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Phytopathogenic agents associated with *Plukenetia volubilis* L. in Cuba: symptomatology and observations about their severity and degree of infestation

Agentes fitopatógenos asociados a *Plukenetia volubilis* L. en Cuba: sintomatología y observaciones acerca de su severidad y grado de infestación

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ABSTRACT: The objectives of this work were to determine the presence of phytopathogens in important producing areas of Sacha Inchi (*Plukenetia volubilis* L.) in Cuba and provide some observations of their severity and degree of the crop infestation. The soil and root samples analyzed were from plantations in three different areas of the western region, two from the province of Mayabeque and one from Pinar del Rio. In the field, areas of poorly developed and dead plants were found. The roots of these plants showed several damages, such as corky areas even up to the level of the plant neck, scarce secondary system, galls of variable size, and, in occasions, tumors with or without necrosis. The phytosanitary diagnosis allowed the detection of the nematode species *Meloidogyne incognita* (Kofoid & White) Chitwood, *Meloidogyne arenaria* (Neal) Chitwood, *Trichodorus* spp., *Helicotylenchus dihystera* (Cobb) Sher, *Aphelenchoides bicaudatus* (Inamura), *Tylenchorhynchus* spp., and *Xiphinema* spp., predominating *M. incognita*. The fungi *Fusarium* spp. and *Lasiodiplodia* spp. was also observed. The combined effect of the *Meloidogyne-Fusarium* complex damaged Sacha Inchi seriously with important consequences for its production.

Key words: *Fusarium* sp., *Lasiodiplodia* spp., *Meloidogyne* spp., Sacha Inchi, root-knot nematodes.

RESUMEN: Los objetivos del trabajo fueron determinar la presencia de agentes fitopatógenos en importantes áreas productivas de Sacha Inchi (*Plukenetia volubilis* L.) en Cuba y ofrecer algunas observaciones de la severidad y grados de infestación en el cultivo. Se analizaron muestras de suelo y raíces provenientes de plantaciones de tres áreas de la región occidental, dos de la provincia de Mayabeque y una de Pinar del Rio. En el campo, se encontraron áreas de plantas con poco desarrollo y muertas, daños en las raíces, áreas corchosas, incluso hasta el nivel del cuello de la planta, escaso sistema secundario de raíces y agallas de tamaño variable, en ocasiones, formación de tumores con o sin necrosis. El diagnóstico fitosanitario permitió detectar las especies de nematodos *Meloidogyne incognita* (Kofoid & White) Chitwood, *Meloidogyne arenaria* (Neal) Chitwood, *Trichodorus* spp., *Helicotylenchus dihystera* (Cobb) Sher, *Aphelenchoides bicaudatus* (Inamura), *Tylenchorhynchus* spp., y *Xiphinema* spp., con predominio de la primera especie. Se observó también la presencia de los hongos *Fusarium* spp. y *Lasiodiplodia* spp. El efecto combinado del complejo *Meloidogyne-Fusarium* causó serios daños, con importantes consecuencias para la producción de Sacha Inchi.

Palabras claves: *Fusarium* spp., *Lasiodiplodia* spp., *Meloidogyne* spp., Sacha Inchi, nematodos agalleros.

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INTRODUCTION

Plukenetia volubilis Lin. (Sacha Inchi) is an oilseed plant species in the family Euphorbiaceae. It is native to tropical South America and the Caribbean. Its fruits are star-shaped with a variable number of lobes. The seeds are dark brown, oval, and high in oil (54%) and protein (27%). Its oil is rich in unsaturated fatty acids, particularly in alpha-linolenic acid (omega 3), linoleic acid (omega 6), and oleic acid (omega 9) with 45, 35, and 8%, respectively (1). In addition, it contains antioxidant compounds, such as α, γ, and δ tocopherols, and phytosterols, such as stigmasterol and β-sitosterol (2).

This crop is highly susceptible to the attack by the root knot nematode *Meloidogyne* spp. (3). The yield and productive life of the crop are reduced because of the different types of damages this nematode produces in the root system, and the physiological processes in the plants are affected. This contributes to high mortality in the second year of production.

Sacha Inchi cultivation was introduced into Cuba in December 2015, while in 2016, the regionalization studies of the crop began being carried out at Productive Basic Units (UBP) belonging to the Center for Research on Protein Plants and Bionatural Products (CIPB) (4).

Symptoms of pest infestations were detected in the areas planted with this crop in Cuba. Rodríguez & Hernández-Ochandia (5) informed the presence of *Meloidogyne arenaria* (Neal) Chitwood in Sacha Inchi plantations; nevertheless, further research works are needed to achieve more information about the phytosanitary condition of the crop in Cuba and possibilities of the pest management.

Sacha Inchi productions have decreased worldwide as a consequence of one of the main threats to the crop: the presence of *Meloidogyne-Fusarium* complex, both in seedling nurseries and in plantations (6). These effects in the plantations have considerably reduced the life- time of the crop, which makes it necessary to carry out a diagnostic research.

Therefore, the objectives of this work were to determine the presence of the most aggressive phytopathogenic agents associated with the cultivation of *P. volubilis* in Cuba and provide some observations of their severity and degree of the crop infestation.

MATERIALS AND METHODS

Prospections were carried out in nurseries and plantations belonging to the Production Base Unit (UBP) "Nazareno" of San José and "50th Anniversary of the Battle of Playa Girón", both in the province of Mayabeque, and "El Pitirre" in Los Palacios, province of Pinar del Rio (Table 1). Soil, substrate and root samples were randomly taken in nurseries and plantations.

Sacha Inchi seeds were sown in plastic bags containing 1 kg of substrate. The substrate for nursery consisted of a mixture of soil, manure, and rice husk in the proportion 2:1:1.

Plant Parasitic Nematodes

Extraction and identification

The soil and substrates were divided into two parts. One part was processed by the Baermann funnel technique, and the other part was used to perform the bioassay using indicator plants for the genus *Meloidogyne* (7).

The bioassay with indicator plant method was used to determine the damage in the plantations and evaluate the degree of infestation. The test plant used in the study was pumpkin (*Cucurbita pepo* L.), which was harvested for analysis after 35 days. Roots of the indicator's plants were assessed by the modified Zeck scale of 0-5 grades (8); whereas, roots of Sacha Inchi from the field were also examined by the Baermann funnel technique.

The detected nematodes were killed and fixed with hot 2% formalin (TAF) (9), prepared in temporary mounts, and identified morphologically and morphometrically using different taxonomic keys (10, 11, 12, 13).

Plant Pathogenic Fungi

For identification of fungal pathogens, samples of 10 to 20 cm in length and diameters ranging from 1 to 10 cm were taken from stems and roots. In each case, the area of growth of the damage was superficially disinfected with 1% sodium hypochlorite for 3 minutes and washed 3 times with abundant sterile distilled water (14).

Table 1. Details of the areas planted with Sacha Inchi in the three UBPs under study in the western region of Cuba / Detalles de las áreas plantadas con Sacha Inchi en las tres UBPs bajo estudio en la región occidental de Cuba

Parameter	UBP 50th Anniversary	UBP El Pitirre	UBP Nazareno
Geo-reference	22º 57´57´´ N, 82º 14´01´´ W	22º 66´66´´ N, 83º 25´ W	22º 57´59.22´´ N, 82º 14´03´´ W
Soil type		Lixiviated Red Fersialytic	
Cultivar		Inca-1	
Age of the sampled plantation		between 7 months and 2 years	
• Sowing type • Sowing distance • Number of plants per hectare		Creeping sowing (3 x 1.5) m; 2222 plants per hectare	
Previous crop to Sacha Inchi	Plantain (*Musa* spp.)	Tithonia (*Tithonia diversifolia* (Hemsl.) A. Gray)	Plantain (*Musa* spp.)

Available in: https://tierra.tutiempo.net/cuba/el-pitirre-cu012725

Subsequently, tissue fragments of 3 to 4 mm^2 were removed with a scalpel and placed separately in Petri dishes. Plates (100 x 15 mm) were prepared with Potato Dextrose Agar (PDA) and H media (14), supplemented with oxytetracycline (0.4 mg. ml^{-1}), and incubated at 26 ± 2˚C in the dark. When the development of colonies of the fungus that predominated in the sowing points was observed, this fungal growth was transferred to test tubes containing the same medium for its conservation and study of the morphometric and morpho-cultural characteristics. The developed fungal structures were observed under a phase contrast microscope (640X). The fungi were identified by comparative sequence analysis, as well as by contrasting with the existing literature and using taxonomic keys (15, 16).

Pathogenicity assay

Two isolates obtained from symptomatic Sacha Inchi plants, identified as *Fusarium* spp., were tested for pathogenicity for Sacha Inchi. The plants to be inoculated were washed thoroughly in sterile distilled water before being challenged with a concentrated spore suspension (17). The spore suspension was prepared from fully colonized plates of PDA and adjusted to a density of 10^6 spores.ml^{-1} in sterile water. Control plants were treated with sterile water instead of a spore suspension. Inoculated and control plants were left at temperatures ranging from 27 to 30°C and relative humidity around 75%. Two weeks later, the plants were rated on a semicontinuous 1 to 5 scale, where: 1 = no symptoms; 2 = drying and death of older leaves; 3 =symptomatic older leaves accompanied by wilting of younger leaves; 4 = severe foliar symptoms; 5 = dead plant.

RESULTS AND DISCUSSION

Initial rot symptoms were observed on Sacha Inchi seedlings in nursery (Fig. 1A). Nurseries of “50 Anniversary” and “El Pitirre” showed some aerial fungal symptoms on seedlings (Fig. 1A). Symptoms of collar rot were also observed (Fig. 1B) in the above nurseries. Only one seedling showed small galls with root-knot nematodes.

Different types of symptoms, such as dry and regressive death of branches (Fig. 1C and D), were observed on the aerial part of adult plants in Sacha Inchi plantations. On the other hand, plants were observed with rot on roots and necks (Fig. 1E), corky areas even up to the level of the plant necks, strong deterioration, a scarce secondary root system, and with galls of variable size, which sometimes formed tumors with and without necrosis (Fig. 1 D and E).

Plant Parasitic Nematodes (PPN)

Nine species of plant parasitic nematodes were found in the sampled areas (Table 2). The endoparasitic root-knot nematodes *Meloidogyne arenaria* (Neal) Chitwood and *Meloidigyne incognita* (Kofoid & White) Chitwood are considered among the most important nematodes worldwide (18). Similar results to those previously reported (19, 20) were observed in all the sampled sites; they were associated with deterioration of necks and roots of plants and the presence of knot- shaped galls. As mentioned above, Rodríguez & Hernández-Ochandia (5) had already reported the presence of *Meloidogyne arenaria* in Cuban Sacha Inchi plantations.

The three sampled’ sites showed heavy infestations of mainly *M. incognita* in soil and roots (4 to 5 grades of infestation), deterioration of adult plants, decreased production, the presence of knot-shaped galls, loss of secondary roots, necrosis, and neck rot. These results agreed with other authors (21, 22), who also reported poor plant growth, foliage yellowing, low production and decreased fruit quality in Sacha Inchi.

The phytosanitary diagnosis of the crop showed deterioration of the neck and roots of the adult plants and the presence of knot-shaped galls, which confirmed the existence of nematodes. Several of these results agree with those previously reported (22, 23). The most complex situation was at the UBP “50th Anniversary of the Victory of Playa Girón”. Six species of parasitic nematodes were found in the soil and root samples from the “50 Anniversary”.

In general, the root-knot nematodes *M. incognita* and *M. arenaria* were the species that showed the highest population levels and those most strongly associated with root damage.

In the case of the UBP "El Pitirre", five species of parasitic nematodes were found in the soil and root samples. The nematodes *M. incognita* and *M. arenaria* once again presented the highest population levels and were strongly associated with root damage. The ectoparasite *Xiphinema* spp. was detected only in this UBP, with a medium level population in a plantation and a low population in other. This species can also damage other crops and should be further assessed, since its symptoms on the roots can be confused with those caused by *Meloidogyne* sp. Due to the importance of the other two species determined (*R. reniformis* and *Criconemoides* spp.) as causal agents of damage on other crops, they should also continue to be evaluated.

In the case of the UBP "Nazareno", three species of parasitic nematodes were found in the soil and root samples. The nematodes *M. incognita* and *M. arenaria* once again presented the highest population levels and were closely associated with root damage. This marked a serious deterioration of the entire root system.

The root knot nematodes *M. incognita* and *M. arenaria* appeared in all locations with a significant level of attack. Nevertheless, in spite of these findings, these results will be confirmed by molecular analysis based on PCR amplification.

According to table 1, plantain was the previous crop planted in UBPs “50 Anniversary” and “Nazareno”. This crop is highly susceptible to nematodes, which may justify the high incidence of these plagues. However, the previous crop planted in the UBP “El Pitirre” was *Tithonia diversifolia* H.W. Schott. No reference on susceptibility level of Tithonia to nematodes was found. Ramírez-Pedroso (Pers. Communication) evaluated indicators, such as fresh and dry matter, botanic seed production, flowering density, and yield of *T. diversifolia* associated with *Cannavalia eusiformis* de Geer and did not observe any effect due to nematodes or the presence of galls.

The phytosanitary diagnosis carried out in the three UBPs of the CIPB showed deterioration of adult plants, decreased production, the presence of knot-shaped galls, loss of secondary roots, necrosis and neck rot.

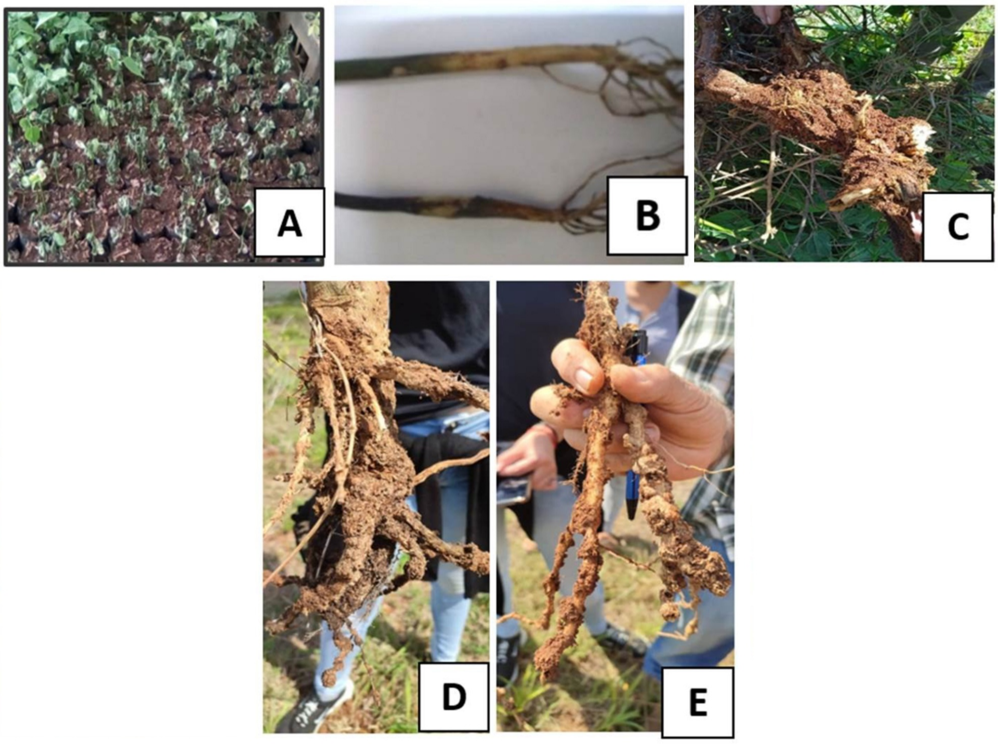

Figure 1. A) Initial symptoms of seed rot of Sacha Inchi in nursery; B) Neck rot symptoms in nursery; C) Symptoms of root rot in adult plants in plantation. D and E) Sacha Inchi roots with strong nematode involvement eight months after planting. Note the abundant presence of galls. / A) Síntomas iniciales de pudrición de la semilla de Sacha Inchi en vivero; B) síntomas de pudrición del cuello en vivero; C) Síntomas de pudrición de la raíz en plantas adultas en plantación. D y E) Raíces de Sacha Inchi de ocho meses luego de plantadas, con fuerte presencia de nematodos. Observe la abundante presencia de agallas.

Table 2. Species of parasitic nematodes found in Sacha Inchi planted in different localities in the Cuban western region. / Especies de nematodos parasíticos encontrados en Sacha inchi plantado en diferentes localidades estudiadas en el occidente cubano.

Nematode species	**Locality**		
	”50th Anniversary”	“El Pitirre”	“Nazareno”
Aphelenchoides bicaudatus Inamura	**X**		
Criconemoides spp.		**X**	
Helicotylenchus dihystera (Cobb) Sher	**X**		**X**
Meloidogyne arenaria (Neal) Chitwood	**X**	**X**	**X**
Meloidogyen incognita (Kofoid & White) Chitwood	**X**	**X**	**X**
Rotylenchulus reniformis Linford & Oliveira		**X**	
Trichodorus spp.	**X**		
Tylenchorhynchus spp.	**X**		
Xiphinema spp.		**X**	

These results agreed with those already reported (20), which also included low plant growth, foliage yellowing, low production, and decreased fruit quality in the Sacha Inchi crop studied.

It is convenient to mention that these are the three most important farms of Sacha Inchi production in Cuba because of the extension of land planted with this crop.

Although in all cases the cultivar Inca-1 was used, the damage caused by the presence of nematodes, particularly *Meloidogyne* spp., have been reported to depend on the plant cultivar (3). In other works, it was also mentioned that the growth rate of plants and the number of nodules per root were directly proportional to the level of infestation (20, 21). Cultivar Inca-1 planted in the three UBPs, like many of the cultivated accessions of Sacha Inchi, has a typical behavior as susceptible to *Meloidogyne* spp., with the consequence that productivity diminishes and the plants die in a few years after plantation (20).

Xiphinema sp. is an ectoparasitic species with economic importance in different crops (22). High populations of this plant-parasitic nematode (more than 150 nematodes per 100 g of soil) were detected only in UBP “El Pitirre”. It was associated with the presence of big rounded and chained galls on the roots. Because of its importance for many crops and the relative similarity of its symptoms on roots with those by *Meloidogyne* spp., which can be confused with it (23), studies about its distribution and pathogenicity must be continued.

Low populations of other species, such as the ectoparasitic nematodes *A. bicaudatus*, *Criconemoides* spp., *Trichodorus* spp., *Tylenchorhynchus* spp., and the semi-endoparasitic *R. reniformis*, were also found, and they will continue to be followed because of their importance in some other crops. All the reports of the nematode species, including *M. incognita*, in this paper are new for this crop in Cuba.

Additionally, the wounds caused on plant roots by plant-parasitic nematodes also act as entry routes for secondary pests and pathogens (23).

Plant Pathogenic Fungi

Regarding the phytopathogenic fungi, the symptoms of neck rot on Sacha Inchi seedlings pointed to the presence of *Fusarium* spp. (Fig. 2A). Other symptoms detected in the plantations were dry branches (Fig. 2B) and necrosis of xylem in main branches (Fig. 2C and D). Necrosis of xylem, as well as sectorial death in branches, pointed to the presence of the fungus *Lasiodiplodia* sp.

Figures 3A and B showed the presence of fruiting bodies of this phytopathogenic fungus. Typical conidia of this genus were also observed (Fig. 3C).

By observations under the optical microscope, microconidia and typical conidia of the genus *Fusarium* sp. were revealed from the mycelium produced when the fungus present on stems and branches was grown in humid chamber (Fig. 4A and B). The observed phytosanitary damages agree with those reported by Acosta- Ramírez (24), who reported the presence of these two fungi besides other fungal genera and species not found in this work. Other fruiting bodies were observed, but these fungi have not yet been identified. The presence of these two fungi are also new reports for the crop in Cuba. In the case of *Lasiodiplodia* spp., it could be the first report not only for Cuba. Our request allowed finding only a previous report for the rest of Sacha Inchi producing countries (24). In order to identify these fungal species, their molecular characterization is in progress by means of PCR amplification using highly- conserved sequences, such as Internal transcribed spacers (ITS) region, Translation and elongation factor (TEF-1α) coding region or β-tubulin genes.

It is important to mention that the presence of fungi is expected once the nematode causes a root wound, since it opens a gateway for fungi, which increase the potential of destruction and accelerate the plant death (25).

Figure 2. A) Neck rot symptoms caused by *Fusarium* sp.; B) Symptoms of dry branches in plantation; C and D) Necrosis of xylem in the main branches of Sacha Inchi / A) Síntomas de pudrición del cuello, causados por *Fusarium* sp.; B) Síntomas de ramas secas en plantación; C y D) Necrosis en el xilema de las ramas principales de Sacha Inchi.

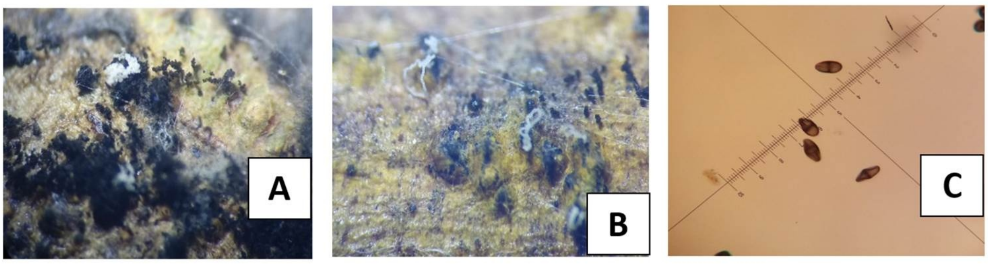

Figure 3. A and B) Fruiting bodies of *Lasiodiplodia* sp. on stems and branches of Sacha Inchi; C) Conidia of *Lasiodiplodia* spp. present on stems and branches. /A y B) Cuerpos fructíferos de *Lasiodiplodia* spp. en tallos y ramas de Sacha Inchi; C) Conidios de *Lasiodiplodia* spp. presentes en tallos y ramas.

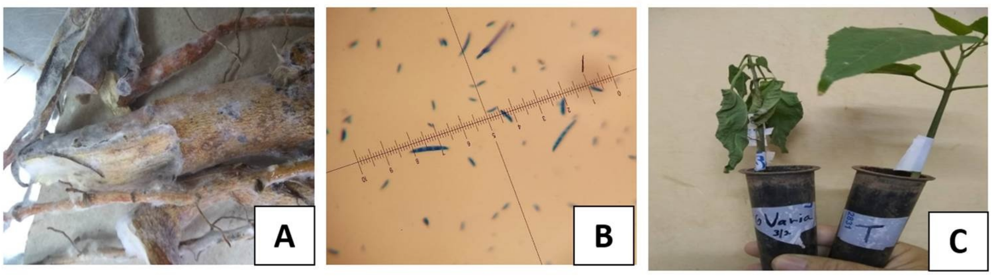

Figure 4. A) Sacha Inchi roots in a humid chamber showing the presence of *Fusarium* sp.; B) Macroconidia and microconidia of *Fusarium* sp. present on stems and branches; C) Pathogenicity test using monosporic isolates of *Fusarium* spp. Observe the damage on the treated seedling compared with the control (T). /A) Raíces de Sacha Inchi en cámara húmeda mostrando la presencia de *Fusarium* spp.; B) Macroconidios y microconidios de *Fusarium* spp. presentes sobre tallos y ramas; C) Ensayo de patogenicidad usando aislados monospóricos de *Fusarium* spp. Observe el daño sobre la plántula tratada comparado con el control (T).

Pathogenicity assay

Concerning the pathogenicity test, the artificial inoculations allowed identifying the damage caused by the fungus *Fusarium* sp. on the Sacha Inchi seedlings (Fig. 4C). The affected tissue can be distinguished by comparing the treated plant with the control one, which does not show any signs of involvement. The Sacha Inchi seedlings inoculated with the assayed isolates developed symptoms that ranged between 3 and 4 into the 1 to 5 scale. That means symptomatic older leaves accompanied by wilting of younger leaves to severe foliar symptoms.

The diagnosis also made it possible to detect the presence of the *Meloidogyne-Fusarium* complex, with the majority presence of the root knot nematode *Meloidogyne incognita*. As previously mentioned, the presence of typical microconidia and conidia of the genus *Fusarium* spp. were observed (Figure 4C).

The *Meloidogyne-Fusarium* complex, which was found to affect these plants, adds a further complication to the sanitary situation. This complex has been reported to cause serious damage to Sacha Inchi plantations (6, 19). The combination of both agents reinforces their effect because the wounds caused by the nematodes serve as entry routes for soil-borne plant phytopathogenic fungi l, particularly such as those of the *Fusarium* genus (26). The combined effect of these two phytopathogenic agents is far more destructive than when they act separately. It drastically reduces the yield and lifespan of this crop (3). This is the first study reporting *Fusarium* spp., *Lasiodiplodia* spp. and the *Meloidogyne-Fusarium* complex in Cuba.

The phytopathogens detected in this work are also important in different crops and, given the observed effects, it was recommended to deepen these studies and apply management measures compatible with the crop.

CONCLUSIONS

- The root-knot nematode infestation was high (between 3-5 degrees), and the most affected areas were the neck of the stem and the roots, due to the formation of galls that limited or prevented nutrient absorption.
- The diagnosis allowed detecting the presence of nematodes: *M. incognita, M. arenaria, Trichodorus* spp., *Helicotylenchus dihystera, A. bicaudatus,* and *Xiphinema* spp., with the majority presence of *M. incognita*.
- Phytopathogenic fungal species of the genera *Fusarium* and *Lasiodiplodia* and the *Meloidogyne* - *Fusarium* complex were detected.

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