Can the northern root-knot nematode, *Meloidogyne hapla,* be controlled by the fungus *Clonostachys rosea*?

- *An experimental study*

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Kan rotgallnematoden *Meloidogyne hapla* bekämpas med hjälp av svampen *Clonostachys rosea?*

- *En experimentell studie*

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Abstract

The northern root-knot nematode, *Meloidogyne hapla,* is causing great economical losses to growers worldwide. There is a need for alternative control measures that are environmentally sustainable and effective against the nematode. Biological control is one promising control measure and there are multiple biological control agents on the market against fungal pathogens and insects. One such biocontrol agent is the fungus *Clonostachys rosea*. In this experiment the biocontrol effect of *C. rosea* against the root-knot nematode *M. hapla* was evaluated. The investigation included an antibiosis *in vitro* experiment and a pot experiment with carrot and lettuce under controlled conditions. Additionally, the effect from drought on *M. hapla* infection and *C. rosea* biocontrol was tested. Drought increased the infection by *M. hapla* on carrot and caused a significant ($P < 0{,}001$) decrease in fresh and dry root weight and shoot dry weight, indicating a higher parasitic effect on plant mass of infected plants during dryer periods in cultivated fields of carrot. No biological control effect from *C. rosea* on *M. hapla* was detected, nor any growth promoting effects from the fungus on the plants. However, plants treated with *C. rosea* showed an increased tolerance towards drought stress by not showing significant distinction between plant mass under normal and drought conditions, which was seen in the other treatments. Though, an error due to a high buildup of roots in the peat soil made it difficult to count the galls on plant roots, affecting the result of biocontrol effect and actual nematode infection. The use of sand might have simplified the rinse of the roots and a more accurate result could have been obtained.

Keywords: Biological control, Carrot, Clonostachys rosea, Drought, Lettuce, Meloidogyne hapla, Nematodes, Pot experiment

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Abbreviations

RKN	Root-knot nematode
SNA	Synthetic low-nutrient agar
PDB	Potato dextrose agar
J2(3/4)	Juvenile stage two (three/four)
BCA	Biocontrol agent

1. Introduction

1.1 Background

Todays' agriculture faces multiple problematic issues that scientists aim to seek sustainable solutions to. A changing climate, the banning of effective pesticides, evolution of pesticide resistance, stricter political provisions, and an increasing human population, demands a more sustainable and effective production of food all over the world. We need to produce an increased amount of food on the same farmed area as today to cater the growing population of humans, which is predicted to peak at 9 billion by the mid - 21st century (Godfray et al. 2010). The nematode genus *Meloidogyne*, the root-knot nematodes (RKNs), contains some of the world's most economically important plant-parasitic nematodes in agriculture, which can infect almost all living species of higher plants (Moens et al. 2009). One particularly problematic and severe species is *Meloidogyne hapla* (the Northern root-knot nematode). *Meloidogyne hapla* has a wide distribution and is found on almost all continents in the world (CABI 2002), and it is problematic in both horticultural and agricultural crops as it parasitizes most cultivated dicotyledonous cash crops, including carrots, potatoes, lettuce, rapeseed, and sugar beets (Aaltjesschema 2022). In Sweden, *M. hapla* is one of four species of RKNs with a main distribution in the southern parts of the country. Especially crops such as carrots and potatoes are affected where the nematode cause considerable economic losses in the form of deformities of the crops and unfarmable nematode-infested soils (Andersson 2003). The three other RKN species in Sweden are *M. chitwoodi*, *M. fallax* and *M. naasi*, and so far, they are much less common than *M. hapla.* When controlling *M. hapla* it is not strictly managed under the control of the Swedish board of agriculture such as the two quarantine pathogenic RKN species *M. fallax* and *M. chitwoodi*. However, the species is still capable of severe damage in fields and affects farmers economically in Sweden (The Rural Economy and Agricultural Societies 2019).

The most effective control measure against *M. hapla* and many other nematode species, are chemical nematicides. However, these have in later years become severely restricted or totally banned in many countries due to the grave risk of

human and environmental health and damage. This due to the toxicity towards the nematicide applier, nematicide resistance within the nematodes and the spread of the chemical compounds in nature (Sánchez-Moreno et al. 2009). Another important risk linked with the application of nematicides is the effect on non-target organisms. The chemical compounds are not only targeting the nematodes but also a wide range of other soil-living organisms that cannot be avoided during the treatment of nematode-infested soil (Perry & Moens 2013). The use of integrated pest management, including prevention of pest infestations, action thresholds and minimal chemical use (*Växtskyddsåtgärder i din odling* 2022) is an important approach to prevent damage of pests and pathogens in the field with low ecological and economic impact. The practice is based on a system where different control measures are graded by their ecological/environmental/sustainable impact. Preferred first options include control by prevention (crop rotation, use of resistant crops) followed by cultural practices, mechanical, biological, and at last (least favourable practice) chemical practises (*Växtskyddsåtgärder i din odling* 2022). The use of biological control is currently receiving increased interest as a potential measure that can replace or complement the use of chemical pesticides. An example of an effective fungal biocontrol agent (BCA) is the mycotrophic fungus *Clonostachys rosea*, a fungus known for its suppressing abilities towards a range of fungal pathogens such as the fungus *Botrytis cinerea* which causes grey mould in strawberries (Boff et al. 2002; Jensen et al. 2021a). It has been shown to also have potential biological control effects against different plant-parasitic nematodes (Iqbal et al. 2018b). However, its effect towards diseases caused by root-knot nematodes is not well studied.

There is a need of safe alternatives and effective measures for controlling plant diseases caused by nematodes. Knowledge-based improvements of biocontrol requires a mechanistic understanding of the interaction between biocontrol agents, plants, and nematodes.

1.2 Literature study

1.2.1 Meloidogyne hapla

Meloidogyne hapla is a species in the genus *Meloidogyne*, root-knot nematodes. It is a sedentary endoparasitic roundworm, which means that the species reproduces and lives most of its life inside the root tissue of plants (Andersson 2018). The only stage found in soil (in the water film surrounding soil particles), is its migrating juvenile stage. The typical symptom of infection from *M. hapla* is the galling of the roots, which shows as bumps on both secondary and primary roots. Infection of *M. hapla* can also result in hyperproduction of roots. This is typically described as bearded appearance of roots, e.g., in carrot taproots (Perry et al. 2009). Most damage in potatoes and carrots comes in the form of unsellable and physically undesirable harvest, but also in the form of negative effects on growth and yield (Andersson 2018).

The life cycle and morphology of Meloidogyne hapla

As the popular genus name of the nematode indicates, the life cycle of this nematode is highly dependent on the production of knots, or galls, on the host roots for survival. This trait is used by all the *Meloidogyn*e species including *M. hapla*. The life cycle starts in a gelatinous egg sack composed of eggs and an enclosing glycoprotein matrix. The egg sack is often located on the outside of the galled roots but can also be found embedded inside the root tissue nearby the swollen nematode female. Until fully grown, the nematode occurs in four different juvenile stages. Two of these stages start already in the egg where juvenile-stage 1 (J1) molts into juvenile-stage two (J2). The infective J2 nematode then hatches, and via the water film of the soil particles, moves towards the targeted roots, either already galled or not galled (Andersson 2018). The J2 stage is vulnerable, with a limited supply of nutrients to survive on when in soil, and therefore needs to find a suitable host fast. The nematode penetrates the root by the root tip with the force of a special evolved mouth part, the stylet, and by excreting cell-wall degrading enzymes. It then moves inside the root until it finds a suitable place to settle. When settled, it starts feeding on the root tissue and induces a production of giant cells from which the nematode can get a constant flow of nutrients. The giant cells are a result of exudates in the form of different enzymes from the nematode which manipulates the cell division in the plants parenchyma cells, inducing a large number of multinucleated cells to be produced to cater the nematode (Perry et al. 2009). During this period the J2 nematode molts into juvenile-stage three (J3) and then juvenile-stage four (J4) before reaching the adult stage of either a male or a female. As the nematode molts, it goes from being a migrating nematode to a sedentary nematode. This as a result of the fact that when the nematode is molting it changes in size and swells up

gradually for molting. When reaching adult-stage, the male reverts back to the thin worm shape and continues as a migrating living nematode, while the swollen, pear shaped female stays feeding on the giant cell as a sedentary nematode. Each gall can contain multiple females, making the galls vary in size. The female later produces an egg sack, often outside the root but still attached to the nematode. As the egg sack matures, the female dies and the egg sack gets detached from the female and root, and ends up in the soil where the cycle can start over (see figure 1) (Perry et al. 2009). *Meloidogyne hapla* has different reproductive abilities and can use a so-called parthenogenesis which means there is no need for a male to be present and it can produce offspring by itself (Liu et al. 2007).

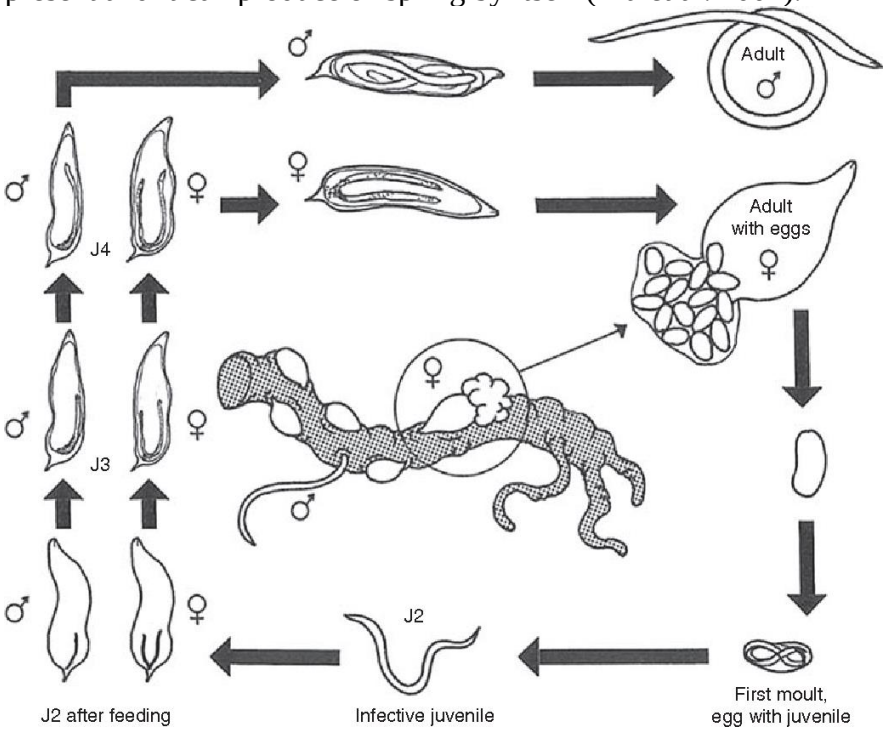

Figure 1. "Diagram of the life cycle of the root-knot nematode, Meloidogyne. J2: Second-stage juvenile; J3: third-stage juvenile; J4: fourth-stage juvenile. (Adapted from Karssen & Moens 2006.)" (Moens et al. 2009).

Temperature has a considerable effect on the development of the nematode, and the length of the life cycle can vary in different temperatures (Inserra et al. 1983). Wong & Mai (1973) showed that temperature can affect the egg production, where a 10°C span from lowest to highest temperature can more than double (from 20 to 54 days) the reproductive time from J2 to eggs in a cooler climate. In Sweden *M. hapla* can have up to three generation a year depending on location (inland or coastal regions) (The Rural Economy and Agricultural Societies 2019).

Present measures for control

Meloidogyne hapla has a wide range of hosts, and parasitizes most dicotyledonous plants (Björklund & Boberg 2019). The most common way of controlling *M. hapla* is by using non-host crops such as monocotyledonous crops, e.g. cereals or forage grass. One problematic aspect here is the need of a weed free soil to prevent the multiplication of the nematodes on the weeds, a hard and work intensive measure to uphold. Another used, but economically expensive, measure is to put the field in fallow. Although even here there is a great need of keeping the field weed free. Studies made by Albertsson Juhlin (2010) and Hushållningssällskapet Skåne showed a reduction in *M. hapla* in the field when using non-host and resistant plants. The study showed a reduction in nematodes (J2 in the soil) up to 70% during the years 2008-2010 with oats, which works as a non-host species for *M. hapla*. Though, it also shows a difficulty to reduce *M. hapla* by this method due to the occurrence of nematode-hosting weeds in weaker populations of oats. This is especially problematic in fields with organically produced crops with a higher occurrence of weeds and proportion of legumes in the crop rotation (Albertsson Juhlin 2010). This empathizes the need for alternative sustainable measures for the control of the plant-parasitic nematodes.

Biological control of nematodes

Nematodes have in general many enemies and can be suppressed by multiple organisms in different ways of actions, e.g., by antagonistic fungi, predatory nematodes and insects (Poveda et al. 2020). Certain species of predatory nematodes of the genera *Mononchida* prefer root-knot nematodes when feeding and may act as biocontrol agents. However, an ineffective bulking process due to cannibalism and sensitivity to environmental variations makes it less attractive for the purpose of biocontrol (Bilgrami et al. 2005; Devi & George 2018). However, certain fungi and bacteria has shown promising results as biocontrol agents against nematodes in general (Tian et al. 2007; Poveda et al. 2020). Fungi with biocontrol abilities can suppress nematodes by antagonism (excreting nematicidal compounds such as enzymes and secondary metabolites, and competition of space), as parasites, or as an indirect measure by strengthening the host plant by making nutrients available or uphold water content in the soil by mycelia (Poveda et al. 2020). Bacteria acts in similar ways and can both parasitize nematodes and release nematicidal compounds, as well as promoting plant growth and induce resistance in plants (Tian et al. 2007). There are studies showing rhizobacteria and endophytic bacteria having suppressing abilities towards RKNs, probably via plant health and growth promotion and/or exudation of nematicidal toxins (Munif et al. 2000; Maulidia et al. 2020).

1.2.2 *Clonostachys rosea*

Biological characteristics

Clonostachys rosea is a soil-borne fungal species belonging to the phylum Ascomycota. It was formerly named as *Gliocladium roseum* but later changed to the today practiced name due to differences in e.g., morphology, ecology, and genetic data from the genus *Gliocladium*. *Clonostachys rosea* is a facultative saprotroph which means that it is able to live on dead organic material in the soil, as well as being an aggressive parasite on other fungi (mycoparasitism) and on nematodes (Zhang et al. 2008; Jensen et al. 2021b). It can also colonize plant roots and form beneficial associations with plants that can result in plant growth promotion and induction of systemic defence responses (Jensen et al. 2021b). The fungus has an extensive distribution and can be found almost everywhere over the globe, with a wide range of host plants such as strawberries, wheat, and barley. The fungus has been isolated from different plant parts, flowers, roots, and leaves, with a majority isolated from roots. *Clonostachys rosea* colonies vary from grey/white to orange/yellow depending on light exposure; more colour during lighter conditions (Sun et al. 2020).

The anamorph stage of the fungus produces spores to spread in the environment in two different forms, chlamydospores and conidia. Conidia is produced by conidiophores in the fungus during favourable conditions for fast and easy distribution into the environment, while chlamydospores in the fungal hyphae during harsh conditions (low pH and lower temperature) and act as more resistant resting spores in comparison to the conidia, which are smaller, higher in number and less resistant. In size, the chlamydospores are approximately 5.8*5.0 μm and conidia approximately 2.9*0.3 μm (Sun et al. 2020). There are also certain strains of *C. rosea* producing sexual spores, in a so-called teleomorph stage, referred to by the name *Bionectria ochroleuca*, acting as a homothallic fungus (self-fertilizing) (Schroers et al. 1999).

As a biocontrol agent

Clonostachys rosea is well known today for its role as a biocontrol agent towards numerous pathogens infecting and harming plants in both agriculture and in garden plants. *Clonostachys rosea* is used as biological control in for example strawberry production against the grey mould pathogen *B. cinerea* and multiple other fungal pathogens. For example, the company “Verdera” sells the fungus as a product, under the name “Prestop” which can suppress the infection of fungal diseases on for example strawberry, cucumber, kale, tomato, and trees (Verdera: Prestop 2022). Another company using the fungus is “Biobest” which uses bumblebees as vectors

to get inoculum from the fungus into the targeted flower. Via pollination the fungus is point-applied on the plant (Prestop: Biobest 2022), an efficient way of using biological disease control.

The fungus has a biocontrol effect, as previously implied, against multiple fungal pathogens, but also against many genera of nematodes. The fungus acts in different ways when it comes to its biocontrol effects. It is for example known to have mycoparasitic behaviours, in which it can penetrate and degrade the cells of the fungal prey, e.g., *B. cinerea* (Jensen et al. 2021b). Another important mode of action from the fungus is antibiosis in the competition for space and resources. By exuding secondary metabolites (antibiotics and toxins) and cell-degrading hydrolytic enzymes it can work in antagonistic ways against different pathogens such as fungi, bacteria and in some examples, nematodes (Jensen et al. 2021b). The nematicidial effect of *C. rosea* has a probable origin in the exudation of nematicidial compounds such as secondary metabolites (toxins produced by e.g., fungi and bacteria) and nematode cuticle-degrading enzymes such as proteases, chitinases and glucanase. Song et al. (2016) showed that *C. rosea* exude secondary metabolites such as the alkaloid gliocladin C and an alkylene resorcinol 5-n-heneicosylresorcinol, which had nematicidial effect towards nematodes of the genera *Panagrellus*, *Caenorhabditis*, and *Bursaphelenchus* (Song et al. 2016). Another important group secreted enzymes are serine proteases that have the ability to degrade the cuticle of nematodes, e.g., on the nematode genera *Panagrellus* (Li et al. 2006; Zou et al. 2010). The genome of *C. rosea* strain IK726 contain a large number of serine proteases genes and many of these are actively expressed in a variety of conditions (Iqbal et al. 2018a). Non-ribosomal peptides also play an important role in the nematicidial effect from the fungus. Epipolysulfanyldioxopiperazine nematicidial compounds can be produce by the *C. rosea* strain IK726 (Iqbal 2019) and these compounds has shown to have possible influence of the nematicidial effect on nematodes of the genera *Bursaphelenchus*, *Caenorhabditis* and, *Panagrellus* (Dong et al. 2005).

However, the fungus also has the ability to parasitize nematodes. Zhang et al. (2016) used a *C. rosea* strain expressing the green fluorescent protein to investigate the parasitic procedure by the fungus. The study shows how the spores of *C. rosea* can attach to the nematode cuticle, germinate, and penetrate the nematodes of the genera *Panagrellus* (non-plant parasitic nematode) and kill it by degrading it from within (Zhang et al. 2008). This indicates the ability of *C. rosea* to not only have nematicidial ability via exuding secondary metabolites but also via direct parasitism. Several strains of *C. rosea* can also parasitize eggs from the soybean cyst nematode *Heterodera glycines* (Iqbal et al. 2020).

Not only does *C. rosea* have biological control abilities, but also shows a positive effect on plant disease resistance. The fungus has shown to induce expression of different defence genes resulting in induced systemic resistance against powdery mildew in tobacco leaves (Lahoz et al. 2004) and *F. circinatum*, causing reduced stem lesion in pine (Moraga-Suazo & Sanfuentes 2016). It has also been shown in multiple studies that *C. rosea* has plant growth promoting abilities where plants inoculated with the fungus showed a higher biomass compared with the non-inoculated plants (Johansen et al. 2005). Sutton et al. (2008) shows similar result in roses where cuttings inoculated with *C. rosea* tended to produce roots faster, showed resistant to fungal pathogens and had a less abundance of dying leaves. Treated plants also flowered faster than non-treated controls.

Strain IK726 and nematicidal effect

The IK726 strain of *C. rosea* used in this study/experiment was isolated from barley roots in Denmark and has been widely evaluated for its potential use as a biological control agent (Jensen et al. 2007). Mamarabadi et al. (2008) studied the two gene groups endoglucanase genes and chitinase genes, and their expression patterns when interacting with the plant pathogen *B. cinerea*. The study showed a potential influence in the biocontrol effect of *C. rosea* strain IK726 from three out of four different chitinase genes in strawberry leaves by an *in vitro* antagonism experiment (Mamarabadi et al. 2008).

In a study made by Iqbal et al. (2018b) the same strain is used and is concluded to be a good candidate as a biological control against a wide range of plant-parasitic nematodes. Soil containing a mixture of several plant-parasitic nematodes were collected. The soil, still containing the nematode mixture, was then used in a pot experiment with lettuce and wheat in which *C. rosea* was inoculated. The study resulted in a high mortality of parasitic nematodes while nonparasitic nematodes remained vigorous and alive in the soil. *In vitro* experiments were also performed where the antibiosis effect was tested. This study showed that the probable reason for the nematicidial effect from *C. rosea* is most likely by antibiosis in the form of exudation of nematicidial compounds rather than direct parasitism by the fungus. However, no nematodes of the genus *Meloidogyne* were found in the soil and therefore not included in the experiment (Iqbal et al. 2018b). In a study by Hussain et al. (2016) multiple fungi collected from soil in the area of Semice and Litol in the Czech Republic, were investigated as biocontrol agents for the nematode species *M. hapla.* The study included *in vitro* - and greenhouse experiment with carrots. The result showed a positive response in biocontrol efficacy by *C. rosea* (unknown strain) toward suppressing nematodes, both juvenile stages and eggs. Plant growth promotion was also a positive fungal response found where both roots

and above parts of the plant had a more increased growth in contrast to the controls (Hussain et al. 2016).

1.3 Objectives

The aim of this study is to test the fungus *C. rosea* as a potential biological control agent against the plant-parasitic root knot nematode *M. hapla* in a pot-based assay and evaluating antibiosis in an *in vitro* experiment. In addition, a drought treatment is also included to investigate both how the damage effect from the nematodes and the biological effect of the fungus are affected by water deficiency. The experiment includes both carrots and lettuce plants, two important cash crops that are susceptible to the root-knot nematode *M. hapla* (Viaene & Abawi 1996; Aaltjesschema 2022). My first hypothesis is that *C. rosea* will have a biocontrol effect on *M. hapla* by reducing disease symptoms on carrot and lettuce. Secondly, I hypothesize that production of nematicidal compounds by *C. rosea* contributes to the biocontrol effect. Finally, my third hypothesis is that drought makes carrot and lettuce more prone to infection by *M. hapla* and the beneficial effect of *C. rosea* increase.

2. Material and Method

2.1 Extraction of nematodes

Second stage juveniles (J2s) of the root-knot nematodes species *M. hapla,* to be used in the pot experiment and the antibiosis assay, were collected from infected roots of tomato plants kept in a climate chamber. The roots were dug up from the pots and cleaned from excess soil. They were then cut into 1 cm pieces and put into mail trays onto a net covered with Kleenex wipes functioning as filters. The mail trays were then put onto other trays which were filled with water so that the root pieces were in contact with the water but not completely covered. Every day for a week the trays were emptied of water into a 2 litres beaker, and the trays were refilled with new fresh water. The beaker was then stored in a cold room (4°C) until emptying a new batch of water with nematodes from the trays. Before emptying the new batch into the beaker, the amount of water was reduced in the beaker. The nematodes are slightly heavier than water and therefore sink, which leaves the majority of the water above the bottom part of the beaker nematode free. The excess water, without nematodes, was removed with a 50 ml syringe, leaving a concentrated nematode solution in the bottom, approx. 500 ml. This was repeated before emptying each new batch and at the end of the extraction to get the final nematode solution.

To count the nematodes extracted from the roots, 1 ml of the nematode solution was emptied into a counting disc. By using a stereomicroscope, the nematodes were counted three times and a mean value per ml was calculated. To get the full nematode concentration, this mean value was multiplied by the total volume of the nematode solution. When the wanted concentration of nematodes was reached the nematode solution was applied to the pots in the experiment. The extraction was performed twice for the pot experiment due to a low concentration of nematodes extracted the first time. Each pot was calculated to contain approximately 5000 nematodes after the two applications. To access more nematodes a new pot with rooted tomato plants was prepared in the climate chamber and the extraction was done a third time to have nematodes for the *in vitro* bioassay experiment.

2.2 Determining *C. rosea* concentration by counting CFUs

Clonostachys rosea was supplied as a dry formulation based on wheat bran and peat (Jensen et al. 2000). To determine the concentration of *C. rosea* strain IK726 in the formula, a method of counting colony-forming units (CFUs) was used. To count the CFUs, a series of dilutions were made. 2.5 g of formula and 2.5 ml of 0.85 % NaCl solution were put into a falcon tube with the concentration of 10^-1 g/ml. The falcon tube was shaken every 5 minutes for 30 minutes on a vortex shaker. 100 µL from the falcon tube was added in an Eppendorf tube along with 900 µL of 0.85 % NaCl solution, to reach the concentration of 10^-2, and shaken for a homogeneous mixture. The procedure was repeated until required dilution levels were reached (10^-5, 10^-6, 10^-7). Each of the three required dilution levels were spread on potato dextrose agar (PDA, Sigma-Aldrich, St. Louis, MO) plates with a spreader for maximum coverage of conidia. Three replicates of each dilution level were made. The cultures were then stored at 20°C for two days and followed by counting of the CFUs. The dilution factor with estimate suitable number of CFUs (20-50 visually distinct CFUs) was selected, in this case 10^-6. The colonies were counted on all the three replicates and a mean value was calculated with the following equation:

CFU = Avg. # colonies from 3 plates / (100 µl * dilution factor)

By knowing the concentration of the formulation and the wanted concentration of *C. rosea* in the soil the weight of the formulation needed for the pot experiment could be calculated to 3.5 grams of formula for 64 pots of soil.

2.3 Pot experiment

The pot experiment was performed in a climate chamber under controlled conditions with a temperature set on 21°C and light 12 hours/day to fit both the two different plants and nematode growth and reproduction. Seeds of carrots (type; 'Jeannette' F1) and lettuce (type; 'Lollo Rossa') were planted in 9x9x9-cm pots filled to the top with approximately 61,44 g (DW: Dry Weight) of sowing soil from the company Hasselfors garden (Örebro, Sweden) composed of peat, perlite, mineral fertilizer (NPK 14-7-15 mikro 9 kg/m^3), and with a pH of 6. The experiment contained 8 different treatments (Table 1) with in total 32 control pots containing only soil and plants, 32 pots with plants and *C. rosea*, 32 pots with plants and nematodes, and the remaining 32 pots with both *C. rosea* and nematodes with the

plants. Half of the pots contained carrots and the other half lettuce, and each treatment was subdivided into a treatment of drought.

Table 1. Number of and composition in treatments.

Treatment	Plant	Added Organisms	Nr. Pots Normal	Nr. Pots Drought
1C	Carrot	Control	8	8
2C	Carrot	RKN	8	8
3C	Carrot	C. Rosea	8	8
4C	Carrot	RKN, C. rosea	8	8
1L	Lettuce	Control	8	8
2L	Lettuce	RKN	8	8
3L	Lettuce	C. Rosea	8	8
4L	Lettuce	RKN, C. rosea	8	8

Formulation of *C. rosea* strain IK726 was added with the soil of 64 pots in a plastic bin and was mixed carefully by hand to homogenize the mixture to a final concentration of 10^6 CFU/g soil.

During the germination of the seeds, the soil was watered until maximum water capacity and then maintenance watered with 1 dl of water per pot every three days until the end of the experiment. The water was applied from under the pots in a tray and then sucked by capillary forces by the soil. Nematodes were applied via a syringe on top of the soil in pots in the treatments supposed to contain nematodes. The number of nematodes applied were determined by looking at similar studies such as the study made by Iqbal et al. (2018b). Approximately 3700 nematodes were applied seven days after germination of lettuce and carrots when the plants had an emersion of their first characteristic leaves. Additionally, approximately 1300 nematodes were applied to all the pots after 4 more days. Thus, a total number of 5000 nematodes per pot were added to the pots.

Seven weeks after potting of the seeds, half of the pots were exposed to a drought period of one week. This was performed by not giving the plants in this sub-treatment the maintenance water until the soil was perceived as dry (respectively 5.6, 9.1, 12.7, 29.8 % water content in soil, measured in 4 randomly chosen pots) and the plants had visual stress symptoms in the form of wilting shoots. To get a measure of how dry the soil was in the treatments, soil samples from each treatment were dried at room temperature and both wet- and dry weight was noted. Throughout the experiment, the plants and pots were visually examined, and deviant phenotypes and changes were noted.

After 59 days, harvest was started and conducted for one week. During the harvest pictures were taken the whole process and multiple phenotypic traits were measured.

Carrot plants were measured by number of leaves (one stem with leaves counted as one), shoot length, leaf colour (yellow/green), fresh- and dry weight of both above- and underground plant parts. The roots were tangled and unmeasurable, so only the weight of the carrot tap root was measured and not the smaller roots. This also made it hard to count the galls, which was therefore not counted. Protocol for harvest can be found in appendix (appendix, table 1).

In the lettuce treatments, the roots were washed off and number of galls was counted as well as the fresh- and dry weight were noted. Aboveground plant parts were measured by leaf colouring (red/red-green/green), size of leaves (length and width), and dry-/fresh weight. Protocol for harvest can be found in appendix (appendix, table 2).

For every phenotype that was scored with a number (root development, galling index and leaf colour on lettuce), there was a reference scoring system assembled (appendix 1). Soil samples were taken from all the pots for further analysis of juvenile occurrence.

2.4 Antibiosis assay

An *in vitro* experiment to measure production of nematicidial compounds produced by *C. rosea* strain IK726 was conducted. Strain IK726 was inoculated onto PDA plates by a piece of actively growing mycelium. The PDA plates were then incubated at 20°C in darkness for approximately 30 days. To extract the spores from the PDA plates, sterile water was continuously sucked up and poured on the plate until spores were released from the mycelium. The solution with spores was then diluted 10 times and counted in a hemocytometer.

By using the equation: Spores/ml = (no. of spores/0.02) * (1000*dilution factor), the concentration of spores was determined to 25*10^6 in the solution. The wanted concentration, 10^6, was reached by diluting the solution 25 times.

The solution containing spores was then divided into five flasks containing either 50 ml potato dextrose broth (PDB, Sigma-Aldrich, St. Louis, MO) or synthetic low-nutrient agar broth (SNA, synthetic medium prepared in the lab (Nirenberg 1976)) and placed on a shaker for seven days at 25°C. To extract the fungal exudates and to rinse the solution from fungal residuals such as conidia and mycelium, 5 ml of

culture filtrate media were sucked up via a syringe and pushed through a 0,2 µm ptfe sterile filter. 850 µl of culture filtrate were then added to a glass tube along with 150 µl of a solution with the concentration of approx. 60 nematodes per 100 µl. With other words, each tube contained the media and approximately 90 nematodes. The composites were then incubated at 20°C for 24 hours. For counting of the number of dead or alive nematodes, a counting disc was used, and the nematodes were determined under a stereomicroscope.

2.5 Statistical analysis

Collected data was then analysed via plotting in excel and analysis of variance (ANOVA) in Minitab (one way and two-way ANOVA) to identify significant differences between treatments and experiments (drought/normal conditions) at a $p \geq 0.05$ level. Differences within treatments was identified by the Fisher post-hoc method at $p \geq 0.05$) could be seen. Students *t*-test was performed in excel to identify significand differences ($p \geq 0.05$) between drought and non-drought conditions, within each treatment.

3. Result

3.1 Pot experiment

3.1.1 Carrot

Dry weight of carrot roots (Figure 2) was affected by both drought conditions ($F = 24.19$, $p < 0.001$) and by the applied treatments ($F = 8.20$, $p < 0.001$), although the interaction between these was not found significant. By the performed t-test, all treatments, except treatment three with only *C. rosea*, resulted in significantly lower root weight under drought conditions. Under normal water conditions there is an effect of the presence of *M. hapla where* a Fisher post-hoc test revealed significant differences between the treatment with only *M. hapla* and the treatment with only *C. rosea*, however no significant difference between these and the control treatments was found (Figure 2). The same pattern is also seen under drought conditions.

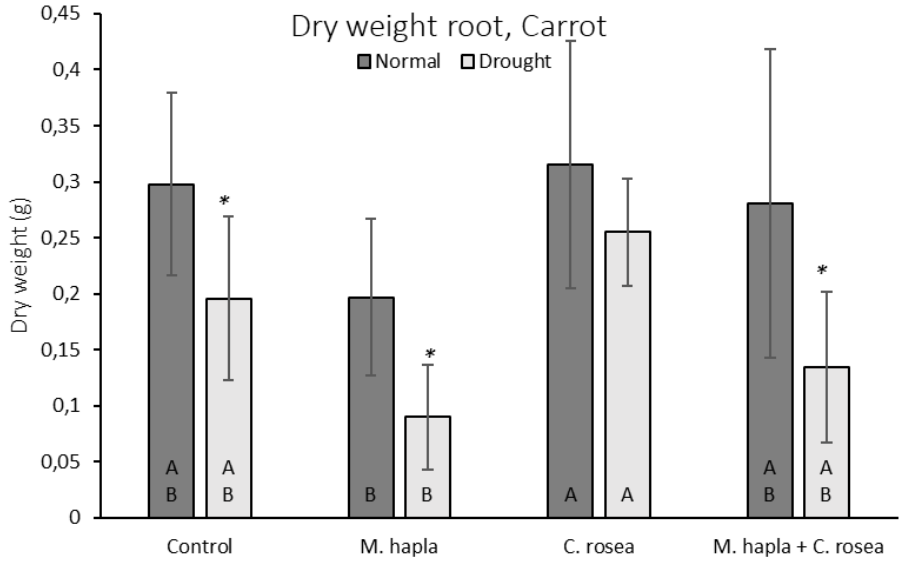

Figure 2. Carrot root dry weight (mean ± SD n = 8) in a pot experiment with addition of M. hapla and C. rosea subjected to normal water conditions or drought. Different letters indicate significant ($p < 0.05$) difference, as determined by the Fisher method, within experimental group. An asterisk indicates a significant ($p < 0.05$) within treatment difference between drought and non-drought conditions, using Students t-test

We found that drought had a significant impact on shoot dry weight ($F = 26.41$, $p < 0.001$) (**Fel! Hittar inte referenskälla.**) as well. As for root weight (Figure 2), there is a drought effect on shoot weight for all the treatments except the treatment

with only *C. rosea* (**Fel! Hittar inte referenskälla.**). If we look at the effect of the treatments, we can see a significant effect on the shoot weight under drought conditions when comparing *M. hapla treatment* with the control treatment and treatment with only *C. rosea*. The drought-resistance effect is also seen here, where plants treated with *C. rosea* have managed the water deficiency treatment better than the rest of the treatments.

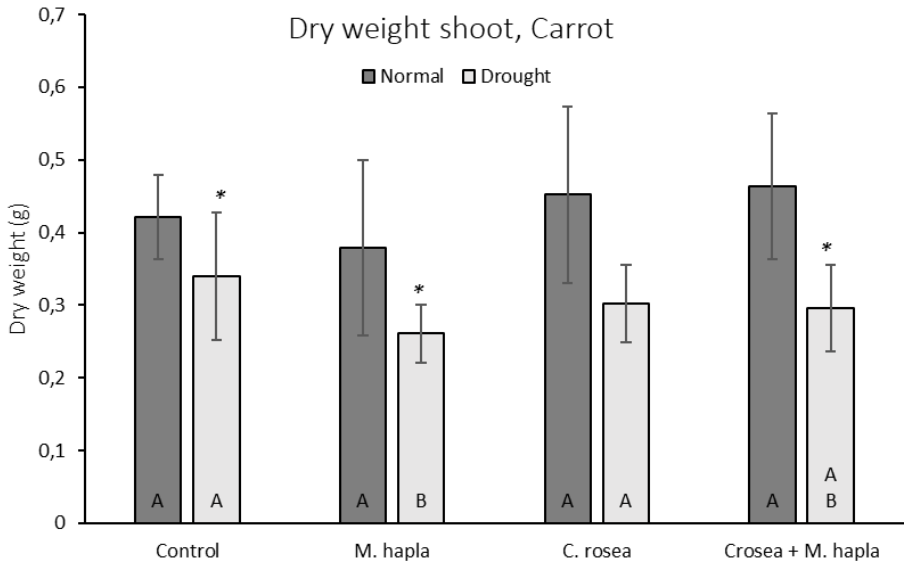

Figure 3. Carrot shoot dry weight (mean ± SD n = 8) in a pot experiment with addition of M. hapla and C. rosea subjected to normal water conditions or drought. Different letters indicate significant ($p < 0.05$) difference, as determined by the Fisher method, within experimental group. An asterisk indicates a significant ($p < 0.05$) within treatment difference between drought and non-drought conditions, using Students t-test

A significant difference was found in dry shoot length for carrot roots (Figure 4) under drought conditions ($F = 26.75$, $P < 0.001$). By the t-test, significant differences between the treatments under drought and normal conditions were found in the control treatment and the treatment with both *M. hapla* and *C. rosea*. There was neither an effect of *M. hapla* treated plants nor *C. rosea* treated plants in comparison to the control. However, under normal water conditions, *M. hapla* treated plants produced significantly shorter plants than the nematode infected plant also treated with *C. rosea*.

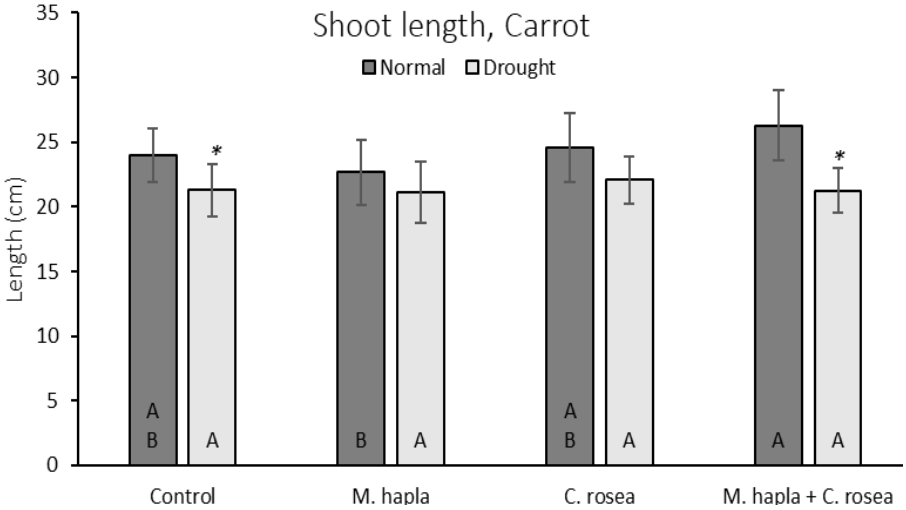

Figure 4. Carrot shoot length (mean ± SD n = 8) in a pot experiment with addition of M. hapla and C. rosea subjected to normal water conditions or drought. Different letters indicate significant ($p < 0.05$) differences, as determined by the Fisher method, within experimental group. An asterisk indicates a significant ($p < 0.05$) within treatment difference between drought and non-drought conditions, using Students t-test.

3.1.2 Lettuce

According to the ANOVA performed, there are neither distinct differences in lettuce root dry weight due to applied fungus/nematode treatment and drought separately nor any interaction between the two. However, via a t-test we found a significantly distinct difference (< 0.05) between the treatment *M. hapla + C. rosea* (Figure 5) when under drought conditions compared to under normal conditions

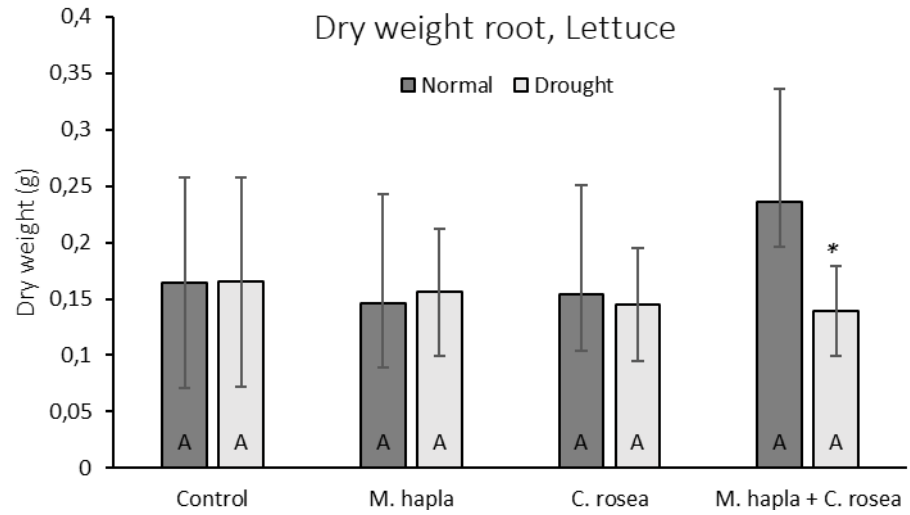

Figure 5. Lettuce root dry weight (mean ± SD n = 8) in a pot experiment with addition of M. hapla and C. rosea subjected to normal water conditions or drought. Different letters indicate significant ($p < 0.05$) differences, as determined by the Fisher method, within experimental group. An asterisk

indicates a significant (p < 0.05) within treatment difference between drought and non-drought conditions, using Students t-test.

We found a significant difference in dry weight of lettuce shoot (Figure 6) under drought conditions (F = 7.87, p = 0.007). By performing a t-test we saw a lower shoot weight in *M. hapla + C. rosea* when under drought conditions compared to under normal water conditions. This indicates a drought effect for nematode infected lettuce plants treated with *C. rosea*.

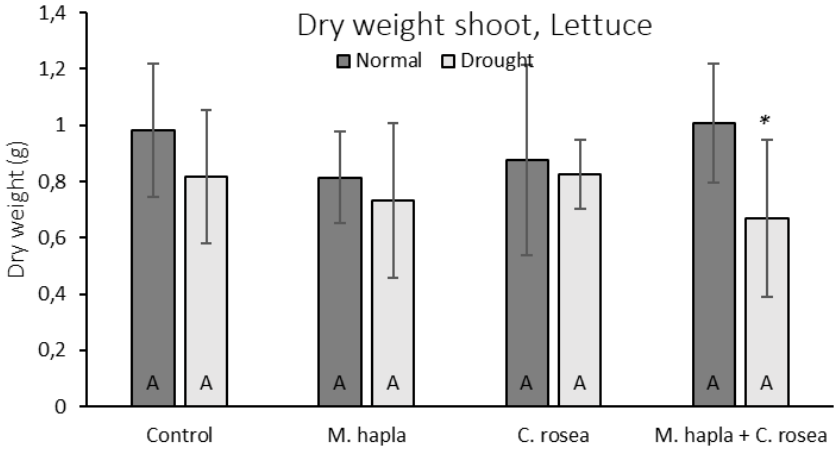

Figure 6. Lettuce shoot dry weight (mean ± SD n = 8) in a pot experiment with addition of M. hapla and C. rosea subjected to normal water conditions or drought. Different letters indicate significant (p < 0.05) differences, as determined by the Fisher method, within experimental group. An asterisk indicates a significant (p < 0.05) within treatment difference between drought and non-drought conditions, using Students t-test.

By the ANOVA performed on lettuce root length (Figure 7), we found that the applied treatment had a significant effect (F = 3.05, p = 0.036) on length of roots. Drought had, however, no significant effect at all. By the Tukey method, we found that nematode infected plants, with added *C. rosea* had significantly (p < 0.05) shorter roots than the plants in the control group under drought conditions. The result for root development (Figure 8) (measured by root development index, Appendix 1) shows a distinction at p = 0,058 (ANOVA, F = 2.65) for nematode infected plants with added *C. rosea* from all the other treatments in the experimental group under normal conditions. Though, there are neither any sign of drought effect on root development nor effect from the nematode infection.

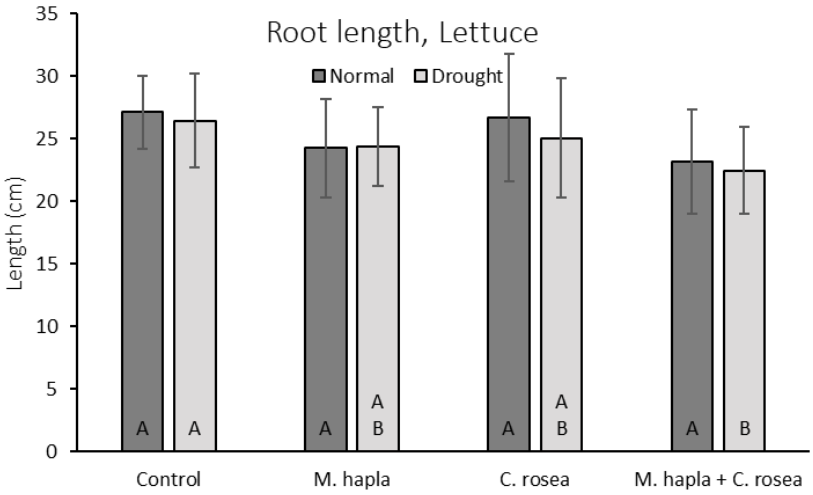

Figure 7. Lettuce root length (mean ± SD n = 8) in a pot experiment with addition of M. hapla and C. rosea subjected to normal water conditions or drought. Different letters indicate significant ($p < 0.05$) differences, as determined by the Fisher method, within experimental group. An asterisk indicates a significant ($p < 0.05$) within treatment difference between drought and non-drought conditions, using Students t-test.

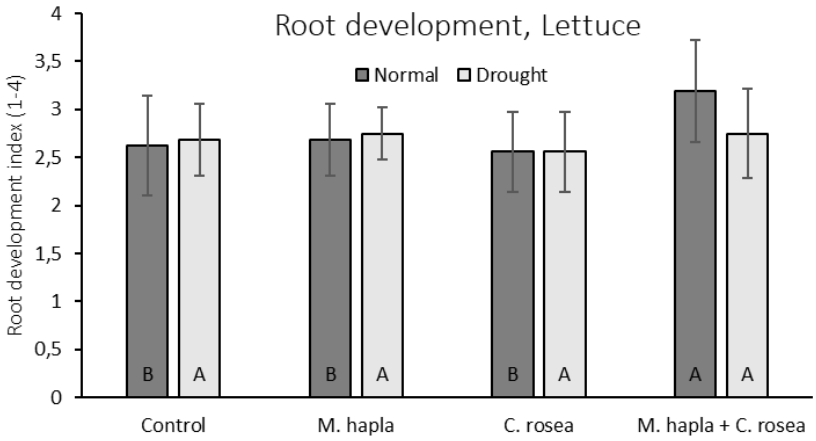

Figure 8. Lettuce root development (mean ± SD n = 8) in a pot experiment with addition of M. hapla and C. rosea subjected to normal water conditions or drought. Different letters indicate significant ($p < 0.05$) differences, as determined by the Fisher method, within experimental group. An asterisk indicates a significant ($p < 0.05$) within treatment difference between drought and non-drought conditions, using Students t-test.

There is a significant difference in leaf width (Figure 9) under drought conditions ($F = 17.57$, $p < 0.001$) and by applied treatment ($F = 3.34$, $p = 0.026$), however no interaction between these. There was a significant difference between plants under normal and drought conditions in control treatment and the treatment infected with only *M. hapla*. In effect of treatment, we saw a distinct difference between *C. rosea* treatments, with and without infection of *M. hapla*, where nematode infected plants

treated with *C. rosea* had significantly wider leaves than non-infected plants with inoculated *C. rosea* under both drought and normal conditions.

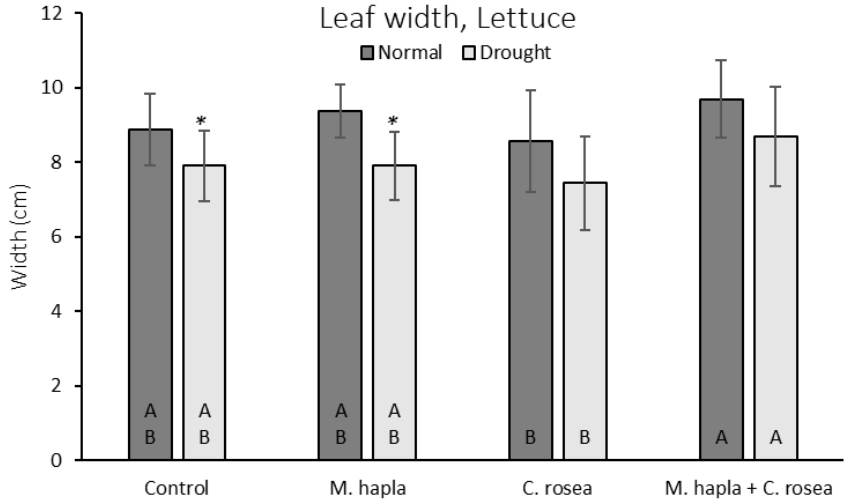

Figure 9. Lettuce shoot width (mean ± SD n = 8) in a pot experiment with addition of M. hapla and C. rosea subjected to normal water conditions or drought. Different letters indicate significant (p < 0.05) difference, as determined by the Fisher method, within experimental group. An asterisk indicates a significant (p < 0.05) within treatment difference between drought and non-drought conditions, using Students t-test.

There is a significant difference in leaf length (Figure 10) under drought conditions ($F = 4.49$, $p = 0.039$). By a t-test this is only shown in the control treatment, while there is no effect of drought in none of the plants treated with *M. hapla* and/or *C. rosea*.

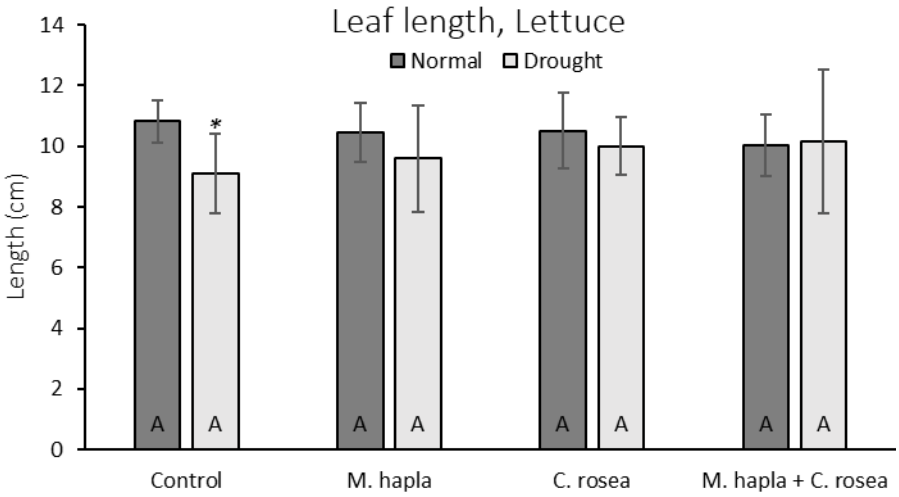

Figure 10. Lettuce leaf length (mean ± SD n = 8) in a pot experiment with addition of M. hapla and C. rosea subjected to normal water conditions or drought. Different letters indicate significant (p < 0,05) differences, as determined by the Fisher method, within experimental group. An asterisk

indicates a significant ($p < 0.05$) within treatment difference between drought and non-drought conditions, using Students t-test.

Galling and nematode infection

There was a visual infection of nematodes on the plants grown in nematode inoculated soil where clearly developed galls were seen on all plants. All the non-nematode treated plants were gall free. None of the nematode treatments were significantly distinct from each other, neither within the experiments nor between the experiments, when looking at the number of galls per plant (Figure 11).

In the experiment under normal conditions the plants treated with *C. rosea* had a significantly ($p < 0.001$) higher gall index (number of galls per root) (Table 2) than the one with only the nematode. There was no significant result showing any difference in gall index in the drought experiment (data not shown).

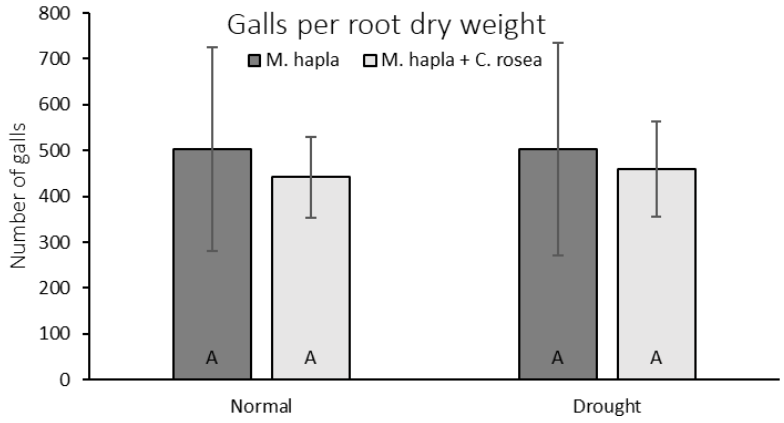

Figure 11. Lettuce, galls per root dry weight (mean ± SD n = 8) in a pot experiment with addition of C. rosea subjected to normal water conditions or drought. Different letters indicate significant ($p < 0.05$) differences, as determined by the Fisher method, within experimental group. An asterisk indicates a significant ($p < 0.05$) within treatment difference between drought and non-drought conditions, using Students t-test.

Table 2. Gall index lettuce, non-drought treatment.

Treatment	Mean	SD
M hapla	2,750	0,463
M. Hapla + C. rosea	4,143	0,690

3.2 Antibiosis assay

In the antibiosis assay (Table 3) we found that all nematodes in the fungal filtrate medias survived, while the majority of the nematodes died in the control samples with only SNA and PDB media.

Table 3. Antibiosis assay pilot experiment. WT: "C. rosea strain IK726", PDB/SNA and M. hapla. Controls: PDB/SNA and M. hapla. The table shows the number of nematodes dead or alive in the media after 24 h in 20 °C

Result chart – Antibiosis assay in vtro experiment					
PDB			SNA		
Sample	Dead	Alive	Sample	Dead	Alive
WT A	1	103	WT A	0	58
WT B	0	78	WT B	0	26
WT C	0	75	WT C	0	52
WT D	-	-	WT D	0	44
WT E	-	-	WT E	-	-
Control A	69	5	Control A	50	0
Control B	61	5	Control B	11	4
Control C	57	0	Control C	28	0
Control D	-	-	Control D	32	1
Control E	-	-	Control E	-	-

4. Discussion

Infection of M. hapla and choice of crops

A clear infection of *M. hapla* in the form of galls on both nematode treated carrot- and lettuce plants could be seen. However, due to the extreme buildup of small roots in the soil of carrot plants, it was impossible to count the number of galls for this crop. The extreme production of roots resembles an effect of the nematode infection on carrot (Perry et al. 2009). This phenotype was also observed in the control treatments, indicating an effect from the used soil type rather than from *M. hapla*. In comparison to the Iqbal et al. (2018) study, where they used a field collected clay soil as substrate, we used soil composed of peat, with a high nutrient and organic matter content (Hasselfors Garden 2020). The fact that the more organic soil contains bigger aggregates with a varying pore size enabled roots to penetrate soil clumps, which made it hard to remove the soil from the roots. Nutrient level and amount of organic matter may also have had an influence on the plant development and speed of its growth. Iqbal et al.'s (2018) study proceeded for 49 days, while my study lasted 59 days. Generally, I had up to 10 times higher plant mass (shoot and root weight) in my experiment indicating other factors affecting this. Choice of cultivar probably played a major role. We used the early summer cultivar "Jeannette" with a maturity time of 8 weeks from sowing, while Iqbal et al. (2018) used a storage cultivar named "Bolero" with a maturity time of 11 weeks. This prevented quantification of galls on the carrot plants in the current study.

Similar problems occurred in the lettuce plants where some smaller and more fragile roots were ripped off during rinsing. Since galls varied in size and roots got tangled and imbedded within peat, it would have been of benefit to count the galls under a dissection microscope as done in the similar study of Hussain et al. (2016). However, this would have been very time consuming but could have had a major impact on the result including root development root length, and gall development. As suggested improvement would be to use sand, which probably is a more practical substrate for an experiment like this. Another improvement is to extract J2 nematodes from the infected roots in the pots to see if *C. rosea* influenced the reproductive abilities of the nematodes in the galls. In this way, we could also have gotten result of nematode infection rate on carrot plants. Hussain et al. (2016) rinsed nematode eggs from the roots by applying 0,5 % NaOCl to count reproductive rate and, this may also be of interest to do in future experiments.

Effect of drought on nematode infection

I found some evidence of water deficit effects in nematode infected plants. This was found foremost in the carrots, where root and shoot weight was significantly lower during both nematode infection and water deficiency. There are some studies showing a decrease in the growth and yield of RKN-parasitized plants. Davis et al. (2014) and Snider et al. (2019) investigated the effect of both infection of *M. incognita* and the stress effect of water deficiency and found a decrease in both yield and growth in cotton . Khanizadeh et al. (1994) did a similar experiment and tested three different water potential levels, and found that a higher galling rate of the species *M. hapla* correlated to lower water content in the soil. However, they found no effect on growth or flowering of the strawberry plants (Khanizadeh et al. 1994). This study speaks in the opposite of our result in which we found an added infection effect from *M. hapla* in foremost carrots, where root and shoot weight was significantly lower during both nematode infection and water deficiency. As there are no previous studies showing the effect, neither positive nor negative, of both drought and nematode infection on carrots to compare with, it could be that the effects are crop specific. This is although an indication of a possible increased phenotypic effect of mass and yield loss, causing bigger damage during drier periods of nematode infection in the field. The mentioned study by Khanizadeh et al. (1994) also reports the opposite result when it comes to the galling of the roots. In the lettuce experiment, where galls were counted, we found no difference in number of galls per root between treatments under drought and with normal water conditions. The fact that the lettuce is another species of plant may of course be one of the factors determining the result. However, it is worth noticing that the water content level of the drought treated pots varied from 5.6-29.8 % (due to uneven trays), which may have affected the result negatively.

Biological effect of C. rosea towards nematode infection and survival

There was no sign of a biological control effect of *C. rosea* towards the species *M. hapla* in our experiment, neither in carrot nor lettuce plants. However, this cannot be statistically tested due to the lack of data for number of galls in the carrot plants. In the study by Hussain et al. (2016), *C. rosea* showed a positive nematicidal effect on *M. hapla* in carrot plants and decreased the reproduction rate of the nematodes. They also found a mycelium mat surrounding both J2 nematodes and egg masses, indicating a parasitic effect via penetration (Hussain et al. 2016). By studying the egg masses from the infected roots, so well on the roots itself, we could have investigated the presence of fungal mycelia on both roots and nematode eggs. Hussain et al. (2016) did not mention in their study which strain of *C. rosea* they used, which would have been important to add valuable additional information for the understanding of our study. High variation between strains in production of

nematicidal compounds are reported for *C. rosea* (Iqbal et al. 2020). It is possible that the strain they used have a nematicidal effect on *M. hapla* while strain IK726 used in my study do not. One possible error in this study is that we did not make sure that the fungus spread throughout the pot. It was hard to mix the formula into the soil and impossible to know when the soil was mixed well enough because of the choice of soil substrate. A lighter substrate needs less formula (3.5 g) while Iqbal et al. (2018) used the much heavier substrate of a clay soil (7 times more formula), making the needed amount of formula needed higher in volume in relation to substrate volume. Therefore, there may have been a risk of uneven distribution of the formula in the pots. The study of Iqbal et al. (2018) did not include RKNs, indicating that morphological differences of nematode genera probably play a big role in potential nematicidal effects.

Antibiosis assays

In the *in vitro* antibiosis assay I did not see any signs of nematicidal effect. In all the samples with the fungus in either SNA or PDB the nematodes were alive and vigorous (*M. hapla*, and non-counted bacterial feeders). In the control samples (only the media without *C. rosea*) almost all the nematodes were dead. This is an indication of a toxicity within both mediums that killed the nematodes, but this was changed from nematicidal to non-nematicidal fungal exudates. Noticeable, only nematodes of the species *M. hapla* were deceased, while the bacterial feeders were not. Compounds such as extracellular serine protease PrC and epipolysulfanyldioxopiperazines were hypothesized to play a role in nematicidal effect in the study by Iqbal et al. (2018). However, my experiment was implemented in the same way, and the same nematicidal compounds should be present in the antibiosis assays, which indicates differential toxicity towards different nematode species. Though, the fact that the nematodes died in the control samples and not in the fungal filtrate media, make it hard to draw any conclusions correlated to presence of nematicidal compounds in this experiment.

Plant growth promoting effects of C. rosea

The plant growth promoting abilities of the fungus *C. rosea* has been studied and shown previously (Johansen et al. 2005). However, in our study we found no sign of any plant promoting effects of the fungus for either carrot or lettuce. Noticeable, there is a pattern in drought resistance in carrot plants when inoculated with *C. rosea*. The carrot plants that were not subjected to drought had a higher shoot and root weight in comparison to the control. This might be an indication of some mutualistic interaction between the fungus and the plant. This has been shown before by Donoso et al. (2008) where they studied mutualistic interaction between the fungus *Trichoderma harzianum* and wheat plants. They found a strong

interaction between the fungus and the plant under drought conditions, where water deficit led to a higher stress tolerance from the plant inoculated with *T. harzianum* in the form of e.g. bigger root systems (Donoso et al. 2008). The same result was also found by Mona et al. (2017) where tomato plants inoculated with *T. harzianum* had an increased growth of roots and shoots under drought conditions in comparison to the controls. Future studies of this are needed and highly relevant for plant protection and plant production.

5. Conclusion

In conclusion, there was an effect of drought on an already initiated infection from the nematode *M. hapla* on carrot plants. The effect was expressed as a loss in plant biomass. No effect could be seen in lettuce plants from neither drought nor nematode infection. There was also no biocontrol effect from the fungus *C. rosea* on nematode infection or reproduction. We saw no nematicidal effect from fungal exudates in the antibiosis experiment; however, the growth medias SNA and PDB caused almost total mortality in *M. hapla*. In my experiments, *C. rosea* did not display any plant growth promoting abilities in regard to plant mass or shoot size/length. It does, however, seem to influence tolerance towards water deficiency in carrot plants.

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Popular science summary

Did you know that there are worms in the soil that can lower the health of farmed plants? These small worms are called nematodes and can be found almost everywhere on the globe. They can parasitize crops and reduce the yield for farmers. To control these nematodes, farmers can use chemicals. However, these chemicals are not good for the environment nor human health, and there is a need of better solutions to control the nematodes. One solution is via so called biological control, where you instead of using chemicals, use natural occurring organisms such as insects, fungi, or bacteria. In this experiment, the fungus *Clonostachys rosea* was investigated as a biocontrol agent towards root-knot nematodes of the species *Meloidogyne hapla*. Infection of this nematode species is seen as knots on the plant roots in which it feeds, the so called "galls". Carrot and lettuce plants grown in pots were inoculated with the fungus and infected by the nematodes to see if the fungus had a supressing effect towards the nematodes. To do this, nematodes were collected from infected tomato plants, where roots were dug up from the pots and put on a net in a tray filled with water. By letting the infected roots soak in water, the nematode eggs hatches, and the nematode juveniles are released into the water. This water was later collected and put in the pots to evenly infect the carrot and lettuce roots. The fungus was added into the pots as a formula consisting of fungal spores, peat, and wheat bran. The pots were then kept in a climate chamber with specific temperature and light settings. After eight weeks, the crops were harvested and their weight, length, and number of galls were measured. After collecting the data, an analysis of variance between treatments was performed to see if there was any effect from drought, nematode infection, and biological control from the fungus in the experiment. We could see that the nematodes have had an impact on carrot plant biomass when it was kept under drought conditions, where the plants were considerably smaller when comparing to the watered carrot plants. However, there was no sign of a biological control effect on the nematodes, which also had infected the roots in the pots with the fungus. One noticeable thing in the experiment was that the carrot plants in the experiment which only was inoculated with the fungus and not infected by the nematodes, grew better under drought conditions than the other plants. This tells us that the fungus, even if not controlling the nematodes, can help the plants to withstand a drought period.

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Appendix

Root galling index was measured by counting galls on each lettuce plant root and was the given an index number based on the index below:
0 = 0 galls
1 = 1-25 galls
2 = 26-50 galls
3 = 51-75 galls
4 = 76-100 galls
5 = >100 galls

Root development index was measure by determining the development of primer and secondary roots and length of the pole root based on the index below:
1 = small pole root, few secondary roots
2 = distinct pole root, few secondary roots
3 = distinct pole root, mediate secondary roots
4 = distinct pole root, lots of secondary roots resulting in bushy roots.

Table 1. Protocol, harvest data for carrot plants in pot experiment.

Treatment	No. of plants	shoot length	Root length	Number of yellow leaves	FW - root	DW - root	FW - shoot	DW - shoot
1CA								
1CB								
1CC								
1CD								
1CE								
1CF								
1CG								
1CH								
2CA								
2CB								
2CC								
2CD								
2CE								
2CF								
2CG								
2CH								
3CA								
3CB								
3CC								
3CD								
3CE								
3CF								
3CG								
3CH								
4CA								
4CB								
4CC								
4CD								
4CE								
4CF								
4CG								
4CH								

Table 2. Protocol, harvest data for lettuce plants in pot experiment.

Treatment	No. of plants	No. of galls/gall index	Root development	shoot length	Root length	Bumps (lettuce)	Leaf color	FW - root	DW - root	FW - shoot	DW - shoot
1LA											
1LB											
1LC											
1LD											
1LE											
1LF											
1LG											
1LH											
2LA											
2LB											
2LC											
2LD											
2LE											
2LF											
2LG											
2LH											
3LA											
3LB											
3LC											
3LD											
3LE											
3LF											
3LG											
3LH											
4LA											
4LB											
4LC											
4LD											
4LE											
4LF											
4LG											
4LH											

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