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[Artigo]

Evaluation of forage sorghum 'BRS Ponta Negra' and plant extracts against *Pratylenchus coffeae*, causal agent of yam dry rot disease

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ABSTRACT

Introduction. *Pratylenchus coffeae* occurs in yam (*Dioscorea* spp.) production areas, reducing tuber yield and quality. **Objective**. To evaluate the host suitability of forage sorghum 'BRS Ponta Negra' to *P. coffeae* and the nematicidal activity of aqueous extracts from *Croton* sp., *Monteverdia rigida*, *Sarcomphalus joazeiro*, *Sideroxylon obtusifolium*, *Byrsonima gardneriana*, *Astronium urundeuva*, and *Tocoyena formosa* against this nematode. **Material and Methods**. In two greenhouse experiments, 37-day-old sorghum plants were inoculated with 250, 500, 750, or 1,000 nematodes (juveniles and adults). Yam plants were used as a susceptible control. Eighty days after inoculation, the number of nematodes in roots and soil, as well as the reproduction factor (RF), were evaluated. Aqueous plant extract concentrations (0% - control; 0.5%; 1.0%; 1.5%; and 2.0%) were tested by adding 200 µL of each extract and 25 nematodes per cavity in Kline plates. After 24 h, immobile specimens were counted and transferred to distilled water for an additional 24 h to determine mortality. **Results and Discussion**. Forage sorghum 'BRS Ponta Negra' was classified as a poor host for *P. coffeae* ($0 \leq RF < 1$). All plant extracts caused immobility and mortality in *P. coffeae*, with the leaf extract of *S. obtusifolium* being the most effective, resulting in mortality rates above 90% at all concentrations tested. **Conclusion**. 'BRS Ponta Negra' shows potential for use in crop rotation systems. The leaf extract of *S. obtusifolium* demonstrates high nematicidal activity against *P. coffeae*.

KEYWORDS: Management. *Dioscorea* spp. Root-lesion nematode. Disease.

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Avaliação do sorgo forrageiro 'BRS Ponta Negra' e de extratos vegetais contra *Pratylenchus coffeae*, agente causal da casca-preta-do-inhame

RESUMO

Introdução. *Pratylenchus coffeae* ocorre em áreas de produção de inhame (*Dioscorea* spp.), reduzindo o rendimento e a qualidade dos rizóforos. **Objetivos**. Avaliar o sorgo forrageiro 'BRS Ponta Negra' e a atividade nematicida de extratos aquosos de *Croton* sp., *Monteverdia rigida*, *Sarcomphalus joazeiro*, *Sideroxylon obtusifolium*, *Byrsonima gardneriana*, *Astronium urundeuva* e *Tocoyena formosa* sobre *P. coffeae*. **Material e Métodos**. Em dois experimentos conduzidos em casa de vegetação, plantas de sorgo com 37 dias de cultivo foram inoculadas com 250, 500, 750 e 1.000 nematoides (juvenis e adultos). O inhame foi utilizado como padrão de suscetibilidade. Oitenta dias após a inoculação, foram avaliados o número de nematoides no sistema radicular e no solo de cada planta e o fator de reprodução (FR). Concentrações aquosas dos extratos vegetais (0% - testemunha; 0,5%; 1,0%; 1,5% e 2,0%) foram testadas em cavidades de placas de Kline, empregando-se 200 µL de extrato e 25 nematoides. Após 24 horas de incubação, foi quantificada a imobilidade dos espécimes. Aqueles imóveis foram transferidos para água destilada por mais 24 horas para avaliação da mortalidade. **Resultados e Discussão**. A cultivar BRS Ponta Negra foi classificada como má hospedeira para *P. coffeae* ($0 \leq RF < 1$). Todos os extratos causaram imobilidade e mortalidade de *P. coffeae*, sendo o extrato foliar de *S. obtusifolium* o mais efetivo, com mortalidade acima de 90%, em todas as concentrações testadas. **Conclusão**. 'BRS Ponta Negra' apresenta potencial para ser empregada em sistema de rotação de cultura. O extrato foliar de *S. obtusifolium* resulta em elevada mortalidade de *P. coffeae*.

PALAVRAS-CHAVE: Manejo. *Dioscorea* spp. Nematoide-das-lesões-radiculares. Doença.

1 INTRODUCTION

In Brazil, yams (*Dioscorea* spp.) represent an important food crop and a valuable source of carbohydrates. In addition, they play a prominent sociocultural role by generating employment and income, with production concentrated mainly in the Northeastern region. In 2024, an estimated 256,678 tons of tubers were produced on 26,216 hectares (FAOSTAT, 2024).

One of the major constraints to yam production is the occurrence of plant-parasitic nematodes. Among them, the migratory endoparasites *Scutellonema bradys* (Steiner & LeHew, 1933), *Pratylenchus coffeae* (Zimmermann, 1898), and *P. brachyurus* (Godfrey, 1929) are known to cause tuber dry rot disease, resulting in severe damage to the crop, mainly through reductions in tuber yield and quality (Moura, 2016). Among these species, *P. coffeae* is widely distributed in yam fields in northeastern Brazil (Muniz *et al*., 2012; Lira *et al*., 2014).

Crop rotation is a primary management strategy for limiting losses caused by plant-parasitic nematodes. However, because *Pratylenchus* spp. have a wide host range, the effectiveness of this practice is limited (Castillo; Vovlas, 2007). Promising results regarding the use of crop management practices for managing yam dry rot disease were reported by Silva *et al*. (2014), who identified plant species such as sunn hemp (*Crotalaria* spp.) and lima bean (*Phaseolus lunatus* L.) as capable of reducing *P. coffeae* populations in yam fields.

Sorghum cultivation contributes to nematode management mainly through the rotation effect, defined as the reduction of nematode populations by growing a poor host or non-host species. Therefore, the effectiveness of sorghum in nematode management depends on the target nematode species and may vary among cultivars (Dover *et al*., 2021). The sorghum cultivar BRS Ponta Negra was classified as a poor host (RF < 1.00) when evaluated as a cover crop for the management of *Meloidogyne ethiopica* Whitehead, 1968 (Ragassi, 2024).

Another promising approach for managing yam dry rot disease is the use of plant extracts (Farias *et al*., 2020; Magalhães *et al*., 2020). This method is characterized by low toxicity, a reduced likelihood of inducing pathogen resistance, and compatibility with other management strategies (Ferraz *et al*., 2010). Numerous plant species can be used to produce extracts with activity against plant-parasitic nematodes due to the presence of bioactive compounds with nematicidal properties (Singh; Prasad, 2014). Native species from the Caatinga biome in the Brazilian Northeast region, e.g. *Tocoyena formosa* (Cham. & Schltdl.) K. Schum and *Croton* spp., have shown the presence of secondary metabolites, including tannins and saponins, in phytochemical analyses (Mesquita Neto *et al*., 2010; Cesário *et al*., 2018). Given the high plant diversity in this biome, further studies are warranted to identify new species with nematicidal potential.

Crop rotation can be combined with other management strategies, such as the use of plant extracts, to reduce *P. coffeae* populations and mitigate their impact on yam tubers. In this context, the objectives of this study were to evaluate the host suitability of the forage sorghum cultivar BRS Ponta Negra for *P. coffeae* and to assess the nematicidal activity of aqueous extracts from seven Caatinga plant species against this nematode.

2 MATERIAL AND METHODS

2.1 Location, climate and characteristics of the experiments

The experiments were conducted in the Nematology Laboratory and greenhouse at the Center for Agrarian Sciences (CECA) of the Federal University of Alagoas (UFAL), Rio Largo, AL, Brazil. During the *in vivo* assays, the average temperature was 28.1 ± 3.8 ºC, and the relative air humidity was 79.6 ± 11.3%, as recorded using a datalogger (Instruterm® HT-500).

Populations of *P. coffeae* were obtained from infected yam tubers collected in the state of Alagoas, and nematodes were extracted according to Coolen and D'Herde (1972). Nematode identification was based on morphological characteristics (Gonzaga *et al*., 2016).

2.2 Experiment with forage sorghum 'BRS Ponta Negra'

Seeds of forage sorghum, *Sorghum bicolor* (L.) Moench cv. BRS Ponta Negra, were sown in a commercial substrate composed of composted and carbonized pine bark, vermiculite, limestone, and NPK additives (Maxfertil®). Thirty days after sowing, seedlings were transplanted into 3.8-L pots containing soil sterilized in a forced-air oven (100°C for 24 h). Seven days after transplanting, plants were inoculated with 250, 500, 750, or 1,000 *P. coffeae* individuals (juveniles and adults), applied to the soil near the base of each plant. Sprouting yam seed tubers (*Dioscorea cayenensis* Lam. cv. Da Costa) were included as a susceptible control and were planted four months prior to sorghum due to differences in growth cycle. These plants were inoculated 30 days after planting with a suspension containing 1,000 nematodes. The experiment was conducted twice, independently, in a completely randomized design, with four *P. coffeae* inoculum densities and eight replications.

Eighty days after inoculation, sorghum roots were collected, and nematodes were extracted according to Coolen and D'Herde (1972). Additionally, 100 cm³ of soil per pot was processed using the method described by Jenkins (1964). The susceptible control was evaluated seven months after inoculation following the same procedures.

After extraction, nematodes were counted in triplicate using Peters slides under a light microscope. The total number of nematodes per replication was used to calculate the reproduction factor [RF = final population (number of *P. coffeae* in sorghum root system + soil or yam root + tuber peel + soil)/initial population (inoculum density of *P. coffeae*)], allowing classification of plants as good hosts (RF > 1), non-hosts (RF = 0), or poor hosts (RF < 1) (Seinhorst, 1965; Njekete *et al*., 2025). Data were subjected to analysis of variance ($P < 0.05$).

2.3 Bioassay with plant extracts

For the *in vitro* assay, stock solutions were obtained from seven plant extracts prepared at the Chemistry Laboratory of Centro Universitário CESMAC, Maceió, AL, Brazil. The plant materials included leaves of *Croton* sp. (alecrim-de-vaqueiro), *Monteverdia rigida* (Mart.) Biral (bonome), *Sarcomphalus joazeiro* (Mart.) Hauenschild (juazeiro), and *Sideroxylon obtusifolium* (Roem. & Schult.) T.D. Penn. (quixabeira); bark of *Byrsonima gardneriana* A. Juss. (murici); leaves and bark of *Astronium urundeuva* (M.Allemão) Engl. (aroeira); and leaves and fruits of *T. formosa* (jenipapo). All extracts were obtained by ethanolic maceration for 5 days, with solvent replacement every 24 h, followed by concentration using a rotary evaporator to obtain the crude extract.

The extracts were diluted in distilled water to obtain concentrations of 0% (control), 0.5%, 1.0%, 1.5%, and 2.0% (v/v). In Kline plates, 200 µL of each extract concentration was added, and 25 nematodes (juveniles and adults) were individually transferred from the suspension to each well. Plates were then placed in plastic boxes containing filter paper moistened with distilled water to maintain humidity and incubated at 24 ± 1 ºC in a growth chamber. After 24 h, immobile nematodes were counted under an inverted light microscope (Labomed TCM 400) at 100× magnification and subsequently transferred to distilled water. Individuals that did not regain motility after 24 h in water were considered dead.

The bioassay was conducted in a completely randomized design in a 9 × 5 factorial scheme (nine extracts × four concentrations for each extract, plus the control treatment with distilled water), with five replications. Data were subjected to analysis of variance, and means were compared using the Scott-Knott test at 5% probability. Linear regression analyses were performed to evaluate the effect of extract concentration on nematode immobility and mortality. Statistical analyses were conducted using the Sisvar software, version 5.8 (Ferreira, 2019).

3 RESULTS

3.1 Experiment with forage sorghum 'BRS Ponta Negra'

The analysis of variance in both experiments showed no significant effects, indicating that the sorghum cultivar did not differ in response to the inoculum densities tested. Populations of *P. coffeae* in sorghum 'BRS Ponta Negra' were low, ranging from 0 to 0.35 and 0 to 0.125 (Trial 1), and from 0 to 0.18 and 0 to 3.75 (Trial 2) in roots (1 g) and soil (100 cm^3), respectively (Table 1). Based on the reproduction factor (RF), this cultivar was classified as a poor host ($0 \leq RF < 1$) in both experiments, whereas the susceptible control exhibited RF = 6.26 (based on population density in tuber peel + root + soil).

Table 1: Host suitability of sorghum 'BRS Ponta Negra' 80 days after inoculation (DAI) with different *Pratylenchus coffeae* densities, in two experiments under greenhouse conditions.

Treatment (nematodes/plant)	250	500	750	1,000
Experiment I				
Nematodes in roots	0[1]	0	0.35	0.20
Nematodes in soil	1.25	0	0	0
Rf (Pf/Pi)	0.005	0	0.007	0.003
Experiment II				
Nematodes in roots	0	0.10	0.11	0.18
Nematodes in soil	0	0	0	3.75
Rf (Pf/Pi)	0	0.003	0.002	0.006

[1]Mean of eight replications.

Source: Research Data, 2024.

3.2 Bioassay with plant extracts

The analysis of variance revealed a significant interaction ($P < 0.05$) between plant extracts and concentrations for both immobility and mortality of *P. coffeae*. The efficacy of the extracts varied according to concentration, except for the foliar extract of *A. urundeuva* (Table 2). The extracts induced nematode immobility ranging from 24 to 99.2% and mortality from 20 to 99.2%. The extract from leaves of *S. obtusifolium* was the most effective treatment at all concentrations tested, resulting in more than 90% immobility and mortality. This was followed by the extract from *T. formosa* fruits, which caused 92.8% immobility and 92.0% mortality, respectively, at higher concentrations (Table 2).

Table 2: Immobility and mortality of *Pratylenchus coffeae* after 24 hours of exposure to aqueous extracts from seven plant species, followed by incubation in water.

Plant species/Plant part	Immobility (%)			
	0.5%	1.0%	1.5%	2.0%
Croton sp. (leaf)	29.6 c[1] ± 3.2*	76.8 cb ± 1.6	76.0 d ± 0.0	80.8 c ± 1.6
Astronium urundeuva (bark)	55.2 b ± 8.2	67.2 d ± 8.9	72.8 d ± 5.3	70.4 d ± 2.0
A. urundeuva (leaf)	28.0 d ± 3.6	30.0 f ± 4.4	32.0 f ± 4.4	30.4 e ± 4.1
Monteverdia rigida (leaf)	53.6 b ± 2.0	65.6 d ± 3.2	49.6 e ± 2.0	73.6 d ± 2.0
Tocoyena formosa (leaf)	32.8 c ± 4.7	88.0 b ± 5.7	87.2 c ± 6.4	88.0 b ± 2.5
T. formosa (fruit)	32.0 c ± 2.5	88.0 b ± 2.5	92.8 b ± 3.0	92.0 b ± 4.4
Sarcomphalus joazeiro (leaf)	50.4 b ± 4.8	54.4 e ± 3.2	53.6 e ± 3.2	69.6 d ± 2.0
Byrsonima gardneriana (bark)	24.0 d ± 2.5	61.6 d ± 3.2	56.0 e ± 10.4	74.4 d ± 2.0
Sideroxylon obtusifolium (leaf)	92.8 a ± 4.7	96.8 a ± 3.0	98.4 a ± 2.0	99.2 a ± 1.6
CV (%) = 8.28				
Plant species/Plant part	**Mortality (%)**			
	0.5%	**1.0%**	**1.5%**	**2.0%**
Croton sp. (leaf)	21.6 d ± 6.0	76.0 c ± 0	72.5 c ± 1.6	76.0 d ± 2.5

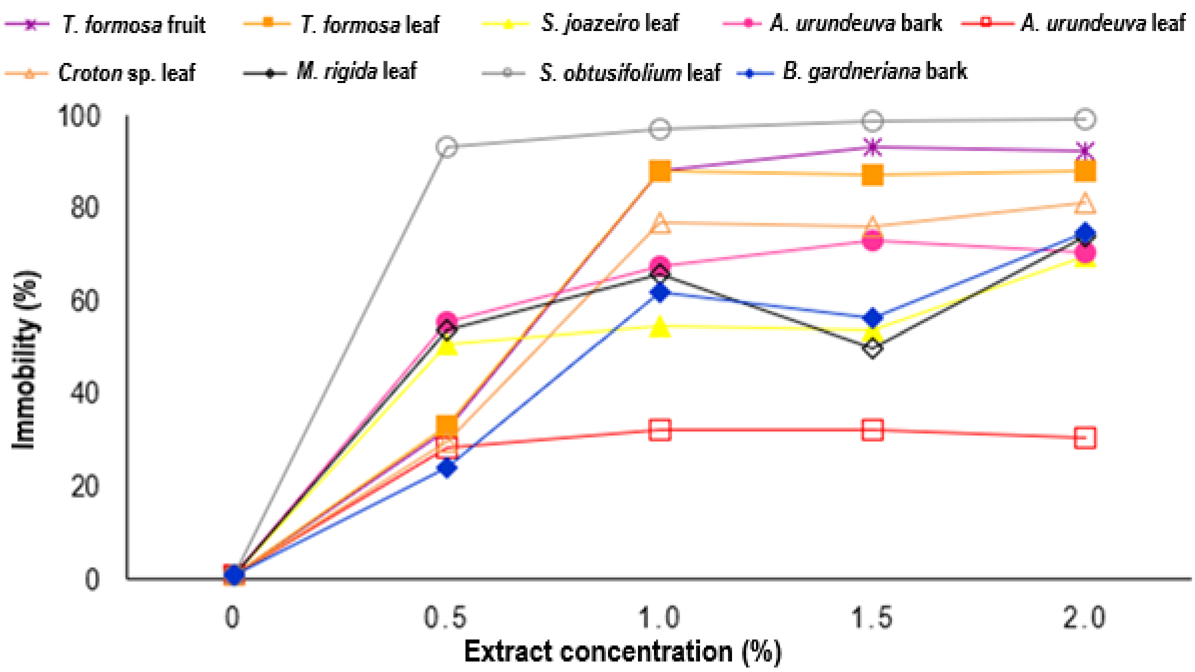
Astronium urundeuva (bark)	48.0 b ± 8.4	57.6 d ± 9.7	62.4 d ± 6.5	64.8 e ± 3.9
A. urundeuva (leaf)	20.0 d ± 5.7	28.8 f ± 3.0	28.8 g ± 6.9	29.6 f ± 4.8
Monteverdia rigida (leaf)	48,0 b ± 3.6	62.4 d ± 4.1	41.6 f ± 2.0	69.6 e ± 2.0
Tocoyena formosa (leaf)	30.4 c ± 6.5	79.2 c ± 6.4	77.6 c ± 8.2	84.0 c ± 2.5
T. formosa (fruit)	28.0 c ± 5.1	87.2 b ± 3.0	92.0 b ± 2.5	92.0 b ± 4.4
Sarcomphalus joazeiro (leaf)	43.2 b ± 6.9	51.2 e ± 3.0	53.6 e ± 3.2	66.4 e ± 2.0
Byrsonima gardneriana (bark)	20.0 d ± 3.6	60.8 d ± 3.0	55.2 e ± 9.9	65.6 e ± 3.2
Sideroxylon obtusifolium (leaf)	92.8 a ± 4.7	96.8 a ± 3.0	98.4 a ± 2.0	99.2 a ± 1.6
CV (%) = 10.23				

[1]Means followed by the same letters within the column do not differ significantly by the Scott-Knott test at 5% probability; CV= coefficient of variation; *Standard deviation.

Source: Research Data, 2024.

Linear and polynomial regression analyses indicated that, in general, higher rates of immobility and mortality of *P. coffeae* were associated with increasing extract concentrations, particularly between 0.5% and 1.0% (Figures 1 and 2).

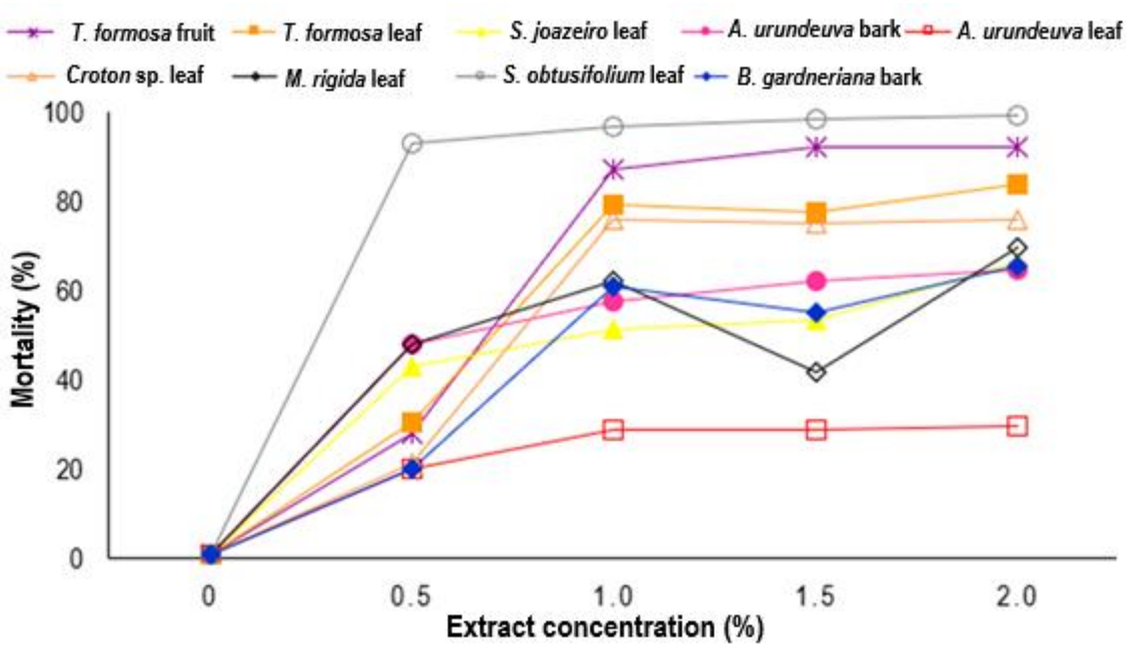
Figure 1: Effect of concentrations of aqueous extracts obtained from seven plant species on the immobility of *Pratylenchus coffeae*.



Regression equations: *T. formosa* fruit: $y = 48.64x + 12.48$, $R^2 = 82.53\%$; *A. urundeuva* bark: $y = -34.286x^2 + 99.931x + 4.7771$, $R^2 = 96.18\%$; *M. rigida* leaf: $y = -24.457x^2 + 77.234x + 8.0914$, $R^2 = 78.41\%$; *T. formosa* leaf: $y = 45.76x + 13.6$, $R^2 = 79.92\%$; *A. urundeuva* leaf: $y = -17.6x^2 + 47.84x + 3.2$, $R^2 = 91.58\%$; *S. obtusifolium* leaf: $y = -52.8x^2 + 146.08x + 10.72$, $R^2 = 88.36\%$; *S. joazeiro* leaf: $y = 28.16x + 17.6$, $R^2 = 72.16\%$; *Croton* sp. leaf: $y = 41.28x + 11.52$, $R^2 = 82.87\%$; *B. gardneriana* bark: $y = 35.84x + 7.52$, $R^2 = 88.17\%$.

Source: Research Data, 2024.

Figure 2: Effect of concentrations of aqueous extracts obtained from seven plant species on mortality of *Pratylenchus coffeae*.



Regression equations: *T. formosa* fruit: y = 49.28x + 10.72, R^2 = 82.98%; *A. urundeuva* bark: y = -26.971x^2 + 82.423x + 4.7543, R^2 = 95.11%; *M. rigida* leaf: y = 54.4x^3 - 184.23x^2 + 185.26x - 0.4343, R^2 = 96.30%; *T. formosa* leaf: y = 42.72x + 11.68, R^2 = 83.28%; *A. urundeuva* leaf: y = -13.029x^2 + 39.337x + 1.8057, R^2 = 97.77%; *S. obtusifolium* leaf: y = -13.2x^2 + 99.44x - 75.52, R^2 = 88.36%; *S. joazeiro* leaf: y = 28.32x + 14.72, R^2 = 79.95%; *Croton* sp. leaf: y = 40.8x + 9.12, R^2 = 79.81%; *B. gardneriana* bark: y = 32.96x + 7.52, R^2 = 83.45%.

Source: Research Data, 2024.

4 DISCUSSION

4.1 Experiment with forage sorghum 'BRS Ponta Negra'

The results indicate that *P. coffeae* was unable to multiply in sorghum 'BRS Ponta Negra'. Therefore, this cultivar shows potential for use in crop rotation systems in yam-producing areas where this nematode is prevalent as the causal agent of dry rot disease. Sorghum has a well-developed root system and produces abundant root exudates, which may promote beneficial interactions with soil microorganisms, indirectly enhancing plant defense against soilborne pathogens (Han *et al*., 2025). However, it is essential to identify the predominant nematode species, as the response of sorghum genotypes may vary. For example, when evaluating sorghum genotypes, including 'BRS Ponta Negra', against *P. brachyurus*, this cultivar was classified as susceptible, resulting in significantly higher nematode population densities (Paes, 2024).

Under greenhouse conditions, Lira and Moura (2016/2017) evaluated grass genotypes for crop rotation in yam fields infested with *P. coffeae* and reported that grain and forage sorghum were classified as non-host and poor host, respectively; however, the cultivars were not specified, limiting direct comparisons. Additionally, 'BRS Ponta Negra' is reported to be resistant to anthracnose [*Colletotrichum graminicola* (Ces.) G.W. Wils.], rust (*Puccinia purpurea* Cooke), and cercosporiosis [*Catenulocercospora fusimaculans* (G.F. Atk.) C. Nakash., Videira & Crous], and moderately resistant to Helminthosporium leaf disease [*Exserohilum turcicum* (Pass.) K.J. Leonard & Suggs] (Santos *et al*., 2007). In addition to its potential role in managing *P. coffeae* in yam production systems, the cultivar 'BRS Ponta Negra' is an important forage resource adapted to the Brazilian Northeast and exhibits favorable agronomic characteristics (Santos *et al*., 2007). However, farmers should be aware that such management strategies should not be used in isolation, as the use of non-host crops reduces, but does not eliminate, plant-parasitic nematode populations in the soil (Silva *et al*., 2022).

Although the results are promising, further studies are required to confirm that 'BRS Ponta Negra' consistently behaves as a poor host for *P. coffeae*. This is particularly relevant given reports of this nematode causing root rot in sorghum (hybrid Jinza 210) (Qin *et al*., 2025). These findings highlight the importance of understanding genotype-specific responses to the pathogen, and currently no sorghum cultivar can be broadly recommended for the management of yam dry rot disease.

4.2 Bioassay with plant extracts

Sideroxylon obtusifolium is widely distributed in northeastern Brazil and is commonly found in the Caatinga biome, being the only species of this genus occurring in the country (Alves-Araújo, 2020). Its ethanolic extract exhibits a phytochemical profile rich in flavonoids, tannins, and saponins (Carnevale Neto *et al*., 2017), compounds that have been associated with nematicidal activity (Desmedt *et al*., 2020; D'Addabbo *et al*., 2022). Similarly, hydroalcoholic leaf extracts of *T. formosa* contain flavonoids, tannins, saponins, and phenolic acids (Cesário *et al*., 2018). Extracts from *Croton* spp. have also been reported to contain bioactive constituents such as saponins (Mesquita Neto *et al*., 2010; Lima *et al*., 2019).

The use of plant extracts for managing plant-parasitic nematodes has been widely investigated, with positive results reported for root-knot nematodes (*Meloidogyne* spp.) (Adegbite; Adesiyan, 2005; Kuhn *et al*., 2015), the burrowing nematode *Radopholus similis* (Cobb, 1893) (Jesus *et al*., 2015), and the reniform nematode *Rotylenchulus reniformis* Linford & Oliveira, 1940 (Gardiano et al., 2011) in economically important crops.

With regard to *Pratylenchus* spp., leaf extracts from various plant species, including *Azadirachta indica* A. Juss., have been effective in reducing *P. coffeae* populations and significantly increasing yield when applied as soil treatments in banana cultivation (Sundararaju; Padmanaban; Sathiamoorthy, 2003). In another study, under *in vitro* conditions, extracts from medicinal plants also showed efficacy against *P. coffeae* (Nguyen; Jung, 2014). Furthermore, reductions in the reproduction factor of mixed populations composed of *S. bradys* and *Pratylenchus* sp. or *P. coffeae* in yam (*Dioscorea* spp.) were observed under greenhouse conditions using pyroligneous extract from *Cocos nucifera* L. (Farias *et al*., 2020) and aqueous leaf extract from *Annona squamosa* L. (Magalhães *et al*., 2020). Similar effects were reported for mixed nematode populations with predominance of *Pratylenchus* sp. in banana plantlets (Paz Filho *et al*., 2021). These findings reinforce the potential of plant extracts as a strategy for controlling *Pratylenchus* species.

5 CONCLUSIONS

In the present study, the sorghum cultivar BRS Ponta Negra was classified as a poor host for *P. coffeae* ($0 < RF < 1$), indicating its potential use in crop rotation systems. The leaf extract of *S. obtusifolium* resulted in high *P. coffeae* mortality even at the lowest concentration tested (0.5%). However, further studies are required to evaluate its nematicidal activity under *in vivo* conditions and to determine its effectiveness in reducing *P. coffeae* population densities in yam production areas. Furthermore, further investigations are required in yam production areas with a high prevalence of *P. coffeae* to confirm the response of sorghum cv. BRS Ponta Negra. Additional studies under both greenhouse and field conditions are also necessary to evaluate the effectiveness of the plant extracts tested herein for managing yam dry rot disease, including infections caused by *S. bradys*.

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CONFLICT OF INTEREST. The authors declare no conflict of interest.
ETHICAL APPROVAL. Not applicable.

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