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A pathogen complex between the root knot nematode *Meloidogyne incognita* and *Fusarium verticillioides* results in extreme mortality of the inka nut (*Plukenetia volubilis*)

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Summary

The combined infection of the root knot nematode *Meloidogyne incognita* and two *Fusarium* species led to high plant mortality of inka nut (*Plukenetia volubilis*) seedlings in pots after 80 days growth in two independent inoculation experiments. Inoculation of *M. incognita* juveniles and conidia of *F. solani* or *F. verticillioides*, simultaneously, increased plant mortality by 25-30%, compared with *M. incognita* alone (5-10% mortality). When inoculated with *F. verticillioides* at 20 days post nematode inoculation, plant mortality increased to 55%. Either of the *Fusarium* spp. alone caused some plant mortality, but less than when combined with *M. incognita*. The synergistic interaction of *M. incognita* and especially *F. verticillioides* demonstrates a lethal outcome for inka nuts. It appears that *M. incognita* creates favorable conditions that lead to enhanced effect and damage by the *Fusarium* species, especially *F. verticillioides,* leading to devastating levels of plant death.

Key words: sacha inchi, root diseases, synergistic effects.

Introduction

The inka nut (*Plukenetia volubilis*) is a perennial vine belonging to the Euphorbiaceae family and native to much of tropical South America, where it is also known as sacha inchi, sacha peanut, mountain peanut or Inca-peanut. In the Amazon Rainforest and the high Andes Mountains of Peru, the aerial seeds of this plant have been eaten and have formed part of the Inca diet for millenia. It is also now cultivated commercially elsewhere, especially in South East Asia, and most notably in Thailand. It is among the richest sources of omega fatty acids (ω3, ω6 and ω9) (MAURER et al., 2012) and is additionally rich in proteins (22-30%), vitamin E (tocopherols and tocotrienols) and natural antioxidants (WANG et al., 2018). These proprieties have generated a high demand for the crop on the global market, for nutritional and pharmaceutical purposes.

An emerging constraint to the production of the inka nut, however, results from a root disease complex, caused by the combined infection of root knot nematodes (*Meloidogyne* spp.) and fungal wilt pathogens (*Fusarium* spp.), which becomes most evident in the second year of production (MANCO, 2004). The root disease complex occurs in plantations when inka nut plants are either monocultured or grown in mixed cropping systems, such as when intercropped with maize (*Zea mays*), beans (*Phaseolus vulgaris*), groundnut (*Arachis hypogaea*), or other locally important annual crops (personal observations, Juan Carlos Guerrero-Abad). However, the disease complex is not evident on plants that have naturally seeded and grow wild in native environments, even on plants aged 20 years or more (personal observations, Juan Carlos Guerrero-Abad).

Combinations of pathogens and parasites on the same host plant may lead to susceptibility through predisposition and many examples of nematodes predisposing plants to disease attack have been documented. In particular, numerous reports have demonstrated that *Meloidogyne* spp. and *Fusarium* spp., when present together, form pathogenic complexes that result in much greater damage to host plants, than when present in isolation (ULLOA et al., 2016; MWANGI et al., 2019). The first such record of a nematode-fungus interaction was reported by ATKINSON (1892), who observed that on cotton (*Gossypium hirsutum*) the wilt disease caused by *Fusarium oxysporum* was more severe in the presence of *Meloidogyne* spp. Other examples have since been reported from other crops, such as eggplant (*Solanum melongena*), tomato (*Solanum lycopersicum*), coffee (*Coffea arabica*), and chili peppers (*Capsicum annuum*) (VILLAIN et al., 2013; MONTEIRO et al., 2016; VILLAIN et al., 2018). In all cases, an initial symptom of this synergistic interaction between *Meloidogyne* and *Fusarium* spp. results in leaf chlorosis. Subsequent symptoms include wilting, suppressed growth and ultimately plant death. The plant reaction is essentially biochemical and physiological and it is likely that nematode parasites influence the host response to many soil borne pathogens (WEBSTER, 1975).

In Yunnan, Southwestern China, CHAI et al. (2017) identified *F. oxysporum* as a potential root pathogen of the inka nut, further to WANG et al. (2014) reporting the presence of *M. javanica* in the same plantations. In our ongoing studies in inka nut plantations aged 2-4-years in San Martín, Peru, at least four *Meloidogyne* species were detected, with *M. incognita* the most frequent and abundant species (personal communication, Juan Carlos Guerrero-Abad).

Given the apparent absence of the root disease complex on naturally growing inka nut plants, it is hypothesized that they may be better protected by proximity to other native plants, or association with microbial communities, such as arbuscular mycorrhizal fungi or antagonistic microflora and microfauna (e.g. SONG et al., 2019; CORAZON-GUIVIN et al., 2019; 2020). The identification and use of antagonistic microorganisms therefore, may provide a possible strategy to address the issue. The current study was undertaken, however, to establish whether a *Meloidogyne*/*Fusarium* interaction is an important factor explaining the plant mortality of inka nut in the field, as both these pathogens were confirmed to be present in our plantations (Fig. 1).

Materials and methods

Collection and germination of inka nut seeds

Inka nut seeds cv. Shanantina were collected in 2018 from a plantation located in Lamas Province, Peru (06°26'47.3''S, 076°31'44.00''W;

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382 m a.s.l.). Seeds were selected for homogeneity, based on morphological characteristics, such as color (dark brown), size (1.5-2.0 cm) and shape (ovoid and bulging seed). Seeds were planted into autoclaved coarse sand in 3 L pots size and irrigated daily. Six-day-old seedlings of uniform size were then used for the experiments.

Meloidogyne incognita inoculum

Root knot nematode egg masses were removed from galled inka nut roots collected from near Aucaloma, Lamas province, Peru (6°24'46.9"S, 76°25'59.4"W; 855 m a.s.l.), placed in Petri dishes in distilled water (Atamian et al., 2012). Hatched second-stage juveniles (J2) were then used for propagation, inoculating 100 J2 per plant onto 15-day-old inka nut plants grown in pots filled with sterilized substrate. After 60 days, J2 were extracted from the galled roots according to Atamian et al. (2012), quantified using a dissecting microscope 5X (Carl Zeiss) and then used as inoculum in the experiment.

Isolation and multiplication of *Fusarium* spp.

Fusarium solani (M32C1) and *F. verticillioides* (M33C1) were isolated from roots collected from inka nut plants showing typical symptoms of the disease complex. The plants originated from the same plantation as above, located in Aucaloma (6°24'46.9"S, 76°25'59.4"W; 855 m a.s.l.). The fungal isolates were purified by monosporic isolation in PDA culture medium and stored at -80 ºC in the fungal collection at National University of San Martin. For fungal multiplication single discs of 0.5 cm diameter from a potato dextrose agar (PDA) plate containing mycelia and conidia were transferred to Erlenmeyer flasks containing 150 ml potato dextrose broth (PDB; Leslie and Summerell, 2008), incubated on a shaker at 25 ºC and 180 rpm for four days and then filtered through nested 180 µm and 38 µm sieves. Conidia collected on the 38 µm sieve were counted at 100 × magnification using a Neubauer counting slide.

Morphological and molecular identification of *Meloidogyne* and *Fusarium* spp.

For molecular identification, DNA was extracted from female and J2 root knot nematodes and *Fusarium* mycelium using a modification of the Cetyl Trimethyl Ammonium Bromide (CTAB) method (Doyle and Doyle, 1987). Species-specific primers INC-K14F/INC-K14R were used to determine the identity of *M. incognita* (Randig et al., 2002), which amplify a 399 bp fragment, while for the morphological identification, perineal sections of the female nematodes were prepared (Hartman and Sasser, 1985) and examined using the taxonomic key described by Eisenback and Triantaphyllou (1991) (Fig. 2). For *Fusarium* spp. the species-specific primers TEF-Fs4f/TEF-Fs4r (*TEF-1α* gene) for *F. solani*, (Arif et al., 2012) and FV-F2/FV-R (*gaoB* gene) for *F. verticillioides* (Faria et al., 2012) were used, which amplify a 658 and 370 bp fragment, respectively, while the morphological identification was based on the presence and structures of the macro and micro-conidia (Leslie and Summerell, 2008).

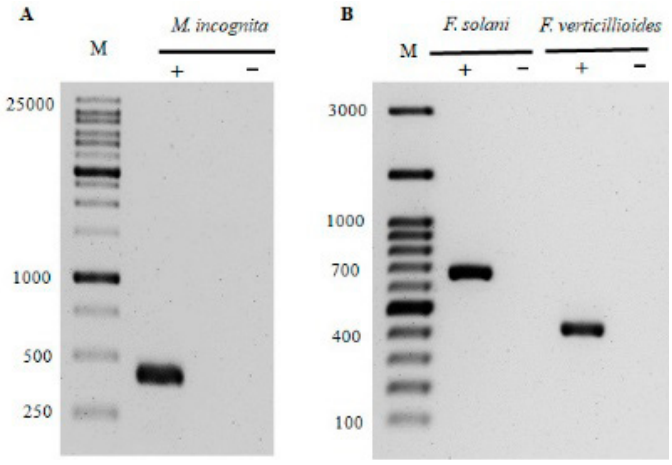

Fig. 2: Gel electrophoresis after PCR reaction: A) PCR reaction, Carril 1: 250-bp molecular marker (Invitrogen, USA), Carril 2: 399 bp fragment of *Meloidogyne incognita* DNA, Carril 3: (-) negative control reaction, no DNA was added; B) PCR reaction, Carril 1: 100-bp molecular marker (Invitrogen, USA), Carril 2: 658 bp fragment of *Fusarium solani* DNA, Carril 3: (-) negative control reaction, no DNA was added, Carril 4: 400 bp fragment of *F. verticillioides* DNA, Carril 5: (-) negative control reaction, no DNA was added.

Experimental design and arrangement

The first experiment was established in September 2018 in the greenhouse at maximum, medium and minimum temperatures of 38.2, 29.0, and 21.4 ºC, respectively; and maximum, medium and minimum relative humidity of 74, 64 and 48%, respectively, and conducted for 80 days until November 2018. The experiment was repeated from March until May 2019. Pre-germinated seeds (with emerged radicle) were transplanted into 3 L sized pots filled with 3 kg of sterilized substrate. The seeds were positioned with the hilum directed downwards for optimum root development in the substrate. The substrate comprised field soil:quartz sand (1:1), which was autoclaved for 1 h per day over three consecutive days. Substrate particles incubated on PDA in Petri dishes for three days at 25 °C confirmed the sterility of the substrate. Upon emergence, seedlings were inoculated with 10 ml of *Fusarium* sp. and 10 ml of nematodes as final volume according to the treatments, as summarized in Tab. 1, into

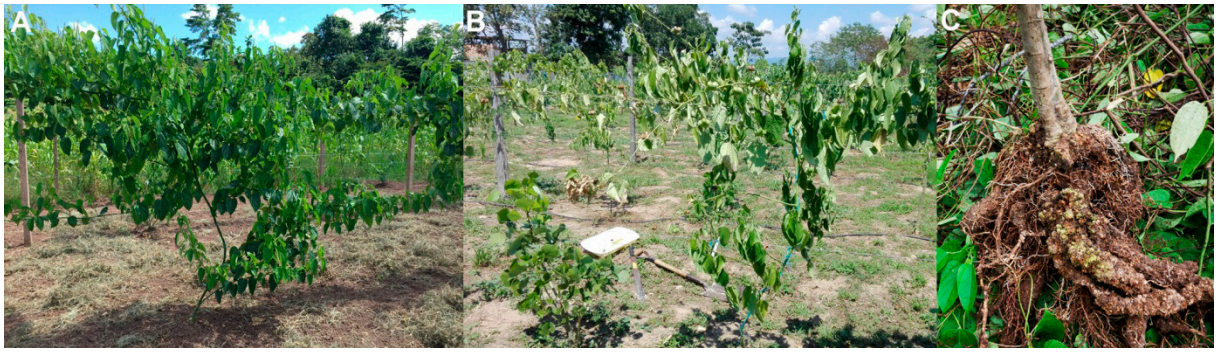

Fig. 1: *Plukenetia volubilis* plantation in San Martin (Perú), A) Healthy inka nut plant; B) Plant infected with nematodes and *Fusarium* spp. with yellowing and wilting symptoms; C) Nematode-*Fusarium* complex in the radicular system of the inka nut.

Tab. 1: Experimental treatments of this study

Ctr	Non inoculated control
M	Inoculation with nematode *Meloidogyne incognita*
Fs	Inoculation with *Fusarium solani*
Fv	Inoculation with *Fusarium verticillioides*
M + Fs_0	Simultaneous inoculation with *M. incognita* and *F. solani*
M + Fv_0	Simultaneous inoculation with *M. incognita* and *F. verticillioides*
M + Fs_{20}	Inoculation with *F. solani* 20 days after inoculation with *M. incognita*
M + Fv_{20}	Inoculation with *F. verticillioides* 20 days after inoculation with *M. incognita*

4 holes, approximately 0.5 cm in diameter and 4.0 cm in depth, at equal distance around each seedling. Nematodes were inoculated at a rate of 3000 J2 per pot (1 J2 g^{-1} soil) and each *Fusarium* sp. at a rate of 8×10^3 microconidia per g of soil. In total, 160 pots were prepared comprising 8 treatments, each with 20 replicates, arranged in a completely randomized design.

Data collection

Data were collected from each of the two independent experiments (see above). The parameters included i) plant height (cm) measured form the soil level to apical meristem, ii) root galling determined according to the scale proposed by Bridge and Page (1980), iii) leaf number per plant measured by counting the total leaves, iv) number of chlorotic leaves evaluated based on a direct observation, and v) plant mortality determined at 10-day intervals over 80 days. In addition, chlorophyll content (SPAD – Soil Plant Analysis Development) was measured from completely expanded leaves, by using a chlorophyll analyzer (SPAD-502, Minolta Camera Co. Ltd., Osaka, Japan), and leaf area index (cm^2) was calculated using ImageJ (https://imagej.nih.gov/), measured at experiment termination at 80 days.

Statistical analyses

The results of the two independent experiments showed only minor numerical differences, but no statistical differences ($p>0.05$) for each parameter recorded. Thus, the data for the two experiments were combined for analyses. Measured variables were evaluated for normality and homogeneity using Shapiro-Wilk (Shapiro and Wilk, 1965) and Levene's (Levene, 1960) tests, respectively. ANOVA analyses followed by Tukey's HSD to test for significant differences among treatments at $p<0.05$ significance level (Snedecor and Cochran, 1980). The data were analyzed using INFOSTAT version 2012.1 software (Di Rienzo et al., 2012).

Results

Identification of *Meloidogyne* and *Fusarium* spp.

Using the prepared perineal patterns *M. incognita* was identified as the species present, which was confirmed from the molecular approach using specific primers for *M. incognita*. Likewise, two species of *Fusarium* were detected, and identified as the fungal pathogens *F. solani* and *F. verticillioides* from damaged roots of *Plukenetia volubilis* plants using species-specific primers (Fig. 2).

Inka nut plant growth

At 30 days after the initial inoculation most crop growth parameters began to relatively differ between the treatments (Figs. 3, 4, 5, 6) with all parameters differing significantly ($p<0.05$) from the untreated control at termination. Plants inoculated with *F. verticillioides* 20 days after *M. incognita* had the poorest growth in terms of plant height (1.4 m), leaf number (27), leaf area (200 cm^2), and plant mortality (55%), when compared to the control plant height (2.8 m), leaf number (52), leaf area (450 cm^2), and plant mortality (0%; Fig. 3-6). In particular, the delay by 20 days in inoculating *F. verticillioides* led to significantly lower leaf areas compared to its simultaneous inoculation with *M. incognita*, and which corresponds to a leaf area decrease of 56% compared to the control. Inoculation of *M. incognita* or *F. solani* alone did not affect plant height or leaf area compared with control plants, although *F. verticillioides* alone generally reduced growth compared with control plants. The combined inoculation of *M. incognita* and *F. verticillioides* or *F. solani* increased plant mortality, which was particularly marked following the 20-day delay in inoculating *F. verticillioides*, with 55% of plants dying before termination of the experiment (Fig. 6). There were no dead plants recorded in the non-inoculated control or *M. incognita* alone treatments.

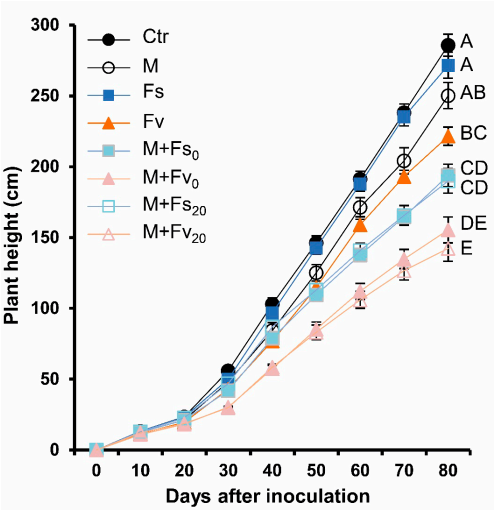

Fig. 3: Effect of *Meloidogyne incognita* and *Fusarium* spp. on the plant height of inka nut plants under different treatments during 80 days of vegetative growth measured at 10-days intervals. Error bars indicate standard deviation (± S.D.). Columns with the same letter are not significantly different ($p<0.05$). Ctrl = control, M=Inoculation with *M. incognita*, Fs = Inoculation with *F. solani*, Fv = Inoculation with *F. verticilloides*, M + (Fs_0 or Fv_0) = Simultaneous inoculation of *Fusarium* species with *M. incognita*, M + (Fs_{20} or Fv_{20}) = Inoculation of *Fusarium* species 20-days after inoculation with *M. incognita*.

Nematode root galling damage

No root galling damage was observed on plants inoculated solely with *F. verticillioides* and *F. solani* and the non-inoculated control (Fig. 7). Plants in all treatments inoculated with *M. incognita*, however, recorded root galling damage, which differed ($p<0.05$) in severity between treatments. The treatments inoculated solely with *M. incognita* recorded the lowest level of damage, with a gall index score of 5, while the treatment with *F. verticillioides* inoculated 20 days after *M. incognita* was severely galled with a score of 10.

Chlorosis and chlorophyll content

The non-inoculated control plants were healthy throughout the experiment, with no chlorotic leaves recorded (Fig. 8a). After 80 days, 15% of plant leaves were chlorotic in the treatment solely inoculated with *M. incognita*, similar to most other inoculated treatments. The combination of *F. verticillioides* and *M. incognita*, however, resulted in the most chlorotic leaves per plant, especially

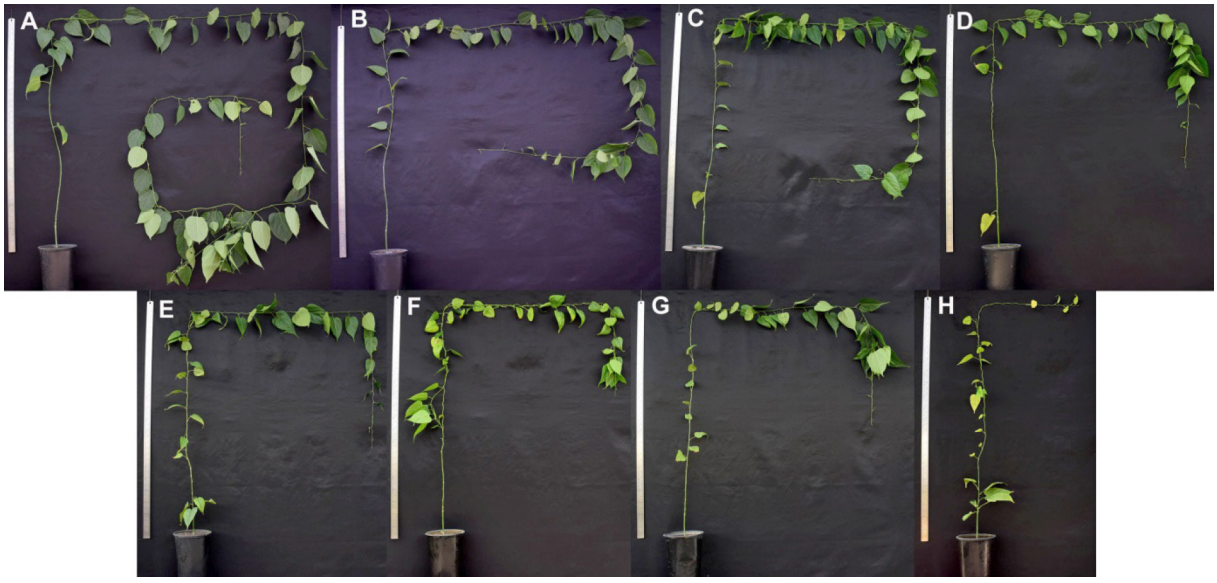

Fig. 4: Plants of the inka nut (*Plukenetia volubilis*), grown for 80 days in the greenhouse. A) Non-inoculated control; B) inoculated with *Meloidogyne incognita*; C) inoculated with *Fusarium solani*; D) inoculated with *F. verticillioides*; E) simultaneously inoculated with *M. incognita* and *F. solani*; F) simultaneously inoculated with *M. incognita* and *F. verticillioides*; G) inoculated with *F. solani* 20 days after inoculation with *M. incognita*; H) inoculated with *F. verticillioides* 20 days after inoculation with *M. incognita*, scale = 100 cm.

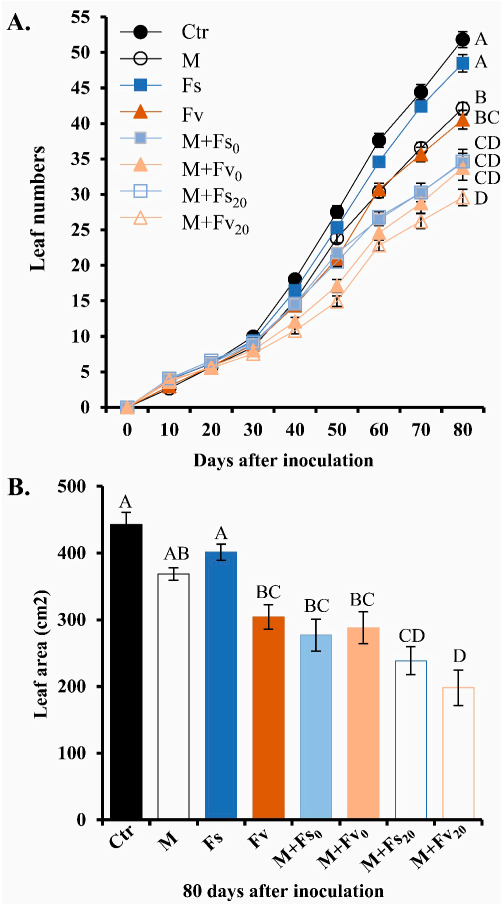

Fig. 5: Leaf number and leaf area index (cm^2) of inka nut plants during the presence of *Meloidogyne incognita* and *Fusarium* spp. A) Leaf number of inka nut plants under different treatments measured between intervals of 10-days until three months; B) Leaf area of inka nut plants after 80-days of treatment. Error bars indicate standard deviation (± S.D.). Columns with the same letter are not significantly different (p<0.05).

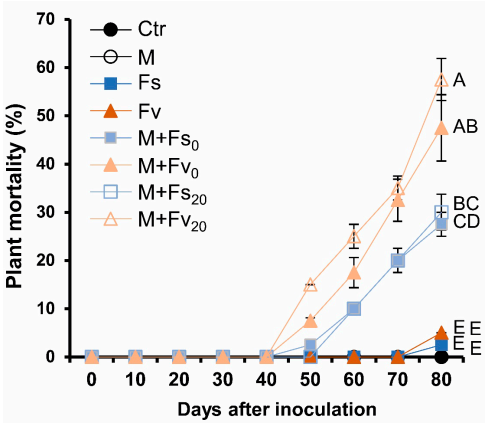

Fig. 6: Percentage plant mortality of inka nut plants at the presence of *Meloidogyne incognita* and *Fusarium* spp. measured at 10-day intervals for 80 days. Error bars indicate standard deviation (± S.D.). Columns with the same letter are not significantly different (p<0.05).

when *F. verticillioides* inoculation was delayed by 20 days, which had significantly more (p<0.05) chlorotic leaves (25%) than all other treatments. Similarly, the chlorophyll leaf content was also highest in the non-inoculated controls, similar to the single inoculated treatments, while plants in the combination of *F. verticillioides* and *M. incognita* had the lowest leaf chlorophyll content, which was 38% lower than the control (Fig. 8b).

Discussion

The current study was undertaken to diagnose the pathology behind the damage being experienced in inka nut plantations in San Martín (Peru), where plant growth was suppressed and plant death occurred. Uprooted plants in the field exhibited extreme galling damage and root rot. Pathogens mostly isolated from this plantation were identified as *M. incognita*, *F. solani* and *F. verticillioides* and thus were the focus of the study, which found that all three pathogens independently and in combination can play a key role in

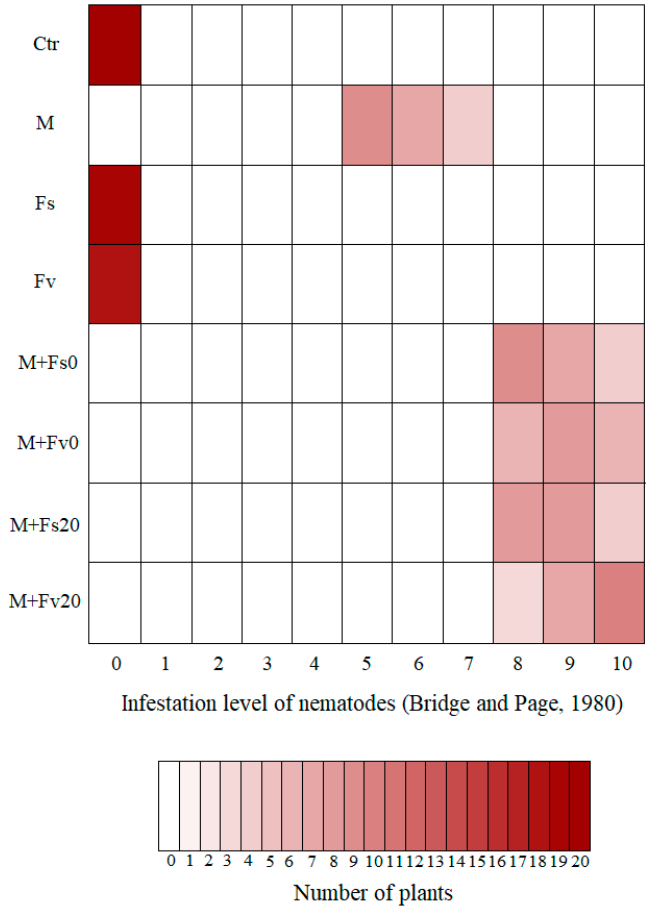

Fig. 7: Heat map showing the infection level of nematodes (BRIDGE and PAGE, 1980) of the treatments under study.

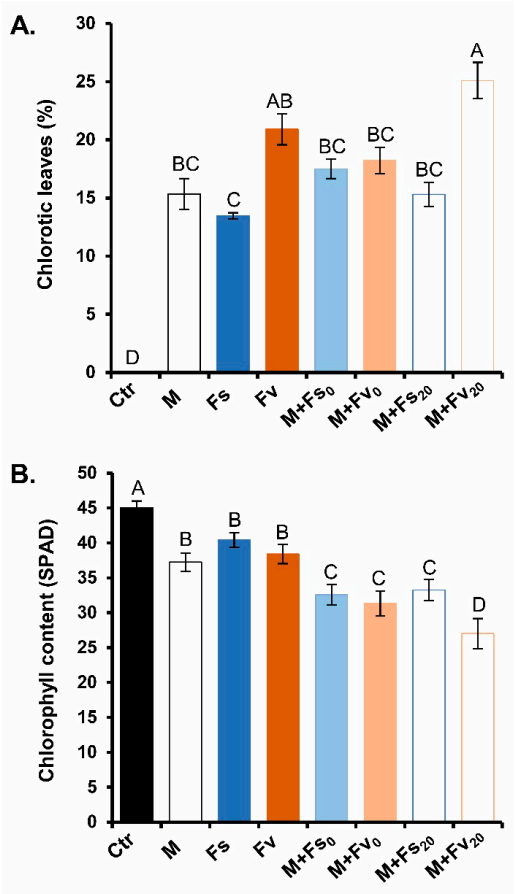

Fig. 8: Percentage of chlorotic leaves of inka nut plants during the presence of *Meloidogyne incognita* and *Fusarium* spp. A) Percentage of chlorotic leaves measured at intervals of 10-days for 80 days after initial inoculation, mean and standard deviation; B) Chlorophyll content in leaves of inka nut plants during the presence of *M. incognita* and *Fusarium* spp., after 90-days of treatment. Error bars indicate standard deviation (± S.D.). Columns with the same letter are not significantly different ($p<0.05$).

the suppression of the inka nut plant growth. The combination of *M. incognita* and *F. verticillioides*, however, appears to be particularly deadly, with half of test plants killed after just three months growth, with the remainder stunted and chlorotic. Similar disease complexes comprising combinations of *Meloidogyne* spp. and Fusarium wilt pathogens have previously demonstrated synergistic interactions, which create a greater than additive damage to crop production (BELL et al., 2017; TAHER et al., 2017). In the current study, the delay in inoculation of *F. verticillioides* following *M. incognita* led to the most harmful damage. Comparable results have been reported from coffee using a combination of *Meloidogyne arabicida* and *Meloidogyne exigua* with *Fusarium oxysporum* (BERTRAND et al., 2000). KUMAR et al. (2017) inoculated tomatoes with *M. incognita* and *F. oxysporum* f. sp. *lycopersici*, and also showed that plant growth suppression was greatest when the nematodes were combined with the fungal pathogens. Examples from inka nut are scarce but CHAI et al. (2017) observed suppressed growth, leaf wilting and chlorosis in inka nut plantations in Yunnan, China, which were then associated with rotted and galled roots caused by *M. javanica* (WANG et al., 2014). There is so far no other report for inka nut.

It is well acknowledged that nematode infection of plant roots can facilitate secondary infections and give rise to root pathogen complexes between soil nematodes and microorganisms (OERKE, 2006). Root infection by nematodes disrupts access to and supply of water and nutrients to the host plant through disruption of cell division and growth, deterioration of root tissue and root death even (KARSSEN et al., 2013). Root knot nematodes are ubiquitous pests, especially in tropical and subtropical environments, where they cause substantial damage to crop production (COYNE et al., 2018). In many crops, root knot nematodes are associated with various wilt-causing pathogens, such as *Fusarium* spp., the combination of which appears to result in much greater damage than when either pathogen is present alone (VILLAIN et al., 2018; MWANGI et al., 2019; DA SILVA et al., 2019).

The present study was undertaken under controlled conditions in sterilized media in pots. Recently, the inka nut was found to form associations with a broad range of arbuscular mycorrhizal fungi (CORAZON-GUIVIN et al., 2019; 2020). Under field conditions, therefore, reaction of the host plant to the presence of pests and pathogens will likely differ, as microorganisms present in the microbiome, including arbuscular mycorrhizal fungi, can provide host protection against such biotic threats. However, after 80 days in the screenhouse, over half of the plants in the treatment combining *F. verticillioides* and *M. incognita* had died. Although under sterile conditions in pots, this compares with zero plant death in the un-inoculated control and demonstrates a particularly lethal outcome. It is interesting, however, that a delay in the inoculation of *Fusarium* sp. following that of *M. incognita* led to significantly stronger effects on growth and development of inka nut plants than a simultaneous inoculation, leading to plant death. This supports other reports, demonstrating that prior establishment of nematodes increased disease intensity, reducing the morphological traits such as shoot length, total chlorophyll contents, and also endogenous biochemical process as capacity and enzyme activity (MAQSOOD et al., 2020). In our study, all dead plants had

symptoms typical of a disease complex: such as chlorosis, dwarfism, hyperplasia with deep and cracked cortical tissues in the affected root system, leading to necrosis and atrophy of the host root system (Wolfgang et al., 2019). The high level of chlorosis and reduced leaf area is also probably symptomatic of a low capacity for nutrient absorption due to the double invasion of *Meloidogyne* spp. and *Fusarium* spp. (Wolfgang et al., 2019), and which leads to plant death (Villain et al., 2018; Da Silva et al., 2019; Mwangi et al., 2019).

Our results showed that the combination between *M. incognita* with *F. verticillioides* is especially harmful to the inka nut, indicating a higher synergism between both organisms than between *M. incognita* and *F. solani*. We did not however, assess the tripartite combination of *M. incognita, F. solani* and *F. verticillioides*, the three pathogens most isolated from the affected plantation. Nor did we assess other species of *Meloidogyne*, which occur in the plantation, and which may have provided even greater levels of damage, or less. In this context, Back et al. (2002) suggested that a complex between diseases is formed through the synergistic, antagonistic or neutral interactions between different organisms. The association between *M. incognita* and *F. verticillioides* resulted in significantly increased levels of damage and death of inka nut plants than other treatments, reflecting symptoms from the field plantation.

Conclusions

We conclude that the pathogenic complex of root knot nematodes (*M. incognita*) and fungal wilt (*Fusarium* spp.) severely affects the morphological and physiological status of inka nut plants, which led to high levels of plant mortality under greenhouse conditions. This complex was not evident under more natural conditions, probably due to antagonistic interactions with beneficial microorganisms or more biodiverse microbiomes, which can help protect plants against harmful nematodes and pathogens. The mycotrophic dependency of the inka nut on microorganisms common in the microbiomes of inka plants growing under natural conditions should therefore be investigated, including their association with arbuscular mycorrhizal fungi.

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Conflict of Interest:

No potential conflict of interest was reported by the authors.

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