendoza, 1984; Inserra et al., 1985; Tovar Soto et al., 2012;

Cabrera et al., 2016; Tordable et al., 2018) causing considerable economic loss. This nematode exhibits sexual dimorphism, with a life cycle that ranges from 25 to 59 days, varying depending on specific populations, host types, and environmental conditions. This cycle includes four juvenile stages and one adult stage (Doucet and Lax, 2005). In roots affected by *N.aberrans*, differentiated galls that can extend along all the roots are formed, such as those caused by *Meloidogyne* sp., from which its common name "false root-knot nematode" is derived (Lax et al., 2021). The formation of lacerations and necrotic tissues in the infected root is due to the repeated penetration and migration of juvenile forms from the root to the ground and vice versa (Lax et al., 2022). When female nematodes become sedentary within the root, they cause ecomorphological alterations in the plant, which affect cell physiology and cause hyperplasia and hypertrophy responses, resulting in gall formation (Hewezi and Baum, 2013). These changes lead to the rupture of plant vascular tissues (xylem and phloem), altering the normal fluid movement and becoming a precursor of plant wilt, nutrients (N, P, K, Ca, and Mg) deficiency, electrolyte loss, and low stomatal conductance, all of which reduce plant growth, generate yield loss, and cause plant death (Cristobal et al., 2001).

In 2020, the most recent year when the Food and Agriculture Organization (FAO) provided global data, world pepper (*Capsicum annuum* L.) production was 36,136.99 million kg. The area under pepper production in 2020 was 2,069,990 ha, with an average 1.75 kg yield per square meter. This nematode represents a potential risk to various horticultural crops, such as pepper, due to its wide host range. It has been quantified, even in incipient populations (1-15 individuals 100 g-1 of soil), that this nematode can significantly affect crop yield (33% loss) (Lax et al., 2022). Since available information about the damage caused by this nematode in plant material used in the CHLP (Cinturón Hortícola de La Plata) is scarce, this work aimed to study the morpho-physiological and biochemical alterations caused by *N. aberrans* when it parasitizes Paco f1 pepper plants.

Material and Methods

The experimental work was carried out in a greenhouse between August and December at the Institute of Plant Physiology (INFIVE). *C. annuum* L. Paco f1 pepper seeds, a variety commonly selected by producers in the area, disinfected with NaOCl (10%) for 5 min. These seeds were sown in trays of 72 cells, previously filled with a substrate composed of a perlite and vermiculite (2:1 v/v) mixture. The trays were kept in a culture chamber until transplantation, and nutrients were provided using complete Hoagland's nutrient solution (Hoagland and Arnon, 1950). Thirty days after sowing (DAS), one plant was transplanted per pot containing 10 kg of a Tyndallized mixture of soil and sand (1:1 v/v). We inoculated half of the pots with 8000 *N. aberrans* eggs three days after transplantation (DAT). The egg suspension used as inoculum became accessible via centrifugation-flotation (Coolen, 1979) from a mono-xenic culture obtained from a single mass of eggs from the roots of the isolated infected plants. Subsequently, we measured the parameters under a microscope. The egg count facilitated our aim to procure 8000 eggs in 5 mL of water. For inoculation to happen, we made three holes approximately 2 cm in depth next to the seedlings with the aid of a glass rod. Using an automatic pipette, we deposited 5 mL of the egg suspension (8000 eggs) in each hole. Then, we filled and covered the holes with substrates and defined the following treatments:

Not inoculated pepper plants (control), pepper plants inoculated with Nacobbus aberrans (inoculated), seven days after transplanting, weekly measurements of stem height and diameter were started. At the end of the test, 120 days after transplantation, samples were taken from aerial parts and the roots for the quantification of several parameters. These parameters were the number of total eggs, for which the eggs were extracted from the infected roots by the centrifugation-flotation technique of Coolen (1979) and counted using an optical microscope Lancet L2101 YB (China). Nematode reproductive factor (RF) was determined according to Oostenbrink (1966). The RF was calculated from $RF = Fp \cdot Pi - 1$, where: Fp = final population, i.e., the number of eggs and larvae extracted from the roots, and Pi equalled the number of inoculated eggs. Where $RF = 0$, plants were considered immune. Where $RF < 1$, they were considered resistant, and where $RF > 1$, they were considered susceptible.

Root anatomy and histochemistry was determined by double staining with Alcian blue 1% and alcoholic safranin 80% on histological sections (Luque et al., 1996). Histochemical techniques were performed to identify the presence of starch with Lugol's aqueous solution (potassium iodide-iodine) (Zarlavsky, 2014). Observations were made with a Ceti Topic T optical microscope (Belgium), and images were captured with a Gemalux XSZ-H microscope

(China), equipped with a Motic 1000 camera and Motic Image Plus 2.0 software.

Malondialdehyde content (MDA) was measured from 200 mg of leaves and 200 mg of roots using a spectrophotometer (Shimadzu UV - 160 Kyoto, Japan), according to the method of Heath and Packer (1968), as an indicator of peroxidation of cell membranes lipids.

Soluble protein content was determined from 100 mg of leaf sample and 100 mg of roots (fresh weight). The absorbance was read at a wavelength of 595 nm using a spectrophotometer (Shimadzu UV - 160 Kyoto, Japan). Protein concentration was calculated using a standard curve prepared with different concentrations of bovine serum albumin (BSA, SiFMa Chemical Co, USA), according to Bradford (1976). Chlorophyll content was determined from a 0.5 cm diameter leaf disk. N, N-Dimethylformamide was used as the extraction solvent, and the absorbance of the solution was determined at 647, 664, and 480 nm using a spectrophotometer (Shimadzu UV - 160 Kyoto, Japan). Pigment content was calculated according to Wellburn (1994).

Proline content was measured from 100 mg of leaf sample and 100 mg of roots (fresh weight). The absorbance was read at a wavelength of 520 nm, using a spectrophotometer (Shimadzu UV - 160 Kyoto, Japan), and proline content per unit of fresh weight (FW) was calculated as µmol proline g^{-1} FW = [(µg proline mL^{-1} × mL toluene)/115.5 µg $µmol^{-1}$] / [(g FW)/5, according to Bates et al. (1973).

Total and reducing sugar content was measured from 500 mg of fresh material according to the Somogy method (Cronin and Smith, 1979). For total sugar, acid hydrolysis was conducted with 0.1 N HCl in a water bath at 100 °C, and the subsequent reaction was obtained using a cupric reagent. The absorbance was measured at a wavelength of 520 nm using a spectrophotometer (Shimadzu UV - 160 Kyoto, Japan). Catalase activity (EC 1.11.1.6) was measured using a spectrophotometer (Shimadzu UV-160 Kyoto, Japan) from 500 mg of leaf sample and 500 mg of roots, according to Maehly and Chance (1954).

Total peroxidase (EC 1.11.17) was measured in a spectrophotometer (Shimadzu UV-160 Kyoto, Japan) from 500 mg of leaves and 500 mg of roots according to Aebi (1984).

Net photosynthesis (PN), sweating (E), and stomatal conductance (CS) were determined using a portable infrared gas analyzer (CIRAS-2® model, PP Systems, USA), which estimates CO2 net assimilation expressed in µmol CO2 m^{-2} s^{-1}. Measurements were carried out at 25 °C with an external CO2 concentration of 360 ppm and 1000 µmol m^{-2} s^{-1} of irradiance. From these data, water use efficiency (WUE) was calculated.

Aerial and root dry weights were determined at the end of the test by drying the material at 80 °C in an oven until a constant weight was achieved.

The assay was carried out following a completely randomized experimental design, with ten replications. Data were analyzed by ANOVA. Mean values were compared using the LSD test ($P < 0.05$) using the Infostat 2018 statistical software.

Results

After transplantation, 8000 nematode eggs per pot were inoculated. At the end of the trial, 154,750 eggs were counted. Thus, inoculation was effective and test conditions were favorable for nematode multiplication. Pepper is a species susceptible to this pest according to the Oostenbrick reproduction factor exceeding one. The histological analysis of the uninoculated plant roots showed a normal pattern, with an ordered tissue structure. The normal roots are tetrarchs (Figs. 1A and B). On the other hand, the roots with galls presented nematodes in the cortical parenchyma and destruction and/or deformation of the phloem tissue, showing holes occupied by mature females (syncytium). Parenchyma hyperplasia and hypertrophy associated with the vascular tissue were observed, rendering the second xylem small (Figs. 1C and D). The accumulation of starch granules was observed in the regions corresponding to the entry and localization sites of the nematodes, thus characterizing the areas of root damage caused by the mechanical action of *N. aberrans* (Figs. 1E and F). In this study, nematodes were also found in the pericycle and the cortical parenchyma (Figs. 1G and H).

While the application of nematode eggs to the pots did not generate differences in the dimensions of the seedling stem, the aerial and root dry weights decreased by 20% and 22%, respectively (Table 1), as well as the chlorophyll content (49%) in plants without inoculation (Table 1). A similar pattern was observed in the soluble protein content, which was higher in the leaves than in the roots. In the leaves, the value was significantly higher ($p < 0.05$) in control plants (5.601 µg mg-1 of FW) compared to those inoculated with the nematode (2.785 µg mg^{-1} of FW). In the roots, the protein content did not change significantly (Fig. 2).

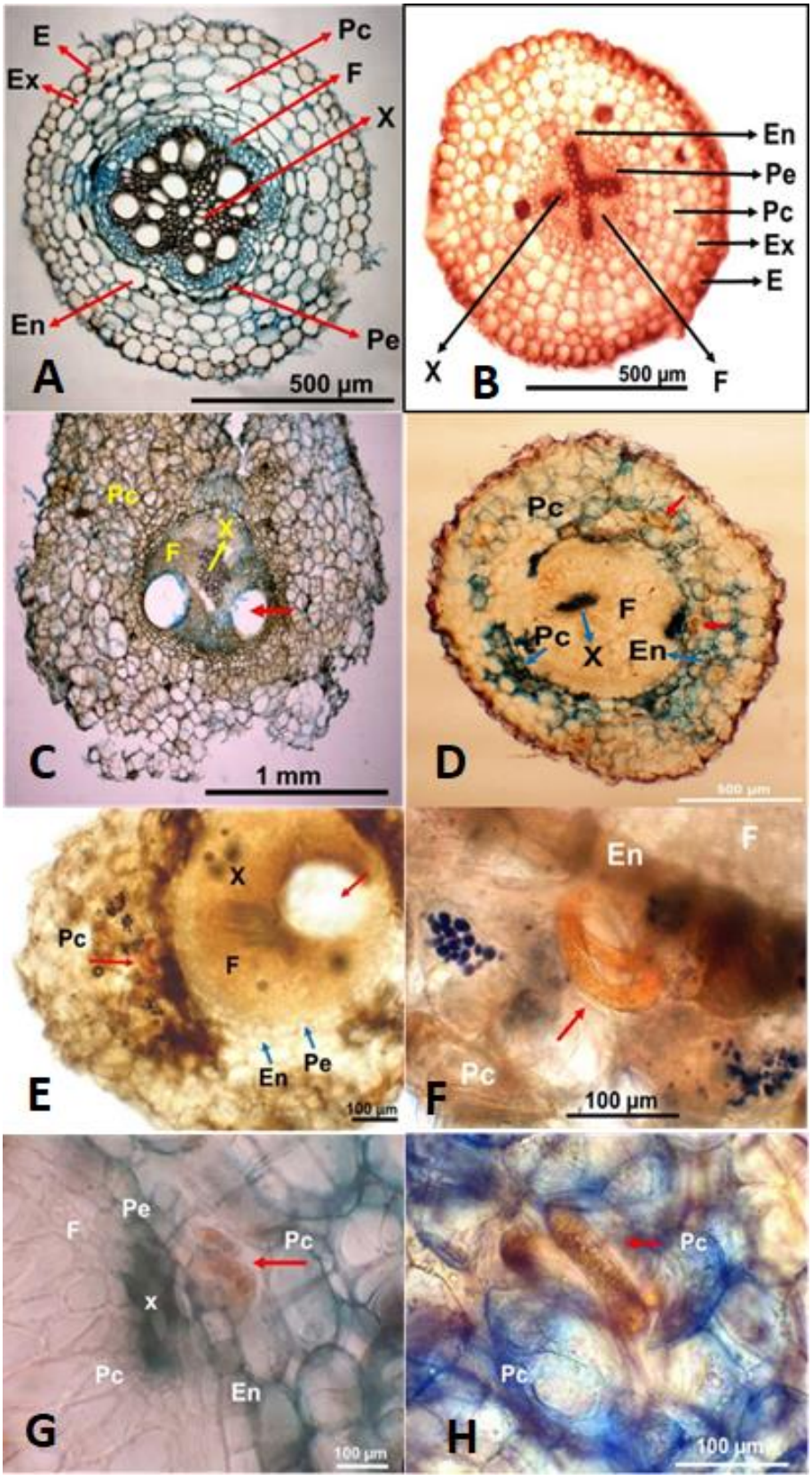

Fig. 1. Control root cross section (A-B) and a root with deformations due to the presence of galls (C-H) and their tissue patterns. Note the presence of nematodes in association with cortical cells with starch grains. E: epidermis, Ex: exodermis, Pc: cortical parenchyma, In: endodermis, Pe: pericycle, F: phloem, X: xylem, red arrow: presence of nematodes.

Table 1. Aerial and root dry weight, chlorophyll in pepper plants not inoculated (control) or inoculated with *N. aberrans*.

Treatments	Air dry weight (g)	Root dry weight (g)	Chlorophyll (µg cm^{-2})
Control	27 ± 2.41^{a}	22.80 ± 1.43^{a}	60.42 ± 8.61^{a}
Inoculated	21.83 ± 3.94^{b}	17.40 ± 1.69^{b}	29.85 ± 8.89^{b}
CV %	15.66	15.19	22.68
P- value	0.0460	0.0368	0.0003

Different letters indicate significant differences ($p < 0.05$).

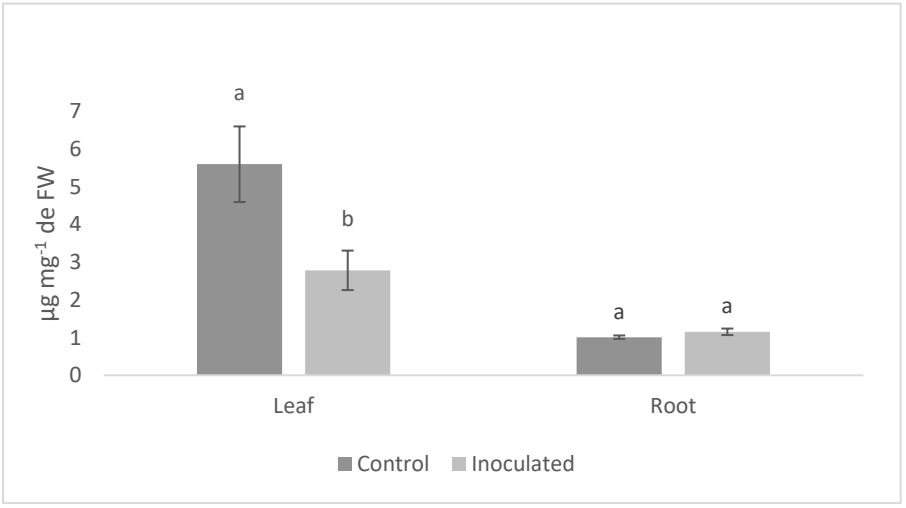

Fig. 2. Soluble proteins content of pepper plants not inoculated (control) or inoculated with *N. aberrans*. Different letters indicate significant differences ($p < 0.05$).

Net photosynthesis and transpiration decreased significantly, leading to lower water use efficiency in the inoculated plants compared to the control. Stomatal conductance in the inoculated plants was also significantly lower compared to the control plants (Table 2).

Table 2. Net photosynthesis (NP), transpiration (E), stomatal conductance (CS) and water-use efficiency (WUE) of pepper plants not inoculated (control) or inoculated with *N. aberrans*.

Treatments	NP (µmol m^{-2} s^{-1})	E (mmol m^{-2} s^{-1})	CS (mol m^{-2} s^{-1})	WUE (µmol mol^{-1})
Control	13.77 ± 3.56^{a}	2.47 ± 0.31^{a}	59.83 ± 8.44^{a}	6.5 ± 0.12^{a}
Inoculated	4.43 ± 1.27^{b}	0.94 ± 0,29^{b}	13.95 ± 2.54^{b}	4.4 ± 1.05^{b}
CV (%)	12.41	12.14	16.50	12.64
p- value	0.0146	0.00397	0.00390	0.0014

Different letters indicate significant differences ($p < 0.05$).

Proline content values showed significant differences in the leaves and roots. In the leaves, the proline content increased significantly in plants exposed to the nematode, while the opposite was observed in the roots (Table 3). Total and reducing sugar contents were significantly higher in the leaves than in the roots. In the leaves, the total and reducing sugar contents were significantly higher in plants infected with *N. aberrans*. Conversely, in the roots, no significant differences were observed in the results (Table 3).

Table 3. Proline content (P), total sugars (TS), and reducing sugars (RS) of pepper plants not inoculated (control) or inoculated with *N. aberrans*.

Treatments	P (µmoles g^{-1} FW)		TS (µg g^{-1} FW)		RS (µg g^{-1} FW)	
	Leaves	Roots	Leaves	Roots	Leaves	Roots
Control	3.53 ± 1.61[b]	4.29 ± 1.53[a]	495.69 ± 87.28[b]	108.07 ± 31,54[a]	419.62 ± 87.88[b]	77.67 ± 26.78[a]
Inoculated	6.61 ± 2.14[a]	3.46 ± 0.24[b]	830.06 ± 115.60[a]	154.17 ± 43,67[a]	557.43 ± 35.81[a]	43.12 ± 16.09[a]
CV (%)	12.27	8.73	12.93	16.50	27.44	16.04
p- value	0.0049	0.0271	0.0120	0.3138	0.0049	0.0852

Different letters indicate significant differences ($p < 0.05$).

Malondialdehyde (MDA) content was significantly higher in the leaves than in the roots. In the leaves, the highest values were observed in plants inoculated with the nematode (7.28 nmol MDA g^{-1} FW) compared to the control plants (5.66 nmol MDA g^{-1} FW). The same happened in roots, where the inoculated plants had higher MDA content than non-inoculated ones, with statistically significant differences (Table 4). Catalase activity was higher in the roots than in the leaves. In both cases, a significant increase in enzyme activity was observed in plants infected by *N. aberrans* compared to the control (Table 4). Regarding peroxidase activity, despite no significant differences observed, its values were higher in the roots than in the leaves. The highest peroxidase activity occurred in plants infected by the nematode (Table 4).

Table 4. Malondialdehyde content (MDA), catalase activity (CAT), and total peroxidase activities (POX) of pepper plants not inoculated (control) or inoculated with *N. aberrans*.

Treatments	MDA (nmol g^{-1})		CAT (U mg $prot^{-1}$)		POX (U mg $prot^{-1}$)	
	Leaves	Roots	Leaves	Roots	Leaves	Roots
Control	5.66 ± 0.50[b]	1.19 ± 0.16[b]	0.010 ± 0.0003[b]	0.018 ± 0,0002[b]	0.23 ± 0.11[a]	1.52 ± 0.43[a]
Inoculated	7.28 ± 0.29[a]	1.79 ± 0.22[a]	0.017 ± 0.0049[a]	0.027 ± 0.005[a]	0.44 ± 0.17[a]	1.69 ± 0.24[a]
CV (%)	12.14	5.44	24.17	15.66	10.85	22.48
p- value	< 0.0001	0.0009	0.015	0.0460	0.8025	0.6346

Different letters indicate significant differences ($p < 0.05$).

Discussion

Plants exposed to nematodes show wilting, decreased growth, yield losses, and even death (Cristobal et al., 2001). In this research, we assessed the impact of *N. aberrans* infection on Paco f1 pepper plants growing under experimental conditions. In the roots, the damage produced by the nematode was due to mechanical

and enzymatic mechanisms caused by its entrance to the root. Damage to the aerial tissues might be related to the dehydration processes suffered by the plant due to the alteration of radical tissues. *N. aberrans* has esophageal glands that secrete xylanase and polygalacturonase enzymes that induce gene reprogramming of the root cells to form the feeding sites (syncytes) where mature females will lodge. Syncytes are characterized by having dense cytoplasm, a large nucleus, and thick walls (Perry and Moens, 2011; Palomares-Rius et al., 2018). Thus, the connection between the phloem and xylem is lost, causing disorganization and reduction of the vascular system (Jones et al., 2013). In this study, the formation of syncytes in the vascular cylinder displaced the phloem and xylem. The cortical parenchyma proliferated, and its cells accumulated starch, a typical feature of *N. aberrans* infection (Lorenzo et al., 2001). Similar conditions were reported by Chavarro-Carrero et al. (2017) where, although they did not observe syncytia formation, juveniles of *N. aberrans* belonging to stages 3 and 4 occurred in the pepper pericycle, which was associated with the accumulation of starch granules. Similar results appeared in tomato, beet, and potato, where female *N. aberrans* formed syncytes in the central cylinder, which were associated with both hypertrophy and hyperplasia of the vascular tissue cells (Tovar Soto et al., 2012; Cabrera et al., 2016; Tordable et al., 2018). Due to the infection, the plants exposed to the nematode suffer water deficit (Hewezi and Baum, 2013). Low absorption of water and nutrients in hosts susceptible to nematodes may have links with a reduction in apical growth, resulting in severe growth stunts in plants, chlorosis, and leaf curl (Jatala, 1985). In this study, certain parameters, such as stem height and diameter, remained unaffected by the nematodes in the roots, as previously reported by Chaves et al. (2011) in tomatoes. They noted that the phytoparasite did not influence plant height in highly susceptible genotypes. After several generations, the nematode population reached high levels, and the damage began to affect growth (Manzanilla Lopez et al., 2002). However, this is also dependent on the population load in the soil, the susceptibility of the host crop, and environmental conditions. Chaves et al. (2011) indicated that the severity of the disease in tomato plant genotypes classified as susceptible and highly susceptible decreases the fresh weight of stem and roots, in agreement with our results where the dry weights of the aerial part and the root were lower in pepper plants inoculated with the nematode. This can be true since galling caused by the nematodes in the roots alters the absorption of water and nutrients and negatively affects plant growth and biomass accumulation (Cristobal et al., 2001). Chlorophyll and soluble protein contents in leaves decreased in plants infected by *N. aberrans*. Al-Yahya et al. (1998) also found a reduction of chlorophyll content in wheat infested by the nematode *Heterodera avenae*. The same was observed in tomato infested with *Meloydogine* sp., accompanied by a reduction of the nutritional status (Nayak and Moharty, 2010) and in pumpkin plants infected with *Meloidogyne incognit*a (Mahaprata and Nayak, 2019). In our work, the protein content in roots was slightly higher in plants infected by the phytoparasite. It could be due to the synthesis of proteins related to pathogenesis. These proteins, called PR proteins, are a variable group that accumulates in plants during and after pathogen infection (Madriz Ordeñana, 2002). Plants infested by the nematode showed an effect on leaf photosynthesis and transpiration, with the stomatal conductance significantly lower than in plants without infection. The same happened with net photosynthesis and transpiration. In tomato plants inoculated with *M. incognita*, photosynthesis, and stomatal conductance were severely affected by inoculation compared to non-inoculated and inoculated and grafted plants (Strajnar et al., 2012). After inoculating susceptible pepper with *M. incognita*, leaf photosynthesis was affected by altered stomatal regulation and photochemical efficiency compared to pepper with rootstock resistant to *Meloidogyne* sp. (Galvez et al., 2019). A decrease in transpiration rate reportedly occurred in various crops, e.g., tomato, potato, and mint, only with phytomatodes that cause alterations in root cells, such as *Meloidogyne sp*. and *Globodera sp*. (Gúzman-Piedrahíta, 2020). Plant infection by nematodes causes a systemic increase in associated substances, such as osmolytes, which can be useful as “indicators” of water stress (Moreno, 2009). Due to this stress type, solutes such as glycerol, sugars, betaines, and proline accumulate. They maintain cell turgor by osmotic adjustment (Wahid et al., 2007; Nagesh and Devaraj, 2008). In this work, both proline and total and reducing sugar contents were higher in plants inoculated with *N. aberrans* compared to the control group without nematode. It confirms previous findings by Garita et al. (2018) that grafted and ungrafted tomato plants in the presence of *N. aberrans* had higher proline content. Mahapatra and Nayak (2019) reported similar results in pumpkins, where the plants inoculated with *M. incognita* had a greater accumulation of proline and total sugars in the shoots and roots.

The stress produced by the nematode on plants also affected their normal growth and development due to the difficulty in absorbing water and nutrients. This stress causes lipid peroxidation, accelerating plant senescence and causing cell death (Göbel et al., 2003). In this work, penetration of mobile forms of N. aberrans with gall formation induced by females in the roots caused alterations in their cell membranes, evidenced by different markers of oxidative stress, such as the increase in the content of MDA. It is a by-product of lipid peroxidation in the membranes, and its concentration can assist as a marker of the type of stress to which plants are exposed (Arbona et al., 2003). According to our results, the MDA content increased in the leaves and roots of plants inoculated with N. aberrans, coinciding with El-Beltagi et al. (2012), who observed that in tomato plants inoculated with M. incognita, there was an increase in the accumulation of MDA, both in the leaves and roots. Ruscitti et al. (2015) observed that the MDA content in tomato plants varied between 1.4 and 2.4 nmol g-1 FW in plants growing at field capacity and those under drought stress, respectively. However, one of the fastest defense responses following nematode recognition is the so-called oxidative explosion, which consists of reactive oxygen species (ROS) at the nematode invasion site (Melillo et al., 2011). Catalase activity was significantly higher in pepper plants parasitized by N. aberrans in the leaves and roots. The activity was higher in leaves than in roots, which may be because catalase activity comes both locally and systemically (Arias et al., 2009). Similar results reportedly occurred in tomato plants inoculated with M. incognita where the enzymatic activity, particularly catalase and peroxidase activity in shoots and roots, was higher than in uninoculated plants (El-Beltagi et al., 2012). A similar response was observed in Vitis vinifera plants infested with M. incognita and Rotylenchus reniformis, where their MDA content increased (Kesba and El-Beltagi, 2012), thus confirming the results in this work. An increase in peroxidase activity has been associated with the pathogen infection to avoid the accumulation of hydrogen peroxide (H2O2) and a toxic cellular environment. However, these responses in plant peroxidase levels may also be associated with tissue lignification, one of the defense mechanisms indicated at the stem level (Hernandez and López, 2019). In this work, the total peroxidase activity was high in the plants inoculated with N. aberrans, although its levels did not show significant differences between treatments. Anjum et al. (2012) found an early increase in catalase levels and total peroxidase activities due to drought stress on pepper plants. Likewise, in tomato roots infested with gall-forming nematodes, local activation of defense genes encoding enzymes such as peroxidases, chitinases, lipoxygenases, and proteinase inhibitors, was also observed within the first 12 h after inoculation (Lambert, 1995). In resistant and susceptible tomato cultivars inoculated individually with M. javanica and Fusarium oxysporum, individually or combined, catalase and peroxidase activities and phenolic compounds and total sugar contents were higher in the inoculated plants (Lobna et al., 2016). However, Anjum et al. (2012) reported an opposite response in pepper plants. They observed that as the drought period extended, lipid peroxidation and electrolyte release through plant membranes increased compared to control plants. Therefore, cell damage is not only caused by the mechanical action of the nematode but also by water-deficit stress, which limits normal water absorption. This research offered valuable information about the morphological and biochemical responses of Paco f1 pepper plants to the incidence of N. aberrans infestation from the horticultural belt of La Plata. Thus, it contributes to our knowledge of this pest and may help in the design of appropriate management programs that reduce the population density of N. aberrans in the CHLP and expand studies to search for cultivars resistant to these nematodes as an alternative to the use of soil disinfectants and chemical products.

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Conflict of Interest

The authors indicate no conflict of interest in this work.

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