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CROP PROTECTION

Occurrence and pathogenicity of the stem and bulb nematode in onion crops in Rio Grande do Sul State, Brazil

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Ocorrência e patogenicidade do nematoide-do-amarelão em cebola no estado do Rio Grande do Sul

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ABSTRACT: The stem and bulb nematode (SBN) *Ditylenchus dipsaci* is one of the major problems affecting alliaceous crops in different cultivated areas in Brazil. However, reports on the occurrence of this phytonematode in onions are scarce. Recently, SBN was detected in onion crops in São José do Norte, city localized at the southern end of the coast of the state of Rio Grande do Sul (RS), Brazil, where parasitized plants showed reduced size and soft rot in the bulbs. Therefore, this study reported the occurrence of *D. dipsaci* in onion crops in Rio Grande do Sul State and characterized the nematode population morphologically, morphometrically, and molecularly through amplification of the 28S rDNA region. Pathogenicity was also evaluated under greenhouse conditions using *'Bola Precoce'* onion plants inoculated with 500 specimens of *D. dipsaci* (initial population) and uninoculated controls. Morphological, morphometric, and molecular analyses confirmed the identity of the population as *D. dipsaci*. In both trials, inoculation reduced root fresh mass, bulb diameter, and bulb fresh mass. The mean reproduction factor (RF = final population/initial population) was 5.18.
Key words: *Allium cepa*, host, *Ditylenchus dipsaci*, damage, reproduction.

Ocorrência e patogenicidade do nematoide-do-amarelão em cebola no estado do Rio Grande do Sul

RESUMO: O nematoide do amarelão do alho *Ditylenchus dipsaci* (SBN) é um dos principais problemas que afetam aliáceas em diferentes áreas cultivadas no Brasil. No entanto, os relatos de ocorrência deste fitonematoide em cebola são escassos. Recentemente, o SBN foi detectado em lavouras de cebola de São José do Norte, cidade localizada no extremo sul do estado do Rio Grande do Sul (RS), onde as plantas parasitadas apresentaram tamanho reduzido e podridão mole nos bulbos. Nesse sentido, o objetivo deste estudo foi relatar a ocorrência de *D. dipsaci* em lavouras de cebola, no Rio Grande do Sul e realizar a caracterização morfológica, morfométrica e molecular da população por meio da amplificação da região 28S do rDNA. Adicionalmente, a patogenicidade do SBN foi testada em cebola '*Bola Precoce*' em condições de casa de vegetação em plantas inoculadas ou não com 500 espécimes de *D. dipsaci* (população inicial). Os resultados mostram que de acordo com as caracterizações morfológicas, morfométricas e moleculares da população do nematoide estudada, a espécie *D. dipsaci* foi confirmada. Nos dois ensaios de patogenicidade, o SBN reduziu a massa fresca das raízes, o diâmetro dos bulbos e a massa fresca dos bulbos das plantas inoculadas. O nematoide apresentou fator de reprodução (FR = população final/população inicial) médio de 5,18.
Palavras-chave: *Allium cepa*, hospedeiro, *Ditylenchus dipsaci*, danos, reprodução.

INTRODUCTION

Ditylenchus dipsaci [(Kühn, 1857) Filipjev, 1936] is one of the most important pathogens of alliaceous crops (*Allium* spp.), especially in temperate regions, due to its wide host range and survival capabilities (JONES et al., 2013). Damage caused by this nematode in Brazil is almost exclusively associated with garlic crops; it is commonly known as the stem and bulb nematode (SBN). Although exist more than 30 races of *D. dipsaci* (STURHAN & BRZESKI, 1991), only the garlic/onion race has been detected in Brazil to date (FERRAZ & BROWN, 2016), causing economic losses of up to 100% in garlic crops (PINHEIRO et al., 2014). At 15 ºC, *D. dipsaci* completes its life cycle in 19 to 23 days, and a single female can lay up to 500 eggs (SCHWARTZ & MOHAN, 2008). In the extreme south of Rio Grande

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do Sul, conditions of cultivate susceptible garlic genotypes for several years in the same nematode infested area (they survive via anhydrobiosis process) has caused problems in its production (CORREIA et al., 2022).

Ditylenchus dipsaci infection in onions can cause symptoms such as stunted growth, atrophy, leaf curling and thickening, and changes in the rigidity and color of the scales, which become soft and light gray. The injured soft bulbs often progress to soft rot accompanied by a strong odor, exacerbated by secondary invaders such as bacteria and fungi, resulting in bulbs that are dehydrated, shriveled, and with low density (SCHWARTZ & MOHAN, 2008).

Although there are *D. dipsaci* populations that attack only onion (*A. cepa* L.) (AFTALION & COHN, 1990), the garlic/onion race completes its life cycle and reproduces effectively on *Allium* species, including garlic (*A. sativum* L.) (DOUDA, 2005). *D. dipsaci* multiplies well on onion in Europe, and is pathogenic to various cultivars (YAVUZASLANOĞLU, 2019). Onion bulb yield is negatively correlated with the initial nematode population present in the soil (YAVUZASLANOĞLU et al., 2015). However, the occurrence of *D. dipsaci* associated with onion has only been reported in the state of Santa Catarina to date (BECKER, 1993), with limited information available regarding the damage and pathogenicity of the nematode on the crop.

In this context, the present study reported the first occurrence of *D. dipsaci* in Rio Grande do Sul state onion crops and to evaluate its pathogenicity to onion under controlled environment crop production.

MATERIALS AND METHODS

In December 2022, onion plants (cv. *'Bola Precoce'*) exhibiting stunted growth and shriveled, rotten bulbs (Figure 1) were observed on a farm in the municipality of São José do Norte, in southern Rio Grande do Sul State (31°56′39.0″S, 51°58′25.8″W). Symptomatic plants were subsequently sampled, and 1 kg of rhizosphere soil (0-20 cm depth) was collected following the procedure described by TIHOHOD (2000) and the collected material was sent to the Nematology Laboratory at Embrapa Clima Temperado (Pelotas, RS) for analysis. The plant material was processed by cutting bulbs and roots into fragments (< 1 cm), which were homogenized, weighed, and subjected to extraction using the Petri dish method (IPPC, 2016), as adapted by CORREIA (2021). Additionally, a 250 cm^3 soil subsample was processed (JENKINS, 1964) to extract and quantify the soil nematode community (MAI et al., 1996). A portion of the symptomatic plant sample was chopped and mixed with steam-sterilized soil (120 ºC) at a ratio of 10 g of plant material per kg of soil; this mixture was transferred to pots containing onion plants *'Bola*

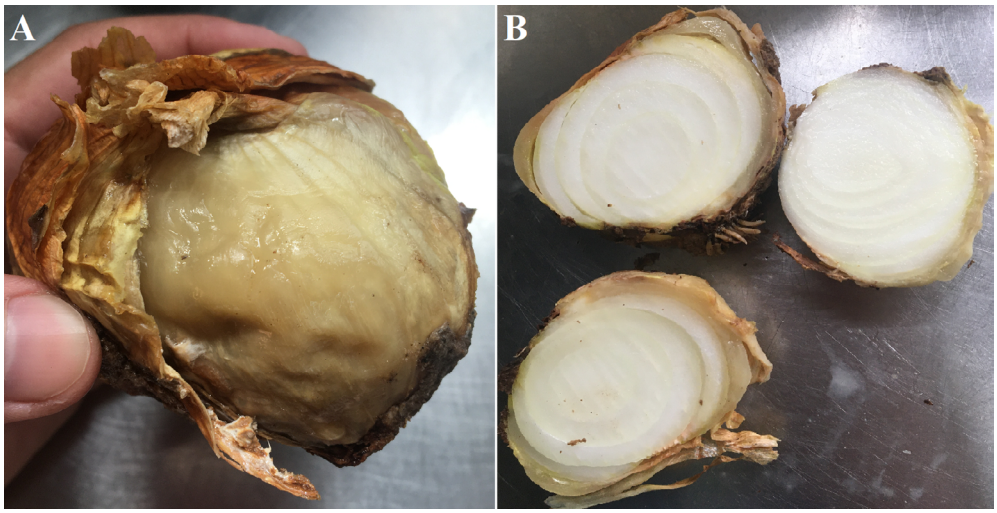


Figure 1 - Symptoms of *Ditylenchus dipsaci* on cv. '*Bola Precoce*' in onion bulbs observed in a crop in the municipality of São José do Norte, RS, Brazil: (A) external scales of the bulb showing softening and darkening; (B) internal portion of the bulb.

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Precoce' and maintained in a greenhouse (25 ± 2 ºC) for nematode multiplication and subsequent studies.

Morphological and morphometric analyses were conducted by preparing temporary slides using 20 live females and 20 live males recovered from onion plants. Each specimen was photographed using a video camera attached to a light microscope (Leica DM 1000) at 10×, 20×, 40×, 60×, and 100× objective lenses and subsequently measured using LAS Core software. These data were used to obtain the standard morphometric measurements and allometric indices described for *Ditylenchus* by STURHAN & BRZESKI (1991).

In addition, molecular characterization of the population detected in onion was performed. Thus, five specimens were individually transferred to 0.2-mL microtubes containing 10.5 µL of nuclease-free water. Next, each nematode was disrupted using the tip of an entomological pin to release the DNA. Subsequently, D2A (5´-ACAAGTACCGTGAGGGAAAGTTG-3´) and D3B (5´-TCGGAAGGAACCAGCTACTA-3´) primers at a concentration of 10 mM were added to amplify the D2 and D3 expansion segments of the 28S rDNA region, following the method of DOUDA et al. (2013). The PCR reaction was performed in a final volume of 25 µL, consisting of 12.5 µL of GoTaq® Green Master Mix 2× (Promega®), 1 µL of each primer, and 10.5 µL of the sample. The PCR profile was adapted from DOUDA et al. (2013), with an initial denaturation step of 5 min at 95 ºC, followed by 35 cycles (30 s at 95 ºC, 30 s at 55 ºC, and 70 s at 72 ºC), and a final extension of 5 min at 72 ºC. Finally, 5 µL of the PCR product was loaded onto an agarose gel and compared against a 1 Kb Plus DNA Ladder (Invitrogen), while the remaining product was sent for sequencing. The obtained sequences were aligned using Clustal W (available in MEGA 11 software), and the phylogenetic tree was constructed via Maximum Likelihood analysis (500 bootstrap replicates). Seventeen additional sequences of *Ditylenchus* spp. and one sequence of *Pratylenchus brachyurus* available in GenBank (NCBI - <https://ncbi.nlm.nih.gov/>) were included in the analysis.

Pathogenicity tests were conducted from May to December 2023 in two separate trials. Seventy-day-old *'Bola Precoce'* onion plants were individually transplanted into pots containing 3 L of sterilized sandy loam soil and inoculated with 1 mL of a suspension containing 500 specimens of *D. dipsaci*. The plants were maintained in a greenhouse at 25 ± 2 ºC and 80 ± 5% relative humidity. The nematode suspension was applied into three holes around each plant, and the pots were kept in a humid chamber for three days. Uninoculated plants were included as controls. Each trial followed a completely randomized design with seven replicates per treatment, with one plant per pot. The plants were then removed from the pots at 111 days post-inoculation, washed, and evaluated for number of leaves (NL), shoot length (SL), root length (RL), shoot fresh mass (SFM), root fresh mass (RFM), bulb fresh mass (BFM), and bulb diameter (BD). Furthermore, total chlorophyll content (TC) in the leaves was quantified according to the method described for onion crops (GHODKE et al., 2018); content was estimated using a 0.05 g (weight - W) sample from the fourth fully developed leaf in 10 mL (volume - V) of DMSO (dimethyl sulfoxide). Test tubes containing the samples and DMSO were placed in a water bath at 65 ºC for 30 minutes, protected from light. Absorbance (A) of the samples was subsequently recorded using a spectrophotometer (645 and 663 nm), and then the TC was calculated using the formula $[(20.2 * A_{645} + 8.02 * A_{663}) * V * W]$.

D. dipsaci specimens were then extracted from the shoot and bulb tissues of each plant; each section was cut into small pieces (< 1 cm) and subjected to the Petri dish method, with the dishes incubated in a BOD chamber for 24 hours at 25 ºC (IPPC, 2016). The material was then poured through 60- and 635-mesh sieves, and the resulting suspension was transferred to a 50-mL tube for quantification. The soil from each pot was weighed, and a 250-cm^3 subsample was processed according to Jenkins (1964). Nematodes recovered from the soil, shoot tissues, and bulb tissues were quantified separately. The number recovered from the soil subsample was extrapolated to the total soil volume in each pot. The estimated numbers recovered from the soil, shoot tissues, and bulb tissues were summed to determine the final population in each experimental unit. The reproduction factor was calculated as RF = final population/initial population (OOSTENBRINK, 1966). Finally, the number of individuals per gram of fresh mass was determined separately for the bulb and shoot parts based on the total number of specimens per plant.

Data for the plant-response variables NL, SL, RL, SFM, RFM, BFM, BD, and TC were analyzed separately for each trial by analysis of variance. When significant treatment effects were detected, means were compared using Tukey's test at the 5% probability level in SAS OnDemand (<https://www.sas.com/pt_br/software/on-demand-for-academics.html>).

RESULTS

A total of 184 specimens were detected per 250 cm^3 of soil and 1,370 nematode specimens per

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plant according to nematological analysis of soil and onion plant samples collected in the field.

Under microscopic examination, relaxed or dead specimens had a straight to arcuate body (Figure 2A). The labial region was continuous with the body contour, and the median bulb showed thickening of the lumen walls (Figure 2B). The lateral field had four lines. In males, the bursa extended over approximately three-quarters of the tail, and the curved spicules measured 23-28 µm (Figure 2C). The tails of both sexes were conical and pointed (Figures 2C and 2D). These characteristics agreed with the descriptions of ESCUER (1998) and IPPC (2016).

Morphometric measurements are presented as mean ± standard deviation. For males (n = 20), the values were: L (mm) = 1.16 ± 0.25; St (µm) = 10.94 ± 1.19; a = 41.85 ± 3.89; b = 6.48 ± 0.41; c = 14.79 ± 1.40; c' = 4.21 ± 0.54. For females (n = 20), the values were: L (mm) = 1.22 ± 0.15; St (µm) = 11.15 ± 1.12; a = 37.29 ± 4.26; b = 6.88 ± 0.44; c = 14.71 ± 1.09; c' = 4.67 ± 0.35; V (%) = 81.05 ± 1.63.

Amplification of the nematode DNA yielded a single 759-bp amplicon for the *D. dipsaci* species analyzed. Comparison of the D2-D3 region sequenced from specimens isolated from onion in our study (PV425949) with other *Ditylenchus* populations sequenced and available on NCBI (Figure 3) revealed that this population clustered within the *D. dipsaci* clade (500 bootstrap replicates), thereby reinforcing the reliability of the morphological and morphometric identification by confirming the species identity.

Based on the pathogenicity assays, *D. dipsaci* negatively affected several plant-growth variables (Table 1). The mean percentage reductions calculated from Trials 1 and 2 were 26.62% for bulb diameter, 40.58% for bulb fresh mass, and 68.52% for root fresh mass. Root length was reduced by 39.40% in inoculated plants in Trial 2. No significant differences were observed for the remaining variables. The reproduction factors were 4.59 and 5.77 in Trials 1 and 2, respectively.

DISCUSSION

The morphometric values obtained fall within the ranges observed for the species *D. dipsaci* by STURHAN & BRZESKI (1991) and align with the recent description of this nematode (CORREIA et al., 2022) in garlic samples showing symptoms of yellowing disease (*amarelão*) in a production area within the same region. The D2-D3 marker of the 28S-rDNA region is widely used for the molecular

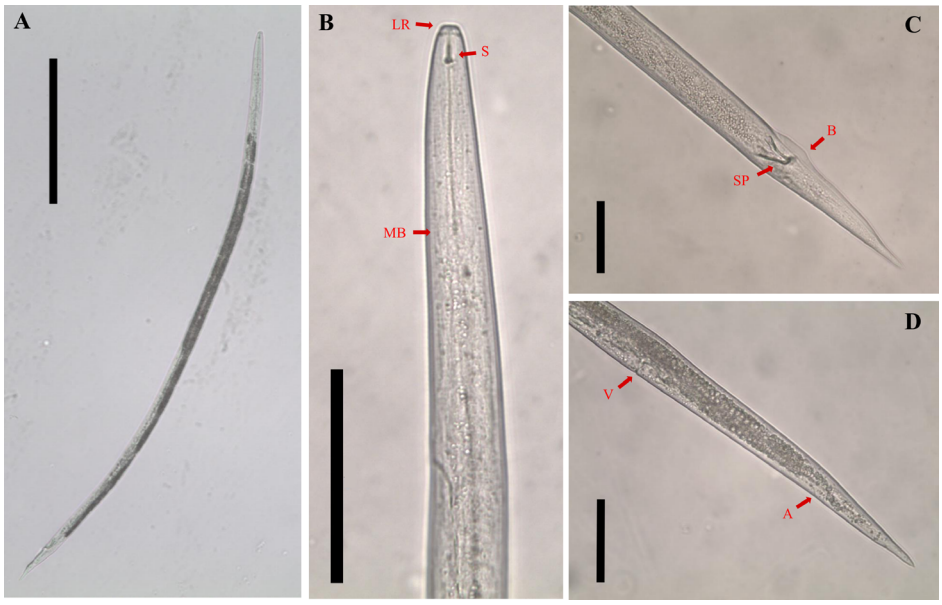


Figure 2 - Photomicrographs of *Ditylenchus dipsaci* recovered from onion plants from São José do Norte, RS, Brazil: (A) General view of the body; (B) anterior region, showing the labial region (LR), the stylet (S) and the median bulb (MB); (C) posterior region of the male, highlighting the bursa (B) and the spicule (SP); and (D) posterior region of the female, showing the vulva (V) and anus (A). Arrows indicate the main morphological structures. Scale bars: 200 µm in (A) and 50 µm in (B-D).

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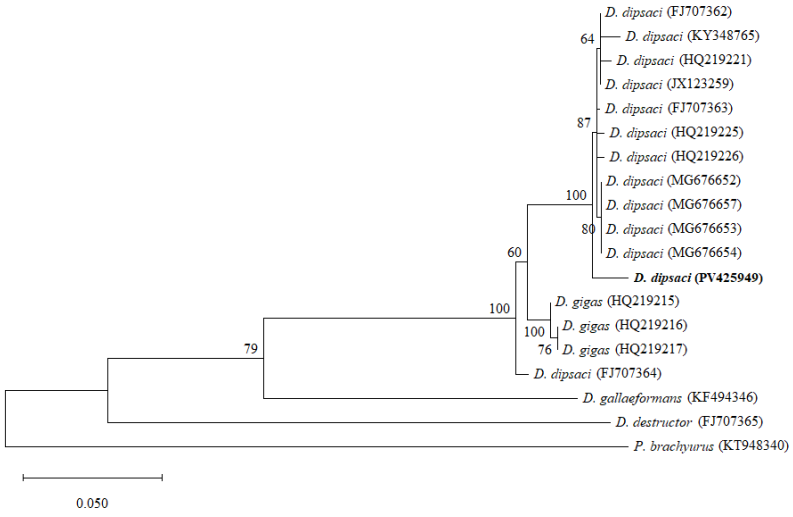


Figure 3 - Maximum-likelihood phylogenetic tree based on sequences of the D2–D3 region of 28S rDNA from *Ditylenchus* spp. The sequence generated in this study (PV425949) is shown in bold. *Pratylenchus brachyurus* (KT948340) was used as the outgroup. Numbers at the nodes indicate bootstrap support values based on 500 replicates, and numbers in parentheses indicate GenBank accession numbers.

diagnosis of *D. dipsaci*; however, its utility is limited to species identification, as molecular analyses cannot currently distinguish among biological races (STORELLI et al., 2021).

Although the D2-D3 marker is a valid tool, the literature also describes the use of other regions, such as ITS (YAVUZASLANOĞLU et al., 2018) and COI (VOVLAS et al., 2011), to confirm the identity of *D. dipsaci*. According to VOVLAS et al. (2011), *D. dipsaci* can be distinguished from *D. gigas* by the females' shorter body length (1.0-1.7 vs. 1.6-2.2 mm) and greater vulva-to-anus distance (202-266 vs. 132-188 µm). Such comparisons are important because both species may coexist in the same location, helping to avoid diagnostic errors, particularly during the pre-planting stage.

These findings corroborate those of YAVUZASLANOĞLU et al. (2015), who observed that *D. dipsaci* has a significant impact on the onion yield, recording yield losses of up to 15.33% in cultivars susceptible to the nematode. The relatively high coefficients of variation observed for root fresh mass in Trials 1 and 2 (45.47% and 39.15%, respectively) may reflect the inherent biological variability of pathogenicity assays involving *D. dipsaci*. The high numbers of specimens recovered per plant may be explained by the ability of *D. dipsaci* to reproduce within host tissues and complete several generations during the onion crop cycle (DUNCAN & MOENS, 2013). According to YAVUZASLANOĞLU et al. (2015), *D. dipsaci* presents an extremely low economic damage threshold in onions, since initial densities between 0.2 and 1 nematode per 100 g of soil, as well as approximately 5 individuals per plant, are already sufficient to cause economically relevant yield losses. The pathogenicity of SBN in other Brazilian onion cultivars has not yet been evaluated. Therefore; although, ecological conditions are favorable to *D. dipsaci* in the south of the country in onion growing areas (also traditionally occupied with garlic), genetic differences among cultivars may partly explain the limited detection of the disease in onion crops; however, this hypothesis requires further investigation.

The RF values ranged from 4.59 to 5.77 and were associated with significant reductions in bulb diameter and bulb fresh mass (Table 1). The RF values observed are in accordance with those obtained by YAVUZASLANOĞLU (2019) and YAVUZASLANOĞLU & AKSAY (2021) in onion cultivars susceptible to *D. dipsaci* (5.2 to 7.35). Furthermore, according to the same authors, garlic and tomato (in addition to onion) were also considered good hosts for the tested populations of *D. dipsaci*. In this sense, investigations into the resistance/susceptibility of other vegetables as well as commercial onion cultivars to SBN are important. This research aimed advancing knowledge in integrated

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Table 1 - Mean values for number of leaves (NL), shoot length (SL; cm), shoot fresh mass (SFM; g), root length (RL; cm), root fresh mass (RFM; g), bulb diameter (BD; mm), bulb fresh mass (BFM; g), total chlorophyll (TC; $mg.g^{-1}$), reproduction factor (RF) and number of specimens per gram of bulb fresh mass (N/gB) and shoot fresh mass (N/gS), evaluated in the pathogenicity test on cv. '*Bola Precoce'* onion plants inoculated with *D. dipsaci* and uninoculated control.

	----------Trial 1----------			----------Trial 2----------		
	Inoculated	Control	CV (%)	Inoculated	Control	CV (%)
NL	10.20^{ns}	10.00	16.71	4.50^{ns}	5.25	31.05
SL	60.80^{ns}	67.40	8.89	41.50^{ns}	44.45	7.72
SFM	40.60^{ns}	45.00	14.42	5.15^{ns}	6.29	35.33
RL	10.10^{ns}	11.52	24.45	5.23 b	8.63 a	17.09
RFM	1.40 b	3.48 a	45.47	0.05 b	0.22 a	39.15
BD	17.29 b	25.36 a	24.12	18.65 b	23.73 a	7.63
BFM	6.34 b	10.02 a	29.08	5.53 b	9.95 a	19.34
TC	1.59^{ns}	1.56	16.20	2.59^{ns}	2.55	13.86
RF	4.59	-	-	5.77	-	-
N/gB	292.74	-	-	105.74	-	-
N/gS	10.60	-	-	438.37	-	-

Within each trial, means in the same row followed by different letters differ significantly according to Tukey's test ($P \leq 0.05$). ns = not significant ($P > 0.05$); CV = coefficient of variation.

management in areas infested by the aforementioned phytonematode, since SBN has high potential for dissemination via stored bulbs and propagative material, and it can survive in an anhydrobiotic state in plant tissues for long periods (FIELDING, 1951), being easily transported to new areas and establishing itself in the soil after the planting.

CONCLUSION

This study confirmed the first occurrence of the stem and bulb nematode, *Ditylenchus dipsaci*, in onion crops in Rio Grande do Sul State, Brazil, and demonstrates its pathogenicity to cv. *'Bola Precoce'* under greenhouse conditions.

DATA AVAILABILITY STATEMENT

Data are contained within the article.

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DECLARATION OF CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

AUTHORS' CONTRIBUTIONS

All authors contributed equally to the conception and writing of the manuscript. All authors critically reviewed the manuscript and approved the final version.

DECLARATION OF USE OF ARTIFICIAL INTELLIGENCE

The authors declare that artificial intelligence tools were used to review and improve the textual quality of the manuscript.

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