Ministry of Environment of Denmark
Environmental Protection Agency

CombiControl

Combining above- and belowground biological control agents for improved pest control in strawberry tunnel production

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Sources must be acknowledged

Contents

1. Preface

The experiments of the project were conducted in laboratory and greenhouse facilities at the Department of Plant and Environmental Sciences, University of Copenhagen. The project was carried out in collaboration with HortiAdvice A/S and two commercial strawberry producers; Søren Olesen and Morten Christensen. The study was funded by the Danish Ministry of Environment's Research Program for Pesticides (J. no. 2019-15110) with the Danish title "*Kombination af biologiske bekæmpelsesmidler til forbedret bekæmpelse af skadedyr i jordbær i tunneler – CombiControl".*

The present project aimed to explore the effects of combining biocontrol agents on pest population build up and infestation in strawberry tunnels. The project further aimed to address the regulation of selected well-known defence proteins, glucanases and chitinases, that are important pathogenesis related (PR) proteins in strawberries to evaluate their potential contribution to any observed negative effects against spider mites. Despite several attempts by various protocols to extract RNA from strawberry leaflets, the efforts were without success. This part of the project therefore generated no data, but the experimental setup and protocols used are added to the report as an appendix (Appendix 1), which can be used as reference for future work.

We thank for constructive discussions and feedback on the project during the meetings with the steering committee (Følgegruppen for bekæmpelsesmiddelforsknings-projekterne inden for indsatsområdet "Jordbrug") chaired by Henrik F. Brødsgaard, Danish Environmental Protection Agency (Danish EPA). We are particularly grateful for comments and critical reviews of the first draft of this report from Mette Sønderskov (Aarhus University) and Vibeke Langer (University of Copenhagen), which contributed to significant improvements of the final report.

2. Summary and Conclusion

Strawberry (*Fragaria* x *ananassa* Duch.) production in Denmark is increasingly conducted on tabletops in tunnels, which can ensure more predictable conditions for plant growth than traditional outdoor field production. However, under these protected conditions, growers experience huge problems with aboveground arthropod pests such as the two-spotted spider mite (*Tetranychus urticae*) and different species of thrips.
There are currently only few chemical and biological pest control options available for Danish strawberry growers. Spider mites and thrips can be targeted by release of biological control agents such as predatory mites or predatory bugs. Thrips pupae are mostly located in the growth substrate on the table tops, in the soil beneath the table tops, and occasionally the thrips are also able to pupate in foliar plant crevices, depending on species. In all cases, these thrips are out of reach for the predators released in the canopy. The concealed insect stages may be targeted by application of the entomopathogenic fungus (EPF) *Metarhizium brunneum*, which can establish in the substrate and await the opportunity to infect and kill different soil dwelling insects. The EPF can also target the larvae of the black vine weevil (*Otiorhychuns sulcatus*) which can be an important pest in strawberry production. The EPF *M. brunneum* can establish and remain active against insects for long periods in the belowground environment. In addition, the fungus has also been shown to negatively affect the development of arthropod herbivores feeding on plants aboveground. It has previously been documented that EPF, including *M. brunneum*, when inoculated to the root system of different plants, can modify the plant defences in aboveground tissues, causing negative effects on herbivorous insects and mites feeding on the leaves, thus reducing their population growth rates. The belowground EPF therefore do not infect the aboveground herbivores, but affect them indirectly by causing accumulation of plant defence compounds in the leaves. Such effects are not yet described in strawberry plants, but if these mechanisms are at play, belowground application of *M. brunneum* to the substrate could be exploited for adding to the effects of aboveground biological control agents, such as predators, as a combined strategy for enhanced control of arthropod pests in strawberry production. The first aim of the present project was therefore to evaluate the effect of single and combined use of a macrobiological control, the predatory mite *Neoseiulus cucumeris*, and microbiological control, the EPF *M. brunneum*, applied above- and belowground, respectively, on the population growth of spider mites, *T. urticae*, on a common strawberry cultivar in greenhouse experiments. The second aim was to evaluate the potential control of the combined use of above- and belowground biological control agents, on some of the major pests in two commercial strawberry tunnels in Denmark.

The belowground application of the entomopathogenic fungus *M. brunneum* to strawberry plants (cv. 'Rumba') in coir substrate showed reduction of spider mite population growth aboveground, but the effect was isolate dependent. Out of the three isolates tested, KVL16-36 (obtained from the commercial product Met52) was the only isolate to have a significant negative impact on spider mite egg and nymph numbers in greenhouse experiments, while the effects of two Danish *M. brunneum* isolates did not differ from control treatments. The combination of *M. brunneum* KVL16-36 with predatory mites *N. cucumeris* on whole plant experiments with spider mite infestations indicated an additional effect of the two biocontrol agents compared to single applications, when spider mite nymphs were considered at a short time span of 24 h. During field trials in two commercial strawberry tunnels, spider mite infestations were reduced in treatment plots with *M. brunneum* treatments, and in combination with predatory mites, at both sites. The abundance of thrips was also reduced in plots with application of biocontrol agents at one of the sites with moderate thrips infestation. At this site, a reduced fruit damage was also observed in the biocontrol plots later in the season. No effects of the biocontrol treatments were found at the other site due to very high thrips infestation.

The biocontrol effect of the strategy is likely to vary among application scenarios, with a potential for reduction in pest infestation. Besides, there was no indication of an increase in pest levels in the biocontrol treatment plots. The application of *M. brunneum* to the substrate indicated to represent an added value of the combined treatment, potentially enhancing the plant defences by causing sub-lethal effects against a spectrum of aboveground arthropod herbivores, although the mechanisms behind the effects remain unknown. While the application of *M. brunneum* showed effects against different pest species in strawberry production, there was no indication of negative effects against predatory mites. The EPF *M. brunneum* retained its biological activity in the substrate throughout the season and was able to infect and kill coleopteran larvae five month after application. The strategy may therefore provide the cropping system with resilience towards pest challenges above-ground and be a standing defence against insect stages in the substrate during the production period. However, severe infestations of thrips cannot be controlled by the biocontrol agents alone, and additional measures are needed to control such attacks by non-chemical means in strawberry tunnels.

3. Sammendrag og konklusion

Jordbærproduktionen (*Fragaria* x *ananassa* Duch.) i Danmark sker i stigende grad i tunneler på tabletops, hvilket giver mere forudsigelige vækstbetingelser sammenlignet med produktion på åben mark. Dog står avlerne over for betydelige udfordringer med skadedyr såsom væksthusspindemiden (*Tetranychus urticae*) og forskellige arter af trips, under de beskyttede forhold i tunnelerne. I Danmark er der kun få kemiske og biologiske bekæmpelsesmuligheder til rådighed for jordbæravlere mod disse skadedyr. For eksempel kan spindemider og trips bekæmpes ved at introducere biologiske bekæmpelsesmidler som rovmider og rovinsekter. Dog kan trips forpuppe sig i vækstsubstratet oppe i tabletops, i jorden under disse, eller endda i sprækker på planterne, hvilket gør dem utilgængelige for rovinsekter, der primært er aktive oppe på planten. Insekter i jorden og i vækstsubstratet kan bekæmpes ved anvendelse af den insektpatogene svamp *Metarhizium brunneum*, som kan etablere sig i substratet og her inficere og dræbe forskellige jordlevende insekter. Svampen kan også bruges mod larverne af væksthussnudebillen (*Otiorhychuns sulcatus*), et vigtigt skadedyr i jordbærproduktion. Svampen *M. brunneum* kan etablere sig og forblive aktiv mod insekter i lange perioder i jorden. Derudover har svampen også vist sig at kunne påvirke udviklingen af skadedyr der lever oppe på planten, negativt. Det er tidligere dokumenteret, at insektpatogene svampe, herunder *M. brunneum*, når tilført plantens rodsystem, kan modificere planteforsvaret i overjordiske væv og forårsage negative effekter på planteædende insekter og mider, og dermed reducere deres populationstilvækst. Den insektpatogene svamp i jorden inficerer dermed ikke de planteædende insekter og mider oppe på planten, men påvirker dem indirekte ved at øge plantens forsvarsmekanismer i bladene. Sådanne effekter er endnu ikke beskrevet i jordbærplanter, men hvis disse mekanismer er i spil, kan en tilførsel af *M. brunneum* til substratet udnyttes til at understøtte virkningerne af biologiske bekæmpelsesmidler udsat oppe på planten, såsom rovinsekter, i en kombineret strategi for forbedret bekæmpelse af skadedyr i jordbær. Det første formål med projektet var derfor at evaluere effekten af enkelt og kombineret brug af makrobiologisk bekæmpelse, rovmiden *Neoseiulus cucumeris*, og mikrobiologisk bekæmpelse, den insektpatogene svamp *M. brunneum*, anvendt over og under jorden, på populationstilvæksten af væksthusspindemiden, *T. urticae*, på en almindeligt brugt jordbærsort i kontrollerede laboratorieforsøg. Projektets andet formål var at evaluere den potentielle bekæmpelse af skadedyr ved den kombinerede brug af biologiske bekæmpelsesmidler over og under jorden på nogle af de vigtigste skadedyr i to kommercielle jordbærtunneler i Danmark.

Anvendelsen af den insektpatogene svamp *M. brunneum* i kokossubstrat med jordbærplanter (cv. 'Rumba') resulterede i en reduktion af populationstilvæksten af væksthusspindemider på planten, hvor effekten afhang af svampeisolatet. Blandt de tre testede isolater var KVL16-36 (fra det kommercielle produkt Met52) det eneste, der havde en signifikant negativ indvirkning på antallet af spindemideæg og nymfer under kontrollerede forhold, mens effekten af de to andre isolater af *M. brunneum* ikke adskilte sig fra kontrollen. Kombinationen af *M. brunneum* KVL16-36 og rovmider *N. cucumeris* viste en yderligere effekt på spindemider sammenlignet med behandlingen hvor kun det ene bekæmpelsesmiddel (KVL16-36) blev anvendt, vurderet over en kort periode på 24 timer. I forsøgene i de to kommercielle jordbærtunneler blev angrebet af spindemider reduceret i behandlede parceller med *M. brunneum* og i kombination med rovmider på begge lokaliteter. Forekomsten af trips blev også reduceret i parceller, hvor biologisk bekæmpelse blev udført, på en af lokaliteterne med moderat tripsangreb. Her blev der også observeret færre skader på frugterne i de behandlede parceller sammenlignet med kontrollen, i opgørelsen foretaget senere på sæsonen. Der blev ikke påvist nogen effekt af biologisk bekæmpelse på den anden lokalitet på grund af meget høje tripsangreb.

Effekten af biologisk bekæmpelse alene og i kombination kan variere afhængigt af lokation og anvendte midler, men generelt var der ingen indikation af en stigning i skadedyrsniveauer. Anvendelsen af *M. brunneum* i substratet repræsenterer en merværdi af den kombinerede behandling, ved potentielt at forbedre plantens forsvar med indirekte virkning på flere vigtige grupper af skadedyr, selvom mekanismerne bag virkningerne forbliver ukendt. Mens anvendelsen af *M. brunneum* viste effekter mod forskellige skadedyrsarter i jordbær, var der ingen indikation af negative effekter på rovmider. Den insektpatogene svamp *M. brunneum* bevarede desuden sin biologiske aktivitet i substratet gennem hele sæsonen og var i stand til at inficere og dræbe billelarver fem måneder efter påføring i substratet. Strategien kan derved understøtte dyrkningssystemets modstandsdygtighed over for udfordringer med skadedyr oppe på planten, samt fungere som et stående forsvar mod skadedyr i substratet. Selvom biologisk bekæmpelse viser effekt mod forskellige skadedyrsarter i jordbær, kan alvorlige angreb af trips ikke bekæmpes alene med disse midler, og yderligere foranstaltninger er nødvendige for at kontrollere sådanne angreb i jordbærtunneler uden brug af kemikalier.

4. Introduction

4.1 Pest control in Danish strawberry tunnel production

Strawberry production in tunnels is one of the primary production methods of strawberry in Europe and in Denmark, where this production method is continuously increasing (current estimate approximately 100-125 ha in DK). This type of production can prolong the growing season considerably and result in yield increases, higher fruit quality and stability (Kadir et al. 2006; Salamé-Donoso et al. 2010). However, tunnel production demands a higher financial input and increases the risk of arthropod pest (hereafter referred to as pest) outbreaks due to favourable climatic conditions for pest development. This is partly due to the extended season using ever-bearing cultivars, where strawberry flowers and fruit are available longer for the pests to feed on and reproduce, and partly due to the higher temperatures inside the tunnels favouring shorter lifecycles and therefore more generations of the pest species per year. It is therefore crucial that pest populations are controlled to avoid significant yield losses.

Several arthropod pest species cause damage to strawberries in Denmark. Some of the major pests in strawberry tunnels are the two spotted spider mite, *T. urticae*; aphids (e.g. *Myzus persicae, Aphis gossypii*, *Macrosiphum euphorbiae*); thrips (e.g. *Frankliniella instonsa*, *Thrips tabaci* (Nielsen et al. 2021)); European tarnished plant bug, *Lygus rugulipennis*; and the black vine weevil, *O. sulcatus* (Cross et al. 2001). While spider mites mainly feed on strawberry leaves, the thrips attack flowers and fruit, thus affecting yield both directly and indirectly.
Currently, control of most of these pest species is mainly achieved by the release of arthropod predators; routinely predatory mites (*N. cucumeris*) and predatory bugs (*Orius majusculus*) (Cross et al. 2001; Kalenius 2016), use of sticky traps, and application of chemical pesticides if the production is not organic. The predatory mite *N. cucumeris* is applied early in the season, sometimes as a preventive measure against thrips, but *N. cucumeris* cannot maintain the pest levels sufficiently low if temperatures increase to an extent where pest reproduction and the in-flight of thrips are occurring faster than the feeding capacity of the predator. In cases of high infestation levels of the two spotted spider mite, the predatory mite *Phytoseiulus persimilis* is effective in controlling the pest. However, this predatory mite is more costly and it is specialized feeders on spider mites (i.e. do not feed on thrips), more difficult to apply, and inefficient at lower temperatures. While *P. persimilis* is not optimal for release early in the season, *N. cucumeris* is, and it can for this reason contribute to the prevention of an early pest population build up, especially in combination with other control measures. In addition, the life strategy of the pests and the crop plant parts attacked vary, and a single application method may not target the entire pest complex in the tunnel. Combining control methods can therefore potentially be a strategy to target more pest species and provide a more resilient control regime for the grower.
Aboveground predators primarily target spider mites, aphids and thrips, while control of the belowground pest stages, such as larvae of the black vine weevil, requires different strategies. Use of entomopathogenic fungi (EPF) is a microbiological control option, which can be applied directly in the plant substrate by dipping the plant roots in spore suspensions before planting (root-inoculation; Canassa et al. 2020a) or applied to the growth substrate as granules or as a liquid suspension. One of the most widely used species of EPF is *M. brunneum* (formerly *M. anisopliae* var. *anisopliae*) (Ascomycota: Hypocreales) (e.g. product names Met52, Lalguard M52, BIPESCO 5), which is authorized for strawberry production in the field, in tunnels and in greenhouses in Denmark (Bekæmpelsesmiddeldatabasen, 2023). Pests such as the black vine weevil and the soil-borne stages of thrips (pupae) can therefore be directly targeted by the application of this microbial control agent to the substrate (Ansari et al. 2007; Shah et al. 2007).

Recent studies have demonstrated that microbial control agents such as EPF applied belowground can provide additional benefits to pest management by affecting population growth of arthropod pests aboveground (e.g. Gange et al. 2019; Rasool et al. 2021a). It was recently shown that spider mite females laid fewer eggs on strawberry plants where roots were inoculated with a range of EPF isolates (Canassa et al., 2020b) and that seed inoculation with EPF in beans caused reduced population growth of spider mites (Canassa et al. 2019). Further, field experiments in Brazil demonstrated reduction in spider mite populations and foliar disease occurrence in different strawberry cultivars with root-inoculation of EPF compared to un-inoculated control treatments over a period of 6 months (Canassa et al., 2020a). Inoculation with EPF belowground could therefore potentially target both pest stages in the soil as well as pests attacking the plant aboveground. However, microbial control agents, such as the EPF *M. brunneum*, have so far only been used to a limited extent in Denmark, and there is currently no knowledge of how such inoculations can be combined with release of arthropod predators.
In addition, not much is known about the mechanisms behind the observed effects of EPF applied belowground on spider mite populations aboveground. It can be expected that spider mites induce the defence system of the plant and potentially the inoculation with EPF can modulate defence responses against pests (Pappas et al. 2018). Recent studies have shown that specific defence compounds in tomato were accumulating more in leaves of plants under spider mite attack, if grown from seeds inoculated with EPF (Rasool et al. 2021b). It can therefore be expected that EPF applied belowground can modulate plant defences aboveground during pest challenge.
Optimizing biological control efforts by combining treatments with different biological control agents to, potentially, obtain synergistic effects will reduce the need for chemical control methods. This will be beneficial for both organic and conventional growers for maintaining yield stability and quality. It will also assist in meeting the goals of integrated pest management (IPM) as well as for meeting consumer demands for products with no chemical residues and produced with consideration for the environment.

The present project aimed to explore the potential of combining macro- and microbiological control agents, above- and belowground, to target several pest species in strawberry production in tunnels. This was investigated in greenhouse experiments for effects on pest reproduction and population growth in combined treatments with a predatory mite commonly applied in Denmark and with high potential as an early relased predator in combination with EPF, *N. cucumeris*, and the EPF *M. brunneum*. The knowledge gained in controlled greenhouse experiments was implemented in field tests of the combinations of predators and EPF at larger scale in commercial strawberry tunnels, hosted by two strawberry growers. The objective was to investigate the effect of combined above- and belowground biological control on natural pest abundance and damage throughout the production season.

Project objectives and hypotheses

The project activities had two main objectives:

a. Evaluate the effect of single and combined use of macro- and microbiological control agents, above- and belowground, on the population growth of spider mites on a common strawberry cultivar in greenhouse experiments. Treatment combinations included root inoculations with the EPF *M. brunneum*, and release of predatory mites, *N. cucumeris*.

b. Evaluate the potential of the combined use of above- and belowground macro- and microbiological control agents, on the control of some of the major arthropod pests in commercial strawberry tunnels in Denmark (spider mites, aphids, thrips), by assessing abundance of organisms and plant damage throughout the production season in different treatment combinations.

The activities aimed to test the following hypotheses:

1. The EPF, *M. brunneum*, applied to the roots of strawberry plants, will strengthen the plant defences and thereby decelerate the population growth of the aboveground pest, the two-spotted spider mite *T. urticae*. Combined treatments of above- and belowground biological control agents, *N. cucumeris* and *M. brunneum*, will reduce *T. urticae* populations more than if the biological control agents were applied individually, in trials under controlled conditions in a greenhouse.

2. The combined use of *M. brunneum* belowground and the commercial predator *N. cucumeris* aboveground will reduce the infestations of commonly occurring pests and reduce plant damage during the production season in tunnel-produced strawberries, compared to treatments with a single biological control agent.

5. Materials & Methods

5.1 Testing above- and belowground biocontrol agents against spider mites under controlled conditions

Experiments under controlled conditions were done on strawberry plants, *Fragaria* x *anannassa* Duch. cv. 'Rumba'. Plants were grown from runners of six month old plants, in pots (10 cm) containing coir (Legro©, the Netherlands), similar to the substrate used in commercial strawberry tabletop production in tunnels in Denmark. Pots were placed in a greenhouse at 15ºC, 16h light: 8h darkness, covered with plastic. The plastic cover was removed from the newly potted runners after two weeks, and the plants were used in the experiments when they had minimum three leaves (nine leaflets) per plant (approximately two months after). Plants were watered as required, with tap water and less frequent with demineralized water to avoid build-up of soluble salts in the coir. Before each experiment, plants were rinsed with water to remove unwanted arthropods, and excess leaves were removed, to three leaves per plant.

Rearings of *T. urticae* (Acari: Tetranychidae) were established on bean plants, *Phaseolus vulgaris*, grown from seeds. Two cages, with fine mesh for ventilation, minimum two plants per cage, were placed in a climate cabinet of 22ºC, 16h light: 8h darkness, and inoculated with *T. urticae*. The bean plants were replaced weekly to maintain the *T. urticae* populations throughout the experimental period. Predatory mites, *N. cucumeris* (Acari: Phytoseiidae), were provided by Borregaard BioPlant© (Denmark) shortly before each repetition of the experiment were initiated. Only adult *N. cucumeris* were used in the experiments.

Three isolates of the entomopathogenic fungus *M. brunneum* were used; KVL12-30, KVL12-37, KVL16-36. The first two isolates were isolated from agricultural soils in Denmark and have previously shown control potential against insects when applied to plant roots (Keyser et al. 2014), while the latter isolate (KVL16-36) was obtained as a pure culture from the commercial product Met52 Granular (Novozymes), which is applied for direct control of below-ground insect pests. The cultures of the three isolates were grown on Sabouraud Dextrose Agar (SDA), in a climate cabinet at 22ºC, in darkness, for approximately one month prior to preparation of spore suspensions for experimental root inoculation.

Spore suspensions were prepared to a concentration of $1x10^7$ spores/ml. Spores were harvested by adding 10 ml of 0.05% Triton-X to the plate of the fungal culture and scraping with a sterile Drigalski spatula. The crude suspension was transferred into a falcon tube (50 ml), vortexed, and centrifuged at 3000 rpm for three minutes. The supernatant was discarded, and the step was repeated once after adding 10 ml of 0.05% Triton-X to obtain the final stock suspension. The stock suspension was diluted (100x dilution) to adjust for the final concentration of $1x10^7$ spores/ml, determined after counting in a Neubauer Improved Haemocytometer (Fuchs-Rosenthal). Two culture plates per isolate were used for each spore suspension, to obtain a sufficient volume for experimental application. The spore viability of each spore suspension was validated by pipetting 100 µl to an SDA plate (two plates per suspension), incubating the plate in 22ºC, in darkness, and observing the frequency of spore germination after 24h under a light microscope (400x magnification), by counting 3x100 spores per suspension. In all cases, spore germination was >97% for the experimental suspensions.

5.1.1 Impact of belowground inoculation of *M. brunneum* on spider mites

Strawberry plants in pots were placed individually in an open plastic box (9 L/328x250x190 mm) under growth light, in a climate controlled laboratory. The experiment consisted of four treatments: plants inoculated with either *M. brunneum* isolate KVL12-30, KVL12-37, or KVL16-36, or a control treatment of 0.05% Triton-X added to the substrate in the same volume as the spore suspensions. Experimental plants were placed in a random block design, with ten plants per treatment. The experiment was repeated at three temporal occasions, thus the total number of plants thereby summed up to 120. In the *M. brunneum* treatments, a spore suspension of $1x10^7$ spores/ml was applied to the substrate around the stem area by adding 20 ml of suspension with a syringe (100 ml). Seven days after fungal inoculation, three adult female *T. urticae* were placed in a clip cage (diameter 3.2 cm) enclosing one leaflet per plant. The clip cage consisted of two rings (insulating foam) covered with fine mesh, placed on each side of the leaflet, attached by three metal clamps. The clamps were supported by wooden sticks mounted in the substrate (Photo 1a). The number of *T. urticae* inside each clip cage was assessed every three days (day 3, 6, 9, 12, 14) during a period of 14 days. At each assessment day, eggs, nymphs and adults in each clip cage were counted by observing through a dissection microscope (16-40x magnification).

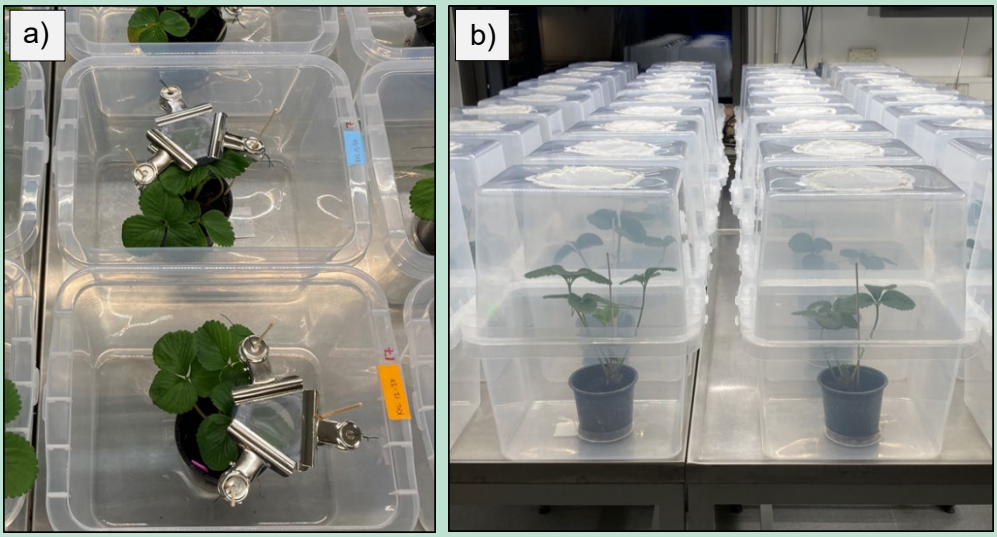

PHOTO 1. Experimental setup for assessment of populations by the two-spotted spider mites, T. urti-cae, on strawberry plants inoculated with the entomopathogenic fungus M. brunneum to the substrate. Three M. brunneum isolates were tested for effects on T. urticae inside clip cages (a). Populations of T. urticae on whole plants ±inoculated with M. brunneum isolate KVL16-36 evaluated before and 24 hours after release of predatory mites, N. cucumeris (b).

At the end of the experiment, strawberry plants were stored (max. 14 days) in a cooling room (4ºC) and subsequently tested for presence of *Metarhizium* colonization in the roots and in the stem. To confirm and quantify the presence of *Metarhizium* in the roots, the substrate with root system of each plant was taken out of the pot and cut through horizontally, 2 cm below the surface (Photo 2a). Below this point, ten pieces of 2 cm of root were cut with scissors from the immediate area surrounding the main stem, placed in a Petri dish and rinsed in water and stored in an Eppendorf tube at 4ºC for 24 h (Photo 2b).

PHOTO 2. Strawberry main root cut horizontally 2 cm below soil surface (a) for retrieval of root pieces for testing Metarhizium root colonization. Ten roots per plant were cut in pieces of 2 cm, and stored in Eppendorf tubes (b) until use.

Hereafter, each root sample of ten root pieces were transferred to a 50 ml glass tube with 10 ml sterile 0.05% Triton-X and homogenized by a custom-fitted pestle mounted on a drilling machine for 1 min. The tube was vortexed and the homogenized root suspension plated by pipetting 100 µl root suspension onto each of two replicate Petri dishes with selective media (Steinwender et al. 2015). The plates were incubated at 22ºC, in darkness, and emergence of *Metarhizium* colonies was checked after five and ten days by counting the number of colony forming units (CFU) on each plate (Photo 3a).

Detection of endophytic fungal establishment in the stems of experimental plants was done by cutting a piece of 5 cm from the uppermost part of the stem below the leaflet. The surface of the stem piece was surface sterilized by dipping it in ethanol (70%) for 90 sec, rinsed in sterile H_2O, dipping in bleach NaClO (1%) for 90 sec, and rinsed twice in sterile H_2O. The surface sterilized stem was cut in four pieces with a sterilized scalpel and placed in a Petri dish on selective media, positioned so that the cut surface of the stem was in contact with the agar medium (Photo 3b). The plates were incubated at 22ºC, in darkness, and checked after five and ten days for emergence of *Metarhizium* at the cut surface.

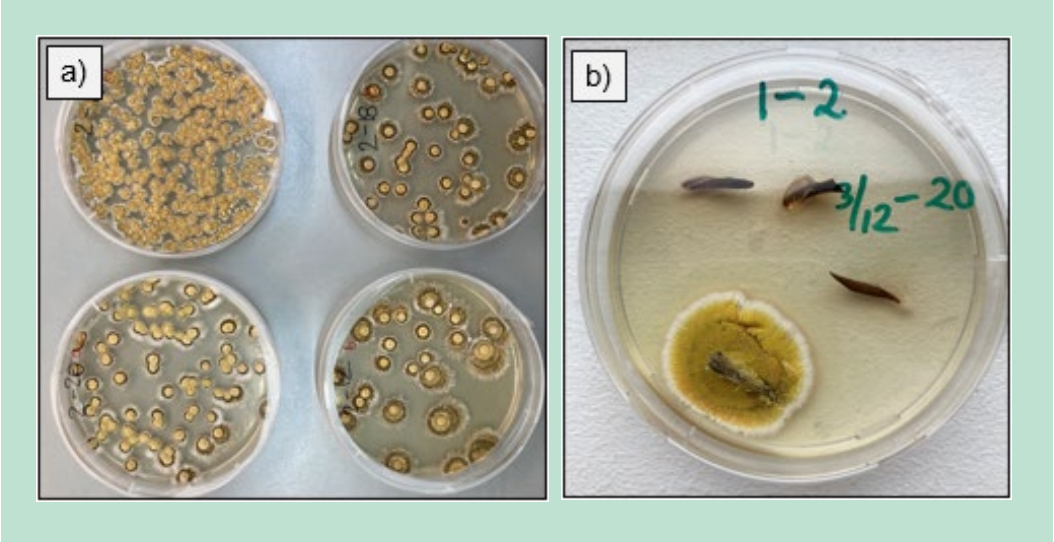

PHOTO 3. Colonies of Metarhizium forming on selective media plates after plating suspensions of strawberry root homogenate from the M. brunneum treatments (a). Surface sterilized stem pieces on selected media with one piece showing growth of Metarhizium (b).

5.1.2 Effects of combined treatments of EPF and predatory mites on spider mite populations

Previous testing of the three *M. brunneum* isolates KVL12-30, KVL12-37, and KVL16-36 on the population growth of *T. urticae* (section 5.1.1) indicated highest potential of KVL16-36 in reducing the population growth of *T. urticae*. Therefore this isolate was selected for the experiment of combining micro- and macrobiological control agents. Strawberry plants were prepared in pots (as described in 5.1), and placed individually inside plastic boxes and under growth conditions as described in 5.1.1. To investigate the effects of the combination of EPF and the predatory mite, *N. cucumeris*, on the population growth of *T. urticae*, four treatments were tested:

- *M. brunneum* KVL16-36
- *N. cucumeris*
- *M. brunneum* KVL16-36 + *N. cucumeris*
- Control (no application of biocontrol agents)

Boxes were placed in a random block design, with ten plants per treatment of three temporal repetitions (n=30 plants per treatment). On day 0, plants with the fungal treatment (KVL16-36), either alone or to be combined with *N. cucumeris*, were applied a spore suspension ($1x10^7$ spores/ml) of 20 ml, by drenching around the stem area. The remaining plants received a similar amount of drenching with 0.05% Triton-X. On day 7, three adult females of *T. urticae* were introduced per whole plant (Photo 1b). The population growth on each plant was monitored by counting of *T. urticae* approximately every four days. On day 20, the numbers of *T. urticae* were counted for each plant, using a magnifying glass, and eight adults of *N. cucumeris* were released per plant. The predatory mites had been starved 24 h prior to the experiment, by placing them individually in 1.5 ml Eppendorf tubes with moist filter paper, at 20°C. At 24 h after release of *N. cucumeris*, the number of *T. urticae* were counted again under a magnifying glass, and subsequently each leaf was placed under a dissection stereomicroscope (16-40x) to enable a more exact quantification of the specific *T. urticae* stages (eggs, nymphs, adults) per plant.
At the end of the experiment, fungal colonization of the roots was checked by placing 2 cm pieces of root from plants from all treatments (collected from plants as described in 5.1.1), onto Petri dishes with selective media, to confirm presence of fungal colonization in a qualitative manner. The plates were incubated at 22ºC, in darkness, and checked after five and ten days for development of *Metarhizium* around the root pieces.

5.2 Testing combined treatments of EPF and predatory mites in commercial strawberry tunnels

5.2.1 Field sites

The combined treatments tested in the laboratory trials was subsequently tested in a field experiment during the entire season of strawberry tunnel production. Two experimental sites were chosen in collaboration with project partner Nauja L. Jensen from HortiAdvice A/S. The requirements to field sites were use of similar strawberry cultivar, tunnel type, and overall management. Both selected sites had plastic tunnels with the cv. 'Favori', placed in tunnels of six rows, on table tops with coir substrate and drip irrigation (five drip holes per m row). The experimental tunnels were located between two neighbouring tunnels at each site (Photo 4). Strawberry plants were planted in late March 2021 (Minitray plants), covered with non-woven fabric until mid-April. Site 1 (Skælskør) had eight plants per running meter, and site 2 (Glumsø) seven plants per running meter.

PHOTO 4. Strawberry plastic tunnel, entrance (Site 1), with six rows of strawberry plants on table tops.

Both sites were managed according to principles of conventional production. During the experimental period, growers were not permitted to apply chemical insecticides or acaricides in the treatment plots, while use of fungicides, fertilization, etc. were permitted. See Table 1 for general management procedures and applications conducted by the grower at each site during the experimental period. Due to a very high level of insect infestation at site 1, it was necessary to apply a chemical insecticide treatment in the middle of the experimental period.

TABLE 5. Management procedures, including chemical applications and active ingredients, conducted by the growers at each of the two sites, during the production season of 2021.

Week	Site 1	Site 2
15	Candit (fungicide, Mildew) Kresoxim-methyl, 0,2 kg/ha	
16		Flexity (fungicide, Mildew) Metrafenone, 0,3 l/ha
17	Flexity (fungicide, Mildew) Metrafenone, 0,3 l/ha	
18	Switch 62.5 WG (fungicide, Grey mould) Cyprodinil+Fludioxonil, 1 kg/ha	Signum (fungicide, Grey mould) Boscalid+pyraclostrobin, 1,8 kg/ha
19	Scala (fungicide, Grey mould) Pyrimethanil, 2 l/ha	Geoxe 50 WG (fungicide, Grey mould) Fludioxonil, 0,5 kg/ha

20		
21	Serenade ASO* (fungicide, mildew/grey mould) Bacillus amyloliquefaciens QST 713, 4 l/ha	
22	Vacciplant (Laminarin, 0,75 l/ha)**	
23		
24		
25		
26	Conserve (insecticide, SWD)***, Spinosad, 0,45 l/ha; leaf thinning	Floramite 240 SC (Acaricide, Spider mite) Bifenazate, 0,4 l/ha (NB: mistakenly applied to plot no. 14 and 16)
27		
28	Conserve (insecticide, SWD), Spinosad, 0,45 l/ha	Leaf thinning

*Applied every two weeks hereafter (max. six times)
**Applied every two weeks hereafter interchangeably (Vacciplant max. seven times)
***Dispensation to apply Conserve against Spotted Wing Drosophila (SWD) from 1 June – 28 September 2021

The experimental design consisted of four blocks, two blocks per site, with each block consisting of four treatment plots (n=16 treatment plots in total). Each block was 20 m long and six rows wide, and the outer two blocks located five meter from the open end of the tunnel. Each treatment plot was 10 meter long and three rows wide (Fig. 1). Outside the open ends of both experimental tunnels, there was an area of 10-20 m of bare soil/grass, followed by a hedgerow of trees and herbaceous plants.

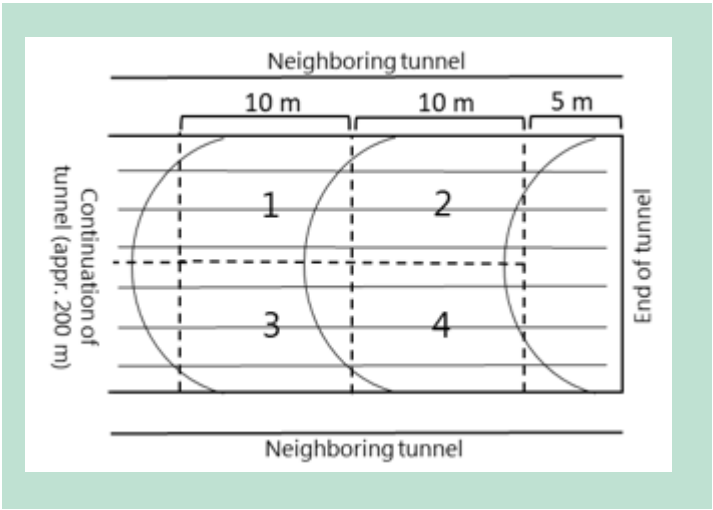

FIGURE 1. Graphical overview of one experimental block with four treatment plots (1-4), indicated by dotted lines, and strawberry rows indicated by full lines within the tunnel. Each plot was ten m long and three rows wide. Outermost plot was five m from the edge of the tunnel.

5.2.2 Production and application of biocontrol agents

The biocontrol agents applied to the treatment plots were those similar to laboratory trial 5.1.2; the EPF *M. brunneum*, strain KVL16-36, and the predatory mite *N. cucumeris*. Treatments included: 1) KVL16-36, 2) *N. cucumeris*, 3) KVL16-36 + *N. cucumeris*, and 4) Control (no application of biocontrol agents). All four treatments were represented within each block (Fig. 2).

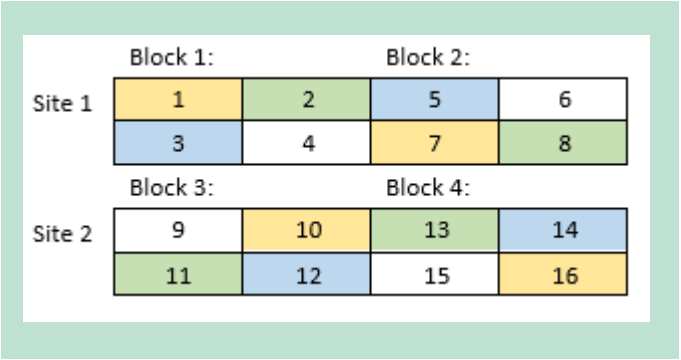

FIGURE 2. Location of treatment plots. Plots are indicated by numbers (1-16), within the two blocks (1-4), at site 1 and 2. Plot 1, 3, 9 and 11 were located near tunnel ends. Placement of the four treatments are indicated with colours: KVL16-36 (green); N. cucumeris (white); KVL16-36 + N. cucumeris (yellow), and Control (no application of biocontrol agents) (blue).

The EPF KVL16-36 was applied as a liquid drench according to permission from the Danish Environmental Protection Agency (J.nr. 2021-6580) to the coir substrate in the beginning of the experimental period, in mid-April, according to the guidelines of the permission. The experimental spore suspension ($1x10^7$ spores/ml, 20 ml spore suspension per plant) was applied around the stem of each strawberry plant in the treatment plots including KVL16-36. The spore suspension was prepared in the laboratory the day prior to field application, by the methodology described in 5.1. Containers were labelled according to the permission guidelines. The predatory mites were released in the following week, and subsequently either every week or every second week, at 400 *N. cucumeris* per m row, based on recommendations of treatment frequency and dose from HortiAdvice A/S (see Table 2 for timing of release). A higher dose than typically released by growers was chosen, to test for potential increased benefits. The predatory mites were provided by EWH BioProdution ApS shortly before field release, in containers of *N. cucumeris* mixed with vermiculite. The predatory mites were applied by evenly distributing the mix throughout all rows of strawberry plants in the treatment plots with predatory mites, from above the plant canopy. The last row meter from the edge of each treatment plot were not treated to create a buffer zone to the neighbouring plot.

TABLE 2. Time schedule for application of biocontrol agents (treatments), arthropod assessments, fruit damage, and sampling of substrate for re-isolation of EPF, in the strawberry production season of 2021, week 15-39. All activities marked with 'X'

	Treatments		**Assessments**			
Week no.	Application of EPF	Release of N. cucumeris	Visual observations, aphids	Leaf sampling, T. urticae	Flower and fruit sampling, thrips	Fruit damage assessment
15	X					
16		X	X	X	*	
17		**				
18		X				
19		X	X	X	X	
20		X				
21		X	X	X	X	
22		X				
23		X	X	X	X	X
24		X				
25		X	X	X	X	
26		X				

27	X	X	X	X	
28	X				
29		X	X	X	
30	X				
31					
32	X	X	X	X	X
33					
34					
35	X	X	X	X	

*sampling was not possible, too early for flower and fruit development.
**missing company delivery of predatory mites.

5.2.3 Field assessments

In the growth season of the strawberries, assessments of arthropods, fruit damage, and end of season detection of EPF presence in the substrate were conducted (Table 2). The arthropod groups included; the two spotted spider mite (*T. urticae*), aphids (Aphididae), thrips (Thripidae), and predators (Insects and Arachnids). The arthropods were monitored on the strawberry plants by different methods, suitable for each arthropod group.

5.2.3.1 Sampling of *T. urticae*, thrips, aphids, and predators

Sampling of *T. urticae* was done nine times during the production season, approximately every second week (Table 2), in the period from before the first flowering until after the final harvest of the season. On each occasion, five samples were collected from each plot, representing all three rows of the treatment plot, with minimum 1 m between samples, and at least 1 m from the edge of the plot. Each sample consisted of 10 fully-grown strawberry leaflets, randomly chosen from one meter of strawberry plants (representing approximately seven plants) across the three rows (at each sampling date per site: n=10 samples per treatment, representing 100 leaflets per treatment). Very young and very old leaves were avoided. One sample, consisting of 10 leaflets, was placed in a paper bag, closed with a pin, and transported to the laboratory in a cooling box. Samples were stored at 4°C for maximum two days. Eggs, immatures, and adults of *T. urticae* were counted separately on each leaflet, under a dissection stereo microscope (16-40x magnification).

Sampling of thrips were conducted eight times by collecting flowers, and seven times from collected fruit, during the production season (Table 2), from the time of first flowering and first fruit set, until the time after the final harvest. On each sampling date, ten flowers and ten fruit were sampled from each experimental plot. Each sample consisted of either ten flowers or ten fruit, randomly collected from each plot, representing plants from all three rows of the plot, with minimum 1 m between the flower/fruit, and at least 1 m from the edge of the plot. Each sample, consisting of ten flowers or fruit, was placed in a falcon tube (50 ml) containing 70% ethanol (at each sampling date per site: n=four samples per treatment; two with flowers and two with fruit, i.e. n=20 flowers and 20 fruit per treatment). In the laboratory, each sample was rotated by hand to release arthropods from the flower/fruit and poured into a funnel with filter paper. After draining the ethanol, immatures and adults of thrips and predatory mites were counted by observing the filter paper under a stereo microscope (16-40x). Previous pilot studies had confirmed that all arthropods would be released from the flower/fruit into the ethanol solution and retained on the filter paper after filtering.

Abundance of aphids and predators were assessed nine times during the production season by visual observations at the same sampling dates as for *T. urticae* and thrips (Table 2). At each

sampling occasion, ten strawberry plants per plot were visually investigated for presence of aphids, predators, and other arthropods of relevance (e.g. larval feeding, mummies of parasitized aphids). Observations were made by randomly selecting ten plants in each plot, representing all three rows of the plot, with minimum 1 m between the observed plants, and 1 m from the edge of the plot. Each selected plant was initially observed without touching the plant, i.e. not causing arthropods to fly or drop from the plant, thereafter by carefully moving leaves, to ensure observation of all plant parts. Finally, the centre of the plant, with newly emerged strawberry leaflets, were observed, as this is often the initial location of aphid colony development. Aphids were identified to species. If on-site identification was not possible, specimens of the aphid were collected and identified in the laboratory under a stereo microscope (16-40x). Predators were observed to main systematic group, order, family or genus, with focus on Araneae (spiders), *Orius* spp., *Anthocoris* spp., *Cantharis* spp., Coccinellidae (ladybirds), larvae of Syrphidae (hoverfly) and *Chrysoperla* spp. (lacewing). Parasitoids (Hymenoptera) were not further identified. At each observation date, the numbers of leaves, flowers and fruit per observed plant were also recorded.

5.2.3.2 Damage assessments

Assessments of strawberry fruit damage were done two times during the production season, in June (week 23) and in August (week 32) (Table 2), shortly before main harvest periods. At each assessment, ten plants were randomly selected per plot, representing all three rows of the plot, with minimum 1 m between plants, and 1 m from the edge of the plot. On each plant, ten fruits, evenly distributed on the plant, were assessed for symptoms of damage within the following categories: deformed, symptoms of disease (fungal, bacterial), and fruit bronzing (typical damage from thrips). For each assessment date sample sizes were: n=100 fruit assessed per plot, n=200 fruit assessed per treatment per site.

5.2.3.3 Re-isolation of EPF from coir substrate in strawberry tunnels

At the end of the production season (in week 37), two weeks after the final assessment of arthropods, samples of roots and the coir substrate from all plots of the experimental strawberry tunnels were collected to test for presence of active *Metarhizium*. The substrate was sampled by randomly selecting one plant per row per plot (n=3 samples per plot) with roots, by cutting out a square around the roots to extract a block of approximately 10x10x10 cm. Any remaining foliage was removed, and the substrate block with roots was placed inside individual plastic bags. The bags were transported back to the laboratory and stored at 4°C until further use (maximum three weeks). Two tests were conducted to detect presence of *Metarhizium* from the field-collected substrate: an insect baiting test of the substrate, and plating of root homogenate suspension on selective agar media.

The insect baiting test was conducted with each sample of substrate, by adding an adequate amount of substrate into a plastic container (155 ml), leaving 1-2 cm airspace between the substrate and the lid of the container. The lid was ventilated by poking holes with a needle. Larvae of *Tenebrio molitor* were obtained from the company Avifauna and kept in a plastic box provided with organic rolled oats and pieces of potato. Ten *T. molitor* larvae were added to each cup, which were kept at room temperature in darkness inside a cardboard box. The cups were inverted approximately every second day to ensure larval movement through the substrate. The samples were checked for larval mortality every week, for three weeks, by transferring the content of the container into a tray, counting and checking viability of all larvae (dead or alive). Dead larvae were removed and potential sporulation identified by individually incubating the dead larvae in medicine cups (33 ml), mounted with lids and provided with a moist filter paper to ensure humidity.

The test of root homogenate suspension was conducted as described in section 5.1.1., with roots from the same samples as the soil baiting test. For both tests, *Metarhizium* colonies were identified based on morphological characteristics of hyphal growth and developing of characteristic green conidia.

5.2.4 Statistical analyses

All statistical analyses are conducted in R, version 4.1.2 (R Core Team, 2021), by generalized linear models (GLM, R package: *base*), or generalized linear mixed models (GLMM, R package: *lme4*) for nonparametric data, unless otherwise stated. Data of CFUs and insect bait from root and substrate tests were conducted in SAS, version 9.4 (SAS Institute 2016). Post-hoc analyses of pairwise comparisons (R package: *emmeans*'), were conducted when the statistical output showed overall differences of dependent variables. Kruskal-Wallis tests were conducted to check for differences between repetitions, by one dependent variable with two or more levels, of non-parametric data. A significance level of $P<0.05$ was used in all analyses.

Impact of below-ground inoculation of *M. brunneum* on spider mites:

A generalized linear mixed model, with a Poisson distribution and logistic regression (log link), was selected to analyse the effect of treatment, with number of *T. urticae* eggs or nymphs as the response variable, treatments (KVL12-30, KVL 12-37, KVL16-36, control) and day (day 0, 3, 6, 9, 12, and 14) as dependent variables, and individual plant as random factor.

The summed numbers (+1) of CFUs on the two replicate selective media plates for each root homogenate suspension (representing 200 µl of suspension) were log10-transformed and analysed as in Klingen et al. (2015) by mixed models in PROC MIXED in SAS with random effects of experimental repetition and adjusting degrees of freedom by Satterthwaite formulae (Littell et al., 1996). Since no CFUs of *Metarhizium* were observed in the plates of the control treatment, these data were omitted for further analyses and only data for the three fungal treatments were analysed. Data were analysed for fixed effects of treatment (fungal isolate KVL12-30, KVL 12-37, KVL16-36). Significant differences were compared by LSMEANS and adjusted by the Tukey-Kramer adjustment to identify pair-wise differences.

Effects of combined treatments of EPF and predators on populations of *T. urticae*:

The analyses of *T. urticae* counts before and after release of predatory mites were based on data at time 0 and 24 h, and data from a final count of *T. urticae* at the end of the experiment. As the number of *T. urticae* were not normally distributed (Shapiro-Wilk normality test: $P<0.0001$), a Wilcoxon signed rank test was chosen to analyse the number of *T. urticae* per plant (all stages combined) before and after release of *N. cucumeris*, with treatment (KVL16-36, KVL16-36+*N. cucumeris*, *N. cucumeris*, control), and time point (0 h, 24 h) as fixed variables, followed by pairwise comparisons. Absolute differences (number of *T. urticae* after, at 24 h minus before, at 0 h) between the four treatments at time 0 h and 24 h, was analysed by a Kruskal-Wallis test.

The final count of *T. urticae*, of specific developmental stages, were analyzed by a GLM with number of *T. urticae* eggs, nymphs, or adults as the response variable, and treatments (KVL16-36, KVL16-36+*N. cucumeris*, *N. cucumeris*, control) as dependent variable, using a Poisson distribution.

Testing combined treatments of EPF and predators in commercial strawberry tunnels:

Differences in arthropod abundance between locations of field sites were analysed separately for each relevant organismal groups (*T. urticae*, thrips, aphids, natural enemies), by a GLMM with a Poisson distribution and logistic regression (log link), with number of organism as response variable, treatment (KVL16-36, KVL16-36+*N. cucumeris*, *N. cucumeris*, control) and time (week) as fixed variables, and experimental plot as random effect. Due to the high variation in data, models were adjusted with nAGQ (number of adaptive Gauss-Hermite quadrature points) when necessary. By this, the interaction with random effects are reduced, but the adjustment provides a better overall model fit. In those cases, pairwise comparisons can be affected by interaction effects and were therefore not considered further.

The number of *T. urticae* were analysed as the total number per leaflet, as well as adults, nymphs and eggs in separate analyses, as the response variable. Thrips and predatory mites were analysed as number per sample (ten fruit or ten flowers), thrips as either adults or larvae, in separate analyses, while aphids and predators (from visual observations) were analysed as

number per plant. In all models, treatment (KVL16-36, KVL16-36+*N. cucumeris*, *N. cucumeris*, and control) and time (week) was included as independent variables, with experimental plot as random effect. Differences between sites were in all cases checked; based on this (significance level $P<0.05$), the data for each site were analysed separately.

Fruit damage assessments were analysed by GLMM with a binomial distribution, as proportion of fruits with symptoms of pest damage (bronzing from thrips) at each time point (June, August), with treatment (KVL16-36, KVL16-36+*N. cucumeris*, *N. cucumeris*, control) as independent variable, total number of fruits assessed as weighted argument, and plot as random effect.

As for the previously described root test of the three fungal isolates, data for *Metarhizium* CFU counts were summed (+1 as log transformation was used) for the two replicate selective media plates for each root homogenate suspension (representing 200 µl of suspension), log10-transformed and analysed by mixed models in PROC MIXED in SAS. Experimental block was included as random factor and degrees of freedom were adjusted by Satterthwaite formulae (Littell et al., 1996). In the model, treatment, location (site 1 and 2) and their interaction were included as fixed factors. Significant differences were compared by LSMEANS and adjusted by the Tukey-Kramer adjustment to identify pair-wise differences.

Proportions of *Metarhizium* infected *T. molitor* larvae (x/10) in each substrate sample were analysed by a logistic regression model with random effect of experimental block using PROC GLIMMIX in SAS as in Meyling et al. (2013). In order to meet model requirements, the proportions were subjected to arcsin-transformation prior to analysis. The model tested for fixed effects of treatment, location and their interaction. Significant effects ($P< 0.05$) were tested by lsmeans and adjusted by the Tukey-Kramer adjustment to identify pair-wise differences.

6. Results

6.1 Combinations of biocontrol agents on pest reproduction and population growth

6.1.1 Effect of fungal isolates on population development of *T. urticae*

Application of the three *M. brunneum* isolates under controlled conditions by belowground-inoculation resulted in overall differences between treatments in the number of eggs laid by *T. urticae* females on strawberry leaflets inside clip cages. On average, fewer eggs were laid on leaflets of plants treated with isolate KVL16-36 compared to the control treatment (P0.0001, z=-3.65). There was no difference in number of *T. urticae* eggs laid between KVL12-37 (P=0.40, z=-0.84) and KVL12-30 (P=0.69, z=0.40) compared to the control. Pairwise comparisons showed that the numbers of *T. urticae* eggs per leaflet were lower on plants treated with KVL16-36 compared to the control (P=0.002, z=3.65), to KVL12-30 (P<0.001, z=4.04), and to KVL12-37 (P=0.03, z=2.80). None of the other treatments were significantly different from each other (control-KVL12-30: P=0.98, z=-0.40; control-KVL12-37: P=0.83, z=0.84; KVL12-30-KVL12-37: P=0.60, z=1.24) (Fig. 3).

During the course of the 14 days, the average number of *T. urticae* eggs peaked at day six after introduction of female adult *T. urticae*, compared to the other time points (P<0.05), where after the number of eggs laid continuously reduced throughout the time period (Fig. 3).

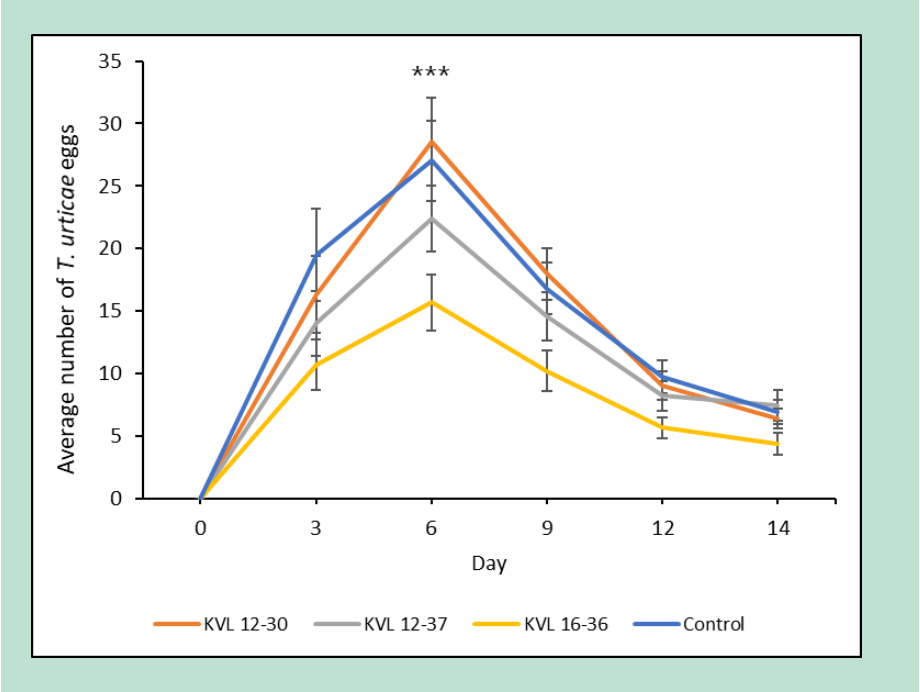

FIGURE 3. Average number ±SE of T. urticae eggs per leaflet inside clip-cage, over time, on strawberry plants with treatments of three isolates of the entomopathogenic fungus M. brunneum: KVL12-30, KVL12-37, and KVL16-36, or control treatment (no fungus). Females were intro-duced on day 0, and eggs were counted on day 3, 6, 9, 12, and 14. ***Pairwise comparisons between time points, peak at day 6: P<0.0001.

Similar to the results of *T. urticae* eggs, there were fewer nymphs in the clip cages on plants treated with *M. brunneum* isolate KVL16-36 compared to the control treatment (P=0.009, z=-

2.58), while there was no difference in number of *T. urticae* nymphs between the control and KVL12-30 (P=0.92) and KVL12-37 (P=0.45) (Fig. 4).

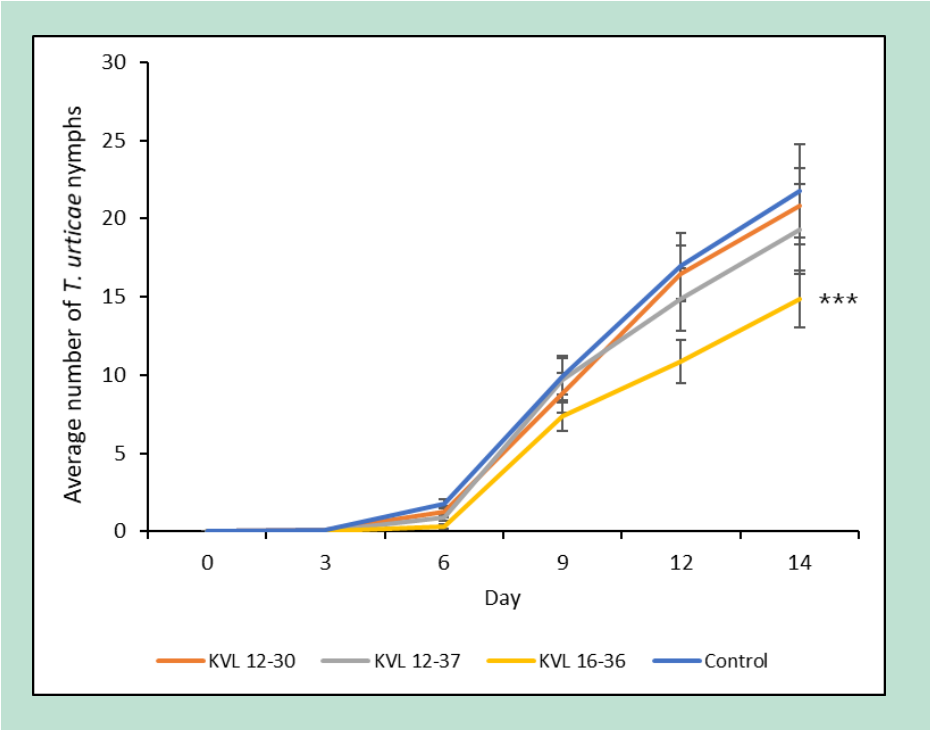

FIGURE 4. Average number ±SE of T. urticae nymphs per leaflet in clip-cage, over time, on strawberry plants with treatments of three isolates of M. brunneum: KVL12-30, KVL12-37, and KVL16-36, or control treatment (plants without fungus). Females of T. urticae were introduced on day 0, and nymphs were counted on day 3, 6, 9, 12, and 14. *** Overall differences between treatments (KVL16-36 lower than the three other treatments: P=0.009, z=-2.58).

6.1.2 Fungal colonization of strawberry roots

Selected washed root pieces were assessed for presence of active *Metarhizium* after homogenizing and plating of standardized suspension on selective agar media in duplicate, which allowed for semi-quantification of fungal density by counting colony forming units (CFU) after incubation. No *Metarhizium* CFU were observed on the selective media plates of root homogenate collected in the control treatment, while *Metarhizium* CFU were observed on all plates from the three treatments of different *M. brunneum* isolates. Hence, the control treatment observations were not included in the statistical analysis where the three *M. brunneum* isolates could be compared.

The observed *Metarhizium* CFU numbers ranged between 1 and 194 CFU per plate for KVL12-30, between 11 and 500 CFU per plate for KVL12-37, and between 11 and 486 CFU per plate for KVL16-36. A significant effect of *M. brunneum* isolate was found when observations were transformed by log10 (CFU+1) and analysed by generalised mixed linear model with inclusion of the three experimental repetitions as random factor (F=5.77; d.f.=2.85; P=0.0045). The mean values are presented in Figure 5 showing that the quantity of CFU for the KVL12-30 isolate was significantly lower than for the two other *M. brunneum* isolates, KVL12-37 and KVL16-36.

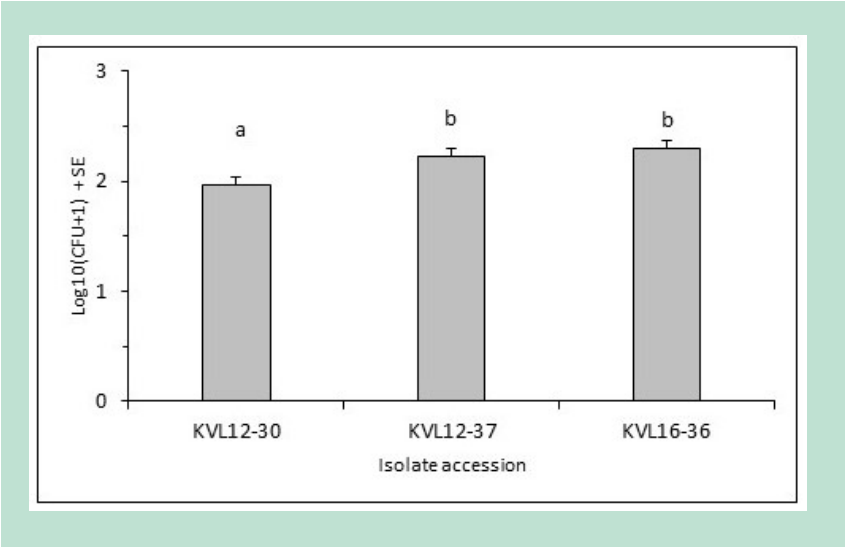

FIGURE 5. Mean value (+SE) of log10 (CFU+1) of Metarhizium observed per 200 µl root homogenate suspension plated on selective agar media. Different letters above bars indicate significantly different values (Tukey-test, P<0.05).

6.1.3 Effects of *M. brunneum* inoculation belowground and release of predators aboveground

There was variation in the numbers of *T. urticae* on each plant in all four treatments, while a trend within each treatment could be detected with few outliers indicating reduced *T. urticae* numbers after 24 h exposure to the predatory mite *N. cucumeris*. Across all treatments, data ranged from 22 to 263 *T. urticae* per plant at time 0 h, and from six to 232 *T. urticae* per plant at time 24 h.

Among treatments, fewer *T. urticae* were found in the treatment of KVL16-36+*N.cucumeris* ($p<0.0001$), and in the treatment with *N. cucumeris* ($p<0.0001$), at 24 h after release of the predatory mites than at time 0 h (Fig. 6a). There was no differences between the two time points in the treatments with KVL16-36 ($p=0.053$) and the control ($p=0.280$) where no predatory mites were released.

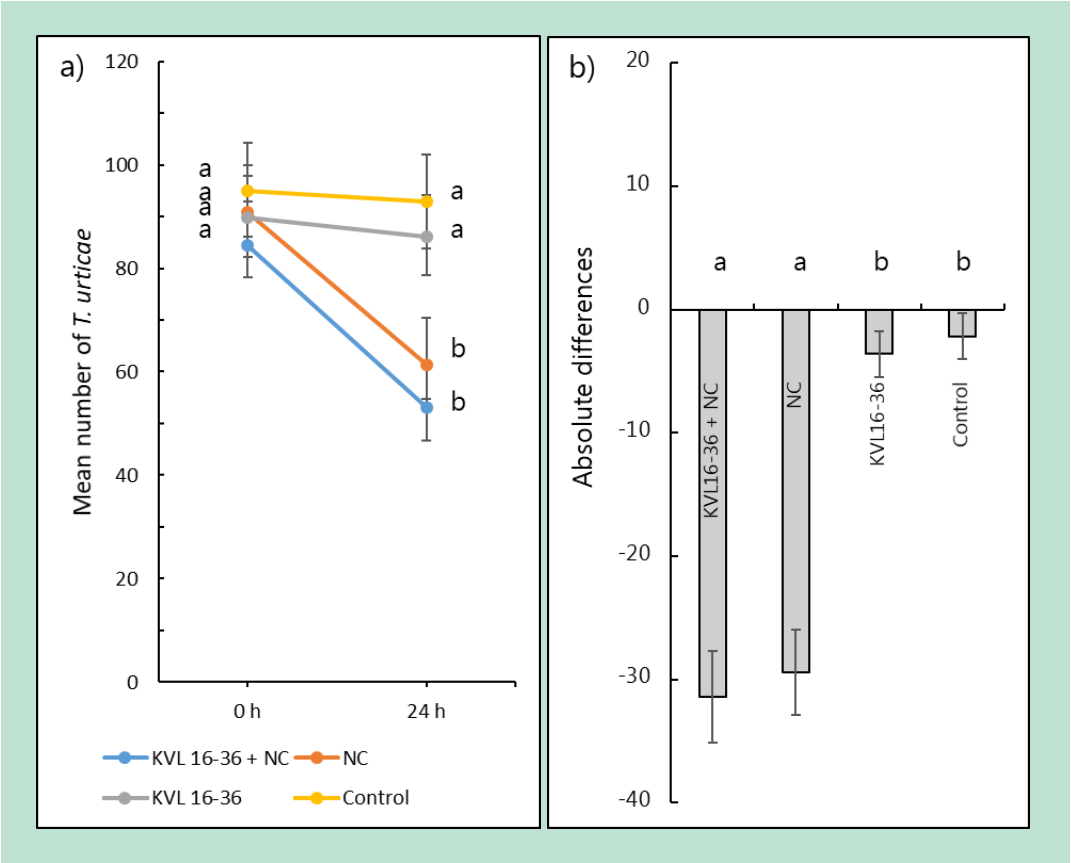

FIGURE 6. Mean number ±SE of T. urticae per plant before (0 h) and after (24 h) release of the predato-ry mite N. cucumeris (NC) (a), and absolute differences ±SE in number of T. urticae before and after release of the predatory mite N. cucumeris (NC) (b), in four different treatments: 1) KVL16-36+NC, 2) NC, 3) KVL16-36, 4) Control. Different letters at points indicate significant-ly different values (P<0.05).

Due to the variable population sizes of *T. urticae* at time 0 h, the difference in numbers between time points on each plant was considered as a response variable as well (Fig. 6b). The number of *T. urticae*, analysed as the difference in number from time 0 h to 24 h, i.e. the absolute differences (the number of *T. urticae* after 24 h subtracted by the number of *T. urticae* at 0 h) was lower at 24 h than at 0h (P<0.0001). The difference was significant between the treatment with *N. cucumeris* and the control (P<0.001), between KVL16-36+*N. cucumeris* and the control (P<0.001), between *N. cucumeris* only and KVL16-36 only (P<0.001), and between KVL16-36 only and the combination KVL16-36+*N. cucumeris* (P<0.001) (Fig. 6b). There were no differences between KVL16-36 and the control (P=0.97), and between KVL16-36+*N. cucumeris* and the treatment with *N. cucumeris* only (P=0.83).

At the end of the experiment, after 24 h, the final count of the specific stages of *T. urticae* (eggs and nymphs) under a dissection stereomicroscope showed an overall lower number of *T. urticae* eggs on plants treated with *N. cucumeris* (P<0.0001), and the combined treatment KVL16-36 + *N. cucumeris* (P<0.0001) compared to the number of *T. urticae* eggs on the control plants. In contrast, the treatment with KVL16-36 alone contained same number of *T. urticae* eggs as in the control treatment (P=0.16) (Fig. 7a).
However, when evaluating only *T. urticae* nymphs, there were significantly fewer nymphs on plants treated with KVL16-36 (P<0.0001), *N. cucumeris* (P<0.0001), and with the combined treatment KVL16-36 + *N. cucumeris* (P<0.0001) than on the control plants. A gradual reduction of *T. urticae* nymphs was observed in the pairwise comparisons, i.e. KVL16-36+*N. cucumeris* < *N. cucumeris* < KVL16-36 < Control (P<0.001) (Fig. 7b).

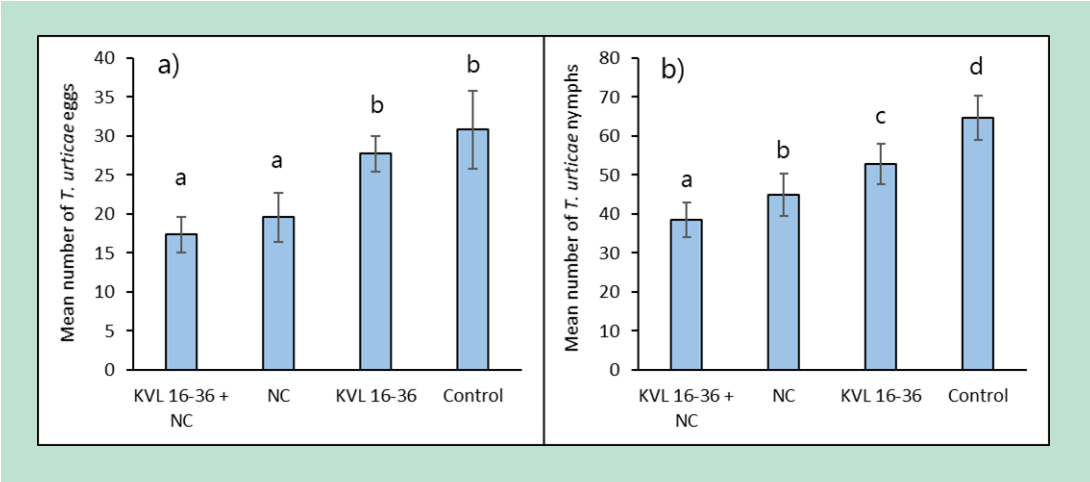

FIGURE 7. Mean number ±SE of T. urticae eggs (a), and nymphs, per plant (b), in the treatments: KVL16-36+NC, NC, KVL16-36, and the control. KVL16-36=M. brunneum, NC=N. cucumeris. Different letters above bars indicate significant differences between treatments

6.2 Effects of combined and single biological control on pest populations in commercial strawberry tunnels

6.2.1 The two spotted spider mite, *T. urticae*

Overall, more *T. urticae* individuals were sampled at site 2 (22.7±1.1 *T. urticae* per sample) than at site 1 (7.0±0.3 *T. urticae* per sample) (P<0.0001), and thus analysed separately. See table 3 for the distribution of the mean number of *T. urticae* per sample between treatments.

TABLE 3. Mean number ±SE of *T. urticae* per sample (one sample=ten leaflets), across the entire sampling period, at site 1 and site 2 in each treatment: Control, KVL16-36, NC, and KVL16-36+NC. KVL16-36=*M. brunneum*, NC=*N. cucumeris*.

	Control	KVL16-36	NC	KVL16-36 + NC
Site 1	8.3 ±0.8	5.1 ±0.5	8.5 ±0.7	5.8 ±0.6
Site 2	28.7 ±2.4	18.2 ±1.7	15.0 ±1.4	28.8 ±2.8

At site 1, there were fewer *T. urticae* (total number of adults, nymphs and eggs) in plots treated with KVL16-36 than in the control (P=0.036), while there was no difference between plots treated with *N. cucumeris* (P=0.906) and KVL16-36+*N. cucumeris* (P=0.175) compared to the control (Fig. 8). The number of *T. urticae* started to increase around week 23 in the control plots, around week 25 in plots treated with *N. cucumeris*, and finally increased around week 26-27 in plots with the two treatments with KVL16-36 (Fig. 8).

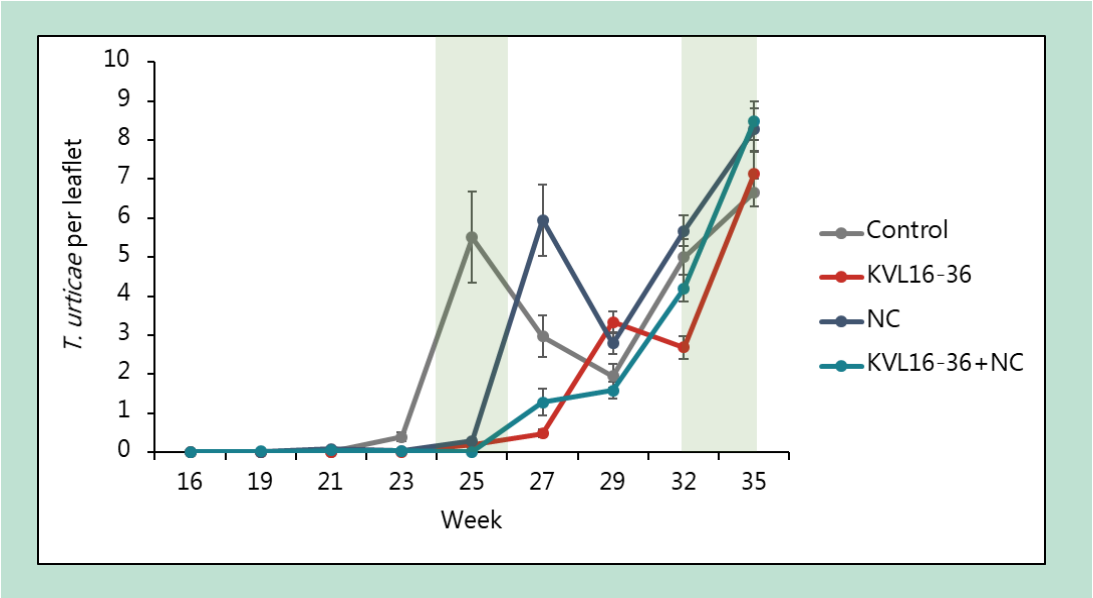

FIGURE 8. Mean number of T. urticae per leaflet (±SE) in strawberry tunnels, site 1. Abundance of spider mites was recorded every two or three weeks, from week 19 to 35, in plots of treatments: KVL16-36+NC, NC, KVL16-36, and Control. KVL16-36=M. brunneum, NC=N. cucumeris. Green areas indicate peak harvest times.

Considering the developmental stages of *T. urticae*, fewer adults were found in the treatments KVL16-36 (P<0.001) and KVL16-36 + *N. cucumeris* (P=0.002) compared to the control (Table 4). No differences were found between the control and the treatment with *N. cucumeris*.
The number of *T. urticae* nymphs did not differ between treatments (KVL16-36: P=0.12; KVL16-36 + *N. cucumeris*: P=0.10; *N. cucumeris*: P=0.95), compared to the control, nor was there any differences found between weeks (P=0.99-1.0) (Table 4).
Fewer eggs of *T. urticae* was sampled from plots treated with KVL16-36 compared to the control (P=0.03), while no differences were found between KVL16-36 + *N. cucumeris* (P=0.32) and *N. cucumeris* (P=0.90), and the control (Table 4).

TABLE 4. Mean number of adults, nymphs and eggs of *T. urticae* per leaflet ±SE, at site 1, sampled in week 16-35, in treatments KVL16-36+NC, NC, KVL16-36, and Control. KVL16-36=*M. brunneum*, NC=*N. cucumeris*.

	Week	16	19	21	23	25	27	29	32	35
Control	Adults	0	0	0	0.3 ±0.09	0.8 ±0.17	0.1 ±0.02	0.1 ±0.01	0.2 ±0.02	0.4 ±0.03
	Nymphs	0	0	0	0.1 ±0.02	1.7 ±0.51	0.7 ±0.14	0.9 ±0.13	1.3 ±0.08	1.1 ±0.1
	Eggs	0	0	0	0.1 ±0.01	3.1 ±0.63	2.1 ±0.37	1.0 ±0.18	3.5 ±0.39	5.3 ±0.3
KVL16-36	Adults	0	0	0	0	0	0	0.1 ±0.01	0.1 ±0.02	0.4 ±0.03
	Nymphs	0	0	0	0	0.1 ±0.03	0.2 ±0.04	1.4 ±0.11	0.7 ±0.08	1.0 ±0.1
	Eggs	0	0	0	0	0.1 ±0.02	0.3 ±0.07	1.8 ±0.2	1.8 ±0.21	5.8 ±0.45
NC	Adults	0	0	0	0	0	0.5 ±0.08	0.1 ±0.02	0.2 ±0.02	0.6 ±0.04
	Nymphs	0	0	0	0	0.1 ±0.02	1.2 ±0.19	1.3 ±0.14	2.1 ±0.18	1.9 ±0.12

	Eggs	0	0	0	0.1 ±0.01	0.2 ±0.06	4.3 ±0.65	1.3 ±0.17	3.3 ±0.23	5.8 ±0.42
KVL16-36 + NC	Adults	0	0	0	0	0	0	0.1 ±0.01	0.2 ±0.03	0.4 ±0.04
	Nymphs	0	0	0	0	0	0.2 ±0.03	0.7 ±0.14	1.1 ±0.08	1.0 ±0.08
	Eggs	0	0	0	0	0	1.1 ±0.31	0.8 ±0.12	2.9 ±0.28	7.1 ±0.47

At site 2, there were overall fewer *T. urticae* (total number of adults, nymphs and eggs) in plots treated with KVL16-36 (P=0.02) and in plots treated with *N. cucumeris* (P=0.002) than in the control, whereas there was no difference between plots treated with KVL16-36+*N. cucumeris* (P=0.80) and the control (Fig. 9).

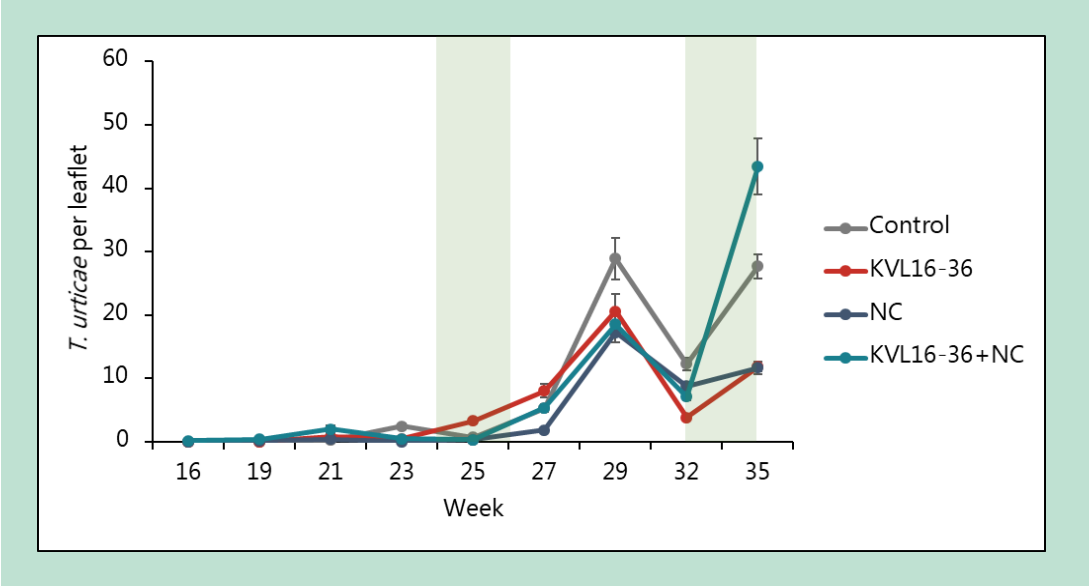

FIGURE 9. Mean number of T. urticae per leaflet (±SE) in strawberry tunnels, site 2. Abundance of spider mites recorded every two or three weeks, from week 19 to 35, in plots of the treatments: KVL16-36+NC, NC, KVL16-36, and Control. KVL16-36= M. brunneum, NC=N. cucumeris. Green areas indicate peak harvest times.

There were no overall differences in the number of *T. urticae* adults in the treated plots compared to the control plots at site 2 (KVL16-36: P=0.28; *N. cucumeris*: P=0.08; KVL16-36+*N. cucumeris*: P=0.65) (Table 5).
The number of *T. urticae* nymphs were lower in the plots treated with *N. cucumeris* than in the control plots (P=0.02), while there was no difference between treatments KVL16-36 (P=0.44), and KVL16-36 + *N. cucumeris* (P=0.71), compared to the control (Table 5).
Finally, fewer eggs of *T. urticae* were found in the treatment plots of KVL16-36 (P<0.001) and *N. cucumeris* (P=0.002), compared to the control, while no differences were found between KVL16-36 + *N. cucumeris* (P=0.91) and the control (Table 5).

TABLE 5. Mean number of adults, nymphs and eggs of *T. urticae* per leaflet ±SE, at site 2, sampled in week 16-35, in treatments KVL16-36+NC, NC, KVL16-36, and Control. KVL16-36= *M. brunneum*, NC=*N. cucumeris*.

	Week	**16**	**19**	**21**	**23**	**25**	**27**	**29**	**32**	**35**
Con-trol	Adults	0	0	0	1.1 ±0.32	0.1 ±0.02	0.2 ±0.04	1.4 ±0.08	0.9 ±0.08	7.5 ±0.76

	Nymphs	0	0	0.2 ±0.06	0.4 ±0.08	0	3.3 ±0.33	9.4 ±1.48	3.3 ±0.19	4.4 ±0.3
	Eggs	0	0	0.1 ±0.02	1.0 ±0.17	0.6 ±0.1	1.8 ±0.17	18.2 ±1.78	8.2 ±0.8	15.8 ±1.11
KVL16-36	Adults	0	0	0.2 ±0.03	0.2 ±0.05	0.4 ±0.07	1.5 ±0.25	1.1 ±0.09	0.3 ±0.03	1.0 ±0.08
	Nymphs	0	0	0.4 ±0.08	0.1 ±0.02	0.2 ±0.03	2.3 ±0.34	10.1 ±1.73	0.8 ±0.09	3.5 ±0.24
	Eggs	0	0.1 ±0.02	0.3 ±0.04	0.3 ±0.06	2.7 ±0.31	4.3 ±0.56	9.5 ±1.0	2.8 ±0.35	7.4 ±0.48
NC	Adults	0	0	0	0	0	0.2 ±0.03	1.4 ±0.07	0.5 ±0.04	1.1 ±0.08
	Nymphs	0	0	0	0	0	0.5 ±0.08	3.2 ±0.35	2.6 ±0.2	3.2 ±0.25
	Eggs	0	0.2 ±0.05	0.4 ±0.14	0	0.3 ±0.04	1.2 ±0.25	12.7 ±1.17	5.8 ±0.48	7.5 ±0.76
KVL16-36 + NC	Adults	0	0	0	0.4 ±0.11	0	0.7 ±0.12	1.4 ±0.15	0.6 ±0.05	10.1 ±1.2
	Nymphs	0.1 ±0.02	0.3 ±0.08	0.1 ±0.02	0	0	2.1 ±0.3	5.2 ±0.66	2.3 ±0.23	7.8 ±1.2
	Eggs	0.1 ±0.04	0.1 ±0.03	1.9 ±0.56	0.1 ±0.02	0.3 ±0.09	2.5 ±0.3	11.9 ±1.12	4.1 ±0.33	25.5 ±2.71

6.2.2 Thrips

The infestation level of thrips was generally higher at site 1 than at site 2 (Table 6). Flowers and fruit were sampled separately with the expectation of a higher abundance of adult thrips in flowers and larval thrips on fruit.

TABLE 6. Total number of thrips adults, thrips larvae, and predatory mites, sampled from flowers and fruit in strawberry tunnels, at site 1 and 2, during one production season (n=640 flowers per site, n=560 fruit per site).

		Thrips adults	Thrips larvae	Predatory mites
Site 1	Flower	1226	1140	37
	Fruit	111	723	362
Site 2	Flower	325	283	110
	Fruit	39	154	335

The total number of thrips larvae (P=0.029), but not adults (P=0.285), was different between site 1 and site 2. Taking into account the application of the insecticide Conserve mid-season at site 1, all thrips data was analysed separately for the two sites.

At site 1, where the thrips infestation was generally more severe throughout the season compared to site 2, there was no effect of treatment on the total number of thrips (KVL16-36: P=0.87; NC: P=0.89; KVL16-36+NC: P=0.55, versus control) (Table 5).

The mean numbers of thrips adults per 10 flowers did not differ in any of the treatments; KVL16-36 (P=0.23), *N. cucumeris* (P=0.18), and KVL16-36 + *N. cucumeris* (P=0.76), compared to the control (Fig. 10a). Likewise, there was no effect of the treatments KVL16-36 (P=0.64), *N. cucumeris* (P=0.25), and KVL16-36 + *N. cucumeris* (P=0.26), compared to the control on the number of larvae in the flowers (Fig. 10b).

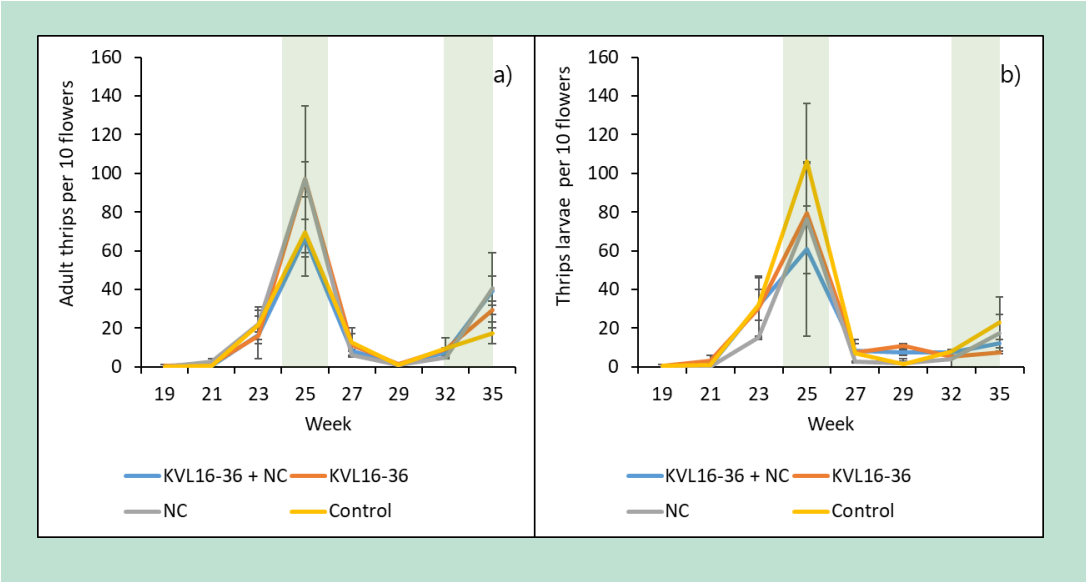

FIGURE 10. Number of adult thrips (a), and thrips larvae (b), per 10 flowers (=one sample), at site 1. Thrips were assessed at eight time points during the production season (week 19-35), in plots of the treatments: KVL16-36+NC, NC, KVL16-36, and Control. KVL16-36= M. brunneum, NC=N. cucumeris. Green areas indicate peak harvest times.

In the strawberry fruit, at site 1, the number of adult thrips per 10 fruit was similar across all treatments; KVL16-36 (P=0.96), *N. cucumeris* (P=0.42), and KVL16-36 + *N. cucumeris* (P=0.55), compared to the control (Fig. 11a). Similarly, the number of thrips larvae per 10 fruit were not affected by treatment; KVL16-36 (P=0.16), *N. cucumeris* (P=0.31), and KVL16-36 + *N. cucumeris* (P=0.27), compared to the control (11b).

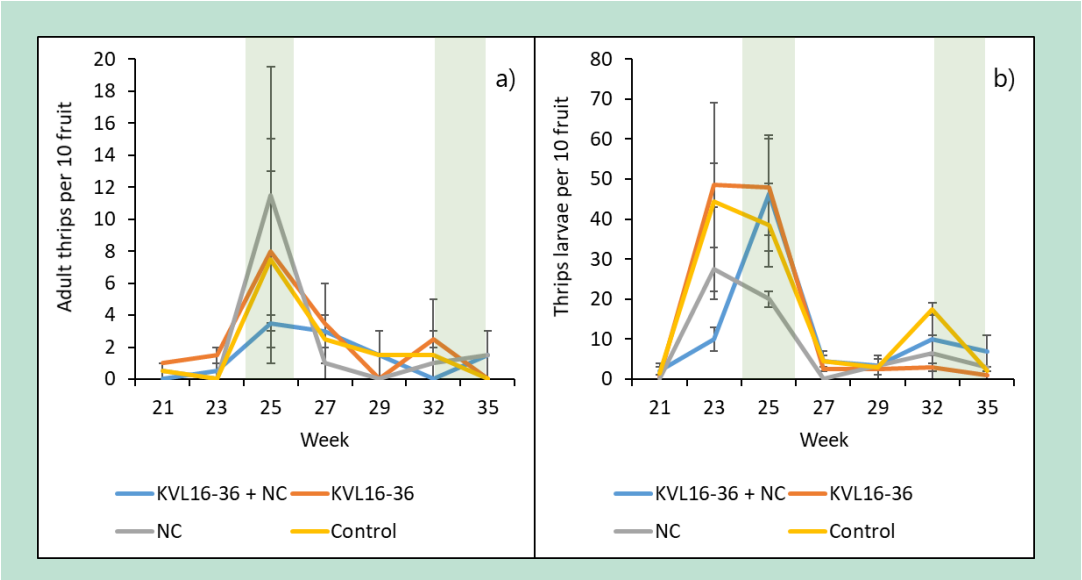

FIGURE 11. Number of adult thrips (a), and thrips larvae (b), per 10 fruit (=one sample), at site 1. Thrips were assessed at seven time points during the production season (week 19-35), in plots of the treatments: KVL16-36+NC, NC, KVL16-36, and Control. KVL16-36= M. brunneum, NC=N. cucumeris. Green areas indicate peak harvest times.

At site 2, the total number of thrips (adults and larvae) in the flowers were lower in plots treated with KVL16-36 (P=0.04) and KVL16-36 + *N. cucumeris* (P<0.001), compared to the control.

There was no difference in the total number of thrips between plots treated with *N. cucumeris* and the control (P=0.20).
Considering adults only, there were fewer adult thrips in flowers in plots with the combined treatment of KVL16-36 + *N. cucumeris*, than in the control (p<0.001), while no differences were found between plots treated with KVL16-36 (P=0.22), and plots with *N. cucumeris* (P=0.89), compared to the control (Fig. 12a).
Concerning the thrips larvae in flowers at site 2, fewer larvae were present in all treatment plots compared to the control (KVL16-36: P=0.02; *N. cucumeris*: P=0.04; KVL16-36 + *N. cucumeris*: P=0.01) (Fig. 12b).

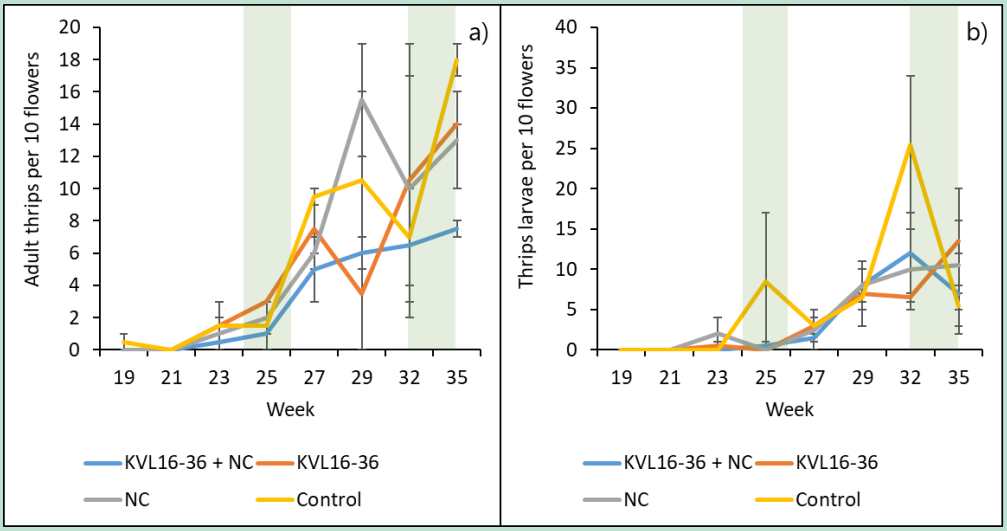

FIGURE 12. Number of adult thrips (a), and thrips larvae (b), per 10 flowers (=one sample), at site 2. Thrips were assessed at eight time points during the production season (week 19-35), in plots of the treatments: KVL16-36+NC, NC, KVL16-36, and Control. KVL16-36= M. brunneum, NC=N. cucumeris. Green areas indicate peak harvest times.

There were more thrips (total of adults and larvae) in the fruit of the control plots at site 2, compared to all other treatments (KVL16-36: P=0.03; *N. cucumeris*: P<0.0001; KVL16-36 + *N. cucumeris*: P<0.001). There were no differences in the number of adult thrips on fruit, between the treatments and the control (Fig. 13a), while fewer thrips larvae were found on the fruit in all treated plots compared to control plots (KVL16-36: P=0.002; *N. cucumeris*: P<0.0001; KVL16-36 + *N. cucumeris*: P=0.005) (Fig. 13b).

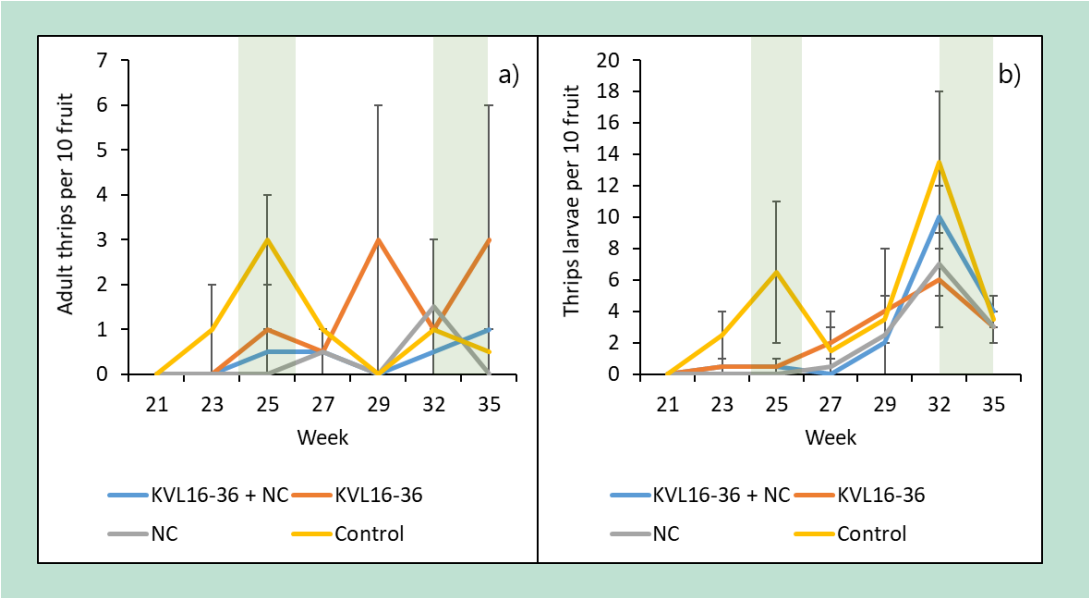

FIGURE 13. Number of adult thrips (a), and thrips larvae (b), per 10 fruit (=one sample), at site 2. Thrips were assessed at seven time points during the production season (week 19-35), in plots of the treatments: KVL16-36+NC, NC, KVL16-36, and Control. KVL16-36=M. brunneum, NC=N. cucumeris. Green areas indicate peak harvest times.

6.2.3 Aphids

The aphids visually observed at the two sites were *M. persicae* (the green peach aphid), *A. gossypii* (the cotton aphid), and *M. euphorbiae* (the potato aphid) (Table 7).

TABLE 7. Total number of aphids of each species visually observed on strawberry plants at site 1 and site 2, during the production season (n=720 plants per site).

	Myzus persicae	Macrosiphum eu-phorbiae	Aphis gossypii	Total
Site 1	1660	50	3	1713
Site 2	395	21	358	774

Similar to the other groups of arthropod pests, the mean number of aphids per plant (considering all aphid species), assessed by visual observations, were different between site 1 and site 2 ($p<0.001$).

At site 1, no overall differences in aphid abundance were found between the control plots and any of the treated plots (KVL16-36: P=0.12; *N. cucumeris*: P=0.78), although results showed a tendency for a difference between the control and the combined treatment of KVL16-36+*N. cucumeris* (P=0.06) (Fig. 14). Aphid abundance peaked around week 23 in the control and *N. cucumeris* treatments, and in week 29 for all treatments (Fig. 14). After this time, aphids naturally migrate from the crop due to decreasing temperatures and day length.

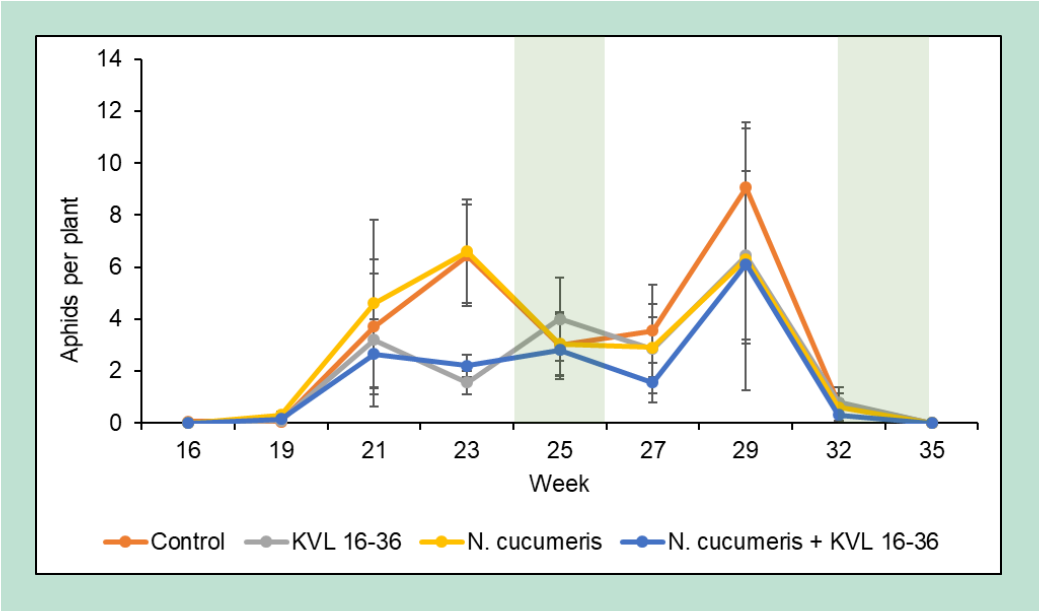

FIGURE 14. Mean number ±SE of aphids per plant, at site 1, assessed by visual observations from week 16 to 35, in plots of the treatments: control, KVL16-36, N. cucumeris, and KVL16-36+N. cu-cumeris. Green areas indicate peak harvest times.

At site 2, overall aphid abundance was higher in the control plots compared to all treated plots (KVL16-36: P=0.01; *N. cucumeris*: P=0.01; KVL16-36+*N. cucumeris*: P=0.02), with a peak in aphid abundance around week 29 in the control (Fig. 15).

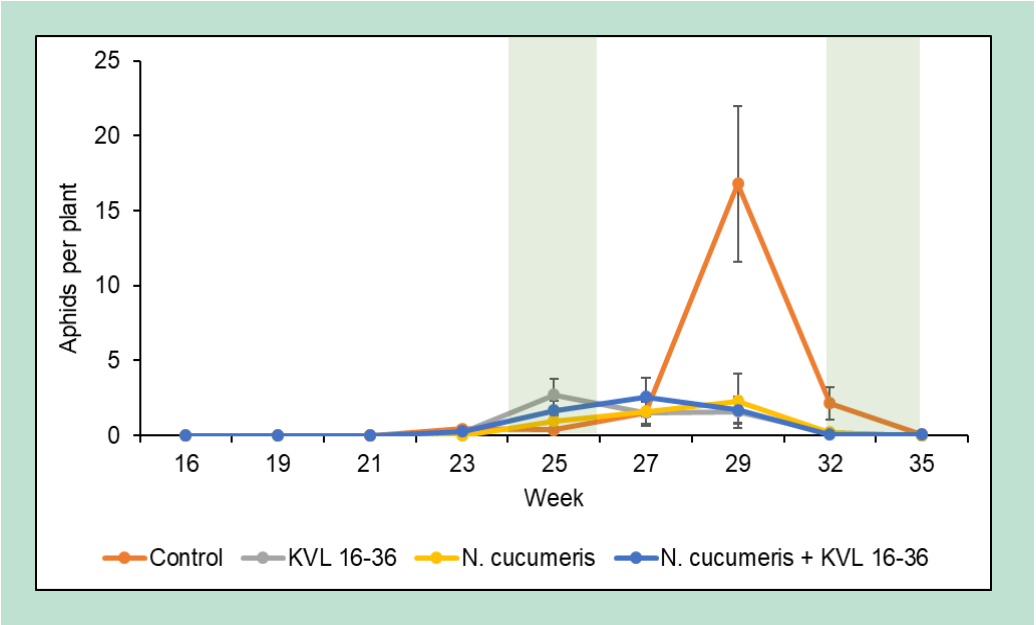

FIGURE 15. Mean number ±SE of aphids per plant, at site 2, assessed by visual observations from week 16 to 35, in plots of the treatments: control, KVL16-36, N. cucumeris, and KVL16-36+N. cu-cumeris. Green areas indicate peak harvest times.

At each assessment time of visual observations, plant characteristics were recorded as well (Table 8). This was to provide an indication of potential differences in plant growth between sites. The additional information confirmed that plant growth, fruit development and harvest periods were similar between the two sites.

TABLE 8. Average number of leaves, flowers, and fruit, estimated per plant, at site 1 and site 2, recorded in week 16-35, concurrently with the visual observations.

	Week								
Site 1:	**16***	**19****	**21**	**23**	**25*****	**27**	**29**	**32**	**35**
Average no. of leaves per plant:	9	11	15	17	18	18	20	22	22
Average no. of flowers per plant:	2	7	10	6	5	11	20	17	6
Average no. of fruit per plant:	-	1	16	16	9	10	14	25	23
Site 2:									
Average no. of leaves per plant:	8	11	12	15	18	20	22	18	19
Average no. of flowers per plant:	3	7	9	5	6	17	18	13	5
Average no. of fruit per plant:	-	1	14	17	10	12	14	22	20

*no fruit developed, **fruit very young, ***majority of fruit harvested.

6.2.4 Arthropod natural enemies

6.2.4.1 Predatory mites in strawberry flowers and fruit

Predatory mite nymphs and adults were counted from the same strawberry flowers and fruit sampled for thrips abundance, with the assumption that the majority, if not all, of the predatory mites were of the species *N. cucumeris*. See Table 9 and Table 10 for number of predatory mites found at each of the two sites in each of the four treatments.

At site 1, there were overall more predatory mites in the flowers in plots where only *N. cucumeris* was applied than in the control plots (P=0.006). There was no overall difference in predatory mite numbers between treatments of KVL16-36 (P=1.00) and KVL16-36 + *N. cucumeris* (P=0.57) compared to the control.

On the fruit sampled at site 1, there were fewer predatory mites in treatment plots of KVL16-36 compared to the control (P=0.03), whereas no differences in predatory mite abundance was found between the control plots and plots treated with *N. cucumeris* (P=1.00), and KVL16-36 + *N. cucumeris* (P=0.13).

TABLE 9. Mean numbers of predatory mites per 10 flowers or fruit ±SE, at site 1, in the treatments: control, KVL16-36, *N. cucumeris*, and KVL16-36+*N. cucumeris*.

	Week:	**19**	**21**	**23**	**25**	**27**	**29**	**32**	**35**
Control	Flower	0	0	1 ±1.0	1.5 ±1.5	0	0	0	0
	Fruit	-	8.5 ±8.5	0	8 ±4.0	7 ±2.0	1.5 ±1.5	2 ±2.0	12 ±4.0
KVL16-36	Flower	0	0	0	0	2.5 ±1.5	0	0	0
	Fruit	-	0.5 ±0.5	5.5 ±3.5	8.5 ±3.5	12 ±6.0	7.5 ±2.5	1 ±1.0	10 ±0.0
NC	Flower	2 ±1.0	2 ±2.0	2 ±2.0	2.5 ±2.5	1.5 ±1.5	0	0	0
	Fruit	-	3 ±3.0	14.5 ±1.5	13 ±1.0	8 ±0.0	1.5 ±1.5	1 ±1.0	7 ±3.0
KVL16-36 + NC	Flower	0	1 ±1.0	0	0	2.5 ±2.5	0	0	0
	Fruit	-	6.5 ±1.5	17.5 ±2.5	8.5 ±4.5	7 ±1.0	7 ±1.0	1 ±1.0	1.5 ±1.5

At site 2, there were more predatory mites in the flowers in plots treated with KVL16-36 + *N. cucumeris* than in the control plots (P<0.001), while no differences were found between treatments KVL16-36 (P=0.25) and *N. cucumeris* (P=0.07) compared to the control, although the latter suggested a near significant trend of more predatory mites in plots treated with *N. cucumeris,* than in the control. On the fruit, there were more predatory mites in all plots treated with biocontrol agents compared to the control treatment (P<0.001), while no differences were found between treatments of KVL16-36, *N. cucumeris*, nor their combination.

TABLE 10. Mean numbers of predatory mites per 10 flowers or fruit ±SE, at site 2, in the treatments: control, KVL16-36, *N. cucumeris*, and KVL16-36+*N. cucumeris*.

	Week:	19	21	23	25	27	29	32	35
Control	Flower	0	0	0	1 ±1.0	6.5 ±1.5	0	0	0
	Fruit	-	0.5 ±0.5	0.5 ±0.5	7.5 ±2.5	6 ±3.0	1 ±1.0	0	4.5 ±1.5
KVL16-36	Flower	0	1.5 ±1.5	0	2 ±2.0	3.5 ±1.5	0	0	0
	Fruit	-	0	2 ±1.0	4 ±1.0	8.5 ±0.5	0	1 ±0.0	8.5 ±1.5
NC	Flower	3 ±2.0	5.5 ±2.5	3.5 ±0.5	3 ±2.0	0.5 ±0.5	1 ±1.0	0	1 ±1.0
	Fruit	-	10 ±0.0	25 ±10.0	12.5 ±0.5	14 ±3.0	5 ±3.0	0.5 ±0.5	3.5 ±1.5
KVL16-36 + NC	Flower	4 ±3.0	7 ±1.0	6.5 ±2.5	3 ±2.0	2.5 ±0.5	0	0	0
	Fruit	-	11.5 ±4.5	22 ±1.0	3.5 ±3.5	7 ±2.0	3.5 ±0.5	2 ±1.0	3.5 ±0.5

6.2.4.2 Predator abundance by visual observations

A total of 239 individual predators were observed on strawberry plants during the production season at site 1, and a total of 248 individual predators at site 2. This was primarily spiders (Araneae), at both sites, followed by *Orius* spp. (nymphs and adults), *Anthocoris* spp. nymphs, *Chrysoperla* spp. larvae, ladybird larvae and adults (Coccinellidae), and hoverfly larvae (Syrphidae) that were observed (Table 11). All predators present was naturally occurring, i.e. there had been no release of other predators than *N. cucumeris*.

TABLE 11. Total number of each arthropod predator group observed visually in strawberry plants during a production season, at site 1 and site 2 (n=720 plants per site), n=nymph, a=adult, l=larvae.

Predator order/genus/family:	Site 1	Site 2
Araneae	162	154
Orius spp.	37	64
Anthocoris spp. (n)	6	11
Cantharis spp. (a)	2	3
Syrphidae (l)	9	2
Coccinellidae (a)	4	2
Coccinellidae (l)	10	3
Chrysoperla spp. (l)	8	9
Forficula spp.	1	0

The number of arthropod predators observed increased during the season (Table 12). At early and mid-season it was particularly spiders that were observed, and towards the end of the season, spiders and *Orius* spp. were the predominant predators observed.

TABLE 12. Total number of arthropod predators observed visually on strawberry plants at site 1 and site 2, from week 16 to 35, in plots of the treatments: control, KVL16-36, *N. cucumeris*, and KVL16-36+*N. cucumeris* (n=20 plants per time point).

		Week								
Treatment	**Site**	**16**	**19**	**21**	**23**	**25**	**27**	**29**	**32**	**35**
Control	1	1	4	5	9	4	8	7	9	25
	2	2	4	7	4	4	2	6	13	22
KVL16-36	1	0	3	4	7	2	13	9	7	16
	2	2	3	9	3	2	6	14	16	18
NC	1	0	3	3	7	2	4	4	9	23
	2	3	1	6	1	1	6	6	18	29
KVL16-36 + NC	1	2	1	6	10	4	3	4	10	19
	2	0	2	3	4	0	1	6	19	12

Aphid mummies were frequently observed later in the season, and therefore included in the visual observations of aphids and predators, providing an estimation of the presence of parasitoids. Generally, the aphid mummies started to appear in week 25, with an increase towards the end of the season (week 35) (Table 13). A total of 293 aphid mummies were observed at site 1, and a total of 166 aphid mummies at site 2, during the nine assessments dates.

TABLE 13. Total number of parasitized aphids (aphid mummies), observed visually on strawberry plants at site 1 and site 2, from week 16 to 35, in plots of the treatments: Control, KVL16-36, NC (*N. cucumeris*), and KVL16-36+NC (n=20 plants per value, of treatment and time).

		Week								
Treatment	**Site**	**16**	**19**	**21**	**23**	**25**	**27**	**29**	**32**	**35**
Control	1	0	0	0	0	4	20	29	12	30
	2	0	0	0	0	0	1	14	11	19
KVL16-36	1	0	0	0	0	1	17	24	16	24
	2	0	0	0	0	0	3	8	15	13
NC	1	0	0	0	0	2	11	18	11	19
	2	0	0	0	0	0	2	13	11	17
KVL16-36 + NC	1	0	0	0	0	0	10	17	13	15
	2	0	0	0	0	0	4	14	7	14

6.2.5 Fruit damage

At both time points of fruit damage assessment, in June and August, there was a difference in the proportion of damaged fruit by bronzing (thrips damage) between site 1 and site 2 ($P<0.001$). Considering the differences in thrips abundance between sites, and the necessity to treat with a chemical insecticide mid-season at site 1, the fruit damages recorded at site 1 are only considered to a minor extent, and each site were analysed separately.

At site 1, there was no differences in fruit damage among treatments, neither in June nor in August (no visual outline). At site 2, there was no effect of treatment on fruit damage in June, while a lower proportion of damaged fruit with bronzing in all plots treated with biocontrol agents was found in August, compared to the control plots (KVL16-36: $P=0.01$; *N. cucumeris*: $P=0.01$; KVL16-36 + *N. cucumeris*: $P=0.03$) (Fig. 16).

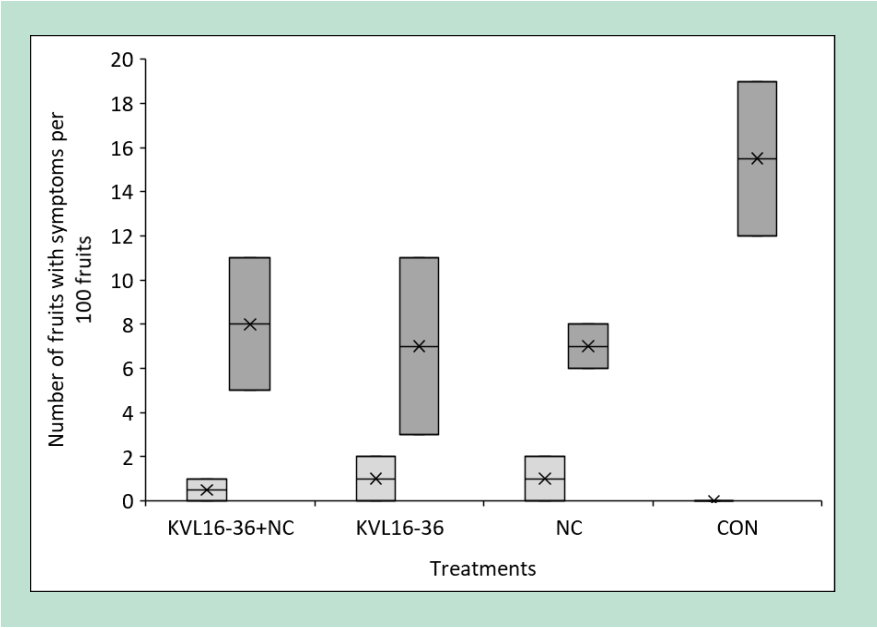

FIGURE 16. Number of strawberry fruit with symptoms of thrips in strawberry tunnels, at site 2, in June (light grey) and in August (dark grey), in the treatments: KVL16-36+NC, KVL16-36, NC, and Control (CON). KVL16-36=M. brunneum, NC=N. cucumeris. Boxplots display mean and SE of damaged fruit, per 100 fruit.

6.2.6 End of season re-isolation of *M. brunneum*

Selected root systems and samples of the growth substrate from the two commercial strawberry tunnels were evaluated for presence of active *Metarhizium* at the end of the growing season in two different ways; by plating root homogenate suspension onto selective agar media, and by baiting the substrate with susceptible insect larvae.

When suspensions of root homogenate were plated on selective agar media, *Metarhizium* CFU were observed in all treatments, except for the control and the *N. cucumeris* treatments at site 2 (Fig. 17). At site 1, CFU numbers ranged between 0-2 per plate in the control and the *N. cucumeris* treatments. The log10-transfomed values of CFU were significantly affected by the treatment ($F=51.73$; d.f.=3,39; $P<0.0001$) and by the site ($F=12.51$; d.f.=1,39; $P=0.0011$). The interaction between treatment and site was not significant ($F=1.05$; d.f.=3,39; $P=0.3826$), indicating that comparable patterns of CFU distributions existed between the two locations, but the overall level of CFU were different between the two sites. There was no difference in the mean values of CFU between the two treatments where *M. brunneum* KVL16-36 had been applied (Fig. 17). The observed values of CFU in the treatment where *M. brunneum* was applied alone ranged between 0 to 14 CFU per plate at site 2, and between 1 to 44 CFU at site 1, while in the combined *M. brunneum* KVL16-36 and *N. cucumeris* treatment, the values ranged between 2 to 31 CFU per plate at site 2 and between 4 to 76 CFU at site 1.

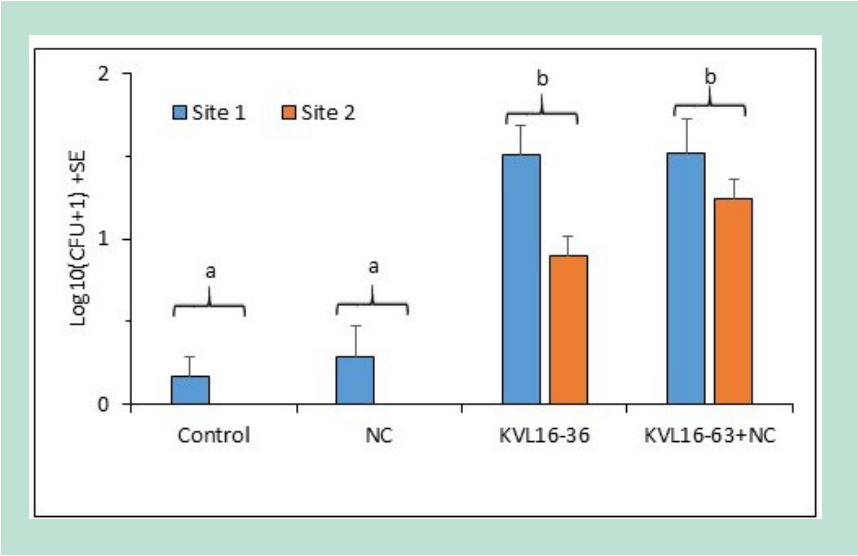

FIGURE 17. Mean value (+SE) of log10-transformed counts of Metarhizium colony forming units (CFU) on two replicated selective media plates after spreading a total of 200 µl root homogenate sus-pension. Strawberry plants had been inoculated with M. brunneum (KVL16-36) or 0.05% Tri-ton-X, and/or with predatory mites, N. cucumeris (NC). Data are presented separately for the two experimental sites, 1 and 2. Different letters above bars indicate significant differences.

The collected substrate samples were each baited with 10 larvae of *T. molitor*. All fungal infected cadavers were showing symptoms of *Metarhizium* sporulation. The frequency of infected cadavers was only affected by the treatment ($F=93.66$; d.f.=3,37; $P<0.0001$), while no effect was found between sites ($F=0.01$; d.f.=1,37; $P=0.9156$) or the interaction between treatment and site ($F=0.48$; d.f.=3,37; $P=0.6973$). The mean frequencies of *Metarhizium* infections were 71.7-81.7% of the larvae per sample at site 1 and 66.7-88.3% of the larvae per sample at site 2 in the treatments, which had received *M. brunneum* inoculations in April (Table 14). No infections were seen in larvae from the substrate of the control treatments, but in the substrate from site 2 there were two infected larvae found in a single sample from the *N. cucumeris* treatment.

Table 14: Mean proportions (±SE) of *Metarhizium*-infected *T. molitor* larvae (x/10) per substrate sample at the end of the growing season. The proportions are presented for the four treatments and two locations (site 1 and 2). Significant differences between treatments are indicated by different letters in the right column.

	Location		
Treatment (n=6 per site)	**Site 1**	**Site 2**	**Significance groups (P<0.05)**
Control (no biocontrol application)	0.0 (±0.0)	0.0 (±0.0)	**a**
N. cucumeris	0.0 (±0.0)	0.03 (±0.03)	**a**
M. brunneum	0.72 (±0.09)	0.67 (±0.08)	**b**
N. cucumeris + M. brunneum	0.82 (±0.05)	0.88 (±0.06)	**c**

7. Discussion

7.1 Aboveground effects of belowground inoculations with *M. brunneum*

The root inoculations of three isolates of the entomopathogenic fungus *M. brunneum* resulted in different responses of the spider mite population growth on enclosed leaflets on cv. 'Rumba' strawberry plants. The *M. brunneum* isolate KVL16-36, which was obtained from the commercial biocontrol product Met52, was the only isolate resulting in fewer eggs than in the control treatment. Similarly, quantification of spider mite nymphs showed that KVL16-36 resulted in significantly fewer individuals on the leaflets compared to the other treatments. The results emphasize the importance of testing individual fungal isolates and not draw conclusions at the species level. Likewise, Canassa et al. (2020b) found that different isolates within the same species of entomopathogenic fungal species differentially affected egg laying of spider mite females inside clip-cages on strawberry leaves.

In the present study, isolate KVL16-36 was also observed to be the most efficient, together with isolate KVL12-37, in colonizing the root system of the inoculated strawberry plants. The two traits, effects on spider mites and root colonization ability, meant that *M. brunneum* KVL16-36 was considered the most promising isolate to include in the following experiments.

No fungal infections occurred in the spider mite populations on the leaflets, thus the mechanism behind the observations of reduced population growth after root inoculation with KVL16-36 is likely to be indirect, mediated by systemic physiological changes in the aboveground plant tissues. Indeed, such mechanisms were also hypothesized by Gange et al. (2019) when conducting a meta-analysis on literature published thus far on the effects of endophytic entomopathogenic fungi on herbivores. This assumption is supported in the current study by the lack of endophytic colonization by *Metarhizium* in the aboveground plant tissue (the stem), while the fungus readily colonized the roots. The fungi are therefore unlikely to come in direct contact with the spider mites and any toxic compounds produced by the fungi in the plant would have to travel systemically to the leaflets. Such a scenario seems unlikely. Also, it was previously shown that inoculation with *M. brunneum*, *M. robertsii* and *Beauveria bassiana* can lead to accumulation of plant defence compounds correlating with reductions of arthropod herbivores on different plant species (Cachapa et al. 2021; Rasool et al. 2021a, 2021b). In strawberry, the accumulation of defence compounds in leaves due to belowground microbial inoculations have so far not been reported, but a recent study on predator-induced defences in strawberry plants found that levels of flavonoids increased in leaves of induced plants, correlating with reduced aphid population growth (Musaqaf, 2022). It remains unresolved if such increases of flavonoids or other defensive compounds were highest in leaflets of plants inoculated with KVL16-36 and negatively correlating with spider mite numbers in the present study, but it could be a possible mechanism to explain the observations.

The present project originally aimed to also study the gene expression of selected plant defence genes in leaves after root inoculation with the three *M. brunneum* isolates on strawberry plants, with and without spider mite exposure. A separate greenhouse experiment was set up for this purpose, with time-course sampling of leaflets. However, the extraction of RNA from the sampled leaflets turned out to be impossible, despite repeated efforts with multiple methods, including a dedicated extraction kit developed for pine needles and strawberry leaves. Although there are reports in the literature of RNA analyses from strawberry leaves (Feng et al. 2020) and fruit (Aristya et al. 2020), it was not possible to complete this part of the project. Given the effort and

relevance to report the difficulties in extracting RNA from strawberry plant tissues, the experimental design and approaches are reported in Appendix A.

7.2 Combination of above- and belowground biological control agents against spider mites

The most promising *M. brunneum* isolate KVL16-36 and the predatory mite *N. cucumeris* were tested individually and in combination to evaluate their ability to reduce spider mite populations on whole strawberry plants in controlled greenhouse settings. In this setup, the root inoculation with *M. brunneum* KVL16-36 did not result in significantly lower spider mite population levels compared to uninoculated plants, as was observed in the first experiment testing the three fungal isolates. However, the assessment method was simpler as it involved direct visual observation on the entire plant and not careful inspection of an enclosed leaflet. Despite this, the addition of predatory mites led to reduced spider mite populations in 24 h, while the number of spider mites on plants without predatory mites remained unchanged over this time period. These results indicate no additional effect of combining the two biocontrol agents on total spider mite numbers, as compared to only using *N. cucumeris*. However, the detailed assessment of spider mite numbers and life stages after the termination of the experiment revealed that the lowest number of *T. urticae* nymphs were found on plants treated with both *M. brunneum* and *N. cucumeris*. In addition, this assessment showed that plants receiving the root inoculation with *M. brunneum* KVL16-36 harboured less spider mite nymphs than the control plants, showing that the fungal treatment alone had an effect on the composition of the spider mite populations, also in this experimental setup.

Although the experiment was conducted within a short timeframe, and with variable results depending on observed life stage, the results suggest that the two biocontrol agents can be applied together with potential increased control effect against spider mite population build-up, and with no apparent negative interactions towards the predatory mites. Canassa et al. (2019) assessed the predatory capacity and searching behaviours of the specialist predatory mite *Phytoseiulus persimilis* on bean leaf discs presented with spider mites from bean plants inoculated with *M. robertsii* and/or *B. bassiana*. No behavioural differences of predation rates were found through laboratory observations (Canassa et al. 2019). Predatory mites may be directly affected in their behaviour by the presence of entomopathogenic fungal conidia, as reported by Wu et al. (2018) where *P. persimilis* avoided leaf areas with conidia of *B. bassiana*, but the overall feeding capacity of *T. urticae* eggs were unaffected by the fungus. However, longer term experiments evaluating the population level effects on spider mites are necessary to assess the consequences for biological control efficacy by the combination of entomopathogenic fungi and predatory mites.

7.3 Combined and single biological pest control in commercial strawberry tunnels

The combination of micro- and macro-biological control agents in the two commercial strawberry tunnels over the full production season from April till September showed some cases of effective control of the main arthropod pests, particularly in terms of delaying pest population build up, although the results varied between the two experimental sites. At the same time, the present study also found that the population control effects depended on factors such as specific pest species, population density, and site specific conditions.

The abundance of spider mites *T. urticae* varied between the two experimental sites, reflecting site specific conditions and thus a difference in efficacy of the applied biological control strategy. At site 1, a lower abundance of *T. urticae* was found than at site 2, in addition, a two week delay in the population build-up of *T. urticae* in the plots with the treatment of *M. brunneum* KVL16-36

and predatory mites *N. cucumeris* compared to untreated control plots was observed at site 1 (Fig. 8). This suggests an effect of the biocontrol treatments at moderate pest abundance levels, as seen at site 1. However, no improved control was seen in the combined treatment. At site 2, the abundance of *T. urticae* increased over time in a comparable pattern for all four treatments, with a common peak in week 29 in all treatments (Fig. 9). Thus, no delay in population build-up as observed at site 2. However, lower overall population levels of *T. urticae* in the treatments of *M. brunneum* KVL16-36 and of *N. cucumeris* indicated an effect of each treatment, even at higher pest infestations. No additional overall effect was seen in the combined treatment though, which could be due to the final population increase in week 35 for this treatment. At this time-point, the strawberry production had ceased and high levels of *T. urticae* will have limited consequences to the grower, because the plants will be destroyed after harvest.
An improved control effect of reduced *T. urticae* populations after root inoculations of entomopathogenic fungi in commercial strawberry fields in Brazil was reported by Canassa et al. (2020a), where bare roots of the strawberry plants were immersed into conidial suspensions of *B. bassiana* or *M. robertsii* and transplanted into the field at three commercial farms. During the following 6 months of plant growth and fruit production, the pest populations were lower in the fungal treated plots compared to control plots, although the population levels varied among sites (Canassa et al. 2020a). The results of the present project further suggests that entomopathogenic fungi can be applied to the coir growth substrate at start of strawberry cultivation in table-tops with potential control effects against spider mites in addition to effects of the usual application of predatory mites. A delay of the spider mite population build-up could be of benefit to the grower, since it would delay the time when it would be necessary to release the specialist predatory mite *P. persimilis*, which is more expensive to apply than *N. cucumeris*. Hence a delay could reduce overall costs for predators although it would add the costs of fungal inoculation.

Thrips are one of the most severe pests in strawberry tunnel production in Denmark, which was also evident from the results obtained in the present study, particularly concerning thrips infestations at site 1. The thrips population at site 1 increased over time, to such high levels that no overall differences between treatments in the flowers could be detected. High infestation levels of thrips in strawberry tunnels are regularly seen in June and July (N.L. Jensen, pers. communication), which is likely due to an influx of adults into the tunnels. Conventional growers can apply chemical insecticides against other pests with side-effects on thrips, while organic producers must aim at keeping the thrips populations low by preventative measures and application of biocontrol agents.
At site 2, the populations of thrips increased later than at site 1 and the overall thrips abundance was lower, which could be regarded as moderate as opposed to the high infestations as seen at site 1. At these moderate thrips infestation levels, lower densities of larvae were observed in plots with applications of *M. brunneum* (KVL16-36), both in flowers and on fruit (Fig. 12 and 13). Similar to the results of *T. urticae*, these observations indicate that the applied biological control agents were potentially more efficient at moderate levels of pest infestation. The combination of the below- and aboveground biocontrol agents, however, provided some control of thrips larvae in the fruit, where both a lower abundance as well as a delayed increase in abundance was found at site 2, despite quite large variation in data. As entomopathogenic fungi can also directly target the soil-dwelling stages of thrips (Ansari et al. 2007), the effect of the treatment could also be due to a combined effect of *M. brunneum* applied to the substrate, and the potential systemic plant defence responses acting against the aboveground stages of the thrips. In this context, Mantzoukas et al. (2022) reported that *B. bassiana* establishing as an endophyte was able to decrease thrips populations in strawberry. Only few studies have investigated the potential of applying plant-associated *Metarhizium* spp. against arthropod herbivores (Jaber and Ownley 2018). In the field study in Brazil conducted by Canassa et al. (2020a), no effects against thrips were reported. The present results indicate the potential of combining *M. brunneum* to the substrate against weevil larvae and thrips pupae with aboveground predators with improved effects on thrips, while also emphasising that control effects are depending on density, thrips developmental stage, and their location on the plant, which must be taken into account.

As expected, the application of predatory mites did not control aphids in the strawberry plants, yet the peak observed in the control treatment at site 2 did not occur in the *N. cucumeris* treatment. There seems to be no obvious explanation for this observation. *Neoseiulus cucumeris* is typically not released with the purpose of aphid control. In contrast, the *M. brunneum* application could be expected to affect aphid populations, as several species of entomopathogenic fungi have shown ability to affect reproduction and population growth of a wide range of aphid species when associating with plants (Francis et al. 2022). Similarly, a root-associated isolate of *M. robertsii* reduced the population growth of different aphid species in wheat and bean plants (Rasool et al. 2021a). Indeed, an early season effect was observed at site 1, where a distinction in aphid abundance between plots with and without *M. brunneum* applications could be observed (Fig. 14). At site 2, no early season peak in aphid abundance was seen, while all three biocontrol treatments kept aphid populations low compared to the peak of the control treatment (Fig. 15). It is reported that *N. cucumeris* does not feed on the aphid *M. persicae* (Messelink et al. 2013), which was found at both sites. In addition, other natural enemies of aphids were observed on the plants. The difference in treatment effects between the two sites is likely also related to the aphid species composition, where *M. persicae* was the dominant species at site 1, and an equal density of *M. persicae* and *A. gossypii* was observed at site 2. Changes in pest densities and species composition, can alter the performance of generalist predators, which typically benefit from mixed diets of pests (Messelink et al., 2008, 2010; Muñoz-Cárdenas et al. 2014). Potentially, combination of *M. brunneum* substrate applications with specific aphid natural enemies may add to biocontrol efficacy in strawberry production.

Application of entomopathogenic fungi such as *M. brunneum* to the substrate at plant establishment would be motivated for controlling larvae of the black vine weevil *O. sulcatus* (Ansari & Butt 2013; Klingen et al. 2015) and potentially for controlling thrips pupae (Ansari et al. 2007). In the experiments in the tunnels, the granular commercial biocontrol product Met52 for substrate application was unavailable (production deficit), so a drench approach was used. However, from 2023 a similar type of product is available, Lalguard M52 GR (Middeldatabasen 2024), for use e.g., against thrips in strawberry, so strawberry growers again have the option of applying this biocontrol agent. The present study showed that *M. brunneum* KVL16-36 can stay infective in the coir substrate throughout the production season, as demonstrated by high fungal infection levels of the *T. molitor* bait larvae in the fungal treated plots. Limited dispersal of the fungi was seen between plots at the end of the experiments, but this is not considered to have had significant impact of the overall results. If growers apply *M. brunneum* as a granular product at plant establishment, they can expect some additional level of control also against aboveground arthropod pests, mainly on delayed population build-up, as well as control against the target insect stages present in the substrate through the growth season.

The fact that more predatory mites were observed on fruit compared to flowers is valuable knowledge for developing efficient pest control in strawberry tunnels. Early in the season, predatory mites were predominantly found in plots where they were released, while later in the season, the numbers of predatory mites increased in other plots indicating dispersal between the experimental plots. This was likely causing a reduction in treatment effects in the final weeks of the field trials, and should be considered in relation to results obtained as well as a methodological consideration for future trials. At the same time, it is also an indication of the dispersing ability of predatory mite species within the production area. At site 1, there was, however, indications of a poor establishment and/or an interference with other organisms, as there were no differences in predatory mite abundance between control plots and the plots treated with *N. cucumeris* early in the season. The predatory mites were occurring at comparable densities at the two sites, primarily on the fruit, where they could target the thrips larvae. This observation could potentially increase the damage threshold, although damage done by adult thrips in the flowers would not be affected by *N. cucumeris*. With this knowledge, additional control measures should focus on biocontrol of adult thrips in the flowers, for example with releases of *Orius majusculus*, as well as preventing influx of thrips into the tunnels in early summer.

Overall, few naturally occurring predators were observed, primarily spiders, which are considered to have had limited contribution to the pest control observed in the experimental plots. The overall low abundance can be related to several factors related to the tunnel production such as late plant establishment (same year as production), management (soil surface below table tops covered in fibre cloth), and consequently lack of available resources inside the tunnel to sustain natural enemy populations. These observations underline the necessity of developing biocontrol strategies based on application to ensure biological pest control in strawberry tunnel production, with additional focus on optimizing input and resources.
While the abundance of naturally occurring generalist predators did not appear to be influenced by the pest population densities in the strawberry plants, the number of aphid mummies increased at the latter part of the season where aphids were also more frequent. This indicates a host density dependent relationship, and the potential importance of naturally occurring parasitoids, as contributing to the natural regulation of aphid populations in strawberry tunnels, despite the scarce environment for parasitoid recruitment. Especially aphid parasitoids could benefit from additional measures of implementation in the tunnels (e.g. increased plant diversity and flowering resources) (Wan et al. 2020), which should also be considered as an infrastructure element for optimal combinations of biocontrol strategies to target pest populations broadly.

Fruit damage, assessed by symptoms of thrips (bronzing), was different between treatments at the second assessment in August, at site 2, correlating with the higher number of thrips in the fruit late in the season. At site 1, the severity in thrips abundance was more evident, and the infestation was so high that a thinning of fruit was necessary to limit the damage and potential spread to neighbouring tunnels. Combined with the application of a mid-season chemical insecticide treatment, the damage assessment at site 1 is not representative in terms of the present scope of the study, and the late damage levels can therefore not be considered an effect of the biocontrol strategy. At the early damage assessment in June however, before insecticide treatments were applied, there was no differences in fruit damage, similar to site 2.

The efficiency of combining below- and aboveground biocontrol agents have shown some potential, while the present project demonstrated that limitations and variability in efficacy occur. In general, effects were more variable in field trials than in laboratory trials. The effect of the two biocontrol agents *M. brunneum* and *N. cucumeris*, both individually and/or in combination, impacted particularly the population build-up of spider mites *T. urticae*, providing some level of resilience to the system. In addition, no apparent negative effects of *M. brunneum* applications on the efficacy of the released predatory mites were seen. In fact, studies on this topic shows that the majority of entomopathogenic fungi tested are generally compatible with arthropod natural enemies (Gonzalez et al. 2016). The study further shows that control effects of the applied biocontrol agents is strongly affected by pest population sizes, and control is more challenging to obtain during early and rapid pest population build-up.

8. Conclusion and Perspectives

The macro- and microbiological control agents, the predatory mite *N. cucumeris* and the entomopathogenic fungus *M. brunneum*, applied above- and belowground, reduced the population growth of spider mites *T. urticae* under controlled greenhouse conditions on strawberry plants, and the results confirmed the importance of evaluating specific isolates within a fungal species. Under controlled conditions, the combination of *M. brunneum* and *N. cucumeris*, versus their individual application, could reduce number of spider mite nymphs, but not eggs. Although considering the short time frame of the experiments, both biocontrol agents have effects against spider mites on strawberry plants and thus potential for control in tunnel production. The mechanisms of the belowground *M. brunneum* is expected to be an indirect effect linked to strengthening of the plant defences or other changes in host plant quality, hampering the population growth of spider mites, but this mechanism remains to be elucidated.

The main groups of pests found in the two commercial strawberry tunnels, where the biocontrol strategies were tested, were spider mites, thrips and aphids. Especially thrips populations reached high levels at one site, confirming the general concern for this pest in strawberry tunnel production. The combined application of *M. brunneum* belowground and the commercial predatory mite *N. cucumeris* aboveground in the strawberry tunnels showed variable potential to reduce pest populations and thus plant damage in the treatment plots. Spider mite population development was at one site delayed by several weeks in the treatment plots with *M. brunneum*, alone and in combination with predatory mites, corroborating the controlled experiments. The most promising effects on thrips were seen on the fruit, by a lower number of thrips when biocontrol agents were combined. The fruit are also where the predatory mites predominantly were found. As seen in other studies, the predatory mites tested in the present study did not have an effect on the adult thrips which are mostly located in the flowers, and additional control methods must be developed to target these pest stages. Development of aphid populations were in some cases delayed and/or reduced in densities in the biocontrol treatment plots containing *M. brunneum*, supporting previous reports that aphids can be affected by belowground applications of entomopathogenic fungi.

The rationale of applying *M. brunneum* to the growth substrate in strawberry production is to target belowground insect pests, i.e. the black vine weevil and pupal stages of thrips. This study showed the added potential of *M. brunneum* by indirectly contributing to regulating the aboveground pest communities. In addition, the application of *M. brunneum* had no apparent negative effects on the predatory mites which are released for direct biocontrol of aboveground pests.

In general, the treatments tested in this project were not efficient at high pest densities. Growers should therefore not rely only on these control methods, but also implement methods preventing pest immigration into the tunnels, particularly of thrips in early season. Current management methods provide limited infrastructure to support populations of indigenous natural enemies in strawberry tunnel production. Further development of biocontrol strategies are needed, e.g., it could be feasible to consider timing of application, use of alternative predator species to supplement *N. cucumeris*, and diversification of the tunnel environment, such as implementation of flower resources, which would provide additional resources for natural enemies, both indigenous and released. Sustaining natural enemy communities as opposed to continuous releases can potentially be more cost efficient long term. However, more knowledge of their interactive effects are needed for optimal combinations and implementation.

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10. Appendix 1

10.1 Experimental setup for RNA extraction from strawberry leaves

Gene expression analyses were planned to evaluate the potential induction of defense responses in strawberry plants inoculated with EPF and/or T. urticae. Specifically, the regulation of selected well-known defense proteins, glucanases and chitinases that are important pathogenesis related (PR) proteins in strawberries (Amil-Ruiz et al. 2011) were selected as targets for evaluation of their potential contribution to any negative effects against spider mites observed in the project.
Nine treatments representing combinations of three fungal isolates, on plants with and without T. urticae, were included in the experiment: KVL12-30 ±T. urticae (n=24), KVL12-37 ±T. urti-cae (n=24), KVL16-36 ±T. urticae (n=24), 0.05% Triton-X ±T. urticae (n=24), and plants with no treatment (n=18). Strawberry plants of cultivar 'Rumba' were prepared and placed in con-trolled conditions as described in section 5.1.1. Similarly, the fungal suspension, of 1x107 conidia/ml, was applied to plants as described in section 5.1.1. The day before application of the fungal suspension (day -1), leaflets from untreated plants were sampled for RNA extrac-tions. Leaflet sampling was done by cutting one leaflet per plant, placing it in a paper bag, and immediately immersing the bag in liquid Nitrogen. Bags were subsequently stored in an ultra-low freezer, at -70ºC. Each plant was used for sampling only once. The following day (day 0), the respective fungal suspensions were applied by drenching, as in section 5.1.1. On day six, leaflets were sampled from plants representing all treatments, and on day seven, three adult female T. urticae was applied to one leaflet per plant in half of the remaining plants per treat-ments, inside a clip cage, as in section 5.1.1. An empty clip cage was placed also on a leaflet of all plants not harbouring T. urticae to eliminate any potential effects of the presence of the cage on the leaflet. Hereafter, leaflets were sampled from plants representing all treatments, on day 8, 10 and 13.

Each time-point (day -1, 6, 8, 10, 13) and treatment combination (drench [4 levels] x spider mite presence/absence [2 levels]) thereby consisted of 1 biological replicate (one leaflet from each individual plant), repeated three times, while sampling from the clean control plants con-sisted of 2 biological replicates for each time point, also replicated three times.

Extraction of RNA from strawberry leaf material proved to be highly difficult and finally unsuccessful. This is despite reports in the literature that standard RNA extraction kits can be used for RNA extraction from strawberry leaves (Feng et al. 2020). Several protocols were attempted, including specialized commercial kits, which are listed in the following. The intent of the information in this appendix is to provide tools for challenging plant samples, which may be useful for others.

Conclusion
Ultimately, the RNA extractions from strawberry leaves proved unsuccessful. The leaf samples are kept as freeze-dried material (ultimo 2023). Potentially, future work could focus on extractions of defence compounds (such as flavonoids), which can be quantified by spectrometry or gas chromatography mass spectrometry.

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10.2 List of RNA extraction protocols

This section provides an overview of each protocol tested and following methodology. Below the list is the procedure for each protocol described in detail. Parts of this was developed in Danish.

1) Protocol for RNA extraction (QIAzol)

2) Double lysis: an integrative time-saving method yielding high-quality RNA from strawberry

3) Protocol developed for extraction of oilseed rape RNA from root samples (Danish version)

4) Plant RNA Extraction Protocol

5-6) Two commercial RNA extraction kits, also tested without success

Spectrum™ Plant Total RNA Kit (Sigma Aldrich)

RNeasy Power Plant Kit (Qiagen)

After RNA extraction

Mål RNA

DNase Behandling

cDNA

Mål cDNA og fortynd ned

MasterMix

real-time PCR (qPCR)

1) Protocol for RNA extraction (QIAzol)

1. Add QIAzol Lysis Reagent to an appropriate vessel for disruption and homogenization and subsequent centrifugation: 500 ul QIAzol Lysis Reagent per 50 mg tissue is required. Add 10 mg of PVP to the above. (The volume of tissue should not exceed 10% of the volume of QIAzol Lysis Reagent.)
2. Place the tube containing the homogenate on the benchtop at room temperature (15–25°C) for 5 min.
3. Add 100 ul chloroform per 500 ul QIAzol Lysis Reagent pipetted in step 1. Securely cap the tube containing the homogenate, and shake it vigorously for 15 s.
4. Place the tube containing the homogenate on the benchtop at room temperature for 2–3 min.
5. Centrifuge at 12,000 x g for 15 min at 4°C.
6. Transfer the upper, aqueous phase to a new tube. Be careful to avoid the interphase. Add 250 µl isopropanol to 500 ul QIAzol Lysis Reagent pipetted in step 1. Mix thoroughly by vortexing.
7. Place the tube on the benchtop at room temperature for 10 min.
8. Centrifuge at 12,000 x g for 10 min at 4°C.
9. Carefully aspirate and discard the supernatant.
10. Add at least 500 ul of 75% ethanol per 500 µl QIAzol Lysis Reagent pipetted in step 1. Centrifuge at 7500 x g for 5 min at 4°C. (Repeat the step twice)
11. Remove the supernatant completely, and briefly air-dry the RNA pellet.
12. Redissolve the RNA in an appropriate volume of RNase-free water. Clean up the RNA using the RNeasy® MinElute® Cleanup Kit or RNeasy Mini, Midi, or Maxi Kit.

(After following all the steps, we expect to have a good quality RNA. Otherwise, we can use the same extracted RNA sample and start the procedure again from step 3 until step 12 in order to extract good quality to continue with PCR).

Another, possible way is to formulate a lysis buffer, which can be used from the beginning in the first step (Optional if we do not succeed with the above method).

2) Double lysis: an integrative time-saving method yielding high-quality RNA from strawberry

Plant materials

Plant tissues were immediately shock-frozen in liquid nitrogen and then stored in -70°C for subsequent extraction of RNA and semi-quantitative RT-PCR.

Buffers

Lysis buffer 1 [13]: 100 mM Tris-HCl, pH 8, 1.4% NaCl, 0.5% (v/v) Triton X-100 and 3% (w/v) CTAB (cetyl trimethyl-ammonium bromide). 2% (w/v) poly-vinyl pyrrolidone (PVP), and 5% (v/v) 2-mercaptoethanol (β-ME) were added just before use. Prewashing buffer (only in case of fruits) [14]: 100 mM Tris-HCl, pH 8, 350 mM sorbitol, 5 mM EDTA, pH 8.0 (the buffer should be stored at + 4 °C), in addition 3% (v/v) β-ME was added immediately before use.

Reagents

Trizol (Ambion, Invitrogene, USA), chloroform:isoamylalcohol (24:1), isopropanol, 75% ethanol, absolute ethanol, and DNase-RNase free water.

RNA

1. Vej maksimalt 0,03 g bladvæv eller næsten 0,5 g frugtvæv.
2. Slib vævet i flydende nitrogen til fint pulver ved hjælp af mørtel og støder.
3. Tilsæt 1 ml lyseringsbuffer (buffer 1 opbevares på køl) frisk tilsat 50 µl β-ME og 0,5µl PVP. Resuspender sedimentet ved at pipettere flere gange med afskårne tips, indtil vævet resuspenderes fuldstændigt, vortex 30 s.
4. Inkuber 10 min. ved 65 ° C, vend rørene forsigtigt et par gange hver 2. minut.
5. Centrifugeres 5 min. ved 17.600 × g ved 4 ° C.
6. Overfør 700 µl af supernatanten til et nyt 1,5ml rør,
7. Overfør 700 µl (vol. 1:1) kloroform: isoamylalkohol (24: 1). Vortex 10 s, lad det derefter afregne ved stuetemperatur i 5 min.
8. Centrifugeres 15 min. ved 21.000 × g ved 4 ° C.
9. Overføre 400 µl supernatanten til nyt 1,5 ml rør og tilsæt 800 µl Trizol (iskold) (vol. 1:2), vortex 30 s, lad den derefter ved stuetemperatur i 5 min.
10. Tilsæt 200 µl kloroform, vortex 10 s, og der inkuberes ved stuetemperatur i 5 minutter.
11. Centrifuger i 15 min. ved 21.000 × g ved 4 ° C
12. Overfør derefter de øvre 750 µl fra den øvre fase til et nyt 1,5 ml rør, hvor 500 µl isopropanol tilsættes, og blandingen blandes derefter ved pipettering flere gange.
13. Inkuber blandingen ved stuetemperatur i 10-15 minutter, og overfør derefter til - 20 ° C i 10-15 minutter.
14. Centrifuger i 15 minutter ved 17.600 × g ved 4 ° C for at opnå et synligt bundfald og kassér supernatanten.
15. Tilsæt 1 ml 75% kold ethanol, vortex 10 s. centrifuger i 3 min. ved 17.600 × g og 4 ° C. Kassér supernatanten.
16. Gentag trin 15. *Tilsæt 1 ml 75% kold ethanol, centrifuger og kasser.*
17. Tilsæt 1 ml absolut ethanol, vortex 10 s, centrifuger i 5 min. ved 17.600 × g ved 4 ° C.
18. Supernatanten kasseres forsigtigt og lad bundfaldet tørre i 5 minutter kun ved stuetemperatur. Mist ikke bundfaldet, da det på det tidspunkt har tendens til at være løst.
19. Tilsæt 30-50 µl DNase-RNase-frit vand (ikke pipetteres eller vortex) og opbevares ved stuetemperatur i 15 min. Opløs derefter de hvide sedimenter forsigtigt ved forsigtig pipettering i nogle få gange. Bemærk: aldrig hvirvel på det tidspunkt, da det vil forstyrre RNA-integritet.

3) Protocol developed for extraction of oilseed rape RNA from root samples (Danish version)

Inden RNA-ekstraktion begynder

Morter bladene med flydende N_2, overføre til RNase/DNase frie rør og gem i -70 grader.

Isopropanol i fryser på 1. dag.
Kloroform skal være stuetemperatur.
Centrifugen skal være 4 grader.
Rengøre arbejdspladsen med RNase/DNase midler.
Arbejde i stinkskab.

RNA-ekstraktion

1. dag.

1. Alikvoter ca. 20 mg plantemateriale til 1,5 rør, (kan gemmes i -70°C til påbegyndelse af RNA-ekstraktion)
2. Tilføj 1 ml Qiazol, vortex i 10-15 sekunder, Inkubere i -70°C til næste dag.

2. dag

1. Optø rørene med ca. 20 mg plantemateriale + 1ml Qiazol i 5 minutter ved 21°C (stuetemp)
2. Tilsæt 200µl kloroform ryst kraftigt i 20 sek i tissue lyzer, men uden hvirvlende
3. Inkubation 3- 5 minutter ved 21° C (stuetemp)
4. Centrifuge 10 minutter ved 14000g ved 4°C
5. Overfør supernatanten ca. 500µl-1ml. (vandig fase) til et nyt rør 1,5 ml – undgå overfladen, gå ca. 2 mm under overflade (DNA og proteinerne er på grænsefladen)
6. Tilsæt 500µl iskoldt isopropanol (Opbevaret ved -20°C)
7. Lad inkubere mindst 1 time ved -20°C
8. Centrifuge 20 minutter ved 14000g ved 4°C for at opnå et synligt bundfald
9. Kassér supernatanten
10. Tilsæt 200µl koldt ethanol 70% og centrifuge 5 minutter ved 14000g ved 4 °C
11. Fjern supernatanten
12. Tilsæt 200µl koldt ethanol 70% og centrifuge 5 minutter ved 14000g ved 4 °C
13. Fjern supernatanten og lad tørre i 5 minutter under stinkskabet
14. opløs pelleten i 100 µl RNase-frit vand eller 0,1 % DEPC-behandlet vand – evt. kom rørene på varmeplade ved 37 °C i et par minutter, hvis pelleten ikke opløses i væsken
15. Opbevares ved -70°C

4) Plant RNA Extraction Protocol

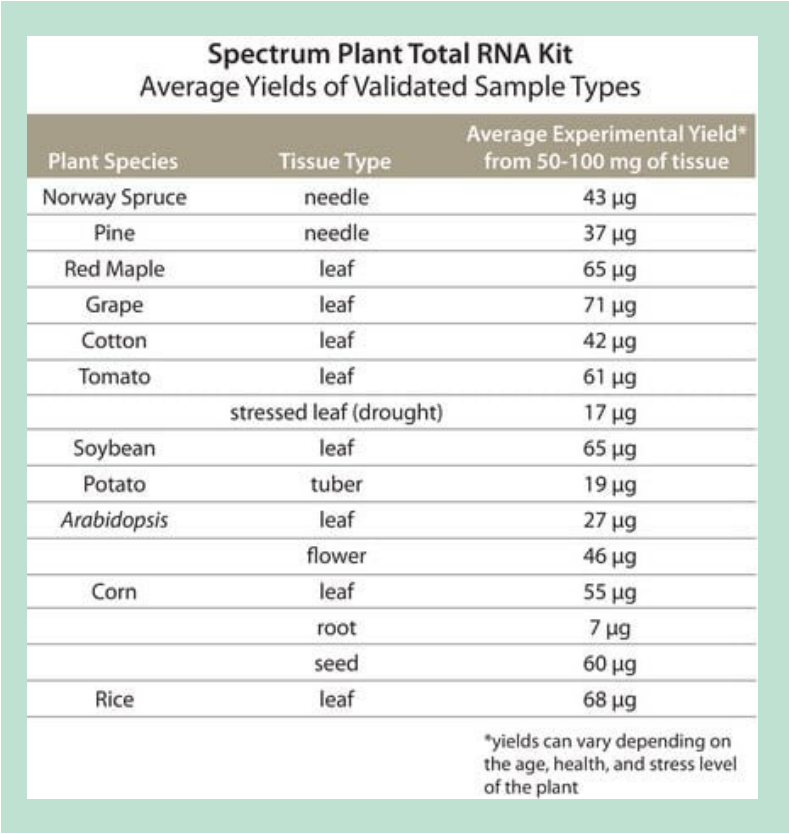
RNA extraction

1. Immediately add 1ml of Qiazol reagent (qiagen). If not using ground tissue, grind 50 mg of tissue in 500ul of reagent (using either mortar and pestle or in a 1.5ml Tube). Once ground add another 500ul of TRIZOL. Transfer to 1.5ml Tube if not already in one.
2. Rotate on daisy wheel rotor for 5mins at room temperature.
3. Centrifuge at 12,000g (11,400rpm) for 10mins at 4 °C to pellet any leftover ground tissue.
4. Transfer supernatant to new 1.5ml Tube.
5. Add 200ul of chloroform and shake vigorously by hand for 15 seconds (or already have the 200ul of chloroform in the new 1.5ml Tube in step 5).
6. Incubate at room temperature for 3mins.
7. Centrifuge at 12,000g (11,400rpm) for 15mins at 4°C (RNA remain in the colourless, upper, aqueous phase).
8. Transfer upper, aqueous phase to a new 1.5ml tube (Do not transfer any interphase material).
9. Add 500ul of isopropanol and mix in the new 1.5ml tube in step 9)
10. Incubate at room temperature for 10 mins.
11. Centrifuge at 12,000g (11,400rpm) for 10 minutes at 4°C (RNA forms pellet on side and bottom of Tube, white to brown in colour)
12. Remove the supernatant with a pipet be careful not to suck up the pellet.
13. Add 1ml of 75% ethanol and mix by vortexing.
14. Centrifuge at 7,500g (9,000rpm) for 5 minuts at 4 °C.
15. Remove the supernatant with a pipet be careful not to suck up the pellet.
16. Leave the 1,5 ml tube open in fume hood to air-dry the pellet for approximately 5-10 minutes (pellet turns from white to clear). Do not over dry as this will decrease the RNA solubility.
17. Resuspend RNA in 25-100ul (30µl) of RNase-free water. Aspirate sample to dissolve RNA pellet. Volume will depend on the amount of RNA which you estimate to be present and whether it is heavily contaminated with DNA. Samples containing less than 10ug of RNA should be resuspended in 25ul.
18. Incubate for 10 minutes at 60°C.
19. Store RNA at -70°C

5-6) Two commercial RNA extraction kits, also tested without success

Spectrum™ Plant Total RNA Kit (Sigma Aldrich):

This kit is designed for extraction from various plant tissues, such as leaf materials (including pine needles), seeds, roots, etc. Target tissues are listed below.



Spectrum Plant Total RNA Kit
Average Yields of Validated Sample Types

Plant Species	Tissue Type	Average Experimental Yield* from 50-100 mg of tissue
Norway Spruce	needle	43 µg
Pine	needle	37 µg
Red Maple	leaf	65 µg
Grape	leaf	71 µg
Cotton	leaf	42 µg
Tomato	leaf	61 µg
	stressed leaf (drought)	17 µg
Soybean	leaf	65 µg
Potato	tuber	19 µg
Arabidopsis	leaf	27 µg
	flower	46 µg
Corn	leaf	55 µg
	root	7 µg
	seed	60 µg
Rice	leaf	68 µg

*yields can vary depending on the age, health, and stress level of the plant

FIGURE A1. List of plant species and tissues suitable for RNA extraction using the commercial kit Spectrum Plant Total RNA Kit. Information in the figure copied from Sigma Aldrich product website: https://www.sigmaaldrich.com/DK/en/product/sigma/strn50 (accessed 28 Dec 2023).

RNeasy Power Plant Kit (Qiagen):

RNeasy PowerPlant Kit kan opbevares ved stuetemperatur (15-25°C) indtil den udløbsdato, der er trykt på æskens etiket.
Opløsning MBL skal opvarmes ved 55°C i 5-10 minutter for at opløse bundfald før brug. Brug Solution MBL, mens du stadig er varmt.
Tilsæt den passende mængde ß-mercaptoethanol (ßME) til opløsning MBL for at frembringe en slutkoncentration på 10 µl/ml MBL/ßME. For hver forberedelse vil der være behov for 600µl MBL/ ßME.

Alternativt kan du tilføje 594µl MBL og 6µl ßME direkte til hvert PowerBead-rør.
F.eks.12 prøver: 7,128 ml MBL + 72 µl ßME i et rør.

1. 50 mg planteprøve i et 2 ml PowerBead-rør.
2. Tilføj 550 µl opløsning MBL/ ßME til PowerBead-røret med planteprøven og 50µl af phenol-separationsopløsningen.

Bemærk: Hvis prøven har et højt indhold af phenoler, og du bruger phenolseparationsopløsningen, skal du reducere opløsning MBL/ßME til 550 µl og tilsætte 50 µl af phenolseparationsopløsningen.

3. PowerBead-røret med planteprøven og opløsningen kommes på Tissuelyser i 45s ved kraftig F/s For at opnå det højeste RNA-udbytte anbefales en kraftig perlepisk.
4. Centrifuger ved 12.000 rpm 2 minutter ved 21 grader.
5. Overfør al supernatanten til et rent 2 ml opsamlingsrør (medfølger). Forvent mellem 500 til 600 µl lysat.
6. Tilsæt 150 µl Solution IRS og vortex for at blande. Inkuber ved 4°C i 5 min. Bemærk: For planteprøver, der stadig indeholder PCR-hæmmere efter RNA-oprensning, kan du prøve at tilføje op til 200 µl Solution IRS.
7. Centrifuger rørene ved 12.000 rpm 2 minutter ved 21 grader. Undgå pelleten og overfør 650 µl af supernatanten til et rent 2 ml opsamlingsrør.
8. Tilsæt 650 µl opløsning PM3 og 650 µl opløsning PM4 til supernatanten. Vortex kort for at blande.
9. Overfør 650 µl supernatant på en MB RNA-søjle og centrifuger ved 12.000 rpm i 1 min. Kassér gennemstrømningen og anbring Spin-søjlen tilbage i 2 ml opsamlingsrøret. Gentag indtil al supernatanten er blevet fyldt på Spin Column.
10. Ryst for at blande opløsning PM5. Tilføj 600 µl opløsning PM5 til MB RNA-kolonnen og centrifuger ved 12.000 rpm i 1 min.
11. Kassér gennemstrømningen, anbring MB RNA Spin Column tilbage i 2 ml opsamlingsrøret, og tilsæt 600 µl opløsning PM4. Centrifuger ved 12.000 rpm i 1 min.
12. Kassér gennemstrømningen og centrifuger igen ved 13.000 rpm i 2 min.
13. Placer MB RNA Spin Filter i et rent 2 ml opsamlingsrør.
14. Tilsæt 50-100µl RNase-frit vand til midten af den hvide Spin Column-membran.
15. Inkuber ved stuetemperatur i 1 min. Bemærk: Eluering med 100µl RNase-frit vand vil maksimere RNA-udbyttet. For mere koncentreret RNA, kan der anvendes minimum 50µl RNase frit vand.
16. Centrifuger ved 12.000 rpm i 1 min. Kassér MB RNA Spin-kolonnen. Opbevares ved -70°C.

According to the manufacturer, this commercial kit is designed to isolate RNA from the most difficult plant tissues, including strawberry leaf, cotton leaf, cotton seeds and pine needles. The kit was tested on leaves of strawberry, rose and cotton plants. The extraction from strawberry leaf was unsuccessful, while rose and cotton leaves did yield RNA visualized on agarose gel (Figure below).

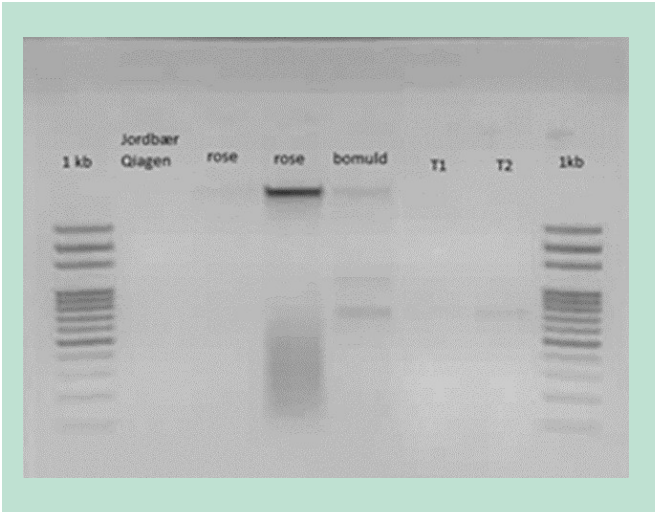


FIGURE 18. Picture of agarose gel after following the protocol of the RNeasy Power Plant Kit from Qiagen, using leaf materials of strawberry (Jordbær), rose, and cotton (bomuld). Only one of the rose leaves and the cotton leaf yielded RNA as visualized by the top band (i.e., large size) on the gel picture.

After RNA extraction

Mål RNA

Mål RNA på Myspec og noter konc. ng/µl – vi skal gerne, som minimum have 100 ng/µl. Tjek med Qubit (RNA BR Assay kit)

Prøve	MySpec ng/µl
2-20	**200 ng/µl**
2-21	**325 ng/µl**
2-22	**158 ng/µl**

DNase behandling

Deoxyribonuclease I, Amplification Grade, invitrogen

Ønsket koncentration 1 µg = 1000ng

$$\mu l\ pip = \frac{\text{ønsket ng konc.}}{\text{RNA målt ng/µl}} = \frac{1000\ ng}{200\ ng/\mu l} = 5\mu l\ RNA$$

Pr. reaktion		Eks.	
RNA (1 µg)	X µl	RNA (1 µg)	5 µl
DNase B	1 µl	DNase B	1 µl
DNase I, amp.	1 µl	DNase I, amp.	1 µl
Nuclear-free H_2O	Op til de 10 µl	Nuclear-free H_2O	3 µl
Total volume	10 µl pr. Reaktion	Total volume	10 µl pr. Reaktion

- Inkubere rørene I 15 min ved 21°C (stuetemp)
- Tilsæt 1 µl 25 mM EDTA for at stoppe DNase.
- Inkubere på PCR-maskinen i 10 min ved 65°C derefter kommes prøverne på cooler

BEMÆRK: Det er vigtigt ikke at overskride inkubationstiden på 15 minutter eller inkubationen ved stuetemperatur. Højere temperaturer og længere tid kan føre til Mg++-afhængig hydrolyse af RNA'et.
For at arbejde med større mængder RNA skal du opskalere reaktionen (inklusive volumen) lineært.

cDNA

iScript cDNA Synthesis kit.

Pr. reaktion		Eks. 12 prøver med 10 µl RNA. Hvis man vil mixe i rør	
5x iScript reaction Mix	4 µl	5x iScript reaction Mix	48 µl
iScript Reverse Transcriptase	1 µl	iScript Reverse Transcriptase	12 µl
Nuclear-free H_2O	5 µl	Nuclear-free H_2O	60 µl

RNA (1ng) efter DNase behandling	10 µl	RNA (1ng) efter DNase behandling	XX µl
Total volume	20 µl pr. Reaktion	Total volume	120 µl mix

Program: PCR maskine: cDNA

Program: PCR maskine: cDNA	
5 min	25°C
20 min	46°C
1 min	95°C
∞	4-12°C

Mål cDNA og fortynd ned

$$FF = \frac{C\ (stam)}{C\ (ønsket)} = \frac{200\ ng/\mu l}{25\ ng/\mu l} = 8$$

$$Vpip = \frac{V\mu l(slut)}{FF} = \frac{20\mu l}{8} = 2{,}5\ \mu l$$

fortyndnings (Nuclear-free H_2O) = Vµl(slut) - Vpip = 20µl - 2,5 µl = 17,5µl Nuclear-free H_2O

fortyndnings (Nuclear-free H2O) + Vpip (cDNA) = 17,5µl H_2O + 2,5µl cDNA

MasterMix

RealQ Plus 2x Master Mix Green Low ROX™

Komponent	Vol./reaktion
RealQ Plus 2x Master Mix	12.5 µl
Primer A (10 µM)	0.5 µl (0.25 – 2 µl)
Primer B (10 µM)	0.5 µl (0.25 – 2 µl)
Nuclear-free H_2O	X µl
Template DNA	X µl
TOTAL volume	25 µl

genomic DNA: 20 ng (1 – 100 ng) : cDNA?

real-time PCR (qPCR)

Primer:

Gene symbol	Primer sequence(5'-3')	Anneline tempera-ture*	Program
ActinA_F (forward)	TGGGTTTGCTGGAGATGAT	56-5=51°C	
ActinA_F (reverse)	CAGTAGGAGAACTGGGTGC	60-5=55°C	

Anealling temperature: T_m = 2(A+T) + 4*(G+C) minus 5°C fra T_m så har man T_a

CombiControl: Combining above- and belowground biological control agents for improved pest control in strawberry tunnel production

The present project explores the effects of combining above- and belowground biological control agents, to target several pest species in strawberry production in tunnels. The effects of single and combined use of macro- and microbiological control agents, on the population growth of spider mites, *Tetranychus urticae*, on a common strawberry cultivar was evaluated in controlled laboratory experiments. Treatment combinations included root inoculations with the entomopathogenic fungus (EPF) *Metarhizium brunneum*, and release of predatory mites, *Neoseiulus cucumeris*. The knowledge gained in the controlled experiments was implemented in field tests in combinations of predatory mites and EPF at larger scale in commercial strawberry tunnels. The objective was to investigate the effect of combining above- and belowground biological control on natural pest abundance, specifically spider mites, thrips, and aphids, as well as fruit damage throughout the production season.

The study found that the population growth of spider mites, *T. urticae*, reduced on strawberry plants with biological control treatments, while the results confirmed the importance of evaluating specific isolates within a fungal species. The combined application of *M. brunneum* belowground and the predatory mite *N. cucumeris* aboveground showed a potential for delaying pest population build up in commercial strawberry tunnel production, while severe infestations of thrips cannot be controlled by the biocontrol agents alone, and additional measures are needed to control such attacks by non-chemical means in strawberry tunnels.

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