Epidemic development of Neozygites floridana (Zygomycetes: Neozygitaceae) in Tetranychus urticae (Acari: Tetranychidae) populations with and without the predatory mite Phytoseiulus persimilis (Acari: Phytoseidae)

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Preface

This master thesis is a part of the BERRYSYS project, with the aim to increase organic and integrated strawberry production in high plastic tunnels. The two- spotted spider mite, its natural enemies (*N. floridana* and predatory mites) and powdery mildew are used as a model system by the use of conservation, inoculation and inundation biological control. The project is run by the Norwegian Institute for Agricultural and Environmental Research and funded by Agricultural Agreement Research Founds (Forskningsmidler over Jordbruksavtalen, JA) and Norwegian Foundation of Research Levy on Agricultural Products (Fondet for forskningsavgift på landbruksprodukter, FFL), project number 190407/ 199.

I would like to thank my external supervisor and project leader at Bioforsk Plant Health and Plant Protection, Dr. Ingeborg Klingen, for sharing knowledge on both academic and technical level. She introduced me to the research idea and the peculiar world of *N. floridana*. Dr. Nina Trandem at Bioforsk Plant Health and Plant Protection/ UMB has been my internal and formal supervisor. She introduced me to Minitab, and gave me valuable advice on how to handle statistics. She also performed valuable proof reading the last days before submitting the thesis. Statistical advice and guidance from Torfinn Torp at Bioforsk was of great help. Karin Westrum, sectional engineer at Bioforsk Plant Health and Plant Protection, is thanked for sharing her brilliant photos of *N. floridana* and thoughts on *N. floridana*. Toril Sagen Eklo, research technician at Bioforsk Plant Health and Plant Protection, gave me advice for preparation and cultivation of bean plants, and for that I am grateful. Thanks also to Katja Hora at Koppert BM for providing me with predatory mites from the Netherlands. Eva, Sigmund and Mari: thank you for entertaining Johan at times when the work load was tremendous. Sigmund and Anniken: thank you for sharing your knowledge and experience on how to refer properly and how to write academically. Finally, and most of all, the patience and support from my beloved Anniken and Johan was of inestimable value.

Abstract

The entomopathogenic fungus *Neozygites floridana* is an important natural enemy of the two-spotted spider mite *Tetranychus urticae*. In order to investigate how the predator *Phytoseiulus persimilis* affects the transmission of *N. floridana* to *T. urticae*, a preliminary experiment on establishing the climatic conditions in a climatic chamber for an *N. floridana* epizootic were conducted. Bean plants (*Phaseolus vulgaris*), *T. urticae* and two different isolates of *N. floridana* were used as a model system in plexiglass cages. Ambient relative humidity (RH) and temperature (˚C) set at a constant of 70% and 18˚C, respectively, led to an epizootic outbreak of a Brazilian *N. floridana* isolate, but not a Norwegian *N. floridana* isolate. Recordings of the microclimatic RH and temperature 5mm underneath the boundary layer of *P. vulgaris* leaves revealed higher RH and temperature compared to the ambient conditions.

In this laboratory experiment, *T. urticae* exposed to *N. floridana* and *P. persimilis* simultaneously seem to have a higher prevalence of *N. floridana* compared to *T. urticae* exposed to *N. floridana* only. Ten days after inoculation with *N. floridana*, 59 % of the *P. persimilis* exposed *T. urticae* contained *N. floridana* hyphal bodies. In comparison, the level of hyphal bodies in the non- exposed *T. urticae* was 29 %. These differences were significant (P= 0.008). Although not significantly, this trend continued throughout the experiment. However, the amount of *T. urticae* with infective capilliconidia attached to the body ten days after inoculation with *N. floridana* was higher in the cages with non- exposed *T. urticae* (18% vs 11%, P= 0.015). This trend continued throughout the experiment, but not significantly except for the last sampling day.

Key words: Neozygites floridana, Tetranychus urticae, Phytoseiulus persimilis, epidemic development, trophic interactions, biological control.

Table of content

1. Introduction

The two- spotted spider mite, *Tetranychus urticae* (Acari: Tetranychidae), is an important pest of many crops in horticulture (Jeppson et al., 1975; Raworth, 1986; Butcher et al., 1987). By penetrating the plant tissue the mite removes cell contents, which in turn negatively affect the processes of transpiration and photosynthesis (Jeppson et al., 1975; Tomczyk & Kropczynska, 1985). Acaricides have been widely available and applied to control *T. urticae* populations. Increased knowledge of the herbivorous mites' rapid development of resistance and reports of negative environmental effects of pesticides since the latter part of 20th century, has intensified the research towards biological control as a part of integrated pest management solutions.

This article aims to further elaborate the potential for biological control of *T. urticae*, by describing a laboratory experiment on the epidemic development of *Neozygites floridana* (Zygomycetes: Neozygitaceae). *N. floridana* is an obligate parasite of *T. urticae,* and is by many authors regarded as a potential mycoinsecticide (Keller, 1997; Chandler et al. 2000; Elliot et al., 2000). The reason for this is its ability to create epizootics in *T. urticae* field populations (Klubertanz et al., 1991). Epizootics are defined as a sudden increase of diseased individuals within a population (Tanada & Kaya, 1993). However, the dynamics of epizootics are complex. Temperature and relative humidity (RH) are two important factors influencing the effectiveness of *Neozygites spp.,* and sporulation and germination is dependent on high RH (Smitley et al., 1986; Klubertanz et al., 1991; Delalibera et al., 2006). However, macroclimatic RH is not always a limiting factor on sporulation and germination due to the fact that these processes occur close to the boundary layer of the leaf surface where RH is high even in periods with low ambient RH (Wekesa et al., 2010; Fargues et al., 2003; Boulard et al., 2002). Additionally, there are significant knowledge gaps regarding the full understanding of epizootics. First, many studies have been undertaken to investigate under what climatic conditions sporulation and germination of *Neozygites spp*. occur (see Carner, 1976; Delalibera et al., 2006; Oduor et al., 1996; Smitley et al., 1986), while there is limited knowledge on the mechanisms of epizootic development. Second, the complexity of interactions between different trophic levels makes this area of studies challenging. Therefore, there are still gaps of knowledge regarding important factors, and it is difficult to conclude generally from the results of single studies. Third, there is a need for more

field studies on the topic. Finally, the knowledge of *N. floridana* isolates originating from the northern hemisphere is limited.

T. urticae infected by *N. floridana* eventually die of mycosis and turn into mycosed cadavers (Carner, 1976). Non- infective primary spores are being actively ejected from the cadavers (Oduor et al., 1996) and spread to nearby surroundings on the leaf (Wekesa et al., 2007), to spider mites close to the cadaver or transported by wind currents to areas within the plant or to other plants. The primary spores then germinate into infective capilliconidia (secondary spores), by which transmission to new *T. urticae* individuals occur (van der Geest et al., 2000).The immobility of the fungus implicates that the transmission process is highly dependent on movement by the host. Factors that increase the movement of *T. urticae* may be various, and the presence of predators may be one of them. *T. urticae* avoids plants and leaves where predators are or have been present (Pallini et al., 1997). Pallini et al. (1999) confirm that this also is the case when the specialist predatory mite *Phytoseiulus persimilis* (Acari: Phytoseiidae) encounters *T. urticae* infected areas. Grostal & Dicke (1998) performed leaf disc tests which demonstrated that, when given a choice, adult *T. urticae* avoided leaf discs that previously (24h) had been exposed to *P. persimilis*. Further, *T. urticae* emigrated more from the predatory exposed discs compared to unexposed discs. Studies done on an aphid- specific entomopathogenic fungus, *Pandora neoaphidis* (Entomophtorales: Entomophtoraceae), revealed that presence of both the coleopteran predators *Harmonia axyridis* (Coleoptera: Coccinellidae) and *Coccinella septempunctata* L. (Coleoptera: Coccinellidae) enhanced transmission of the fungus to aphids compared to a control without predators present (Wells et al., 2011). Similar findings have been reported in other systems with entomopathogenic fungi and foraging predators and parasitoids (Roy et al., 1998; Furlong & Pell, 1996). These findings may lead to the following hypotheses: (1.) *P. persimilis* increases the movement of *T. urticae* on leaves/ plants and (2.) this increased movement of *T. urticae* leads to more rapid development of an *N. floridana* epizootic. To my knowledge, no studies have previously been conducted on the effect of *P. persimilis* on the transmission of *N. floridana* to *T. urticae*.

A greater understanding of the interactions between pathogens and predators of *T. urticae* could help us to determine whether it is advantageous to use *N. floridana* and *P. persimilis* simultaneously in augmentative and/ or in conservation biological control systems. Thus, the

main objective of this experimental study was to investigate whether *P. persimilis* affects the epizootic development of *N. floridana* in a *T. urticae* population. In order to do this, the climatic conditions needed for establishing an epizootic of *N. floridana* had to be investigated. The article therefore presents two different experiments:

- Experiment 1: Establishment of conditions needed for an epidemic development of *N. floridana* in a *T. urticae* population
- Experiment 2: The effect of *P. persimilis* on the epizootic of *N. floridana*

The experiments were carried out in insect cages with bean plants, in a climatic chamber/ culture room at Bioforsk, Ås.

2. Materials and methods

2.1 Preparations for the experiments

2.1.1 Cultivation of plants

Bean plants (*Phaseolus vulgaris*) were grown in pots (Billund Potter, height 120 mm width 80 mm) with soil (L. O. G. Gartnerjord) at 70% RH, 24˚C and a photoperiod of 16 L: 8 D. Ten days after sowing, the first leaf pair unfolded and ten pots containing 3- 5 plants were transferred into transparent plexiglass cages (height 610 mm, width 510 mm) in a climatic chamber at 70% RH, 18˚C and a photoperiod of 18 L: 6 D.

Picture 1. Bean plants were cultivated in pots (left) and transferred to transparent plexiglass cages (right) ten days after sowing. Photo: Ronny Berdinesen.

2.1.2 Two- spotted spider mite (*T. urticae*) stock culture

T. urticae used in the experiments were picked from a laboratory culture reared on bean plants at The Norwegian Institute for Agricultural and Environmental Research (Bioforsk), Plant Health and Protection Division at Ås in Norway. This laboratory culture was earlier collected in a commercial strawberry field in south- east of Norway (59˚ 42´N, 10˚ 44´ E) in 2003 and then reared on bean plants (*P. vulgaris*) in a transparent plexiglass cage at 60% RH, 21˚C and a 16 L:8 D photoperiod.

2.1.3 Predatory mites (*P. persimilis*)

Predatory mites (Spidex ^{TM}) were ordered from Koppert BV (Berkel en Rodenrijs, Holland). By the use of a hand held magnifier (6x Eschenbach ^{TM}), large mites with a more widely rounded posterior could be identified as adult females. They were picked with a small paintbrush and used for the experiment.

2.1.4 *N. floridana* isolates

The *N. floridana* isolates used in experiment 1 had different origins, one Norwegian isolate and one Brazilian isolate. The Norwegian *N. floridana* isolate (NCRI 271/ 04) used was collected from its host *T. urticae* on strawberry, *Fragaria spp*., in a commercial strawberry field in the south- east of Norway (59˚ 42´ N, 10˚ 44´ E) in 2003. The Brazilian isolate (ESALQ 1420) used was collected from its host *T. urticae* on Jack Bean, *Canavalia ensifromis*, at Piraicaba, Sao Paolo- Brazil (22˚ 42´ 30´´ S, 47˚ 38´ 00´´ W).

2.1.5 Production of *N. floridana* infected *T. urticae* cadavers (used to infect *T. urticae* in the experiments)

Leaf discs (12 mm diameter) from *P. vulgaris*, were placed with the underside upwards on 1.5% water agar in a Petri dish (60 mm diameter). On each leaf disc, three *N. floridana* killed *T. urticae* cadavers (referred to as cadavers from now) (stored in dry and cold conditions (4 ˚C)) were placed on a leaf nerve with their dorsal side up in order to enhance the dispersal of primary spores. Five such Petri dishes (with cadavers on leaf discs) were then placed in a plastic box (22x16x7 cm) covered with aluminum foil for darkness, and incubated at 20 ºC, 90% RH and L18:D6 for 24 h. Cadavers were checked for good sporulation and production of capilliconidia after 24 h of incubation. When a good development of capilliconidia was observed, 30-35 living *T. urticae* females were placed on each leaf disc with cadavers for *N. floridana* infection. Some drops of water were poured onto the agar to prevent *T. urticae* from escaping from the leaf discs. Before the plastic container once more was wrapped in aluminum foil for darkness and incubated at RH 90% and 20˚C for 24 hours, the *T. urticae* were exposed to light for one hour in order to increase the movement of *T. urticae* and increase the probability for capilliconidia attachment to mite legs. After 24 hours of incubation, leaf discs with *N. floridana* inoculated *T. urticae* were transferred to bean plants (*P. vulgaris*). These plants were kept under ambient laboratory conditions at 22-25 ˚C, 20-30% RH and continuous light. After 5- 10 days, dry non- sporulating cadavers developed, and could be harvested with a small paintbrush and wrapped in small pieces of cotton cloth. The cotton cloth with the cadavers was packed into a 1, 8 ml NUNC Cryo tube™ and stored in the fridge at 4˚C and constant darkness until used for production of live, *N.*

floridana infected *T. urticae* for the experiment. *T. urticae* adult females were inoculated at the same conditions as described above one day before use in the experiments.

2.2. Experiment 1: establishment of conditions needed for an epidemic development of *N. floridana* in a *T. urticae* population

To experimentally establish light and climatic conditions that would yield an epidemic development of the Norwegian and the Brazilian *N. floridana* isolate in a *T. urticae* population on bean plants in a plexi glass cage, an experiment was conducted in a climatic chamber at The Norwegian Institute for Agricultural and Environmental Research (Bioforsk), Plant Health and Protection Division at Ås in Norway between 15.10.2010 and 16.12.2010.

Ten pots, containing between three and five 10- day old bean plants, were placed into each of 3 plexiglass cages. The pots were arranged as shown in Fig. 1. One of the cages was exclusively used for micro climatic monitoring purposes (see 2.2.3).

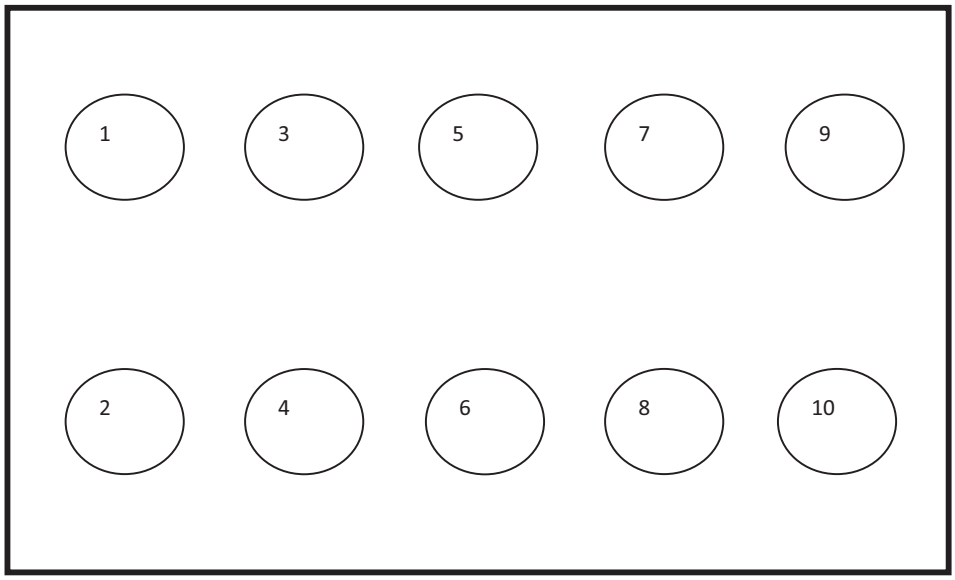


Fig. 1. Bird view of the arrangement of pots of bean plants inside a plexiglass cage. Three identical arranged plexiglass cages were used in the experiment: one cage for the Brazilian isolate, one for the Norwegian isolate and one for monitoring purposes.

In order to infest the plants with *T. urticae*, two bean leaf discs (12 mm diameter) with 25 living *T. urticae* females were placed in the centre on the upper side of each of two fully unfolded cotyledon leaves on one plant in each of the 10 pots. The mites were then left undisturbed for 10 days to establish. To infect the established *T. urticae* population with *N. floridana*, one leaf disc with 20 live, *N. floridana* inoculated *T. urticae* were transferred to the centre of each of two fully unfolded cotyledon leaves in pot 3, 4, 5, 6, 7 and 8. No *N. floridana* inoculated *T. urticae* were transferred to plant 1, 2, 9 and 10. All plants were irrigated when needed and fertilized once a week.

2.2.1 Monitoring the *T. urticae* population

To record the population development of *T. urticae*, living adult females were counted on site with a hand held magnifier (magnification: 6x). This was conducted weekly on plant 3, 4, 5, 6, 7 and 8, from 10 days post transfer of healthy mites until senescence of plants. The sites of counting were the middle leaf of a trifoliate leaf, at three heights in the plant canopy (see Fig. 2). This was done in order to standardize the registration method and to map the vertical differences in *T. urticae* distribution.

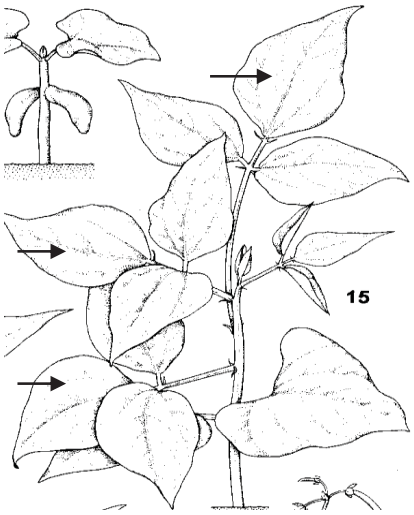


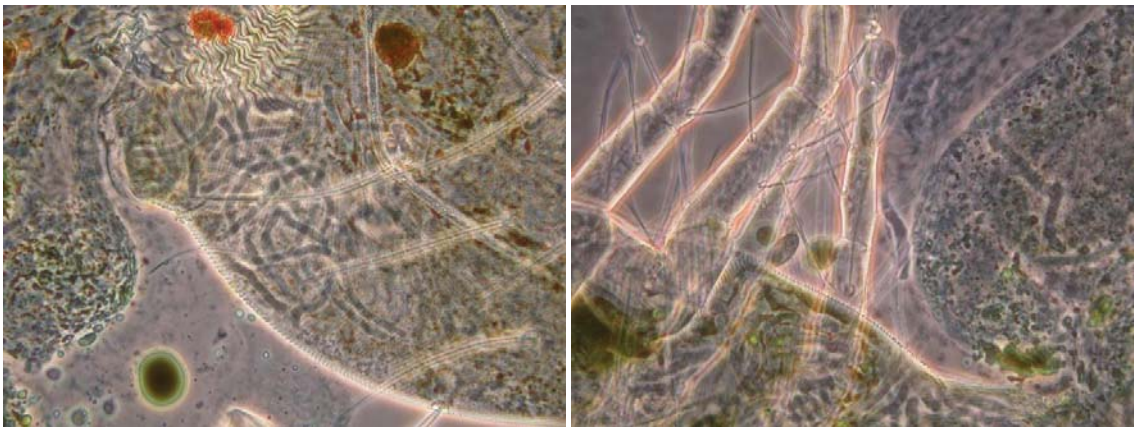
Fig. 2. The three sites used for monitoring the spider mite population. The lower arrow indicates the middle leaf of trifoliate 1, the middle arrow indicates the middle leaf of trifoliate 2 and the uppermost arrow indicates the middle leaf of trifoliate 3. Drawing from Feller et al. 1995.

2.2.2 Monitoring of *N. floridana* killed *T. urticae* cadavers, capilliconidia and hyphal bodies

Registrations and samplings to determine the level of *N. floridana* infection were solely carried out on plant 3, 4, 5, 6, 7 and 8. All registrations and samplings were conducted on the underside of the middle leaf of trifoliate 1, 2 and 3 (see Fig. 2). The infection level was determined by the use of two methods, the first by direct counting cadavers and the second to search for *N. floridana* hyphal bodies and capilliconidia in female *T. urticae* under a microscope. The two methods are described in detail below.

1) Direct counting of cadavers: This was conducted by the use of a hand held magnifier (magnification: 6x). The counting of cadavers started ten days post transfer of *N. floridana* infected mites and was carried out once a week until senescence of plants.

2) Squash mounting of live adult female mites to observe for *N. floridana* hyphal bodies and capilliconidia: The sampling of live adult female *T.urticae* started ten days post transfer of *N. floridana* infected mites and was carried out two times a week until senescence of plants. One adult female mite was collected from each of the three leaves of the trifoliate at all three heights in the plant canopy, giving a total of maximum 9 mites per plant. The mites were stored in 70% ethanol until they were squash mounted on a coverslip in lactic acid cottonblue (50 % lactic acid mixed with 50 % distilled water and 0.075 % cotton blue) and checked for hyphal bodies and capilliconidia as described by Nordengen & Klingen (2006).



Picture 3. *N. floridana* hyphal bodies inside a live mounted adult female *T. urticae* (left) and capilliconidia attached to the legs of a *T. urticae* (right). Photos: Karin Westrum.

2.2.3 Light, macro- and microclimatic conditions

Brazilian and Norwegian *N. floridana* isolates require different conditions for ideal sporulation, especially regarding temperature, as described in Klingen et al. (2009) and Oduor et al. (1996). Due to the fact that only one culture room was available for the experiment, the macroclimatic conditions were, in order to promote an epizootic, set at a level intermediate to the two isolates' requirements; 70% relative humidity and 18˚C, and a photoperiod of 18h L- 6h D. Fluorescent tubes adapted for plant growth and maximum stomata opening were obtained by using Philips Master TL- D 90 Graphica/ 965 and Philips Master TL- D Super 80/ 827fluorescent tubes, arranged 2:1 (TL- D 90: TL- D Super 80) as suggested by plant physiologist, Dr. Anita Sønsteby. The idea was that maximum stomata openings would give a high microclimatic (within the leaf boundary level) RH and hence better conditions for an epidemic development of *N. floridana* in the *T. urticae* population.

Two MSR 145W Modular Signal Recorders were used to measure microclimatic RH and temperature close to the axial leaf surface. The sensors made recordings every 10^{th} minute, and were adjusted to a distance of 5mm from the axial side. The microclimatic measurements were conducted on a leaf on plants in a separate plexiglass cage (see Fig. 1). This was in order to avoid disturbance of the Signal Recorders when monitoring the spider mite population.

Picture 2. The sensors of the Modular Signal Recorder were located underneath the leaf. Photo: Ronny Berdinesen.

2.3 Experiment 2: the effect of *P. persimilis* on the epizootic of *N. floridana*

To evaluate the effect of the predatory mite *P. persimilis* on the epidemic development of *N. floridana* in a *T. urticae* population on bean plants, an experiment was conducted in a climatic chamber at The Norwegian Institute for Agricultural and Environmental Research (Bioforsk), Plant Health and Protection Division at Ås between 28.01.2011- 04.11.2011.

Due to lack of success with establishing the right conditions needed for an epidemic development of the Norwegian *N. floridana* isolate (see section 3.1), this experiment was carried out with the Brazilian isolate only. The experimental set up was as described in 2.2, except that six Plexiglas cages, each with 10 pots, were used in this experiment:

- three cages (cage 1, 2 and 3) were used for treatment 1: *T. urticae* with *N. floridana*
- three cages (cage 4, 5 and 6) were used for treatment 2: *T. urticae* with both *N. floridana* and predatory mites (*P. persimilis*)

Two of the cages (1 and 6) were also used to record the microclimatic relative humidity (RH) and temperature (˚C). The introduction of *T. urticae* and the *N. floridana* inoculation was conducted as described in 2.2. Predatory mites (*P. persimilis*) were introduced into cage 4, 5 and

6 one week after the introduction of *N. floridana* inoculated *T. urticae*. One healthy adult *P. persimils* female was placed on the middle leaf of a trifoliate at the lowest and middle height of the plant canopy (see Fig. 2) on plant 3, 4, 5, 6, 7 and 8.

The experiment was repeated two times, giving a total of 6 cages per treatment.

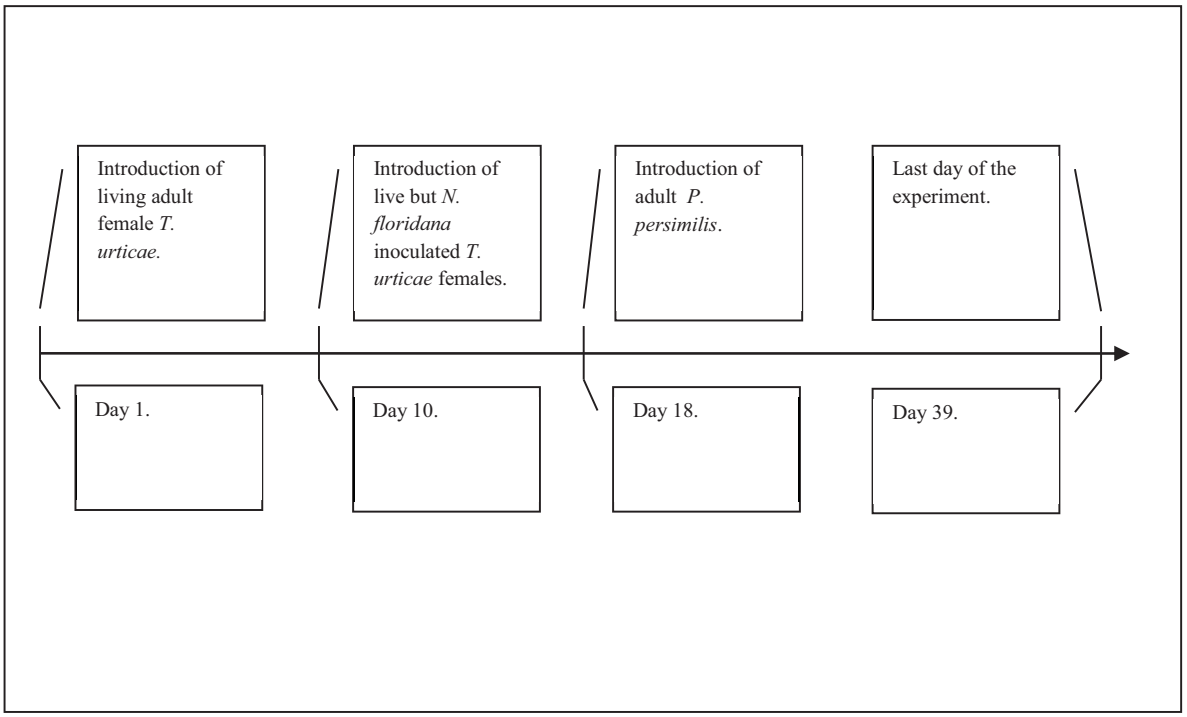


Fig. 3. Timeline showing the days for introduction of healthy adult *T. urticae* females, *N. floridana* inoculated adult *T. urticae* females, and adult *P. persimilis*.

2.3.1 Monitoring of *T. urticae* and *P. persimilis* populations

Registrations of healthy adult female *T. urticae* and *P. persimilis* were carried out as described for *T. urticae* in 2. 2. 1.

2.3.2 Monitoring *N. floridana* (capiliconidia, hyphal bodies and mummified *T. urticae*)

The infection level was determined by the use of two methods (direct counting and squash mounting), as described in 2.2.2. An adjustment in method 2 (squash mounting) had to be made, however: a maximum of five adult female *T. urticae* were collected from each middle leaf of a trifoliate at all three canopy heights on plant 3 and 7 two times a week every second week and two times a week every second week the same was conducted on plant 4 and 8. This gave a total of maximum 15 mites per plant collection, not 9 as described in 2.2.2.

2.4 Statistical analysis

2.4.1 Establishment of conditions needed for an epidemic development of *N. floridana* in a *T. urticae* population

This experiment was carried out in just two cages, solely to find a method suitable for conducting the experiment with the effect of *P. persimilis* on the epizootic on *N. floridana*, and no statistical analyses were performed.

2.4.2 The effect of *P. persimilis* on the epizootic of *N. floridana*

In order to test whether *P. persimilis* had an effect on the transmission of *N. floridana* to *T. urticae*, analysis of variance (ANOVA, general linear model, Minitab Inc, 2010) was carried out for each response variable and week of monitoring the study organisms. Two plants per cage were sampled throughout the experiments, and the response variables were: The number of *N. floridana* killed *T. urticae* cadavers per two plants, the number of adult female *T. urticae* per two plants, the percent of *T. urticae* with hyphal bodies per two plants and the percent of *T. urticae* with capilliconidia per two plants. The data were log transformed ($\log_{10}(n+0.5)$) to obtain a normal distribution of the residuals. The factors in the model were: The treatment effect

(presence of *P. persimilis* (0,1)) and two random effects (identity of trial repetition (1, 2), and cage number (1, 2, 3, 4, 5, 6)).

3. Results

3. 1 Establishment of conditions needed for an epidemic development of *N. floridana* in a *T. urticae* population

When using method 1 (cadaver counts) to estimate the *N. floridana* infection level, no epidemic development in the *T. urticae* population and very few mummified cadavers were found in the cage with the Norwegian *N. floridana* isolate, while the numbers of mummified *T. urticae* cadavers in cage with the Brazilian *N. floridana* isolate seem to increase from day 14 (Fig. 4). The *T. urticae* population inoculated with the Norwegian isolate continued to increase after day 21, while the one with the Brazilian isolate decreased (Fig. 4). No observations were done in the cage with the Norwegian isolate after day 28 due to senescence of the bean plants.

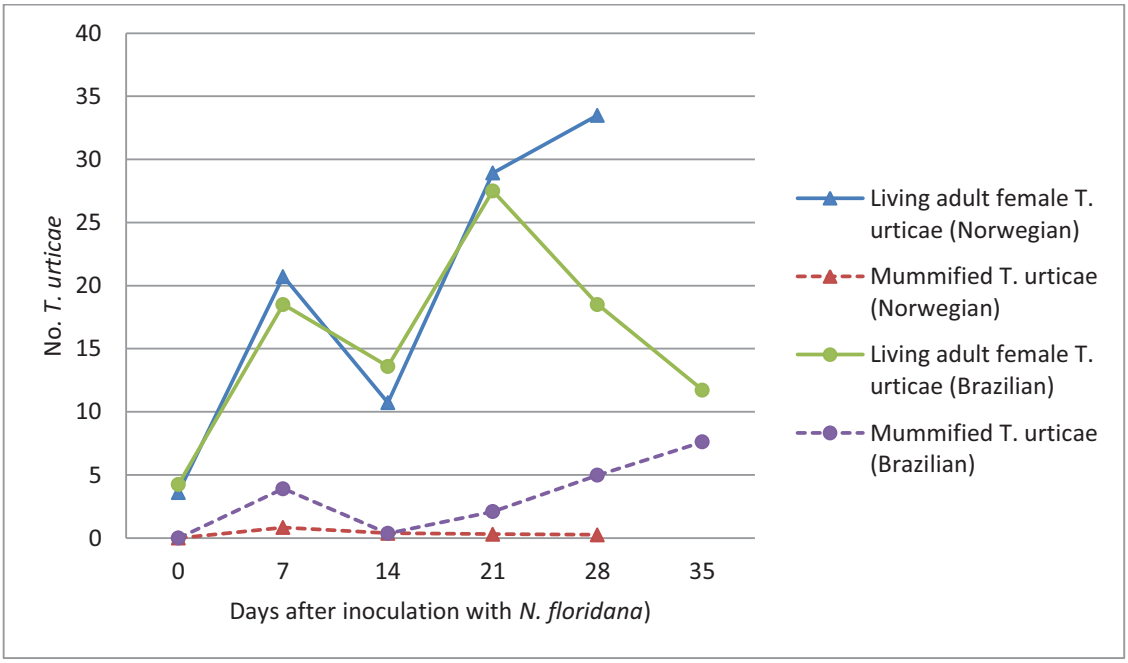


Fig. 4. Numbers of living adult *T. urticae* females and mummified *T. urticae* cadavers (Method 1) in cage with Norwegian and Brazilian *N. floridana* isolates, respectively, at different times after *N. floridana* inoculation.These are mean numbers of three leaves (middle leaf strata 1, strata 2 and strata 3) and 6 plants (plant 3, 4, 5, 6, 7 and 8).

Also when using method 2 (squash mounting) to estimate the *N. floridana* infection level, the Norwegian isolate had a lower infection compared to the Brazilian isolate except for the last sampling day when the infection levels were similar (32.5% and 29.7%, Norwegian and Brazilian isolate respectively) (Fig. 5 and 6).

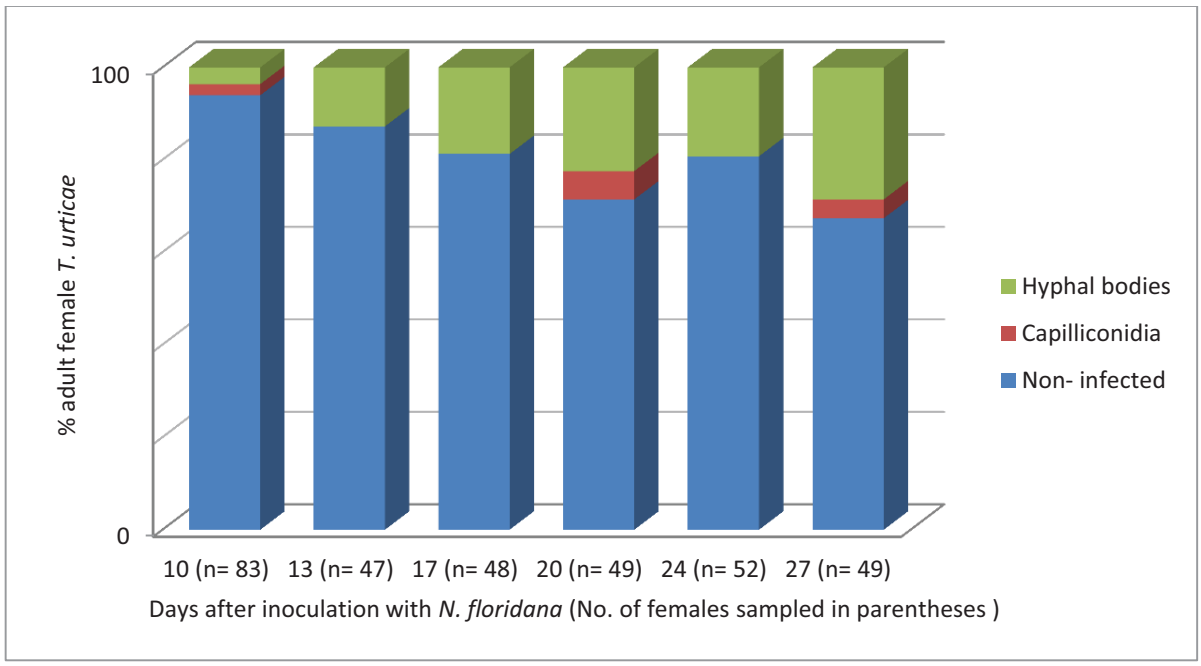


Fig. 5. Percent of live squash mounted *T. urticae* adult females (Method 2) with no *N. floridana* fungal structures (non-infected), with capilliconidia attached to body and with hyphal body of the Norwegian *N. floridana* isolate (NCRI 271/ 04).

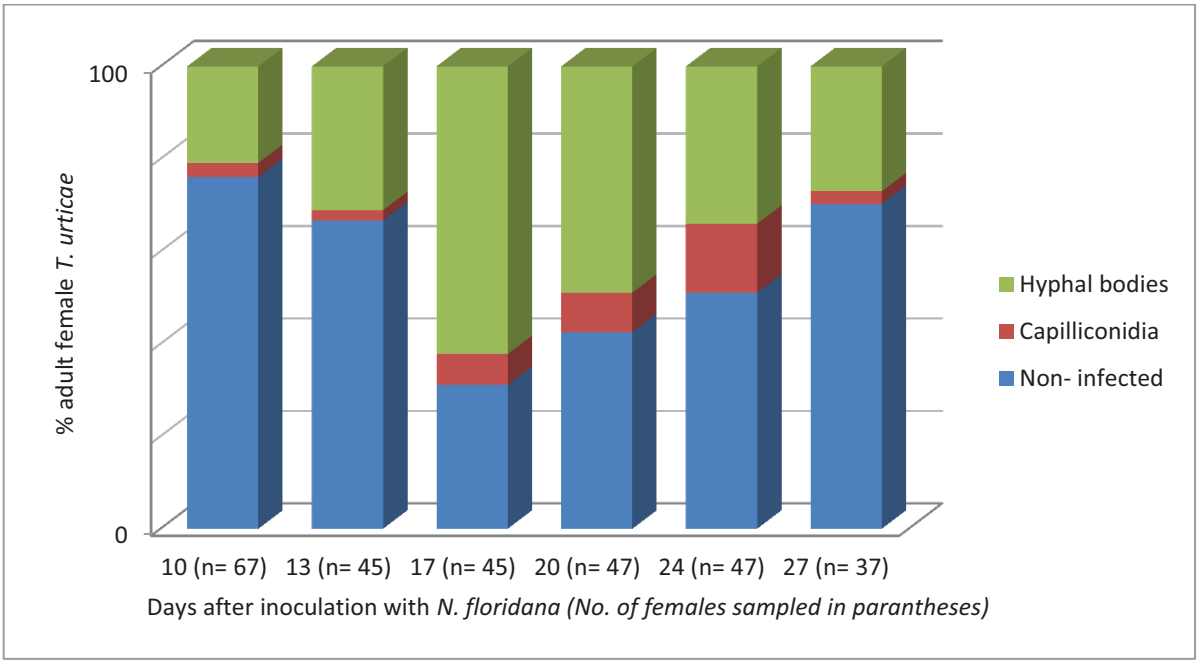


Fig.6. Percent of live squash mounted *T. urticae* adult females (Method 2) with no *N. floridana* fungal structures (non-infected), with capilliconidia attached to body (contaminated) and with hyphal body(infected) of the Brazilian *N. floridana* isolate (ESALQ 1420).

The RH 5 mm below the underside of the leaf did not exceed 95 % until the sixth day after inoculation with *N. floridana* (Fig. 7.), and the microclimatic temperatures were stable throughout the experiment, with night temperatures (with light off) at approximately 18˚C and day temperatures (with light on) at approximately 21˚C (Fig. 7.).

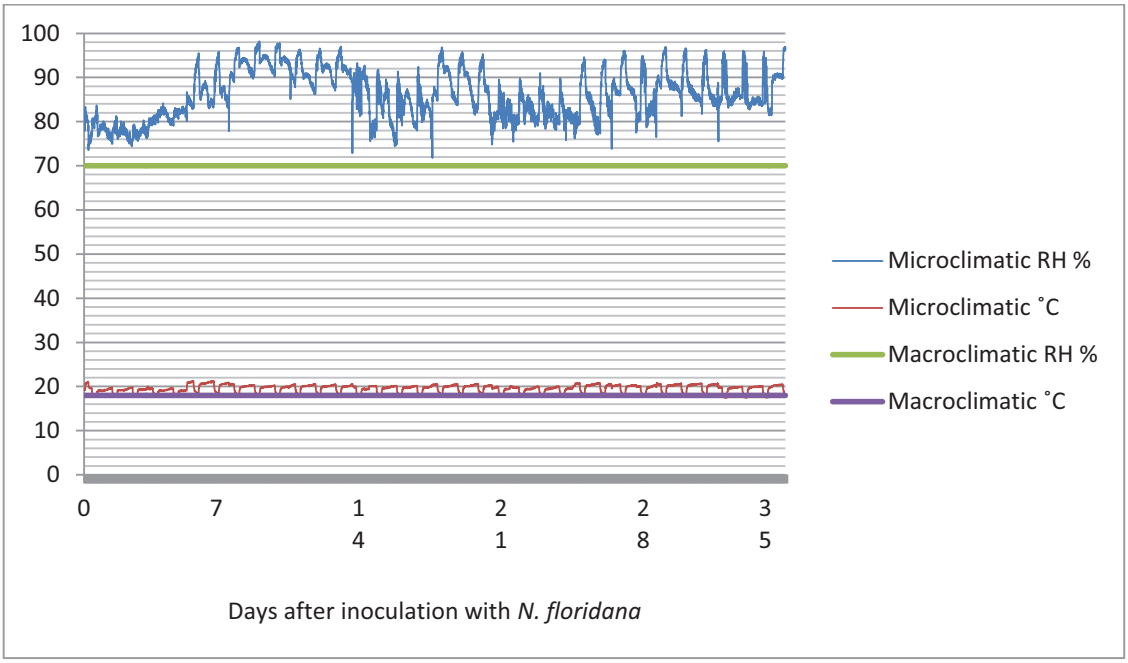


Fig. 7. Microclimatic (5 mm below the underside of the leaf) RH (%) and temperature (C˚) measured every 10th minute throughout the experiment. Straight lines indicate the macroclimatic settings (70 % RH and 18 ˚C).

3. 2 The effect of *P. persimilis* on the epizootic of *N. floridana*

When using method 1 (cadaver counts) to estimate the *N. floridana* infection level, a higher number of cadavers were seen in cages with *P. persimilis* compared to cages without *P. persimilis* at day 8, 15 and 22 (Table 1.), although not significantly (P= 0.971; 0.321; 0.052, respectively). However, the amount of *N. floridana* killed *T. urticae* cadavers were higher in the cages without *P. persimilis* (1545) compared to in the cages with *P. persimilis* (1327) on day 28 (P= 0.697) (Table 1). Living *P. persimilis* were observed all days after the day they were introduced (Table 1.).

Table 1. Total numbers of living adult *T. urticae* females on, mummified *T. urticae* cadavers and living *P. persimilis* in cages with and without *P. persimilis* at different time intervals. N= 12 plants.

Day after inoculation	**# Living adult female *T. urticae***	**# Mummified *T. urticae* (all stages)**	**# Living *P. persimilis* (mobile stages)**
Without *P. persimilis*			
1	89	0	
8	491	14	
15	376	144	
22	449	384	
29	243	1545	
With *P. persimilis*			
1	69	0	0
8	563	17	12
15	328	245	2
22	226	548	8
29	17	1327	18

When using method 2 (squash-mounting adult females) to estimate the *N. floridana* infection level, the results showed that 10 days after inoculation with *N. floridana*, a significantly higher percent of *T. urticae* with hyphal bodies were found in cages with *P. persimilis* than in cages without *P. persimilis* (59.2 vs 28.9%, respectively, p=0.008, figure 8). This trend of higher infection in the presence of *P. persimilis* persisted through the rest of the experiment (day 13= 73.2 vs 54.7%, day 17= 65.6 vs 61.1%, day 20= 68.7 vs 64.2%, day 24= 78.1 vs 58.4% and day 28= 81.2 vs 56.6%), but the difference was not significant for any of the days (P= 0.10; 0.76, 0.13; 0.66; 0.19, respectively). For the percent *T. urticae* found with capilliconidia attached to the body the results were only significantly (P= 0.015) higher in the cages without *P. persimilis* (18.5%) compared to the cages with *P. persimilis* (10.8%) at day 10 after inoculation of *N. floridana*. Day 13= 13.9% vs 10.9%, day 17= 7.4% vs 11.5%, day 20= 14.2% vs 6.2%, day 24= 39.6% vs 6.2% and day 28= 11.1% vs 9.4. However, none of these findings were significant (P= 0.60; 0.46; 0.09; 0.20, day 13, 17, 20 and 24 respectively) except for day 28 (P= 0.037) (Fig. 8 and 9).

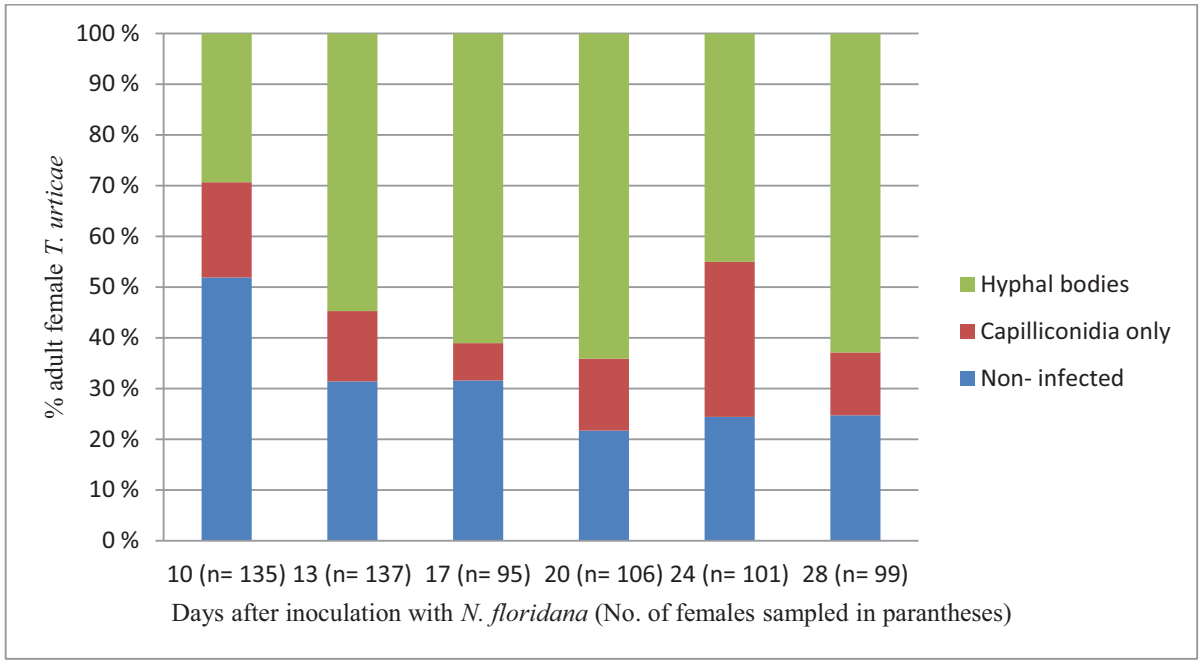


Fig. 8. Percent of live squash mounted *T. urticae* adult females (Method 2) with no *N. floridana* fungal structures (non-infected), with capilliconidia attached to body and with hyphal body in cages without *P. persimilis* at different time intervals.

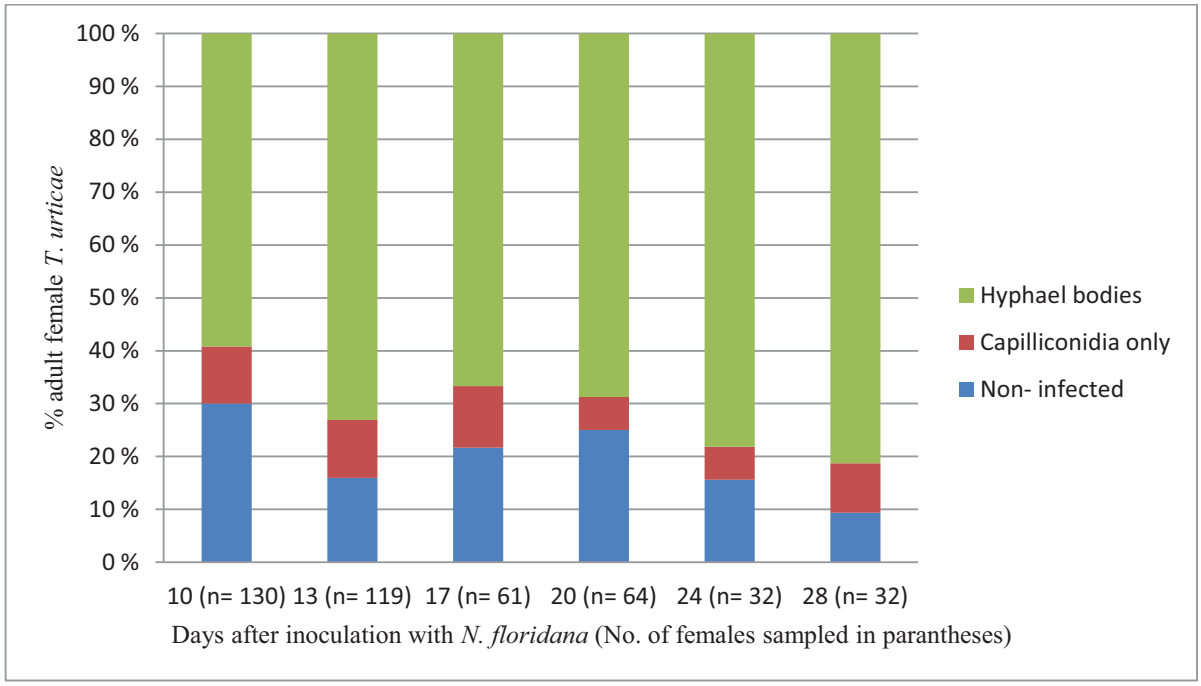


Fig. 9. Percent of live squash mounted *T. urticae* adult females (Method 2) with no *N. floridana* fungal structures (non-infected), with capilliconidia attached to body (contaminated) and with hyphal body (infected) in cages with *P. persimilis* at different time intervals.

The RH % and C˚ measured 5 mm below the underside of a leaf at two different heights in the canopy was different throughout the experiment, as shown in Fig. 11.

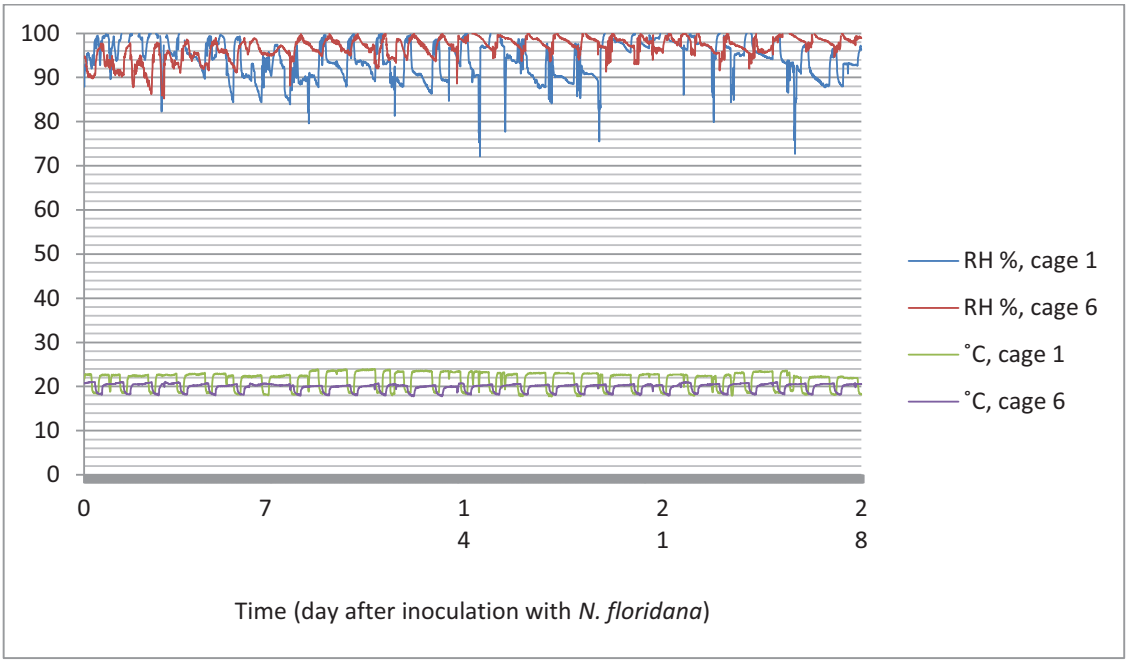


Fig. 11. Microclimatic (5 mm below the underside of the leaf) RH (%) and temperature (˚C) measured at two different heights throughout the experiment in two different cages. In cage 1, the sensors of the micrologger are placed under a leaf 14 cm from top of the cage and in cage 6 under a leaf 24 cm from top of the cage.

4. Discussion

4.1. Establishment of conditions needed for an epidemic development of *N. floridana* in a *T. urticae* population

In this laboratory experiment, the macroclimatic conditions were set at a constant level of 18˚C and 70% RH, and a photoperiod of 18h L- 6h D. This was to attain a level intermediate of the two isolates' assumed requirements. However, microclimatic temperature and RH (5 mm underneath the leaves) were measured to be higher than these macroclimatic settings (Fig. 7). It is likely that both microclimatic temperature and RH are even higher closer to the leaf than 5 mm (Boulard et al., 2002). This may be an important reason why an epizootic occurred with the Brazilian and not the Norwegian *N. floridana* isolate. The two isolates have their origin from

regions with different environmental conditions, and therefore have different requirements to temperature. The Brazilian isolate is probably better adapted to high temperatures. Several authors have reported that *N. floridana* isolates originating from semi- arid regions have better sporulation at temperatures around 23˚C compared to 13˚C, 15˚C, 18˚C, 28˚C and 33˚C (Oduor et al., 1996; Elliot, 1998; Carner, 1976). Wekesa et al. 2010 tested the effect of temperature on sporulation and germination of three different Brazilian *N. floridana* isolates on *T. evansi*, and found that all of them had an optimum at 25˚C. Annual mean temperatures in the area of Piraicaba, Brazil are close to 25˚C. Temperatures inside the cages were thus similar to temperatures found in this semi- arid area of Brazil. However, in Norway, when an epizootic of *N. floridana* is usually observed, average temperatures vary between 8- 14˚C. Klingen et al. 2009 found that the Norwegian isolate NCRI 271/ 04 produced significantly higher amount of spores at 13˚C and 18˚C than at 23˚C.
Regarding RH, the requirements for the two isolates are likely to be less different, as most species of Entomophtoralean fungi discharge conidia only when relative humidity is greater than 90% (Delalibera et al., 2006; Pell et al., 2010).

The points discussed above indicate the need for separate climatic chambers for the two *N. floridana* isolates. This would have given more accurate information about the conditions needed for an epidemic development.

4.2. The effect of *P. persimilis* on the epizootic of *N. floridana*

The introduction of *P. persimilis* seemed to have an immediate effect on the transmission of *N. floridana* to *T. urticae* in this laboratory experiment. The exposure to *P. persimilis* resulted in almost 30% more adult female *T. urticae* infected with capilliconidia and hyphal bodies (Fig. 8 and Fig. 9). The increased transmission of entomophtoralean fungi elicited by the presence of predators has been reported by other authors as well (Roy et al., 1998; Baverstock et al., 2008; Baverstock et al., 2009). One example is the study undertaken by Wells et al. (2011). They tested the effect of two different coccinellids on the transmission of the entomophtoralean fungal pathogen *Pandora neoaphidis* to aphid hosts in single plant arenas. Six days after exposure to inoculum, the treatments with aphids exposed to the predatory coccinellids *H. axyridis* and *C.*

septempunctata showed 15% more fungus- infected cadavers compared to a control with no predatory coccinellids present.

There could be various reasons why the presence of *P. persimilis* increases the transmission of *N. floridana* to *T. urticae*. One mechanism could be that *P. persimilis* is able to vector the infective capilliconidia to new *T. urticae* hosts. However, as other studies on predatory mites and *N. floridana* suggest, this is not likely (Wekesa et al., 2007). A more likely hypothesis is that the presence of *P. persimilis* causes increased movement of *T. urticae,* increasing its exposure to infective capilliconidia. The immobility of the conidia means that infection will depend on movement by the potential host, in this case, *T. urticae*. The density of *T. urticae* is also an important factor. *P. persimilis* potentially decreases the *T. urticae* population, but in this study only two *P. persimilis* were placed on each plant. This low number would probably not have a major impact on the population of *T. urticae*.

An additional reason for higher infection level in *P. persimilis* exposed cages can be due to development of high infection levels earlier in the experiment (see Fig. 9).

This latter point can be of importance for the potential of *N. floridana* as a pest control agent. This is because *T. urticae* is known to cause more yield loss at lower population levels earlier in the season than late in season (Sances et al., 1981). Thus, an early introduction of *P. persimilis* may increase the possibilities of an early epizootic. Although this has been possible to achieve under the controlled laboratory conditions of this experiment, a more realistic study (e.g in a greenhouse or outdoor cultivation system) should be conducted to achieve progress on this field. There are however several challenges to succeed with field studies, among them the need for suitable climatic conditions to provoke an epizootic of *N. floridana*.

The fluorescent tubes used was adapted to maintain plant growth and obtain maximum stomata openings in order to achieve high relative humidity close to the leaf boundary layer where the sporulation and germination of *N. floridana* occur. This seems to have worked the way intended. Droplets of water and were often observed on the underside of the bean plant leaves, which indicates that RH was close to100%. This was more frequently observed in the hours after the lights were switched on. At this time large parts of each plant were covered with droplets. Droplets were also observed at other times of the day, but then only on a few leaves in areas

where leaf density was high. Such high RH may have reduced the movement of both *T. urticae* and *P. persimilis*, and thereby influenced the epizootic development of *N. floridana*. Putman (1970) found that relative humidities of 100% inhibited the activity and development of the European red mite (*Panonychus ulmi*, Acarina: Tetranychidae). Mori et al. (1966) found that both *T. urticae* and *P. persimilis* were less active in high humidities.

To investigate processes involving organisms on a tritrophic level is very complex. There are many factors that may influence the outcome of the results. After conducting an experiment as this, many new questions have showed up. How does the density of the plants influence the movement of *P. persimilis* and the epizootic development of *N. floridana*? Does it differ on a vertical level in the canopy? Do *P. persimilis* act as a vector for transport of the infective capilliconidia? Do high densities of *N. floridana* capilliconidia hamper the movement of *P. persimilis* because of self- grooming behavior? However, experiments conducted in laboratory chambers do exclude many interactions that are happening in natural and farmland ecosystems. This could be an important source of error which has to be taken under consideration when discussing biological control.

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6. Appendix

Appendix I: Number of living adult female *T. urticae* and number of *N. floridana* killed *T. urticae* cadavers counted on two plants in each of 6 cages at different time intervals after inoculation with *N. floridana* (method 1).

Appendix II: Total numbers of *T. urticae* collected and the number of non- infected *T. urticae*, *T. urticae* with capilliconidia*, T. urticae* with hyphal bodies, *T. urticae* with both capilliconidia and hyphal bodies sampled from two plants in 6 cages at different time intervals after inoculation with *N. floridana* (method 2).

Appendix III: Number of *P. persimilis* on 2 plants in 6 cages at different time intervals after inoculation with *N. floridana*.

Appendix I

Number of living adult female *T. urticae* and number of *N. floridana* killed *T. urticae* cadavers counted on 2 plants in each of 6 cages at different time intervals after inoculation with *N. floridana* (method 1).

Identity of trial	Day after inoculation with *N. floridana*	Cage nr.	Plant nr.	Number of living adult female *T. urticae*	Number of *N. floridana* killed *T. urticae* cadavers
1	1	1	5	3	0
1	1	1	6	7	0
1	1	2	5	14	0
1	1	2	6	7	0
1	1	3	5	13	0
1	1	3	6	7	0
1	1	4	5	2	0
1	1	4	6	3	0
1	1	5	5	7	0
1	1	5	6	13	0
1	1	6	5	6	0
1	1	6	6	5	0
2	1	1	5	5	0
2	1	1	6	6	0
2	1	2	5	5	0
2	1	2	6	7	0
2	1	3	5	9	0
2	1	3	6	6	0
2	1	4	5	7	0
2	1	4	6	8	0
2	1	5	5	0	0
2	1	5	6	5	0
2	1	6	5	4	0
2	1	6	6	9	0
1	8	1	5	17	1
1	8	1	6	21	1
1	8	2	5	47	0
1	8	2	6	21	0
1	8	3	5	32	1
1	8	3	6	17	0
1	8	4	5	6	0

1	8	4	6	22	1
1	8	5	5	58	0
1	8	5	6	29	0
1	8	6	5	28	0
1	8	6	6	58	0
2	8	1	5	31	2
2	8	1	6	62	0
2	8	2	5	42	1
2	8	2	6	33	3
2	8	3	5	111	3
2	8	3	6	57	2
2	8	4	5	64	3
2	8	4	6	41	1
2	8	5	5	41	4
2	8	5	6	27	2
2	8	6	5	74	5
2	8	6	6	115	1
1	15	1	5	38	10
1	15	1	6	35	6
1	15	2	5	21	36
1	15	2	6	14	11
1	15	3	5	18	5
1	15	3	6	21	6
1	15	4	5	20	11
1	15	4	6	47	31
1	15	5	5	11	33
1	15	5	6	15	3
1	15	6	5	26	20
1	15	6	6	22	4
2	15	1	5	29	10
2	15	1	6	51	12
2	15	2	5	15	6
2	15	2	6	13	9
2	15	3	5	80	21
2	15	3	6	41	12
2	15	4	5	48	35
2	15	4	6	29	29
2	15	5	5	17	25
2	15	5	6	11	11
2	15	6	5	60	37
2	15	6	6	22	6
1	22	1	5	32	27
1	22	1	6	44	7
1	22	2	5	104	91
1	22	2	6	35	35
1	22	3	5	20	10

1	22	3	6	44	24
1	22	4	5	30	39
1	22	4	6	38	80
1	22	5	5	8	59
1	22	5	6	7	18
1	22	6	5	1	39
1	22	6	6	33	31
2	22	1	5	26	31
2	22	1	6	26	26
2	22	2	5	49	20
2	22	2	6	5	7
2	22	3	5	37	68
2	22	3	6	27	38
2	22	4	5	no data	no data
2	22	4	6	9	50
2	22	5	5	57	197
2	22	5	6	13	21
2	22	6	5	14	21
2	22	6	6	16	33
1	29	1	5	21	156
1	29	1	6	no data	no data
1	29	2	5	20	247
1	29	2	6	23	65
1	29	3	5	69	20
1	29	3	6	30	64
1	29	4	5	no data	no data
1	29	4	6	no data	no data
1	29	5	5	2	63
1	29	5	6	1	91
1	29	6	5	6	45
1	29	6	6	4	54
2	29	1	5	no data	no data
2	29	1	6	32	341
2	29	2	5	5	58
2	29	2	6	1	40
2	29	3	5	30	298
2	29	3	6	12	196
2	29	4	5	no data	no data
2	29	4	6	0	164
2	29	5	5	4	601
2	29	5	6	0	45
2	29	6	5	0	129
2	29	6	6	0	135

Appendix II

Total numbers of *T. urticae* collected and the number of non- infected *T. urticae*, *T. urticae* with capilliconidia*, T. urticae* with hyphal bodies, *T. urticae* with both capilliconidia and hyphal bodies sampled from 2 plants in 6 cages at different time intervals after inoculation with *N. floridana* (method 2).

Identity of trial	Day after inoculation of *N. floridana*	Cage nr.	Plant nr.	Total number of *T. urticae* collected	Number of non-infected *T. urticae*	Number of *T. urticae* with capilliconidia	Number of *T. urticae* with hyphal bodies	Number of *T. urticae* with both capilliconidia and hyphal bodies
1	10	1	3	6	2	4	0	0
1	10	1	8	5	5	0	0	0
1	10	2	3	14	8	2	0	4
1	10	2	8	15	10	2	3	0
1	10	3	3	14	12	1	1	0
1	10	3	8	12	7	3	0	2
1	10	4	3	10	5	0	3	2
1	10	4	8	15	1	2	7	5
1	10	5	3	12	6	1	1	4
1	10	5	8	13	8	1	4	0
1	10	6	3	10	3	0	0	7
1	10	6	8	14	1	1	9	3
2	10	1	3	12	7	0	3	2
2	10	1	8	14	5	5	0	4
2	10	2	3	11	4	2	0	3
2	10	2	8	13	2	1	0	10
2	10	3	3	9	5	3	1	0
2	10	3	8	10	2	2	2	4
2	10	4	3	15	6	4	1	4
2	10	4	8	5	0	0	0	5
2	10	5	3	8	1	0	1	6
2	10	5	8	8	2	0	2	4
2	10	6	3	7	3	1	1	2
2	10	6	8	13	3	4	2	4
1	13	1	4	5	1	1	2	1
1	13	1	7	13	5	3	2	3
1	13	2	4	10	3	4	1	2
1	13	2	7	6	4	0	1	1
1	13	3	4	15	14	0	1	0
1	13	3	7	15	8	0	6	1
1	13	4	4	15	3	3	5	4
1	13	4	7	8	0	0	2	6
1	13	5	4	13	3	1	3	6
1	13	5	7	4	1	1	0	2

1	13	6	4	11	2	0	5	4
1	13	6	7	9	2	0	4	3
2	13	1	4	15	4	3	1	7
2	13	1	7	9	1	3	0	5
2	13	2	4	6	0	0	1	5
2	13	2	7	14	0	2	3	9
2	13	3	4	14	0	2	4	8
2	13	3	7	15	3	1	1	10
2	13	4	4	11	0	0	0	11
2	13	4	7	10	1	0	2	7
2	13	5	4	8	3	1	3	1
2	13	5	7	13	2	4	0	7
2	13	6	4	6	2	0	0	4
2	13	6	7	11	0	3	0	8
1	17	1	3	8	8	0	0	0
1	17	1	8	11	8	0	2	1
1	17	2	3	12	2	0	4	6
1	17	2	8	10	2	2	3	3
1	17	3	3	15	5	0	8	2
1	17	3	8	15	4	2	4	5
1	17	4	3	5	3	0	2	0
1	17	4	8	4	1	1	0	2
1	17	5	3	4	2	1	1	0
1	17	5	8	6	1	2	2	1
1	17	6	3	7	1	1	0	4
1	17	6	8	7	1	0	3	3
2	17	1	3	7	0	0	2	5
2	17	1	8	8	0	2	2	4
2	17	2	3	2	1	0	0	1
2	17	2	8	1	0	0	0	1
2	17	3	3	2	0	0	1	1
2	17	3	8	4	0	1	0	3
2	17	4	3	7	0	1	0	6
2	17	4	8	2	0	0	1	1
2	17	5	3	9	1	1	2	5
2	17	5	8	3	0	0	1	2
2	17	6	3	5	1	0	0	4
2	17	6	8	2	2	0	0	0
1	20	1	4	9	3	2	1	3
1	20	1	7	8	1	2	2	3
1	20	2	4	4	0	1	2	1
1	20	2	7	8	0	2	0	6
1	20	3	4	13	6	0	4	3
1	20	3	7	15	6	1	5	3
1	20	4	4	10	1	1	6	2
1	20	4	7	7	3	0	1	3
1	20	5	4	7	0	1	0	6

1	20	5	7	0	0	0	0	0
1	20	6	4	4	3	0	0	1
1	20	6	7	3	0	2	0	1
2	20	1	4	9	2	2	2	3
2	20	1	7	10	0	0	3	7
2	20	2	4	4	0	0	0	4
2	20	2	7	6	0	1	0	5
2	20	3	4	8	3	1	3	1
2	20	3	7	12	2	3	0	7
2	20	4	4	2	0	0	0	2
2	20	4	7	0	0	0	0	0
2	20	5	4	7	1	0	3	3
2	20	5	7	14	7	0	3	4
2	20	6	4	4	1	0	1	2
2	20	6	7	6	0	0	1	5
1	24	1	3	14	7	0	4	3
1	24	1	8	15	7	0	1	7
1	24	2	3	6	1	1	1	3
1	24	2	8	13	2	1	4	6
1	24	3	3	14	7	2	3	2
1	24	3	8	11	6	1	4	0
1	24	4	3	4	2	0	1	1
1	24	4	8	4	0	0	1	3
1	24	5	3	5	0	1	0	4
1	24	5	8	0	0	0	0	0
1	24	6	3	6	0	0	0	6
1	24	6	8	8	2	1	3	2
2	24	1	3	7	1	1	3	2
2	24	1	8	6	0	0	4	2
2	24	2	3	0	0	0	0	0
2	24	2	8	5	1	0	2	2
2	24	3	3	10	0	4	0	6
2	24	3	8	0	0	0	0	0
2	24	4	3	0	0	0	0	0
2	24	4	8	1	0	0	0	1
2	24	5	3	2	1	0	0	1
2	24	5	8	1	0	0	0	1
2	24	6	3	1	0	0	0	1
2	24	6	8	0	0	0	0	0
1	28	1	4	7	3	0	4	0
1	28	1	7	15	1	0	8	6
1	28	2	4	10	3	1	5	1
1	28	2	7	6	1	1	0	4
1	28	3	4	12	4	1	6	1
1	28	3	7	14	9	0	4	1
1	28	4	4	12	2	3	6	1
1	28	4	7	4	0	0	1	3

1	28	5	4	6	0	0	2	4
1	28	5	7	6	1	0	2	3
1	28	6	4	2	0	0	2	0
1	28	6	7	1	0	0	1	0
2	28	1	4	8	0	3	3	2
2	28	1	7	9	1	1	4	3
2	28	2	4	2	0	0	0	2
2	28	2	7	0	0	0	0	0
2	28	3	4	9	0	3	3	3
2	28	3	7	7	0	1	1	5
2	28	4	4	0	0	0	0	0
2	28	4	7	0	0	0	0	0
2	28	5	4	0	0	0	0	0
2	28	5	7	1	0	0	1	0
2	28	6	4	0	0	0	0	0
2	28	6	7	0	0	0	0	0

Appendix III

Number of *P. persimilis* on 2 plants in 6 cages at different time intervals after inoculation with *N. floridana*.

Identity of trial	Day after inoculation of *N. floridana*	Cage nr.	Plant nr.	Number of *P. persimilis*
1	1	1	5	0
1	1	1	6	0
1	1	2	5	0
1	1	2	6	0
1	1	3	5	0
1	1	3	6	0
1	1	4	5	0
1	1	4	6	0
1	1	5	5	0
1	1	5	6	0
1	1	6	5	0
1	1	6	6	0
2	1	1	5	0
2	1	1	6	0
2	1	2	5	0
2	1	2	6	0
2	1	3	5	0
2	1	3	6	0
2	1	4	5	0
2	1	4	6	0
2	1	5	5	0
2	1	5	6	0
2	1	6	5	0
2	1	6	6	0
1	8	1	5	0
1	8	1	6	0
1	8	2	5	0
1	8	2	6	0
1	8	3	5	0
1	8	3	6	0
1	8	4	5	0
1	8	4	6	0
1	8	5	5	2
1	8	5	6	0
1	8	6	5	1
1	8	6	6	0

2	8	1	5	0
2	8	1	6	0
2	8	2	5	0
2	8	2	6	0
2	8	3	5	0
2	8	3	6	0
2	8	4	5	2
2	8	4	6	1
2	8	5	5	1
2	8	5	6	2
2	8	6	5	2
2	8	6	6	1
1	15	1	5	0
1	15	1	6	0
1	15	2	5	0
1	15	2	6	0
1	15	3	5	0
1	15	3	6	0
1	15	4	5	0
1	15	4	6	1
1	15	5	5	0
1	15	5	6	0
1	15	6	5	0
1	15	6	6	0
2	15	1	5	0
2	15	1	6	0
2	15	2	5	0
2	15	2	6	0
2	15	3	5	0
2	15	3	6	0
2	15	4	5	1
2	15	4	6	0
2	15	5	5	0
2	15	5	6	0
2	15	6	5	0
2	15	6	6	0
1	22	1	5	0
1	22	1	6	0
1	22	2	5	0
1	22	2	6	0
1	22	3	5	0
1	22	3	6	0

1	22	4	5	0
1	22	4	6	1
1	22	5	5	0
1	22	5	6	0
1	22	6	5	0
1	22	6	6	0
2	22	1	5	0
2	22	1	6	0
2	22	2	5	0
2	22	2	6	0
2	22	3	5	0
2	22	3	6	0
2	22	4	5	no data
2	22	4	6	2
2	22	5	5	2
2	22	5	6	0
2	22	6	5	2
2	22	6	6	1
1	29	1	5	0
1	29	1	6	no data
1	29	2	5	0
1	29	2	6	0
1	29	3	5	0
1	29	3	6	0
1	29	4	5	no data
1	29	4	6	no data
1	29	5	5	0
1	29	5	6	1
1	29	6	5	0
1	29	6	6	2
2	29	1	5	no data
2	29	1	6	0
2	29	2	5	0
2	29	2	6	0
2	29	3	5	0
2	29	3	6	0
2	29	4	5	no data
2	29	4	6	0
2	29	5	5	11
2	29	5	6	3
2	29	6	5	0
2	29	6	6	1