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Effect of salt stress on the parasitism of *Meloidogyne enterolobii* in cowpea

Efeito do stress salino sobre o parasitismo de *Meloidogyne enterolobii* em feijão-caupi Interacción

Efecto del estrés salino sobre el parasitismo de *Meloidogyne enterolobii* en el caupí

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Abstract
Cowpea, *Vigna unguiculata* (L.) Walp, is a very important Fabaceae in the diet of families in the northeast region of Brazil. This plant species is among the different cultures affected by the root-knot nematode. In this region, in addition to phytonematodes, another condition that affects cowpea productivity is salinity, one of the main abiotic limiting factors. The cultivar Pitiúba, however, has good adaptability to salt stress conditions. However, information on the behavior of root-knot nematodes in an environment with high levels of salinity for this crop is still scarce. Based on the above, the objective of this work was: 1) to evaluate the hatchability of J2 of *Meloidogyne enterolobii* in 100mM NaCl solution; 2) evaluate the motility and infectivity of J2 of *M. enterolobii* in saline solution at the levels of 0, 25, 50, 75, 100, 125, 150, 175, 200, 300, and 400mM; 3) to investigate the impairment of the development of *M. enterolobii* in cowpea Pitiúba under irrigation with NaCl solution at 100mM. It was found that J2 hatching, motility, and infectivity of juveniles of *M. enterolobii* were not affected by the levels of salinity tested in vitro. In Pitiúba, irrigation with 100 mM saline solution considerably affected the development of juveniles of the pathogen in the roots.
Keywords: Root-knot nematodes; *Vigna unguiculata;* Salinity.

Resumo
O feijão-caupi, *Vigna unguiculata* (L.) Walp, é uma fabácea muito importante na dieta de famílias da região nordeste do Brasil. Esta espécie de planta está entre as diferentes culturas afetadas pelo nematoide das galhas. Nessa região, além dos fitonematóides, outra condição que afeta a produtividade do feijão-caupi é a salinidade, um dos principais fatores limitantes abióticos. A cultivar Pitiúba, entretanto, apresenta boa adaptabilidade às condições de estresse salino. No entanto, informações sobre o comportamento dos nematoides das galhas em ambiente com altos níveis de salinidade para esta cultura ainda são escassas. Com base no exposto, o objetivo deste trabalho foi: 1) avaliar a eclodibilidade de J2 de *Meloidogyne enterolobii* em solução de NaCl 100mM; 2) avaliar a motilidade e infectividade de J2 de *M. enterolobii* em solução salina nos níveis de 0, 25, 50, 75, 100, 125, 150, 175, 200, 300 e 400mM; 3) investigar o comprometimento do desenvolvimento de *M. enterolobii* em feijão-caupi Pitiúba sob irrigação com solução de NaCl a 100mM. Verificou-se que a eclosão de J2, a motilidade e a infectividade dos juvenis de *M. enterolobii* não foram afetadas pelos níveis de salinidade testados *in vitro*. Em Pitiúba, a irrigação com solução salina 100 mM afetou consideravelmente o desenvolvimento de juvenis do patógeno nas raízes.
Palavras-chave: Nematoides das galhas; *Vigna unguiculata*; Salinidade.

Resumen
El caupí, *Vigna unguiculata* (L.) Walp, es una fabaceae muy importante en la dieta de las familias de la región noreste de Brasil. Esta especie vegetal se encuentra entre los diferentes cultivos afectados por el nematodo agallador. En esta región, además de los fitonematodos, otra condición que afecta la productividad del caupí es la salinidad, uno de los principales factores limitantes abióticos. El cultivar Pitiúba, sin embargo, tiene una buena adaptabilidad a las condiciones de estrés salino. Sin embargo, la información sobre el comportamiento de los nematodos agalladores en un ambiente con altos niveles de salinidad para este cultivo es aún escasa. Con base en lo anterior, el objetivo de este trabajo fue: 1) evaluar la incubabilidad de J2 de *Meloidogyne enterolobii* en solución de NaCl 100 mM; 2) evaluar la motilidad e infectividad de J2 de *M. enterolobii* en solución salina a niveles de 0, 25, 50, 75, 100, 125, 150, 175, 200, 300 y 400 mM; 3) investigar el deterioro del desarrollo de *M. enterolobii* en caupí Pitiúba bajo riego con solución de NaCl a 100 mM. Se encontró que la eclosión de J2, la motilidad y la infectividad de los juveniles de *M. enterolobii* no se vieron afectadas por los niveles de salinidad probados *in vitro*. En Pitiúba, el riego con solución salina 100 mM afectó considerablemente el desarrollo de juveniles del patógeno en las raíces.
Palabras clave: Nematodos Agalladores; *Vigna unguiculata*; Salinidad.

1. Introduction

Salinity is a limiting factor for plant development, due to decreased productivity in several agronomic crops decreasing the rate of absorption by the root system, with changes in the assimilation and transport of water and nutrients in the plant, producing disturbances at the physiological and physical level in plants (Sá *et al*., 2018; Souza *et al*., 2019). Soil salinity is a worldwide problem that affects approximately 10% of all soils and half of the irrigated areas in the world (Kamran *et al*., 2020; Singh, 2021). Salinization is a process that occurs in poorly managed soils, usually with poor drainage conditions, indiscriminate use of fertilizers, low-quality water used for irrigation, or natural causes due to the weathering of rocks, which have high levels of soluble salts (Aderaldo *et al*., 2020; Araújo *et al*., 2019). A soil is considered saline when it has an electrical conductivity greater than 4 dS/m (40 mM) (Jesus & Borges, 2020). The high concentration of salts in the soil solution occurs mainly in arid and semi-arid regions in the world (Oliveira *et al*., 2015; Etikala *et al.,* 2021).

In Brazil, in the northeast region, the occurrence of salinity problems in soils is commonly reported, mainly due to the use of low-quality water, with the accumulation of Na^+ and Cl^- ions, in irrigation (Ribeiro *et al*., 2016; Vasconcelos *et al*., 2013).

Research aimed at improving plant species resistance to abiotic factors has been gaining more and more notoriety in the world. Among the plants with a tolerance to salinity, cowpea (*Vigna unguiculata* (L.) Walp) is an example, a species well adapted to the edaphoclimatic conditions of the northeast region of Brazil (Ayers & Westcot, 1999; Silva *et al*., 2020).

In addition to salinity, diseases caused by phytonematodes are also an important problem for cultures worldwide, highlighting the species of the genus *Meloidogyne* Goeldi. In Brazil, among the several species of root-knot nematode that have already been identified in the northeast region, there is the highlight for *M. enterolobii* Yang and Eisenback, commonly associated with the losses caused in the guava tree (*Psidium guajava* L.) (Castro, 2019; Cavalcanti *et al.*, 2021). In addition to this fruit, this pathogen has a wide range of hosts, including grain-producing crops, such as cowpea (Castro, 2019; Guimarães *et al*., 2003), already registered Fabaceae associated with *Meloidogyne* spp. in the northeast region (Sobrinho, 2016). In Ceará state, isolates of *M. enterolobii* were recorded in several agricultural regions in different cultures (Silva *et al*., 2017). The root-knot nematodes can adapt to various environments with variations in temperature, humidity, and salinity (Adrian *et al*., 2009).

In the literature, there are several studies on the effects of salinity on the physical and chemical properties of the soil (Sardinha *et al*., 2003), but few studies focusing on soil microbiota. Information on the behavior of root-knot nematodes in an environment with salinity levels is also scarce.

Thus, the objectives of this work were: i. evaluate the hatching of juvenile of the second stage (J2) of *M. enterolobii* in saline solution at 100 mM NaCl; ii. evaluate the motility and survival of J2 of *M. enterolobii* in saline solution at different levels of salinity; iii. to investigate the infectivity and the development of *M. enterolobii* in cowpea under irrigation with saline

solution at the level of 100 mM NaCl.

2. Methodology

To evaluate the interaction between saline stress and *M. enterolobii* parasitism in cowpea, the cultivar Pitiúba launched by Federal University of Ceará (UFC) was selected, which stands out for its good agronomic characteristics and for having good adaptability to salt stress conditions (Paiva *et al*., 2014).

The population of *M. enterolobii* used in this study came from the collection of phytonematodes from the Phytopathology Laboratory of UFC, whose identification of the isolate was carried out by molecular techniques (Silva *et al*., 2016). The extraction of eggs from the roots for the tests was carried out according to the methodology of Bonetti & Ferraz (1981). The choice of this *Meloidogyne* species was related to its occurrence in several microregions in Ceará state (Silva *et al*., 2016).

To evaluate the in vitro effect of salinity on the hatching of juveniles of *M. enterolobii*, 50 eggs of the phytonematode were added in 3 mL of a solution with 100 mM NaCl, distributed in Petri dishes of 3 cm in diameter. Distilled water was used as a control. The plates were placed in trays and kept in the environment, at approximately 27ºC. The hatching of the J2s was evaluated daily for 15 days after the assembly of the bioassay. The design used was completely randomized, consisting of 2 treatments and 6 repetitions, in which each plate composed one repetition, totaling 12 plates and 600 eggs.

For the in vitro study of motility, survival, and infectivity of *M. enterolobii* in phase J2 in saline solution, juveniles obtained from egg masses taken from Santa Clara tomato roots (*Solanum lycopersicum* L.) were used in a hatching chamber. The bioassay was divided into two stages. The first phase consisted of depositing 50 individuals of J2 of *M. enterolobii* in saline solution in Petri dishes of 3 cm in diameter, in different salt concentrations (0, 25, 50, 75, 100, 125, 150, 175, 200, 300, and 400 mM NaCl). After the 48-hour incubation period at 27 ° C, mobile and immobile juveniles were counted to determine survival and mortality. To confirm mortality, the immobile J2 were transferred from saline solutions to a Petri dish containing distilled water, and after 24 hours the individuals were again evaluated. This bioassay was conducted in a completely randomized design, consisting of 11 treatments with 6 replications, totaling 66 plates and 3,300 juveniles.

Considering that the Pitiúba cowpea, a variety selected for use in the other trials, supports salinity well at the maximum level of 100mM NaCl (Alves, 2015), it was decided to test the infectivity of juveniles submitted to this level of salinity, extending the individuals remained in the 100 mM saline solution for 15 days, during which time they still had motility. Thus, 150 individuals were removed directly from the saline solution and inoculated in Santa Clara tomato seedling, after 45 days, to evaluate the presence, or not, of root-knots on the roots. Juveniles that remained in the water for 15 days were also inoculated in another tomato seedling. In both cases, the tomatoes kept in the greenhouse were irrigated with water.

The cowpea seeds, cultivar Pitiúba, were obtained from the UFC Germplasm Bank. A cowpea seed was planted per pot with a capacity of 1.5 L, containing river sand washed 5 times, to remove salts and clays present, and autoclaved at 120 ° C for 50 min. From 10 days after sowing, the plants were subjected to irrigation with saline or desalinated water. At 12 DAS, 5,000 eggs/J2 of *M. enterolobii* were inoculated, according to the following treatments: A. plants inoculated and irrigated with 100 mM NaCl solution; B. plants inoculated and irrigated with desalinated water; C. uninoculated plants, and irrigated with 100 mM NaCl solution; D. plants not inoculated and irrigated with desalinated water (control), which were examined in two periods, 15 and 18 days after inoculation. The trial was conducted in a completely randomized design, resulting in 4 treatments with 10 repetitions. The desalinated water used in this study was obtained through the WW2 desalinator (Ferran®). Irrigation was performed daily in each pot.

Part of the plants from the 4 treatments was removed at 15 DAI (17 days of irrigation with saline) and the rest of the

plants after 18 DAI (20 days of irrigation with saline). The difference in the evaluation period was due to the chlorotic aspect presented by the plants, which was accentuated at 18 days, with the end of the bioassay. Considering the time elapsed from inoculation at the end of the test, the number of galls (NG) and the nematode development stages present in the roots at 15 and 18 DAI were considered for the evaluation. To identify the phases of the pathogen in the root system, the method of staining root nematodes with acid fuchsin was used (Byrd *et al.*, 1983). The 1 to 2 cm sections of the stained roots were distributed in slides that were then pressed with a coverslip, for observation and recording of the nematode phases present under an optical microscope.

All bioassays were repeated twice. The quantitative data were subjected to analysis of variance (ANOVA) and the averages to the Tukey test at the level of 5% probability, using the Sisvar software (Ferreira, 2014).

3. Results and Discussion

The data for the hatching of *M. enterolobii* from the control treatments (distilled water) and 100 mM NaCl solution can be seen in Table 1.

Table 1. Hatching percentage of second stage juveniles of *Meloidogyne enterolobii* under interference of a 100 mM NaCl solution and desalted water (control) at 3, 6, 9 and 12 days.

Treatments	3º Day	6º Day	9º Day	12º Day
100 mM of NaCl	12	32	94	100
Control	6	28	86	100

Source: Authors.

In the first evaluations (3ºand 6tº day), the use of NaCl in the concentration of 100 mM did not affect the hatching of juveniles of *M. enterolobii*, observing that from the 9th day the number of hatched J2 was similar to the control, occurring in both treatments 100% hatching on the 12º day (Table 1).

Table 2 shows the results for the average number of immobile J2 after immersion in various concentrations of NaCl solution (0-400 mM) and the percentage of J2 that recovered motility after passing in distilled water.

Table 2. Data on the mean of second stage juveniles motility of *M. enterolobii* after 24h and 48h of immersion in saline in different concentrations of NaCl.

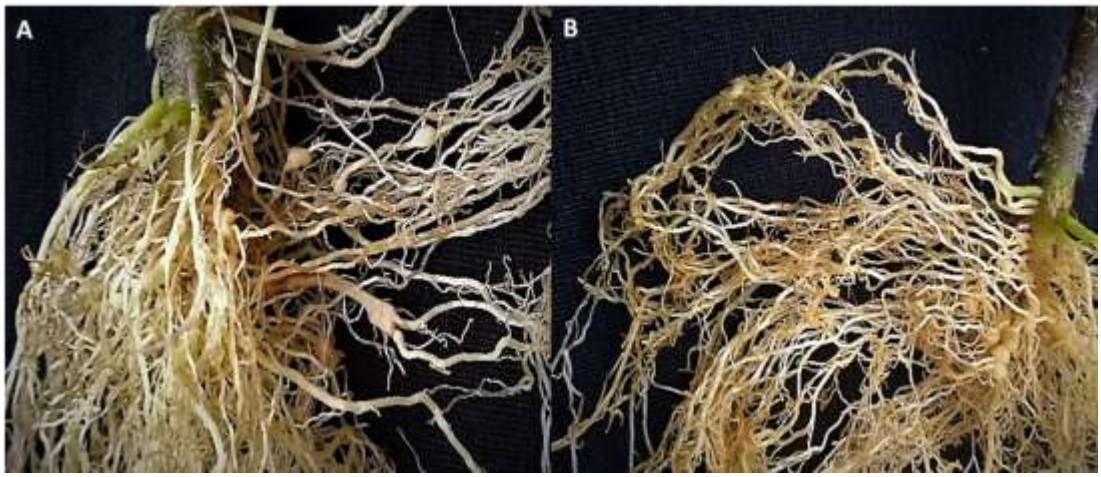
Concentrations of NaCl (mM)	J2 not mobile (24h)	J2 not mobile (48h)	J2 mobile* (%)
0	0.0	0.0 f	-
25	0.0	0.0 f	-
50	0.0	0.0 f	-
75	0.0	0.0 f	-
100	0.0	2.2 ef	100
125	0.0	3.7 ef	100
150	0.0	5.3 e	100
175	0.0	13.5 d	100
200	0.0	24.7 c	100
300	0.0	41.0 b	100
400	0.0	48.7 a	100
CV[1]	-	15.74	-

*After 48h in saline and 24h in distilled water.
[1]CV: coefficient of variation.
Source: Authors.

As shown in Table 2, after 24h of immersion in the 11 treatments, all J2 of *M. enterolobii* were perfectly active. However, after 48 hours, the occurrence of juvenile immotility was verified, which increased with the increase in salinity levels, being higher than 97% in 400 mM. In the control and at the levels of 25, 50, and 75 mM, immotility was not observed in the individuals even after 48h of exposure in saline solution, the reason for which they were not transferred to the distilled water. All immovable J2 in solutions with concentrations between 100 and 400 mM NaCl recovered their movement after being transferred to distilled water (Table 2), indicating a nematostatic effect at concentrations above 100 mM.

In the second part of the trial, when investigating the infectivity of juveniles after 15 days in saline, it was found, after 45 days, the presence of many galls in the roots of inoculated Santa Clara tomato plants, both with juveniles that were exposed to salinity as with those who stayed in the water, thus demonstrating that J2 infectivity was not affected by salt (Fig. 1A-B).

Figure 1. Galls on Santa Clara tomato root 45 days after inoculation of *Meloidogyne enterolobii* with: A. J2 that remained in saline solution for 15 days; B. J2 that remained in water (control).



Source: Authors.

The examination using an optical microscope of the roots stained with acid fuchsin from Pitiúba plants irrigated with 100 mM saline solution and with water showed different results regarding the number and development of nematode phases (Table 3).

Table 3. The average number of *M. enterolobii* individuals present in Pitiúba roots irrigated with 100 mM saline solution for 15 and 18 days.

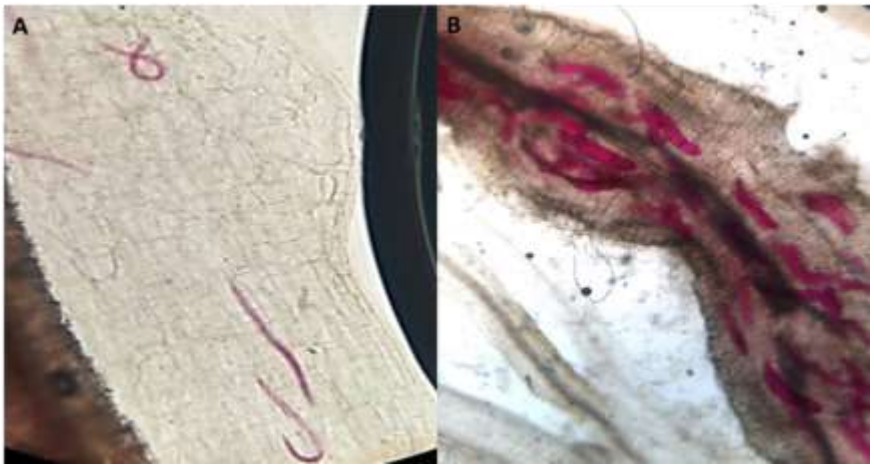
Stages	15º DAI*		18º DAI*	
	Saline solution	Control	Saline solution	Control
J2	24.7	17.3	4.3	19.2
J3	0.0	42.6	0.6	68.4
J4	0.0	40.0	2.1	82.2
Female	0.0	0.6	0.0	4.0

*DAI: days after inoculation.
Source: Authors.

At 15º DAI, the roots of plants irrigated with the saline solution presented average values of 24.7 J2, varying from 10 to 55 individuals/root (Fig. 2A). Phases J3 and J4 were not observed, nor the presence of galls on the roots. For plants irrigated

with desalinated water, the average values were 17.3 (7-35) J2, while for J3 the average was 42.6 (11-98). The mean for J4 was 40 (12-56) and for females 0.6 (0-2) (Fig. 2B). In these plants, an average number of 14 galls was observed.

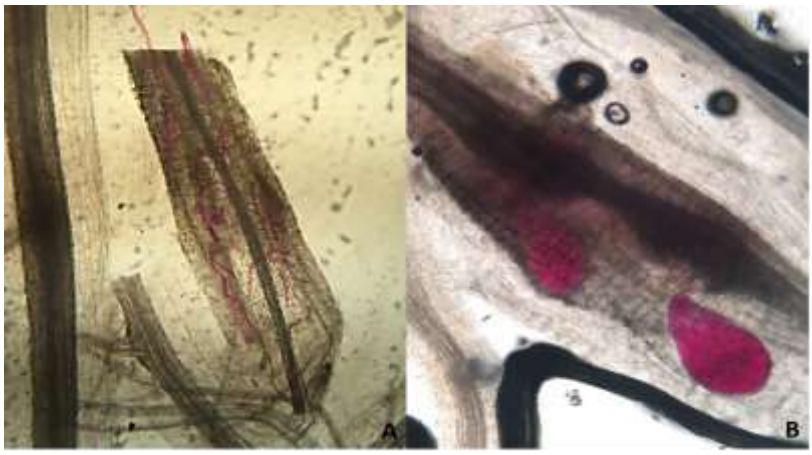
Figure 2. *Meloidogyne enterolobii* 15 days after inoculation in cowpea Pitiúba irrigated with: A) saline solution presenting only second stage juveniles; B) desalinated water with many third and fourth stage individuals.



Source: Authors.

The plants removed at 18º DAI, kept under irrigation with the saline solution presented average values of 4.3 J2 (1-12) (Figure 3A), 0.6 J3 (0-2), 2.1 J4 (3-10), and absence of females. Plants irrigated with desalinated water had an average value of 19.2 J2 (0-47), 68.4 J3 (31-89), 82.2 J4 (44-127), 4.0 females (1-9), and 35.4 galls (Fig. 3B).

Figure 3. Pitiúba cowpea roots 18 days after inoculation with *Meloidogyne enterolobii* presenting: A) only spindly and cylindrical second stage juveniles (saline); B) presence of females (desalinated water).



Source: Authors.

4. Discussion

In the present study, the hatching of *M. enterolobii* was not influenced by the concentration of 100 mM NaCl tested. This result differs from those observed in some works published with this genus of phytonematodes. It has been

reported that the hatching of juveniles of *Meloidogyne* spp. it is affected by salinity, with a decrease in hatching rate as the salinity increases, as exemplified by Dropkin *et al*. (1958), Edongali & Farris (1981), Khan & Khan (1990), and Lal & Yadav (1975). Adrian et al. (2009) revealed that the concentration of 300 mM NaCl can induce J2 quiescence of *M. javanica* and that *M. hapla* is intolerant to 800 mM NaCl. Shepherd & Clarke, 1971 found that the hatching of juvenile nematodes is influenced by different factors, such as temperature, soil moisture, pH, and organic or inorganic chemical components present in the soil water solution. In general, there is a lack of research on the hatchability of *Meloidogyne* spp. in saline conditions in Brazil.

Regarding the infectivity of J2 in the presence of saline solution, similar results to this study were obtained by Edongali & Ferris (1981) who observed that the infectivity of J2 de *Meloidogyne* spp. it was not altered after exposure to NaCl for seven days. According to the authors, the number of galls in tomatoes was higher than the control with water, possibly due to the osmoregulation process in J2.

Khan & Khan (1990) observed that NaCl at a concentration of 5 mM significantly induced the mortality of J2 from *M. javanica* and *M. incognita*. However, in this study, J2 of *M. enterolobii* was not affected in the 100 mM saline solution even after 15 days.

The high permeability of nematodes to ions has a deleterious effect on their metabolism, and this unbalances several biological processes in phytonematodes, including infectivity as reported by Adrian *et al*. (2009) in *M. incognita* inoculated with J2 submitted to different concentrations of NaCl in tomato.

In general, a delay in the development of the J2 phase of *M. enterolobii* in plants irrigated with saline compared to the control was found in this test. Similar results were obtained by Heald & Heilman (1971) for *Rotylenchulus reniformis*, who attributed the delay of *R. reniformis* juveniles to the limitation of the development of the root system of the cotton plant by salinity and not by the direct effect of salinity on nematodes. Corroborating this information, Sumera *et al.* (2015) in experiments involving salinity (1.71 mM NaCl), conducted with eggplant (S*olanum melongena* L.) inoculated with *M. javanica*, observed impairment in the development of juveniles, reporting a greater number of J2 and a lower number of J3 and J4 in eggplant roots when compared to the control with water.

Wallace (1966) mentions that the success of juvenile root infection depends on factors such as an adequate host and the speed of J2 penetration, with the osmotic potential and pH having less influence. Edongali *et al*. (1982), however, found that the penetration of *M. incognita* juveniles into tomato plants was affected by the concentration of saline in the soil, which may have caused the depletion of their body reserves, possibly leading to less infectivity by reducing metabolic activity and movement in the soil, which could inhibit the search for infection sites in the root. The authors also cite a delay in the formation of mature females in saline conditions, but that they may be able to produce eggs. Possibly, the difference in the number and phases of individuals of *M. enterolobii* observed in the roots irrigated with saline solution compared to water, in the two periods evaluated (15 and 18 days), where it was always observed in a greater number of individuals from phase J2, it could be attributed to a supposed delay in penetration coupled with depletion of their bodily reserves, resulting in a delay in the development of juveniles at the roots.

Khan *et al*. (1997) in trials with *M. incognita* race 2 in okra and cucumber mentioned that there was less penetration of juveniles and delayed development in plant roots in saline soils (50 mM). The delay induced by salinity in pathogenicity and the production of females and egg mass can lead to a reduction in the population of the pathogen. According to the authors, the apparent suppression of salinity in the penetration and reproduction of the nematode results in some improvement for the growth of plants.

In studies by Asif *et al*. (2021) and Devran & Baysal (2018), who used the same NaCl concentration in this study (100 mM) to irrigate tomato plants inoculated with *M. incognita*, a reduction in the number of galls was observed, a behavior similar to that verified in this research with *M. enterolobii*.

In contrast to this information, in a study conducted with plants whose citrus rootstocks, both sensitive and resistant to *Tylenchulus semipenetrans* Cobb, were exposed to salinity, Mashela *et al*. (1992) observed that there was a predisposition of plants to parasitism by the nematode. The accumulation of salt and the leaching cycles in the soil would have favored the increase of the population of the pathogen in the roots. According to the authors, similar behavior occurs during the rainy season in infested orchards irrigated with saline water during the dry season.

5. Conclusion

Salinity did not affect hatching and motility of *M. enterolobii* juveniles *in vitro* and the presence of J2 in saline solution for 15 days did not interfere with its infectivity in tomato. However, the irrigation with saline solution at 100 mM NaCl in Pitiúba inoculated with *M. enterolobii* considerably reduced the development of juveniles of the pathogen in tomato roots.

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