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Growth and root-knot nematode infection of tomato are
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intercropping cultivation system with leeks

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Abstract

In a greenhouse experiment, we investigated the influence of leek (*Allium porrum*) intercropping, mycorrhizal fungi and earthworms on the root-knot nematode infection by *Meloidogyne incognita* (RKN) and the growth and development of tomato plants (*Solanum lycopersicum*). Tomato plants were grown in pots, intercropped with leeks or in monoculture, and earthworms and/or arbuscular mycorrhizal fungi (AMF) were introduced. The effects of these two treatments on root-knot nematode infection and tomato plant growth and development were assessed i) by counting the galls induced by the nematodes and the number of egg masses on their roots, and ii) by measuring their height increase, shoot and root dry biomasses and flowering time. Tomato growth was increased with leek compared to the monoculture (e.g., +82 % shoot biomass), probably due to a lower plant-plant competition, although a facilitation effect cannot be excluded. The presence of leeks did not reduce the number of galls (per g of root). The presence of AMF (*Rhizophagus irregularis*) increased the overall infection of *M. incognita* (e.g., +36 % egg masses) and induced more galls with multiple egg masses in the leek-tomato association (+71 %) but did not increase plant biomass. On the other hand, it increased plant height in presence of the compost earthworms (*Eisenia andrei*) compared to earthworms alone with RKN (+2 %). It also delayed the flowering compared to RKN alone (+7.6 days in average). Competition between the two tomato plants in monoculture probably strongly impacted plant growth, flowering time and thereby the RKN infection, but leek-tomato interactions should be further examined. The overall increased number of multiple egg masses per gall in presence of AMF is contrary to its protective effect reported in the literature.

Keywords: Crop association; biological control; soil biodiversity; *Meloidogyne incognita*; *Eisenia andrei*; *Rhizophagus irregularis*

1. Introduction

Root-knot nematodes (RKN) from the genus *Meloidogyne* are considered as pests of primary importance in agriculture and the top-ten nematodes in the world based on scientific and economic importance (Jones et al., 2013). As endoparasites of plants, they carry out a part or all of their life cycle in the root tissues to feed and reproduce (Hoefferlin et al., 2018). These nematodes will induce galls on roots from which egg masses are laid, and eggs are then released into the soil. Under a favourable environment, they develop into juveniles which, as soon as they hatch, will infect the roots of the same or other neighbouring host plants, thereby inducing the formation of new galls (Moens et al., 2009; Eisenback and Triantaphyllou, 2020). Thus, through series of reproduction cycles, the soil infection by these nematodes can reach 100,000 to 200,000 eggs kg⁻¹ of soil (Blancard, 2009). Yield reductions have been estimated between 12 and 21 % for all cultivated plants (Hoefferlin et al., 2018), and up to 100 % yield losses were recorded on untreated susceptible tomato varieties (Wesemael et al., 2011; Jones et al., 2013). Among the methods developed to control plant-parasitic nematodes, chemical control based on pesticides is commonly used in intensive conventional agriculture, despite its cost and unsustainability (Neves et al., 2009; Cheng et al., 2015). However, due to increasing restrictions to limit environmental impacts, physical and/or biological control (a.k.a. biocontrol) alternatives are being developed for a more sustainable agriculture. Biocontrol approaches rely on “natural enemies, antagonists or competitors and other self-replicating biotic entities” (Kenis et al., 2012), and on molecules from natural origin (French Ministry of Agriculture 2020). Among alternatives to chemicals for pest control, intercropping can be considered alone or combined to other practices in integrated pest management (Smith and McSorley, 2000, Djian-Caporalino et al., 2019). The simultaneous presence of several plant species in the same area offers significant advantages, such as improved nutrition or protection against pests (Smith and McSorley, 2000, Xie and Kristensen, 2017). Plant species from the *Allium* genus, for example, are known for their biocidal effects by releasing sulfur compounds such as thiosulfinates and disulphides with methyl (DMDS), propyl (DPDS) or propenyl groups (Arnault et al., 2005). These volatile compounds released in root exudates have an inhibitory effect

on the hatchability of RKN (Li et al., 2018). Extracts can be used alone efficiently against RKN (Tada et al., 1988) and associations of *Allium* sp. with other crops also produced nematicidal effects (Huang et al., 2016).

The diversity of soil organisms is also particularly important and often neglected in pest control (Fournil et al., 2018). Indeed, the diversity of soil organisms induces ecological niches that are more favourable to the development of plants and unfavourable to plant pests (bacteria, fungi or nematodes), whose populations decline or stabilize below a tolerable threshold (Baker, 1991; Barrios, 2007). Endomycorrhizal fungi, and particularly arbuscular mycorrhizal fungi (AMF), establish symbiosis with plant roots, colonizing the majority (74%) of plant species (Smith and Read, 2010; Van Der Heijden et al., 2015). This mutualistic interaction is mainly known to promote water, sugar and nutrient exchanges between the plant and the fungal partners. Besides, mycorrhizal fungi also enhance soil structure and foster diversified and stable microbial communities, preventing the proliferation of pathogenic propagules (Pozo and Azcón-Aguilar, 2007; Sosa-Hernández et al., 2019). AMF can physically protect roots in the rhizosphere acting like a barrier against microorganisms and invertebrates, or acting inside roots by releasing fungal compounds (Simard and Durall, 2004; Song et al., 2010). For example, the AMF *Funneliformis mosseae* (formerly *Glomus mosseae*) has already been shown to reduce RKN infection on mycorrhized tomato roots (Vos et al., 2012, 2013). Understanding the interactions between AMF and nematodes is an important challenge to reduce crop yield losses due to nematodes (Schouteden et al., 2015). Earthworms are also important components of agroecosystems, as they stimulate plant growth by increasing soil fertility via earthworm casts and by promoting microbiological activity (Blouin et al., 2013; van Groenigen et al., 2014). Their presence can also limit the activity of pests such as the epigeic *Amyntas corticis* reducing thrip attacks on tomato plants (Xiao et al., 2019). Rostami et al. (2014) also showed in an *in vitro* experiment that the application of *Eisenia fetida* earthworm products (liquid from vermicompost, coelomic fluids and water-soluble compounds from earthworm skin) inhibited the hatching of *Meloidogyne javanica* eggs in cucumbers and had larvicidal effects. Few studies were conducted on the combined effects of soil organisms and plant-parasitic nematode populations and on

their impact on crops. Among them, Hua et al. (2010) showed that the combined presence of AMF and earthworms reduced the number of plant-parasitic nematodes compared to the fungus alone in a polluted soil, mostly driven by an earthworm effect.

Tomato (*Solanum lycopersicum*) is one of the world's most economically important high-yielding crop and is susceptible to soil-borne pathogens such as RKN (FAO, 2014). In a context of vegetable gardening, agroecological practices relying on plant-plant and plant-soil organisms' interactions could limit the populations of RKN, and/or reduce their impacts on tomato plant growth and production. Among candidates, co-cultivation of tomato crops with another cash crop from the genus *Allium* such as leek (*Allium porrum* L.), in which DPDS is predominant (Auger et al., 1989; Casella et al., 2013), represents a potential tool to control RKN. Using an experimental greenhouse set-up, we investigated the impact of leek, arbuscular mycorrhizal fungi (*Rhizophagus irregularis*) and compost earthworms (*Eisenia andrei*), alone and in combination, on the control of RKN (*Meloidogyne incognita* Kofoid and White) and on the growth and development of tomato plants. This work provides a better understanding of whether co-cultivating tomato with leek is a promising practice and/or whether the presence of organisms such as earthworms and AMF is beneficial for tomato production.

2. Material and methods

2.1. Study system

The tomato cultivar (var. 'Coeur de boeuf rouge'; AGROSEMENS, France) was selected for its economic potential, as it is commonly found on markets and appreciated by consumers. Seeds were organically produced and, according to the supplier, the variety is not known to exhibit any resistance against *Meloidogyne incognita*. The leek cultivar (var. 'Lancia'; AGROSEMENS, France) was selected for its ability to grow during summer in order to be intercropped with tomato. Tomato plants were infected by a solution containing eggs of a pure population of *Meloidogyne incognita*, provided by the Institute Sophia Agrobiotech-INRAE, France.

Rhizophagus irregularis (DAOM 197198 inoculum; Agronutrition, France) was selected to investigate the effect of arbuscular mycorrhizal fungi (AMF). Due to its wide commercial distribution among farmers for field inoculation, this species is likely to be present in many agricultural landscapes (Savary et al., 2018). It can be purchased in high concentration as pure inoculum and can be easily transported in a sterile environment. Finally, the epigeic earthworm species *Eisenia andrei* Savigny (Vers la Terre, Pezenas, France) was chosen to test an ‘earthworm effect’. Known as compost worm, it feeds on decaying organic matter, lives in the soil litter and the top soil and is commonly found in agrosystems due to the use of compost as manure by farmers. Moreover, unlike endogeic and anecic species, this species was observed at the root level in a tomato field experiment in the same sampled soil (pers. observation).

2.2. Experimental design

To measure the effects of leek, AMF and earthworms alone and in combination on the biocontrol of RKN infection, we set up a four-factor randomized experiment. The four factors were 1) the crop Association (*i.e.* intercropping) with two levels: tomato monoculture (TT) or leek-tomato (LT) and, within each of them, 2) the presence of the RKN *M. incognita* alone, 3) the presence of RKN and arbuscular mycorrhizal fungi (AMF) *R. irregularis* (RKN/AMF), and 4) the presence of RKN and the compost earthworm *E. andrei* (RKN/EW). The presence of RKN and AMF and earthworms, thereafter referred to as ‘soil organisms’, was also tested (RKN/AMF/EW). No RKN, AMF or earthworms were introduced into the control pots. The combination of all factors resulted in 10 treatments, with 5 biological replicates ($n=5$), totalling 50 pots. As tomato was the target plant, leek monocropping was not tested.

The experiment was carried out from May to July 2019 at the University of Poitiers, France (46°33'55.3", N 0°22'57.3"E) under ventilated greenhouse conditions (24.5°C average temperature, natural day/night cycle, 45 % average humidity). The experimental soil was collected from the topsoil (0-40 cm) of a grassland in an organic vegetable farm (46°15'07.1"N, 0°45'25.5"E; Adriers, France). The sandy loam soil was sieved through a 4 mm diameter mesh and coarse fragments and macrofauna were manually removed. To prevent the presence of potential nematodes (cysts, eggs, larvae, adults) and other contaminating

organisms, the soil was autoclaved two months before the beginning of the experiment (two cycles of 20 min at 120°C, 1 bar pressure). The initial soil characteristics were: organic matter (OM) = 3.32 %, pH_{KCl} = 6.21, N_{tot} = 0.14 %, C_{tot} = 1.93 %, available P = 94 g kg⁻¹, CEC = 6.28 cmol g⁻¹, Ca^{2+} = 1721 g kg⁻¹, Mg^{2+} = 327 g kg⁻¹, Na^{+} = 25.3 g kg⁻¹, K^{+} = 240 g kg⁻¹.

Tomato seedlings were prepared by sowing seeds on moist filter paper in Petri dishes and transplanted 5 days later in 9 x 9 cm pots filled with autoclaved soil. Upon transplanting, 40 seedlings corresponding to the RKN/AMF and RKN/AMF/EW treatments for the monoculture (TT) and the leek-tomato association (LT) were inoculated with a 1 mL solution containing 1000 spores mL⁻¹ of the AMF *R. irregularis*. Thirty supplementary seedlings were used to weekly check the tomato root colonization by AMF. When tomato root colonization by AMF was confirmed, tomato seedlings were transplanted in the 3.5 L microcosm pots filled with soil infested with nematodes eggs (RKN; 5,000 eggs) or not (Control), with associated plants (tomato or leeks) and with soil organisms (EW and/or AMF). Leek seeds were sown and grown on peat soil for 6 weeks in an organic vegetable farm (Chez Paulet, Adriers) then placed in experimental greenhouse conditions for additional 36 days before being transplanted with the tomato plant. Upon this first transplantation, leaves and roots of leeks were cut to favour their growth in pots, and absence of AMF colonization was checked on leek roots before transplantation.

In order to be as representative of field conditions as possible, where the crop density of a monoculture can be twice as high as that of an intercropped crop, two tomato plants were set in a single pot in the tomato monoculture condition, and only one in the leek-tomato association. No treatment with one plant and no leek was tested, as a co-cropping effect rather than a single-plant effect was tested. Furthermore, the same quantity of inoculum of RKN was applied to pots of both TT and LT, without considering the tomato plant density. 5,000 eggs per pot was estimated to effectively colonize all root systems (Djiani-Caporalino, pers. comm.). At the start of the experiment (t_0), tomato plants (11 weeks old) were individually transplanted into plastic pots (upper diameter 20 cm, lower diameter 16 cm, height 15 cm) filled with homogenized experimental soil with either another tomato plant or two leeks. Before the transplantation, soil of treatments containing nematodes were inoculated with 5,000 eggs of *M. incognita*

by pipetting 1 mL of egg solution (5,000 eggs mL⁻¹; Institute Sophia Agrobiotech-INRAE, France), and soil was mixed to homogenize egg concentration, along with 5 g of dry grasses (*Avena fatua*, *Deschampsia cespitosa*, *Anisantha sterilis* and *Brachypodium sylvaticum*). For AMF treatments, tomato plants had been previously inoculated with *R. irregularis* 8 weeks before being transplanted. Eight individuals of *E. andrei* (total average fresh weight: 0.30 ± 0.04 g) were gently introduced below the soil surface, and 5 g of dry grass litter were added to the soil surface. The earthworm density was determined from the density observed upon sieving the soil, and the rewetted litter provided food and protection for earthworms. It also aimed at reducing evaporation throughout the experiment. Extra tomato plants were prepared to check for RKN infection and completion of their reproductive cycle (7 weeks, 20°C) during the experiment. All pots were transferred to greenhouse conditions and a tomato plant was randomly selected in TT treatments for monitoring changes in plant height. Monitoring started on May 22nd and ended on July 7th. These times were hereafter referred to as t₀ (start) and t₇ (end at week 7), respectively. Plants were watered 2 to 3 times/day as heat conditions increased outside the greenhouse. To prevent the leaching and loss of RKN, plants were watered from the top through 2-3 short water shoots, adjusted according to soil moisture, and underpot dishes were checked for dryness. Due to the colonization time of AMF and the additional time for nematodes to complete their cycle, the experiment was extended after the flowering. No sign of nutrient deficiency was observed.

2.3. Sampling

After the final recording of plant measurements at week 7 (t₇), plants were harvested. Shoots of the monitored tomato plants were cut at the ground level and roots were gently separated from the soil and from other roots systems. Shoots were set aside and oven-dried (70°C, 3 days) and soil samples were collected and stored at 4°C for chemical analysis. Root systems were washed in three consecutive baths and were gently dried on a filter paper. Similar root samples were collected on each root system to check and quantify the AMF colonization. The remaining root systems were then weighed and stored at 4 °C for nematode analyses.

2.4. Measurements

2.4.1. Plant growth and development

All plant measurements were performed on the unique tomato plant in the leek-tomato association, and on the tagged tomato plant in the tomato monoculture. From the start to the end of the experiment (t_0 to t_7), tomato plant height was measured weekly from the stem base to the apical meristem and the number of flowers was counted. The first day of flowering was recorded to assess the time of flowering. To estimate the changes in plant growth through tomato shoots, we calculated the weekly difference between the plant height (t_x) and the initial plant height (t_0). At the end of experiment, shoot and root dry biomasses were weighed and plant height was measured.

2.4.2. Root-knot nematode infection level

After being cleaned from soil, fresh tomato root systems were weighed and colored in a solution of eosin B (15 min, 4.5 g L⁻¹) (Hooper et al., 2005). The solution of eosin B stains egg masses in red and facilitates counting. Root systems were then washed in two successive baths for root and gall discoloration, and both galls and egg masses were counted. Due to the very large number of galls per root system (estimated from 2,000 to 10,000 galls over the entire root system), the measurement of infection by RKN was systematically performed on a sub-sample of the root system (5 % fresh weight of tomato root system). All counts were then multiplied by 20 to consider the infection on the entire root system. The level of RKN infection was measured by counting the total number of galls, the total number of egg masses (*i.e.* eggs that were laid on galls) and the number of galls with multiple egg masses, which indicates that more than one nematode had penetrated and reproduced in the same gall (*i.e.* > 1 egg mass per gall) (Djiani-Caporalino et al., 2019). All those measurements were done blindly with a binocular microscope. Control tomato roots were checked and no RKN was found.

2.4.3. AMF root colonization and earthworm counts

To estimate mycorrhizal colonization, tomato root systems were collected and AMF colonization rates were determined using “the vinegar ink” staining protocol (Vierheilig et al., 1998). Fresh clean root samples were digested in 10% KOH at 70°C for 20 min, rinsed several times with distilled water, stained at 70°C for 15 min in vinegar:ink solution (95:5 by volume) and destained in 8% acetic acid for 5 min. For light microscopy, 30 1-cm-long root fragments were mounted on glass slides. Mycorrhizal rates (frequency of colonization by mycorrhizal fungi [F%], intensity of mycorrhizal fungi colonization out of all 30 root fragments [M%] and intensity of mycorrhizal fungal colonization in colonized root fragments only [m%]) were calculated using the Mycocalc software (<http://www2.dijon.inra.fr/mychintec/Mycocalc-prg/download.html> ; Trouvelot et al., 1986). Recovered earthworms from each mesocosm were counted and weighed.

2.4.4. Soil properties

All soil physicochemical properties were performed by a certified laboratory (Laboratoire d’Analyses Chambre d’Agriculture du Loiret, France). NH_4^+ and NO_3^- were extracted with a KCl solution and measured by colorimetry (540 nm and 660 nm). Organic matter (OM) was measured by dry combustion (international standard ISO NF 14235). pH was determined with water (1:5 ratio; international standard NF ISO 10390). Total C and N (C_{tot} and N_{tot}) concentrations were measured with a CHN pyrolysis micro-analyzer (international standards NF ISO 10694 and NF ISO 13878). Available phosphorus (P) was measured by spectrophotometry using the Olsen method (international standard NF ISO 11263). Cation Exchange Capacity (CEC) was measured with the Metson method (international standard NF X 31-130) and exchangeable cations (K^+ , Ca^{2+} , Mg^{2+} , Na^+) were measured using atomic flame emission from ammonium acetate extracts (international standard NF X 31-108).

2.4.5. Data analysis

All statistical analyses were performed using the version 4.0.2 of R (R Core Team, 2020). Nematode infection variables (*i.e.* total number of galls, total number of egg masses, and number of galls with multiple egg masses) were standardized per root dry biomass. The effects of the Association (LT vs TT), the presence of RKN, the presence of AMF and/or earthworms with RKN and of their interactions on plants and RKN infection were assessed using four-way models, with Association, RKN, EW and AMF as two-level explanatory variables. The effect of soil organisms within each association was also assessed (Supplementary Materials). Linear models were used for continuous variables and Generalized Linear Models (GLM; ‘Poisson’ distribution) were used for discrete variables (e.g., number of flowers, number of galls). Models were checked for normality and homogeneity of variances, and overdispersion for GLMs. When model assumptions were not met, variables were log-transformed in the case of continuous variables, and the ‘negative binomial’ distribution was used for numbers. Interactions were tested in models, and best models were selected based on the lowest Akaike Information criterion (AIC). Because of the numerous explanatory factors, p-values were adjusted with the False Discovery Rate (FDR) correction. Significant differences between treatments were assessed using Tukey’s post-hoc tests.

To assess the overall impact of treatments on plant growth and RKN infection and an eventual link with differences in soil properties, a principal component analysis (PCA) was performed using the *FactoMineR* and the *ade4* R packages (Dray and Dufour, 2007; Lê et al., 2008). Variables that were highly correlated and did not provide any additional information were removed and general patterns of variance were determined from the 8 following variables: 5 measures of plant growth and development (height increase, shoot biomass, root biomass, number of flowers, first flowering day) and 3 measures of RKN infection (total number of galls, total number of egg masses and number of galls with multiple egg masses, all calculated per gram of dry root). 7 soil properties and 3 soil organisms parameters (number of earthworms remaining in pots and AMF colonization of tomato roots) were added as illustrative variables. As the study focused on the biocontrol of RKN and because nematode-free control treatments highly impacted

the results of the PCA analysis, controls were removed from the analyses. A between–within classes analysis was then performed on the PCA to quantify to what extent the association, the combined soil organisms or the interaction of both factors affected plants and root infection by RKN. This method allows hierarchizing factors that contribute to the recorded variation. The between-classes analysis examined the differences between TT and LT or between soil organisms, whereas the within-classes analysis examines the remaining variation after the class effect has been removed (Dray and Dufour, 2007). The statistical significance of the between classes PCA was tested using a Monte-Carlo test based on 999-permutations.

3. Results

3.1. Plant growth and development

The presence of leek (LT) favoured the height increase (+29 %; Fig. 1A, Table 1), the shoot biomass (+82 %), the root biomass (+69 %) and the number of flowers produced (+72 %; Fig. 1D). This higher height increase was observed from the second week of the experiment (Supplementary Fig. S1). Plants grown with leek also flowered before plants grown in monoculture (-7 days; Fig. 1E) (see Supplementary Table S1 for all mean values). The infection of root-knot nematodes (RKN) and associated galls only had an effect on the height increase and the root biomass : plants grew less (-10 % height increase; Fig. 1B), while they produced more root biomass (+35 %) (Table 1). The lower shoot biomass was visible but not significant (Table 1, Supplementary Table S1). These effects were all significant when plants were grown with leek but not in the tomato monoculture (Supplementary Table S2). Particularly, the presence of leek reduced the negative impact of the RKN infection on tomato height increase (Table 1, Supplementary Table S1). The RKN infection had no impact on flowering in TT or in LT (Table 1, Supplementary Table S1). In presence of RKN, the tomato height increase was overall higher when plants were inoculated by both AMF and earthworms (+18 % compared to EW alone ; Table 1, Fig. 1C), and plants inoculated with AMF flowered later (+7.6 days; Fig. 1F). This overall positive interaction with earthworms on height increase was driven by an effect in the tomato monoculture only (Supplementary Table S2). Plants grown

in association with leek grew more when AMF was inoculated (+5 % height increase; Supplementary Table S1). Earthworms had no other effect on tomato growth and development parameters.

3.2. Root-knot nematode infection

Within pots inoculated with RKN, more galls, egg masses and galls with multiple egg masses on tomato roots were found on plants grown with leek than with another tomato (+56 %, +49 %, +71 %, respectively; Table 2, Fig. 2B). This 'leek' effect was absent when numbers were standardized per g of dry root (Table 2). Overall, more egg masses and galls with multiple egg masses were found on root systems of plants inoculated with AMF alone compared to AMF-free root systems (+97 % and +71 %, respectively; Table 2, Fig. 2A, 2C), but impacts varied within associations (Supplementary Tables S3 and S4) : fewer egg masses tended to be found in the tomato monoculture while no trend was visible in the leek-tomato association (Supplementary Table S3). On the other hand, more galls with multiple egg masses were found on roots inoculated with AMF compared to AMF-free roots when plants were grown with leek (+71 %; Supplementary Table S3), and there was no such difference in the tomato monoculture (Supplementary Table S3). An 'AMF' effect was found, with more galls with multiple egg masses when numbers were standardized per g of dry root (Table 2). Adding earthworms had no effect on nematode infection parameters (Table 2, Supplementary Table S3).

3.3. Within-between multivariate analyses

The within-between analyses performed on the Principal Component Analysis (PCA) based on plant responses and the RKN infection showed that the impact of the association was greater than that of soil organisms (Fig. 3, Supplementary Table S5). 30.15 % of the total variation was explained by differences between the TT and LT association, while a weak part of the total variation (9.56 %) was explained by differences between soil organisms (Supplementary Fig. S2 and Table S5). Differences between soil organisms explained 69.48 % of the variation within TT and LT association, although they were not clearly segregated (Supplementary Fig. S2). The root biomass ($r = 0.89$), the shoot biomass ($r = 0.85$), the

number of flowers ($r = 0.74$) and height increase ($r = 0.62$) were strongly and positively correlated to the first axis (41.8 % of the total variance), and the number of galls per gram of tomato root ($r = -0.53$), the number of egg masses per gram of tomato root ($r = -0.49$) and to the first flowering day ($r = -0.49$) were negatively correlated (Supplementary Table S6). This first axis clearly segregated leek-tomato-associated samples from the monoculture samples and was associated with a gradient of plant growth and development. The number of galls with multiple egg masses per gram of tomato root ($r = 0.87$) and the number of egg masses per gram of root ($r = 0.77$) were positively correlated to the second axis (24.3 % of the total variance) (Supplementary Table S6). Although they did not contribute to the PCA axes, soil properties differed with treatments (Supplementary Table S7). The soil content in PO_4^{3-} was positively correlated with the axis 1, and the C:N ratio was negatively correlated with the axis 2. The earthworm number and AMF root colonization were not correlated with any axes, but AMF root colonization was positively correlated to RKN infection variables, while earthworms were positively correlated to plant variables.

4. Discussion

4.1. Plant growth and development

We believe that both plant-plant competition and facilitation were involved in the higher plant growth and earlier development of tomato plants when they were grown with leek. Plant height and root mass are known to be reduced with as little as two plants per pot and as early as 8 days post-transplant (Stamp et al., 2004), and plant density impacts plant flowering (Papadopoulos and Pararajasingham, 1997). In the monoculture, the shade induced by the larger canopy of tomato plants could have increased the competition for light, and thereby reduced the aboveground biomass, increased the flower abortion, reduced the number of flowers or delayed the flower initiation (Papadopoulos and Pararajasingham, 1997). However, the authors also reported that plant height can be increased at high density due to photomorphogenic mechanisms that increase light interception by leaves. Hence, although greenhouse conditions were controlled and pots were regularly spatially reallocated, a direct effect of competition for

light within pots cannot be excluded, particularly on biomasses and plant development. In maize, Hammer et al. (2004) reported no direct effect of the increased canopy architecture through increased density on crop biomass, but suggested an indirect effect through the allocation of assimilates and water and nutrient uptakes. The lower root biomass produced 100 days post-transplant by plants grown with another tomato compared to those grown with leek was likely partially caused by the development of two tomato root systems for an extended period in a restricted substrate volume (3.5 L). Stamp et al. (2004) had indeed observed a reduced root biomass over a 25-day post-transplant period only, in a context of tomato-tomato competition. The length of our experiment was constrained by the required colonization of mycorrhizal fungi and the additional completion of the nematode reproduction cycle. Two or more plants compete for space and nutrients and may develop costly extra roots to capture remaining available resources, thereby increasing the rooting volume, modifying the root architecture and affecting shoot growth and the plant development. The increased rooting and associated cost in presence of competitors is referred to as the 'tragedy of the commons' (Gersani et al., 2001). It has not been tested in tomatoes to our knowledge, and it was also not the objective of our study. However, as suggested by Semchenko et al. (2007), "exploring larger substrate volumes to acquire the same amount of resources in the 'root competition' treatment as in the 'no root competition' treatment may make growth in larger volumes less efficient", and different rooting strategies may have impacted below and aboveground biomasses. The authors found that a competition effect may be confounded with a self-inhibition effect on constrained space for oat plants, and that plants inefficiently placed roots in large substrate volumes shared with roots of competitors (Semchenko et al., 2007). Contrary to previous experiments that compared tomato plant densities of 2 vs. 1, our experiment compared two types of potential competitors: tomato vs. leek. Experiments on leek competition in intercropping are scarce. Yet, they show that shallow-rooted leek is a poor competitor compared to large-rooted celery, and that it tends to have a low N uptake, partly due to its late N demand (Baumann et al., 2001). The leeks' root development in our pot experiment was probably dominated by tomato's root development, which supports the large effect of the leek-tomato treatment on root biomass. Similar soil contents in NO_3^- or NH_4^+ were found in controls of LT and TT associations, which would

indicate that the tomato in presence of leeks took up as much nitrogen as two tomato plants in the monoculture. The higher available phosphorus (P) content in the soil of the leek-tomato association would suggest that the two tomato plants took up more for phosphorus in the monoculture. No sign of P deficiency was observed at any time on leaves. On the other hand, previous experiments showed that the use of *Allium sativum* residues increased the growth of lettuce and eggplant when intercropped (Han et al., 2013; Wang et al., 2015), and that the microbial emission or inoculation of dimethyl disulfide (DMDS) modified the root architecture of *Nicotiana attenuata* and increased root growth of *Arabidopsis thaliana* in sulphur-deficient medium (Meldau et al., 2013; Tyagi et al., 2019). As our experiment targeted the inoculation of soil organisms in a context of association (or monoculture), no tomato was grown alone in pots. Yet, comparing the effect of a single tomato and of leek on the tomato growth and development and measuring root exudates would help disentangle mechanisms of plant-plant interactions.

In this context, plants grew less and produced less biomass after 7 weeks when infected by RKN alone compared to the control treatment, only when grown with leek. It means that the potentially stimulating or biocontrol effect of leek was not sufficient to prevent the negative effect of RKN on tomato growth. This effect can also be explained by patterns of infection on tomato roots in each treatment. The initial population density of *M. incognita* and the level of egg inoculum are important factors of damage to tomato plants (Chindo and Khan, 1988; Refaei et al., 2007). Kayani et al. (2017) showed that cucumber growth was negatively correlated to all inoculum densities, 12 weeks after inoculation of J2 juveniles. Here, we used an equal density of eggs in the nematode inoculum in both TT and LT associations (5,000 eggs per pot) in our 7-week experiment. Nematode populations were not counted, but total galls were 1.5 times more numerous on tomato roots in presence of leeks. Yet, the numbers per g root were similar to those in the monoculture (Supplementary Table S4). Additionally, no correlation was found between the number of galls and egg masses and plant growth measurements (data not shown). Hence, the level of inoculum did not limit the nematode colonization and rather resulted in a particularly large infection (Djian-Caporalino, pers. comm.).

The higher height increase of plants grown in monoculture and in pots inoculated with both AMF and earthworms could be explained by a higher efficiency of N and P uptake (Plenchette et al., 1983; Candido et al., 2015; Bona et al., 2017; Ziane et al., 2017). Yet, according to the PCA (Fig. 3), no relation was found between the AMF colonization and the soil P availability, indicating that a higher colonization did not increase the P uptake. Facelli et al. (2009) showed that *R. irregularis* limited tomato growth and P uptake when it was inoculated alone, which was probably close to our conditions, since no other AMF was inoculated, and the soil was autoclaved two months prior to the experiment to avoid nematode contaminations. On the other hand, the frequency of colonization was associated with the number of galls and, instead of reducing the nematode infection, the root penetration of hyphae may have favored it in a context of tomato-tomato competition, thereby indirectly altering plant growth. The mitigating effect of inoculated AMF in presence of a RKN infection and leeks could not be explained by an enhanced root colonization. It may therefore be due to a modified nutrient uptake efficiency or the production of molecules that increased the plant tolerance to damages induced by RKN (Hol and Cook, 2005), which were not tested here. The presence of AMF also induced a later flowering, which is contrary to previous results that showed an early flowering in presence of AMF (Salvioli et al., 2012; González-González et al., 2020). Finally, the weak negative to no effect of earthworms on plant growth is not consistent with previous studies that showed earthworms increased nutrient mineralization and plant growth (Scheu, 2003; Xiao et al., 2018) and a resistance against herbivorous nematodes (Xiao et al., 2018).

4.2. Root-knot nematode infection

An overall higher total number of galls was found on tomato roots in presence of leeks, due to their more developed root systems. However, no difference on the total number of galls per gram of tomato root was observed between both associations, implying that leeks did not reduce the RKN infection on tomato roots but did not increase it neither. Lower concentrations of allelochemicals produced by tomato in a context of higher plant competition may have caused reduced plant defenses and an increased infection, compared to

a leek-tomato system dominated by tomato (Stamp et al., 2004). Leeks are also known to contain and to release thiosulfinates (Freeman, 1975; Wang et al., 2008) that are rapidly degraded in the soil and are transformed into disulfides such as dimethyl disulfide (DMDS) or dipropyl disulfides (DPDS) (Auger et al., 1989; Dugravot et al., 2005; Arnault et al., 2013; Casella et al., 2013). DMDS and DPDS are more stable in the soil than thiosulfinates, and these three compounds have biocidal and particularly nematicidal effects (Tada et al., 1988; Arnault et al., 2005; Zanón et al., 2014; Yan et al., 2019). Although DMDS was known to be a more effective/toxic pesticide than DPDS (Arnault et al., 2005; Reddy, 2012), DPDS is more predominant in leek (Auger et al., 1989; Casella et al., 2013). Here, disulfides released by leeks into the soil were not sufficient to reduce the tomato root infection by *M. incognita*. The non-significant yet negative effect of growing tomato with leek on the numbers of galls and egg masses per gram of tomato compared to the monoculture should be further explored to confirm a potential effect on the establishment or on the RKN reproductive cycle through an ‘intoxication’ at the larval stage. With the exception of the study by Tada et al. (1988), studies are lacking on the potential effect of disulfides and particularly DPDS on RKN *M. incognita* and on their establishment in tomato roots.

The fact that the inoculation of AMF *R. irregularis* did not reduce but rather increased the root infection by *M. incognita* is not consistent with previous results (Talavera et al., 2001; Sharma and Sharma, 2015). Vos et al. (2012, 2013) showed that inoculating AMF reduced RKN penetration by altering host root exudation and by inducing systemic defence. In our experiment, the introduction of AMF in microcosms was achieved by inoculating a pure AMF spore solution (*in vitro* inoculum) on freshly sprouted tomato roots, while Vos et al. (2012, 2013) used an AMF infected soil (conventional inoculum). Also, Vos et al. (2012, 2013) used the AMF species *Funneliformis mosseae* (formerly *Glomus mosseae* BEG12) while we inoculated *R. irregularis*. Both AMF could have a specific mode of action, with *F. mosseae* being a better inoculum for biocontrol while *R. irregularis* would be a better inoculum to promote plant nutrition. The enhanced growth phenotype of AMF-treated tomato was possibly due to their better acclimation to the RKN infection, to which they would resist longer. This means that more *M. incognita* reproductive cycles can occur in mycorrhized hosts, whereas non-mycorrhized plants will not withstand the development of

RKN, as roots and shoots grow poorly, and plants rapidly die due to the infection. Here, our experiment highlights the current challenge in developing efficient inoculum with appropriate selection of plant host/fungus combinations adapted to the environmental conditions and needs of the farmers (Rouphael et al., 2015).

The presence of earthworms had no effect on tomato root infection by RKN, although a trend in the reduction of egg masses was observed in the monoculture. Previous studies showed a nematicidal effect of the use of earthworms produce *in vitro* (Rostami et al., 2014). The earthworm density (c. 250 individuals m⁻² here) was probably too low to observe their direct impact in natural soil and, although epigeic earthworms were chosen because of their ubiquity in farming systems and their tolerance to higher temperatures (Lee, 1985), stressful greenhouse conditions with a repeated peak of temperature (twice; up to 42°C) likely impacted earthworm behaviour and effects (see Supplementary Table S8 for earthworm data).

5. Conclusion

In this greenhouse experiment, the competition between the two tomato plants grown together reduced plant growth and produced an early flowering compared to plants grown with leek. On the other hand, the use of leek in association with tomato failed at reducing the nematode infection of its root system. Yet, the positive effect of leek-tomato intercropping on tomato growth reduced the otherwise negative effect of the RKN infection. The associated higher production of flowers could also lead to a higher productivity. Analyses of tomato and leek root exudates as well as experiments with a single tomato plant per pot would help to disentangle the facilitating vs. competition effects of leeks. Contrary to previous studies, the inoculation of AMF and earthworms had little effect on plant growth, AMF delayed flowering, and AMF increased the RKN infection. These effects were constrained by the pot experiment, the choice of the AMF species (i.e., *R. irregularis*) and the use of a pure inoculate. Using endogeic earthworm species and allowing natural mycorrhizal fungi to interact with an introduced AMF may show a higher potential to reduce RKN infection.

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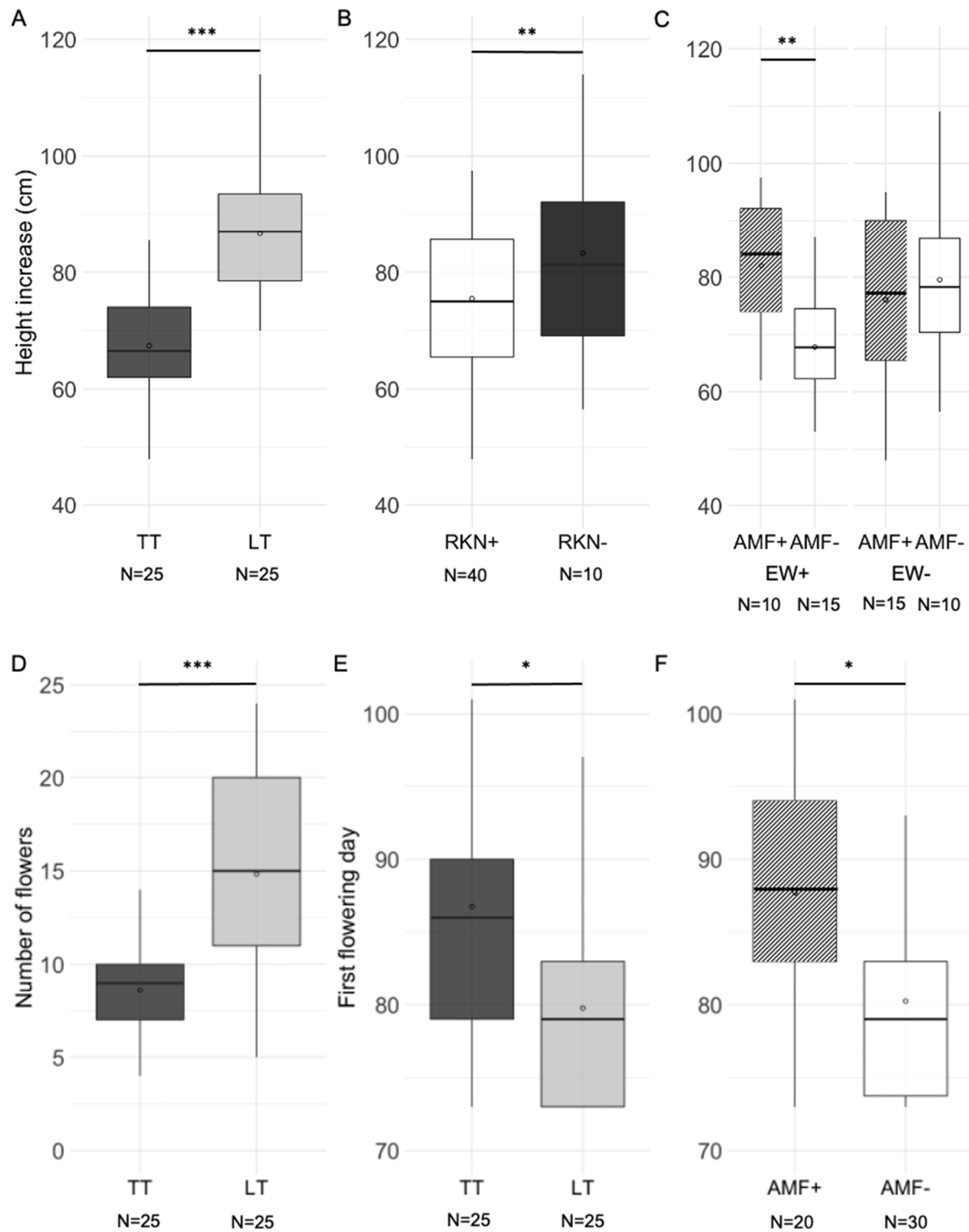
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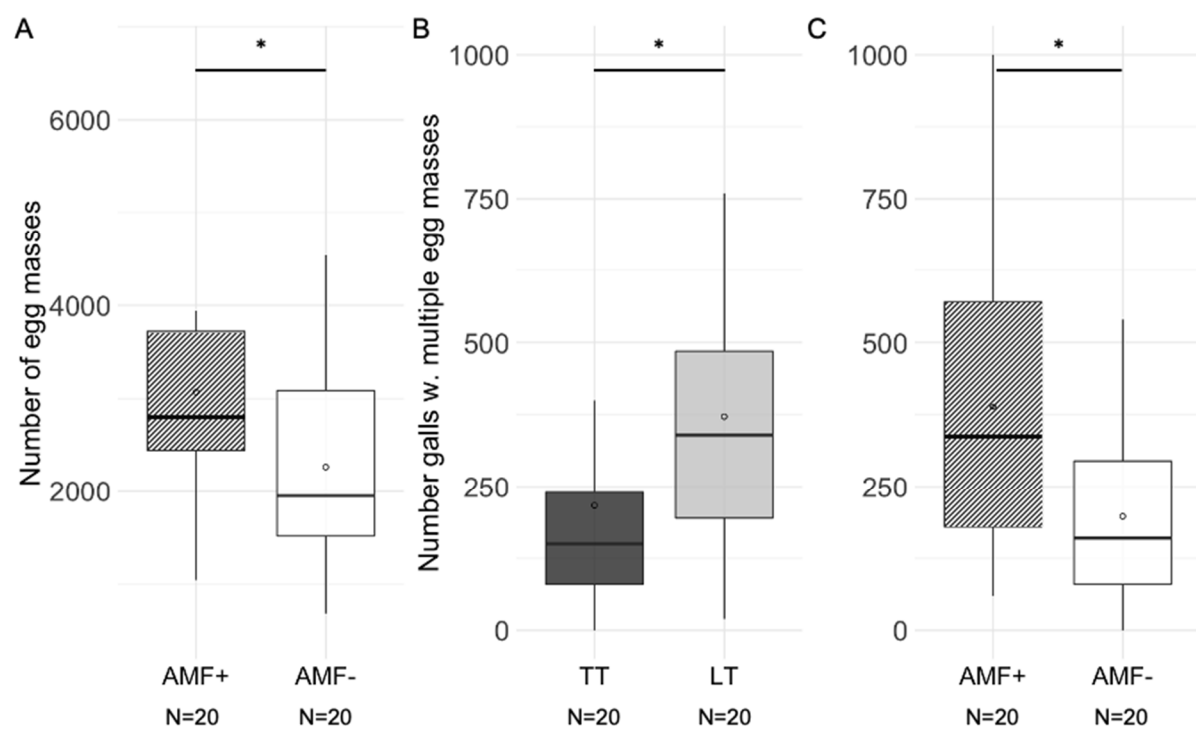
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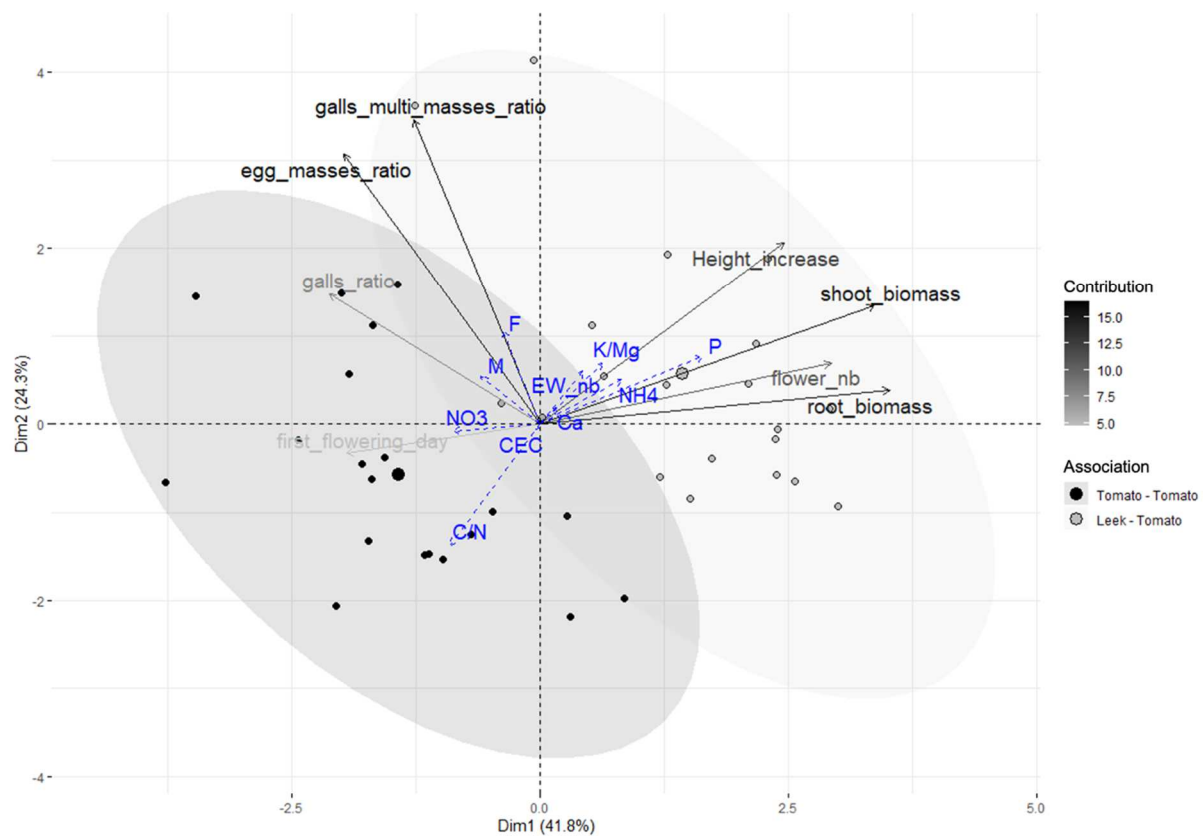


Table 1. Statistical results of effects of the Association (Leek-Tomato vs. tomato monoculture (TT)), the presence (+/-) of root-knot nematodes *M. incognita* (RKN), alone or in presence of earthworms *E. andrei* (EW) and/or arbuscular mycorrhizal fungi (AMF), also alone or in interaction, on plant growth and development parameters. Estimate, standard errors (SE) and adjusted p-values with a False Discovery Rate correction (FDR) are shown, and bold outputs are significant.

Variables	Estimate	SE	p.value	FDR
<i>Height increase</i>				
Association (vs TT)	29.50	5.61	4.32e-06	1.5e-05
RKN+ (vs RKN-)	-13.80	5.06	0.009	0.02
EW+ (vs EW-)	-8.05	3.97	0.049	0.06
AMF+ (vs AMF-)	0.25	3.97	0.95	0.95
Association : RKN	12.80	6.27	0.047	0.06
EW : AMF	14.10	5.61	0.02	0.03
<i>Shoot biomass</i>				
Association (vs TT)	5.93	0.36	1.25e-20	3.12e-20
RKN+ (vs RKN-)	-1.12	0.53	0.04	0.07
EW+ (vs EW-)	0.31	0.40	0.44	0.55
AMF+ (vs AMF-)	-0.22	0.40	0.58	0.58
<i>Root biomass</i>				
Association (vs TT)	0.89	0.11	3.66e-10	1.46e-10
RKN+ (vs RKN-)	0.47	0.16	0.008	0.005
EW+ (vs EW-)	-0.02	0.12	1.0	0.87
AMF+ (vs AMF-)	0.0005	0.12	1.0	1.0
<i>Number of flowers</i>				
Association (vs TT)	0.55	0.10	4.02e-08	1.00e-07
RKN+ (vs RKN-)	-0.20	0.14	0.15	0.19
EW+ (vs EW-)	0.14	0.11	0.20	0.20
AMF+ (vs AMF-)	-0.17	0.11	0.13	0.19
<i>First flowering day</i>				
Association (vs TT)	-0.08	0.03	0.007	0.01
RKN+ (vs RKN-)	-0.01	0.05	0.81	0.94
EW+ (vs EW-)	-0.002	0.03	0.94	0.94
AMF+ (vs AMF-)	0.09	0.03	0.006	0.01

Table 2. Statistical results of the effects of the Association (Leek-tomato vs. tomato monoculture (TT)), the presence (+/-) of root-knot nematodes *M. incognita* (RKN), alone or in presence of earthworms *E. andrei* (EW) and/or arbuscular mycorrhizal fungi (AMF), also alone or in interaction, on nematode infection parameters. Estimate, standard errors (SE) and adjusted p-values with a False Discovery Rate correction (FDR) are shown, and bold outputs are significant.

	Estimate	SE	p.value	FDR
<i>Total number of galls</i>				
Association (vs TT)	0.44	0.08	1.02e-08	5.10-09
EW+ (vs EW-)	-0.7	0.08	0.38	0.38
AMF+ (vs AMF-)	0.15	0.08	0.07	0.06
<i>Number of egg masses</i>				
Association (vs TT)	0.41	0.14	0.006	0.003
EW+ (vs EW-)	0.004	0.14	0.98	0.98
AMF+ (vs AMF-)	0.32	0.14	0.03	0.02
<i>Number of galls with multiple egg masses</i>				
Association (vs TT)	0.56	0.25	0.04	0.03
EW+ (vs EW-)	-0.04	0.25	0.87	0.88
AMF+ (vs AMF-)	0.69	0.25	0.01	0.006
<i>Number of galls g root⁻¹</i>				
Association (vs TT)	-214.2	236.5	0.49	0.37
EW+ (vs EW-)	-161.2	236.5	0.50	0.50
AMF+ (vs AMF-)	281.3	236.5	0.48	0.34
<i>Number of egg masses g root⁻¹</i>				
Association (vs TT)	-0.11	0.17	0.70	0.53
EW+ (vs EW-)	0.04	0.17	0.83	0.83
AMF+ (vs AMF-)	0.34	0.17	0.11	0.06
<i>Number of galls with multiple egg masses g root⁻¹</i>				
Association (vs TT)	0.01	0.27	0.48	0.36
EW+ (vs EW-)	0.02	0.27	0.49	0.49
AMF+ (vs AMF-)	0.71	0.27	0.02	0.01