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**Studies for implementation of *Neozygites floridana* as control agent of two-spotted spider mite in strawberry**

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**Thesis submitted in partial fulfillment for the degree  
of Doctor in Science. Area of concentration:  
Entomology**

**Piracicaba  
2013**

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Bachelor in Agronomy

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**Versão revisada de acordo com a resolução CoPGr 6018 de 2011**

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## RESUMO

### Estudos para implementação de *Neozygites floridana* como agente de controle do ácaro rajado em morangueiro

*Neozygites floridana* é um importante inimigo natural de *Tetranychus urticae* em diversas culturas, incluindo morango. Nesta cultura tem havido uma mudança no sistema de produção, passando de cultivo em campo aberto para túnel baixo. Este novo sistema apresenta benefícios na produção de morango, no entanto pode aumentar problemas com ácaros fitófagos e algumas doenças fúngicas, conseqüentemente aumentando o uso de agrotóxicos. Para a integração de *N. floridana* no manejo de ácaros fitófagos em morango, existem ainda muitos aspectos importantes a serem conhecidos sobre a biologia, epizootiologia do fungo e efeito dos sistemas de cultivo sobre a sua prevalência em campo. Para isso três estudos foram realizados, sendo o primeiro estudo sobre o efeito de sistemas de produção de morango (túnel baixo e campo aberto) e da aplicação de agrotóxicos sobre a dinâmica populacional do ácaro rajado *T. urticae*, seus inimigos naturais (*N. floridana* e ácaros predadores) e doenças de plantas, o experimento foi realizado em Inconfidentes-MG. A população de *T. urticae* atingiu altos níveis em tratamentos com controle químico de pragas, independente do sistema de cultivo, túnel baixo ou campo aberto. O fungo *N. floridana* foi observado no final de ambos os ciclos de cultivos, nos tratamentos com e sem liberações inoculativas, sugerindo que houve incidência natural do fungo. As maiores prevalências do fungo foram observadas em tratamentos com altas densidades de *T. urticae*. Os ácaros predadores ocorreram naturalmente durante os dois ciclos de cultivo, sendo *Neoseiulus anonymus* (Chant) a espécie mais abundante (57.51%). A incidência de doenças fúngicas foi maior em campo aberto do que em túnel baixo, no entanto parece que o sistema de cultivo não afetou a incidência da nova doença vermelhão. No segundo estudo objetivou-se conhecer os fatores abióticos e bióticos envolvidos na formação de esporos de resistência nos hospedeiros *T. urticae* e *Tetranychus evansi*. O isolado brasileiro ESALQ1420 produziu um grande número de esporos de resistência (51.5%) em *T. urticae*, a uma temperatura de 11 ° C, fotoperíodo de 10F:14E, intensidade de luz de 42-46 ( $\mu\text{mol m}^{-2}\text{s}^{-1}$ ), em plantas senescentes. Pequenas percentagens de ácaros com esporos de resistência (0-5%) foram encontrados para o isolado Norueguês NCR1271/04 sob as condições testadas, e muito baixas percentagens de esporos de resistência (até 1%) foram observados em *T. evansi* infectados pelo isolado brasileiro ESALQ1421. O terceiro estudo objetivou analisar filogeneticamente os isolados de *Neozygites* patogênicos a ácaros, e desenvolver ferramenta utilizando PCR em tempo real para a detecção e quantificação de propágulos de *N. floridana* a partir do solo. A árvore filogenética mostrou que *Neozygites* é um grupo de fungos distante dos demais Entomophthoromycota, e que estes fungos apresentaram alta variabilidade intra-específica entre isolados de *N. floridana*. Estes resultados indicam que isolados provenientes de diferentes espécies hospedeiras do gênero *Tetranychus* podem ser de fato diferentes espécies de *Neozygites* não descritas ainda. Desenvolveu-se uma sonda molecular para detecção e quantificação de *N. floridana* no solo utilizando PCR em tempo real. Esta sonda foi eficiente na quantificação de *N. floridana*, em concentrações relativamente altas de DNA inoculado no solo.

Palavras-chave: *Neozygites floridana*; *Tetranychus urticae*; Esporos de resistência; Filogenia; PCR em tempo real; Sistema de produção de morango; Agrotóxicos



## ABSTRACT

### Studies for implementation of *Neozygites floridana* as control agent of the two-spotted spider mite in strawberry

*Neozygites floridana* is an important natural enemy of *Tetranychus urticae* in many crops, including strawberry. In this crop there has been some changes in the production system from open field to low tunnel. This new system presents benefits to strawberry production, moreover this system increases problems with spider mites and some fungal plant pathogens; consequently increasing the use of pesticides. In order to integrate *N. floridana* in the management of spider mites in strawberry there are still many important aspects to be revealed on the biology, epizootiology of the fungus and the effect of crop systems on its prevalence in the field. For this, three studies were performed, being the first one about the effect of strawberry production systems (low tunnel and open field) and pesticide application on population dynamics of two-spotted spider mite *T. urticae*, its natural enemies (*N. floridana* and predatory mites), and plant diseases, the experiment was performed in Inconfidentes-MG. The population of *T. urticae* attained the highest level in treatments with chemical control of pest, independently of the crop system, low tunnel or open field. The fungus *N. floridana* was observed late in both crop seasons, in treatments with and without inoculative releases, and has suggested that it might be a natural occurrence of the fungus. The highest prevalence of the fungus were observed in treatments with high densities of *T. urticae*. The predatory mites occurred naturally during the two crop seasons, and the most abundant species was *Neoseiulus anonymus* (Chant) (57.5%). The incidence of fungal plant diseases are greater in the open field than in low tunnel, however it seems that the crop system do not affect the new disease “vermelhão”. The second study aimed to reveal the abiotic and biotic factors involved in the regulation of resting spores formation in the hosts *T. urticae* and *T. evansi*. The Brazilian isolate ESALQ1420 produced a large number of resting spores (51.5%) in *T. urticae* at a temperature 11 °C, photoperiod of 10L:14D, light intensity of 42-46 ( $\mu\text{mol m}^{-2}\text{s}^{-1}$ ), on non-senescent plants. Small percentages of mites with resting spores (0-5 %) were found for the Norwegian isolate NCRI271/04 under the conditions tested, and very low percentages of resting spores (up to 1%) were observed in *T. evansi* infected by the Brazilian isolate ESALQ1421. The third study was therefore conducted phylogenetic analysis of isolates of *N. floridana* and develops efficient real-time PCR-based analysis for detection and quantification of *N. floridana* propagules from the soil. The phylogenetic three showed that *Neozygites* is a fungal group far from other Entomophthoromycota and that *N. floridana* present high intra-specific variability. This data indicates that the isolates from different *Tetranychus* species may be in fact different *Neozygites* species, not described yet. We developed a real-time PCR probe for detection and quantification of *N. floridana* in the soil. This molecular probe was successful in quantifying *N. floridana* at relatively high DNA concentrations seeded into the soil.

Keywords: *Neozygites floridana*; *Tetranychus urticae*; Resting spores; Phylogeny; Real-time PCR; Strawberry crop system; Pesticides



## 1 INTRODUCTION

Brazil is the largest strawberry (*Fragaria* × *ananassa* Duch) producer in South America (KIRSCHBAUM; HANCOCK, 2000). Strawberries have been cultivated in Brazil since 1960 (CASTRO, 2004), and the production has grown in recent years. The estimated annual production of strawberries in Brazil is 105,000 tons covering approximately 3,500 ha of land (ANTUNES, 2006) most of it located in the states of Minas Gerais, São Paulo and Rio Grande do Sul (RIGON, 2005). This crop plays important social and economic roles in the country since strawberry production is done mostly in small properties and use mainly domestic labor (ANTUNES; DUARTE FILHO, 2005). The main problem of strawberry production is plant health, that is affected by pests such as two-spotted spider mite *Tetranychus urticae* Kock (Acari: Tetranychidae) and fungal plant diseases (MAAS, 1998; MORAES; FLECHTMANN, 2008).

The control of pests and diseases is done mostly with chemical pesticides (ZAMBOLIM; COSTA, 2006). The two spotted spider mite is recognized as a major pest of strawberry and can reduce fruit production up to 80%, when uncontrolled or controlled incorrectly (CHIAVEGATO; MISCHAN, 1981). The damage of this mite appears as stippling of yellow spots that advance into scarring and bronzing of leaves, resulting in lower production, and sometimes plant death (MORAES; FLECHTMANN, 2008). The control of *T. urticae* has been carried out almost exclusively with the use of chemical acaricides causing significant environmental impact (WATANABE et al., 1994). Moreover, the misuse of pesticides is a problem and can induce pest resurgence, due to the elimination of natural enemies (VAN DE VRIE; McMURTRY; HUFFAKER, 1972). For plant diseases the control is done exclusively with fungicides and it is estimated that the number of fungicides applications is approximately 35 per crop cycle (ZAMBOLIM; COSTA, 2006).

Strawberry production in protected environments is becoming increasingly common, and in particular high or low plastic tunnels are used, since they provide protection from unsuitable weather conditions and may reduce some important diseases, as well as an early production with high yield and quality (XIAO et al., 2001, ÖZDEMİR, 2003, ÖZDEMİR; GÜNDÜZ, 2004). Production systems in plastic tunnel creates, however, favorable conditions for the two-spotted spider mite *T. urticae* (SVENSSON, 2006) and powdery mildew (XIAO et al., 2001) caused by the plant pathogenic fungus *Sphaerotheca macularis* (Wall. ex Fries) Jacz f. sp. *fragariae* (Peries). Both of these organisms can lead to major

economic losses for strawberry producers, and yield reductions of up to 25% due to spider mites (WALSH; ZALOW; SHAW, 1998) and 60% to powdery mildew (HORN; BURNSIDE; CARVER, 1972). Studies on macroclimate changes in plasticulture, including high and low tunnel, demonstrate that the temperature becomes higher in plastic tunnel despite the relative humidity decrease in this system when compare with open field (FREEMAN; GNAYEM, 2004; KADIR; CAREY; ENNAHLI, 2006, WIEN, 2009).

Integrated pest management (IPM) is a practice increasingly adopted in different agro-ecosystems. To maintain the intermediate- and long-term sustainability of agriculture in Brazil, an alternative to prophylactic pest control is IPM (BUENO et al., 2011). IPM is a decision support system for the selection and use of pest control tactics, singly or harmoniously coordinated into a management strategy, based on cost/benefit analyses that take into account the interests of and impacts on producers, society, and the environment (Kogan, 1998). In integrated management, one of the most important things is the maintenance of natural enemies in the crop and this can be done through conservation or inundative releases mass produced natural enemies. When necessary, the pesticides used in crop system, must be effective against a pest and selective to natural enemies. These practices should be performed in order to maximize production with minimal environmental impact. The increase of organic production and the demand for alternative control by farmers resulted in increased use of biological control agents.

Several microorganisms (fungi, viruses, bacteria and microsporidia) are pathogenic to mites and at least two fungal species, *Neozygites floridana* (Weiser and Muma) Remaudière and Keller and *Hirsutella thompsoni* Fischer, are known to be important for the natural regulation of phytophagous mites, especially mites from the family Tetranychidae and Eriophyidae, respectively (CHANDLER et al., 2000; VAN DER GEEST et al., 2000). *N. floridana* has been observed infecting several species of spider mites in many crops. These fungi are able to penetrate directly through the cuticle of the host, not requiring intake by the host as it happens with most of the other pathogens (HALL; PAPIEROCK, 1982). Although most entomopathogenic fungi need high relative humidity for germination and infection of the hosts, this condition is probably often met in protected environment such as greenhouse, plastic tunnels or crops that generate a microclimate close to the leaf surface where *T. urticae* live and feed. This was elegantly shown by Fargues et al. (2003) in a study with *Beauveria bassiana* (Bals.) Vuil and *Lecanicillium lecanii* (Zimmermann) Gams & Zare based mycoinsecticides for the control of the greenhouse whitefly, *Trialeurodes vaporariorum*. The genus *Neozygites* has a restricted host range; it is an obligated pathogen and fastidious to

grow in artificial culture media. Epizootics caused by *N. floridana* have been found in a large number of crops in many countries and resulted in drastic reduction of host population, therefore being considered an important factor for the populations regulation of spider mites (KLUBERTANZ; PEDIGO; CARLSON, 1991; HUMBER; MORAES; SANTOS, 1981; DUARTE et al., 2009).

The family Neozygitaceae which belongs to the phylum Entomophthoromycota (Class Neozygitomycetes) was found attacking small arthropods such as mealybugs, aphids, thrips (KELLER, 1991), *N. floridana* is pathogenic to several species of mites (KELLER, 1997). This group of fungus can infect all developmental stages of mites, except egg (SELHIME; MUMA, 1966; NEMOTO; AOKI, 1975; ELLIOT; MUMFORD; MORAES, 2002), with higher infection rates for adult. Carner and Canerday (1970) studied the occurrence of epidemics of *Neozygites* sp. in populations of *T. urticae* and observed that the level of infection differed along the developmental stage of the host where deutonymphs and adults (males and females) had higher infection.

Several factors have led the strawberry crop to have a negative image to consumers, especially the misuse of pesticides, especially non-registered ones and high levels of pesticide residue in strawberry fruits (PARA, 2009, 2011). Understanding the effect of cropping systems and pesticides on pests, plant diseases and their natural enemies is extremely important in order to devise strategies to minimize the use of pesticides and encourage natural enemies in the field.

The effect of fungicides on natural epizootics of entomopathogenic fungi is demonstrated in several species. Fungicides are a group of pesticides with the greatest adverse effect on survival and efficiency of *N. floridana*. For instance, the application of fungicides in the field reduces the incidence of *Neozygites* spp., resulting in an increase of their host density (WELLS et al., 2000; KLINGEN; WESTRUM, 2007; WEKESA; KNAPP; DELALIBERA JR., 2008). For example, the fungicide Captan has a negative effect on sporulation and germination of *N. floridana* thereby reducing the transmission and development of epizootics (WEKESA; KNAPP; DELALIBERA JR., 2008). Klingen and Westrum (2007) tested the effect of the most commonly used fungicides in strawberries in Norway on *N. floridana* and found that all fungicides tested may potentially reduce the survival and efficacy of this fungus. Castro (2011) tested most of fungicides, acaricides and insecticides, used in strawberry in Brazil and Norway, and observed that most of them presented negative effect on *N. floridana*. Therefore the use of fungicides on strawberry can benefit the populations of *T. urticae* by eliminating one of its main natural enemy.

Because of the uncertainties concerning the delimitation of species within the class Neozygitomycetes, all pathogens of this group associated with spider mites were identified as *N. floridana*. Delalibera Jr., Hajek and Humber (2004) compared isolates pathogenic to the cassava green mite, *Mononychelus tanajoa* Bondar and mite pathogenic to two-spotted spider mite, and although morphologically similar, these two groups are distinct genetically, physiologically and pathobiologically. Based on these studies they described the species isolate from the cassava green mite as a new species, *Neozygites tanajoae* Delalibera Jr., Humber and Hajek, setting it apart from *N. floridana* pathogenic to other spider mites. Isolates of *N. floridana* from different spider mites present a high degree of host specificity from which they were collected (RIBEIRO, et al., 2009).

A few members of the *Neozygites* were characterized molecularly. Only six isolates of the species *N. tanajoae*, *N. floridana* and *Neozygites parvispora* (MacLeod and Carl) Remaudiere and Keller had the SSU rDNA region sequenced (DELALIBERA JR.; HAJEK; HUMBER, 2004). Knowledge about the delimitation of species and the evolutionary relationships among species of this genus with other entomopathogenic fungi will be important for future of biological control programs of spider mites using this fungus.

Molecular techniques have been used to understand the degree of diversity of fungi used in biological control of pests (JENSEN et al., 1998; JAMES et al., 2006, BIDOCHKA et al., 1995; GAUTHIER et al., 2007), to clarify the taxonomy of the main entomopathogenic species (DELALIBERA JR; HAJEK; HUMBER, 2004; HAJEK et al., 2003; WHITE et al., 2006; HIBBETT et al., 2007; BISCHOFF; REHNER; HUMBER, 2009; GRYGANSKYI et al., 2012), to evaluate the establishment and spread of pathogens in field (CASTRILHO et al., 2007; CATRILLO; GRIGGS; VANDENBERG, 2008), and characterization of isolates to ensure the commercial patent right.

The fungus *N. floridana* can be found in the field as resting spores remaining in the soil from one year to another (NORDENGEN; KLINGEN, 2006). Different from other entomopathogenic fungi which can be easily isolated in culture media, *N. floridana* is a fastidious fungus to be isolated from the soil. Occasionally it causes drastic reduction in host population, and usually occurs at low levels most of the year. This characteristic makes it difficult to assess the impact of this pathogen in host populations as well as detecting its presence in the soil. Thus, it would be very useful to have molecular tools for quantification of the pathogen in the field. Real-time PCR is a technique that allows the quantification of DNA of target organism by the use of fluorescence detection. This technique has been

employed with species of the phylum Entomophthoromycota in the soil (CASTRILLO et al., 2007; FOURNIER; WIDMER; ENKERLI, 2010).

The phylum Entomophthoromycota consists of obligated fungal pathogens, which present a restricted host range. A host specific pathogen is desired for biological control since it minimizes the risk of non-target effects, however they must have strategies to survive during the absence of the host. Some of the possible strategies used by them for short and long term survival in the environment are: survive inside mummified host (i) through primary conidia, capilliconidia or hyphal bodies of the pathogen (ii) at low rates of infection in the population (iii) (BRANDENBURG; KENNEDY, 1981), and as a resting spore in the soil (iv) (HAJEK, 1997).

*N. floridana* is an obligate mite pathogen and has been suggested that this fungal species form resting spores to survive adverse conditions (e.g., winter, dry season, host absence). Resting spores are thick-walled structures formed either asexually from a hyphal body (azygospores) or sexually from the conjugation of two hyphal bodies (zygospores). The formation type is not of usually clearly visible. According to Keller, (2007), genus-specific resting spores can only be found in *Neozygites*. Their resting spores are zygospores, dark brown to black, spherical or ellipsoid, smooth or ornamented and binucleate. All other resting spores are multinucleate and produce both azygospores and zygospores.

Field studies in temperate climate documented resting spores of *N. floridana* in *T. urticae* populations in late summer, fall and winter (KLUBERTANZ; PEDIGO; CARLSON, 1991; MIETKIEWSKI; BALAZY; VAN DER GEEST, 1993; KLINGEN; WAERSTED; WESTRUM, 2008). In the seventies, Carner (1976) suggested that *Neozygites* resting spores were restricted to northern/ temperate regions where the weather is cold and often below zero during fall and winter. But field studies with *N. tanajoae* showed later that resting spores of *N. tanajoae* in *M. tanajoa* populations were found under tropical condition in Brazil. First at low prevalences were reported, (3.75% of mites with resting spores) (DELALIBERA JR. et al., 2000) and then later at high prevalences (34-38%) (ELLIOT; MUMFORD; MORAES, 2002). Several factors such as photoperiod, temperature, inoculum density, host age, as well as isolate may be important for the induction of resting spores in fungi in the Entomophthoromycota (GLARE; MILNER; CHILVERS, 1989; HAJEK; SHIMAZU, 1996; THOMSEN; EILENBER, 2000; HUANG; FENG, 2008; ZHOU; FENG, 2010; ZHOU; FENG; ZHANG, 2012).

To integrate the use of *N. floridana* in the management of spider mites in strawberry there is still many important questions on the biology, epizootiology of the fungus and the effect of crop systems on its prevalence in the field. For this three studies were performed:

In the first study, our objective was to create a system approach on how to enhance organic and sustainable production of strawberry and reduce the use of pesticides and problems with pests and plant pathogenic fungi in low tunnel and open field in Brazil. We used *T. urticae*, its natural enemies (*N. floridana* and predatory mites) and plant pathogenic fungi as a model system. Two crop systems, were used, low tunnel and open field, within them were used five combinations of the following pesticides regimes using, chemical control of pests, inoculative release of *N. floridana*, biological control of pests with *Metarhizium anisopliae* and *Beauveria bassiana*, chemical control of plant diseases, and biological control of plant diseases with *Trichoderma harzianum*, *Bacillus subtilis* and *Clonostachys rosea*.

Due to lack of basic studies of the mite pathogenic fungus *N. floridana*, two studies were performed, one aimed to determine the factors that induce the resting spores production of *N. floridana* in *T. urticae*. Laboratory bioassays were performed using two isolates of this fungus, one from tropical and another from temperate regions at different regimes of temperature, photoperiod and light. The factors associated to induction of resting spore formation can be useful for the development of techniques that allow the use of this pathogen in the control of mites. In another study, the molecular characterization of *Neozygites* isolates was performed and a phylogenetic analysis was constructed. We also aimed to develop a molecular tool to quantify this fungus in the field using real-time PCR. The development of molecular probes for quantification of *Neozygites* in soil by real-time PCR will be important to understand the population dynamics of this fungus in the field.

This thesis is a part of the BERRYSYS project, aiming to increase organic and integrated berry production in high and low plastic tunnels. The project has a systematic approach towards handling some of the challenges in strawberry production by looking at the interactions between the two-spotted spider mite, its natural enemies (mite pathogenic fungus and predatory mites) and plant diseases.

## References

ANTUNES, L.E.C. Situação da produção integrada de morango (PIMo). In: ENCONTRO NACIONAL DO MORANGO, 3.; ENCONTRO SOBRE PEQUENAS FRUTAS E FRUTAS NATIVAS DO MERCOSUL, 2., 2006, Pelotas. Pelotas: Embrapa Clima Temperado, 2006. v.1, p. 101-104. (Documentos, 167).

ANTUNES, L.E.C.; DUARTE FILHO, J. **Importância da cultura do morango**. Pelotas: Embrapa Clima Temperado, 2005. 118 p. (Sistema de Produção do Morango, 76).

BIDOCHKA, M.J.; WALSH, S.A.; RAMOS, M.E.; LEGER, R.J.; SILVER, J.C.; ROBERTS, D.W. Pathogens in the *Entomophaga grylli* species complex of grasshopper pathogens differentiated with random amplification of polymorphic DNA and Cloned- DNA Probes. **Applied and Environmental Microbiology**, Washington, v. 61, p. 556- 560, 1995.

BISCHOFF, J.F.; REHNER, S.A.; HUMBER, R.A. *Metarhizium anisopliae* lineage multilocus phylogeny of the *Metarhizium anisopliae* lineage. **Mycologia**, New York, v.98, n. 5, p. 737-745, 2006.

BRANDENBURG, R.L.; KENNEDY, G.G. Overwintering of the pathogen *Entomophthora floridana* and its host, the two spotted spider mite. **Journal of Economic Entomology**, Lanham, v.74, p. 428-431, 1981.

CARNER, G.R. A Description of the life cycle of *Entomophthora* sp. in the two-spotted spider mite. **Journal of Invertebrate Pathology**, San Diego, v. 28, p. 245-254, 1976.

CARNER, G.R.; CANERDAY, T.D. *Entomophthora* sp. as a factor in the regulation of the two-spotted spider mite on cotton. **Ecological Entomology**, Southern Gate, v.63, p. 638–640, 1970.

CASTRO, R.L. Melhoramento genético do morangueiro: avanços no Brasil, In: SIMPÓSIO NACIONAL DO MORANGO, 2.; ENCONTRO DE PEQUENAS FRUTAS E FRUTAS NATIVAS, 1., 2004, Pelotas. Pelotas: Embrapa Clima Temperado, 2004.p. 21–36.

CASTRO, T.R. **Estudos para o desenvolvimento de metodologia da produção *in vivo* do fungo *Neozygites floridana* Weiser e Muma para controle do ácaro *Tetranychus urticae* Koch**. 2011. 86p. Dissertação (Mestrado em Entomologia) –Escola Superior de Agricultura “Luiz de Queiroz”, Universidade de São Paulo, Piracicaba, 2011.

CASTRILLO, L.A.; GRIGGS, M.H.; VENDENBERG, J.D. Quantitative detection of *Beauveria bassiana* GHA (Ascomycota:Hypocreales), a potential microbial control agent of the emerald ash borer, by use of real-time PCR. **Biological Control**, Orlando, v. 45, p. 163-169, 2008.

CASTRILLO, L.A.; THOMSEN, L.; JUNEJA, P.; HAJEK, A.E. Detection and quantification of *Entomophaga maimaiga* resting spores in forest soil using real-time PCR. **Mycological Research**, Cambridge, v. 111, p. 324-331, 2007.

CHANDLER, D.; DAVIDSON, G.; PELL, J.K.; BALL, B.V.; SHAW, K.; SUNDERLAND, K.D. Fungal biocontrol of acari. **Biocontrol Science and Technology**, Oxford, v. 10, p. 357-384, 2000.

CHIAVEGATO, L.G.; MISCHAN, M.M. Efeito do *Tetranychus urticae* (Koch, 1836) Boudreaux & Dosse, 1963 (Acari, Tetranychidae) na produção do morangueiro (*Fragaria* sp) cv. 'Campinas'. **Científica**, Botucatu, v.9, n.2, p.257-266, 1981.

DELALIBERA JR., I.; HAJEK, A.E.; HUMBER, R.A. *Neozygites tanajoae* sp. Nov., a pathogen of the cassava green mite. **Mycologia**, New York, v. 96, n. 5, p. 1002-1009, 2004.

DELALIBERA JR., I.; MORAES, G.J.; LAPOINTE, S.T.; SILVA, C.A.D.; TAMAI, M.A. Temporal variability and progression of *Neozygites* sp. (Zygomycetes: Entomophthorales) in populations of *Mononychellus tanajoa* (Bondar) (Acari: Tetranychidae). **Anais Sociedade Entomologica do Brasil**, Jaboticabal, v. 29, p. 523-535, 2000.

DUARTE, V.S.; SILVA, R.A.; WEKESA, V.W.; RIZZATO, F.B.; DIAS, C.T.S.; DELALIBERA JR., I. Impact of natural epizootics of the fungal pathogen *Neozygites floridana* (Zygomycetes: Entomophthorales) on population dynamics of *Tetranychus evansi* (Acari: Tetranychidae) in tomato and nightshade. **Biological Control**, Orlando, v. 51, p. 81-90, 2009.

ELLIOT, S.L.; MUMFORD, J.D.; MORAES, J.G. The role of resting spores in the survival of mite-pathogenic fungus *Neozygites floridana* from *Mononychellus tanajoa* during dry periods in Brazil. **Journal of Invertebrate Pathology**, San Diego, v. 81, p. 148-157, 2002.

FOURNIER, A.; WIDMER, F.; ENKERLI, J. Assessing winter-survival of *Pandora neoaphidis* in soil with bioassays and molecular approaches. **Biological Control**, Orlando, v.54, p. 126-134, 2010.

FREEMAN, S.; GNAYEM, N. Use of plasticulture for strawberry plant production. **Small Fruit Review**, Philadelphia, v.4, n. 1, p. 21-32, 2004.

GAUTHIER, N.; DALLEAU-CLOET, C.; FARGUES, J.; BON, M. Microsatellite variability in the entomopathogenic fungus *Paecilomyces fumosoroseus*: genetic diversity and population structure. **Mycologia**, New York, v. 99, p. 693-704, 2007.

GLARE, T.R.; MILNER, R.J.; CHILVERS, G.A. Factors affecting the production of resting spores by *Zoophthora radicans* in the spotted alfalfa aphid, *Therioaphis trifolii* f. *Maculata*. **Canadian Journal of Botany**, Ottawa, v. 67, p. 848-855, 1989.

GRYGANSKYI, G.P.; HUMBER, R.A.; SMITH, M.E.; MIADLIKOVSKA, J.; WU, S.; VOIGT, K.; WALTHER, G.; ANISHCHENKO, I.M.; VILGALYS, R. Molecular phylogeny of the Entomophthoromycota. **Molecular Phylogenetics and Evolution**, Orlando, v. 65, p.682-694, 2012.

HAJEK, A.E. Ecology of terrestrial fungal pathogens. In: JONES, J.G. (Ed.). **Advances in microbial ecology**. New York: Plenum Press, 1997.v.15, p. 193-249.

HAJEK, A.E.; SHIMAZU, M. Types of spores produced by *Entomophagaimaiga* infecting the gypsy moth *Lymantriadispar*. **Canadian Journal of Botany**, Ottawa, v. 74, p. 708–715, 1996.

HAJEK, A.E.; JENSEN, A.B.; HODGE, K.T.; EILENBERG, J. PCR-RFLP I is used to investigate relations among species in the entomopathogenic genera *Eryopsis* and *Entomophaga*. **Mycologia**, New York, v. 95, p. 262-268, 2003.

HALL, R.A.; PAPIEROK, B. Fungi as biological control agents of arthropods of agricultural and medical importance. **Parasitology**, Cambridge, v. 84, p. 205-240, 1982.

HIBBETT, D.S.; BINDER, M.; BISCHOFF, J.F.; BLACKWELL, M.; CANNON, P.F.; ERIKSSON, O.; HUHDORFF, S.; JAMES, T.; KIRK, P.M.; LÜCKING, R.; LUMBSCH, T.; LUTZONI, F.; MATHENY, P.B.; MCLAUGHLIN, D.J.; POWELL, M.J.; REDHEAD, S.; SCHOCH, C.L.; SPATAFORA, J.W.; STALPERS, J.A.; VILGALYS, R.; AIME, M.C.; APTROOT, A.; BAUER, R.; BEGEROW, D.; BENNY, G.L.; CASTLEBURY, L.A.; CROUS, P.W.; DAI, Y.-C.; GAMS, W.; GEISER, D.M.; GRIFITH, G.W.; GUEIDAN, C.; HAWKSWORTH, D.L.; HESTMARK, G.; HOSAKA, K.; HUMBER, R.A.; HYDE, K.; KÖLJALB, U.; KURTZMAN, C.P.; LARSSON, K.-H.; LICHTWARDT, R.; LONGCORE, J.; MIALIKOWSKA, J.; MILLER, A.; MONCALVO, J.-M.; MOZLEY-STANDRIDGE, S.; OBERWINKLER, F.; PARMASTO, R.; REEB, V.; ROGERS, J.D.; ROUX, C.; RYVARDEN, L.; SAMPAIO, J.P.; SCHUESSLER, A.; SUGIYAMA, J.; THORN, R.G.; TIBELL, L.; UNTEREINER, W.A.; WALKER, C.; WANG, Z.; WEIR, A.; WEISS, M.; WHITE, M.; WINKA, K.; YAO, Y.-J.; ZHANG, N. A higher-level phylogenetic classification of the fungi. **Mycological Research**, Cambridge, v. 111, p. 509–547, 2007.

HORN, N.L.; BURNSIDE, K.R.; CARVER, R.B. Powdery mildew of strawberry. **Plant Disease Reporter**, Washington, v.56, p.368, 1972.

HUANG, Z.; FENG, M. Resting spore formation of aphid-pathogenic fungus *Pandora nouryi* depends on the concentration of infective inoculums. **Environmental Microbiology**, College Park, v.10, n.7, p. 1912–1916, 2008.

HUMBER, R.A.; MORAES, G.J.; SANTOS, J.M. Natural infection of *Tetranychus evansi* (Acarina: Tetranychidae) by a *Triplosporium* sp. (Zygomycetes: Entomophthorales) in Northeastern Brazil. **Entomophaga**, Paris, v.26, n. 4, p. 421–425, 1981.

JAMES, T.Y.; KAUFF, F.; SCHOCH, C.; MATHENY, P.B.; HOFSTETTER, V.; COX, C.J.; CELIO, G.; GUEIDAN, C.; FRAKER, E.; MIADLIKOWSKA, J.; LUMBSCH, H.T.; RAUHUT, A.; REEB, V.; ARNOLD, A.E.; AMROFT, A.; STAJICH, J.E.; HOSAKA, K.; SUNG, G.-H.; JOHNSON, D.; O’ROURKE, B.; ROCKETT, M.; BINDER, M.; CURTIS,

J.M.; SLOT, J.C.; WANG, Z.; WILSON, A.W.; SCHÜBLER, A.; LONGCORE, J.E.; O'DONNELL, K.; MOZLEY-STANDRIDGE, S.; PORTER, D.; LETCHER, P.M.; POWELL, M.J.; TAYLOR, J.W.; WHITE, M.M.; GRIFTH, G.W.; DAVIES, D.R.; HUMBER, R.A.; MORTON, J.B.; SUGIYAMA, J.; ROSSMAN, A.; ROGERS, J.D.; P.STER, D.H.; HEWITT, D.; HANSEN, K.; HAMBLETON, S.; SHOEMAKER, R.A.; KOHLMAYER, J.; VOLKMANN-KOHLMEYER, B.; SPOTTS, R.A.; SERDANI, M.; CROUS, P.W.; HUGHES, K.W.; MATSUURA, K.; LANGER, E.; LANGER, G.; UNTEREINER, W.A.; LÜCKING, R.; BÜDEL, B.; GEISER, D.M.; APTROOT, A.; DIEDERICH, P.; SCHMITT, I.; SCHULTZ, M.; YAHR, R., HIBBETT, D.S.; LUTZONI, F.; MCLAUGHLIN, D.J.; SPATAFORA, J.W.; VILGALYS, R. Reconstructing the early evolution of Fungi using a six-gene phylogeny. **Nature**, London, v.443, p.818–822, 2006.

JENSEN, A.B.; GARGAS, A.; EILENBERG, J.; ROSENDAHL, S. Relationships of the insect pathogenic order Entomophthorales (Zygomycota, Fungi) based on phylogenetic analyses of nuclear small subunit ribosomal DNA sequences (SSU rDNA). **Fungal Genetics and Biology**, Orlando, v. 24, p.325–334, 1998.

KADIR, S.; CAREY, E.; ENNAHLI, S. Influence of high tunnel and field conditions on strawberry growth and development. **HortScience**, Alexandria, v. 41, p. 329–335, 2006.

KELLER, S. Arthropod-pathogenic Entomophthorales of Switzerland. II. *Erynia*, *Eyniopsis*, *Neozygites*, *Zoophthora* and *Tarichium*. **Sydowia**, Horn, v. 43, p. 39-122, 1991.

\_\_\_\_\_. The genus *Neozygites*(Zygomycetes, Entomophthorales) with special reference to species found in tropical regions. **Sydowia**, Horn, v. 49, n.2, p. 118-146, 1997.

\_\_\_\_\_. Fungal structures and biology. In: \_\_\_\_\_. (Ed.) **Arthropod-pathogenic Entomophthorales: biology, ecology, identification**. Luxemborg: CostOffice, 2007. p. 27-54.

KIRSCHBAUM, D.S.; HANCOCK, J.F. The strawberry industry in South America. **HortScience**, v.35, n. 5, p.807-811, 2000.

KOGAN, M. Integrated Pest Management: Historical Perspectives and Contemporary Developments. **Annual Review Entomology**, v. 43, p. 234-270, 1998.

KLINGEN, I.; HAUKELAND, S. The soil as a reservoir for natural enemies of pest insects and mites with emphasis on fungi and nematodes. In: EILENBERG, J.; HOKKANEN, H.M.T. (Ed.) **An ecological and societal approach to biological control**. Dordrecht: Springer, 2006. p. 145-211. (Series: Progress in Biological Control, 2).

KLINGEN, I.; WESTRUM, K. The effect of pesticides used in strawberries on the phytophagous mite *Tetranychus urticae* (Acari: Tetranychidae) and its fungal natural enemy *Neozygites floridana* (Zygomycetes: Entomophthorales). **Biological Control**, Orlando, v. 43, p. 222-230, 2007.

KLINGEN, I.; WAERSTED, G.; WESTRUM, K. Overwintering and prevalence of *Neozygites floridana* (Zygomycetes: Neozygiteaceae) in hibernating females of *Tetranychus urticae* (Acari: Tetranychidae) under cold climatic conditions in strawberries. **Experimental and Applied Acarology**, Amsterdam, v. 46, p.231-245, 2008.

KLUBERTANZ, T.H.; PEDIGO, L.P.; CARLSON, R.E. Impact of fungal epizootics on the biology and management of the two spotted spider mite (Acari: Tetranychidae) in soybean. **Environmental Entomology**, College Park, v. 20. p. 731-735, 1991.

MAAS, J.L. **Compendium of strawberry diseases**. 3<sup>rd</sup>ed. St. Paul: American Phytopathological Society, 1998.138 p.

MIETKIEWSKI, R.; BALAZY, S.; VAN DER GEEST, L.P.S. Observations on a mycosis of spider mites (Acari: Teranychidae) caused by *Neozygites floridana* in Poland. **Journal of Invertebrate Pathology**, San Diego, v. 61, p.317–319, 1993.

MORAES, G.J.; FLECHTMANN, C.H.W. Ácaros encontrados em diferentes espécies vegetais de importância econômica. In: \_\_\_\_\_. **Manual de acarologia: acarologia básica e ácaros de plantas cultivadas no Brasil**. Ribeirão Preto: Holos, 2008. cap.9, p. 106-208.

NEMOTO, H.; AOKI, J. *Entomophthora floridana* (Entomophthorales: Entomophthoraceae) attacking the sugi spider mite, *Oligonychus hondoensis* (Acarina: Tetranychidae), in Japan. **Applied Entomology and Zoology**, Tokyo, v.10, n.2, p.90-95, 1975.

NORDENGEN, I.; KLINGEN, I. Comparison of methods for estimating the prevalence of *Neozygites floridana* in *Tetranychus urticae* populations infesting strawberries. **Journal of Invertebrate Patology**, San Diego, v. 92, p. 1-6, 2006.

ÖZDEMİR, E. Early production of strawberry cultivars grown under plastic houses on sand dunes. **Small Fruit Review**, Philadelphia, v. 2, p. 81-86, 2003.

ÖZDEMİR, E.; GÜNDÜZ, K. Comparison of bag raised bed treatments for strawberry production under unheated greenhouse conditions. **Journal of the American Pomological Society**, University Park, v.58, p. 118-122, 2004.

PROGRAMA DE ANÁLISE DE RESÍDUOS DE AGROTÓXICOS EM ALIMENTOS. **Nota técnica para divulgação dos resultados do PARA de 2008: relatório geral de atividades 2008**. Brasília: Agência Nacional de Vigilância Sanitária, 2009.12p.

\_\_\_\_\_. **Nota técnica para divulgação dos resultados do PARA de 2010:relatório geral de atividades 2010**. Brasília: Agência Nacional de Vigilância Sanitária, 2011. 26p.

RIGON, L.; CORRÊA, S.; REETZ, E.; VENCATO, A.; ROSA, G.R.; BELING, R.R. Pequenas frutas. **Anuário Brasileiro da Fruticultura**, Santa Cruz do Sul, v. 1, n. 1, p. 90-97, 2005.

SELHIME, A.G.; MUMA, M.H. Biology of *Entomophthora floridana* attacking *Eutetranychus banksi*. **Florida Entomologist**, Gainesville, v.49, n. 3, p.161-168, 1966.

SVENSSON, B. Organic strawberries in tunnels with control of powdery mildew and mites. In: NJF SEMINAR 389. PEST, DISEASE AND WEED MANAGEMENT IN STRAWBERRY Ð PROGRESS AND CHALLENGES FOR THE NORDIC PRODUCTIONNJF, 2006, Helsinki.**Abstract...** Helsinki: NJF Report, 2006. Abstract 17.

THOMSEN, L.; EILENBERG, J. *Entomophthora muscae* resting spore formation in vivo in the host *Delia radicum*. **Journal of Invertebrate Pathology**, San Diego, v. 76, p. 127-130, 2000.

VAN DE VRIE, M; MCMURTRY, J.A.; HUFFAKER, C.B. Ecology of tetranychid mites and their natural enemies: a review. III. Biology, ecology, and pest status, and host-plant relations of tetranychids. **Hilgardia**, Oakland, v. 41, p. 387-403, 1972.

VAN DER GEEST, L.P.S.; ELLIOT, S.L.; BREEUWER, J.A.J.; BEERLING, E.A.M. Diseases of mites. **Experimental and Applied Acarology**, Amsterdam, v.24, p. 497-560, 2000.

WALSH, D.B.; ZALOM, F.G.; SHAW, D.V. Interaction of the two spotted spider mite (Acari: Tetranychidae) with yield of day-neutral strawberries in California. **Journal of Economic Entomology**, Lanham, v.91, p.678–685, 1998.

WATANABE, M.A.; MORAES, G.J.; GASTALDO JR., I.; NICOLELLA, G. Controle biológico do ácaro rajado com ácaros predadores fitoseídeos (Acari: Tetranychidae), Phytoseiidae) em culturas de pepino e morango. **CiênciaAgrícola**, Maceio, v. 51, n. 1, p.75-81, 1994.

WELLS, M.L.; McPHERSON, R.M.; RUBERSON, J.R.; HERZOG, G.A. Effect of fungicide application on activity of *Neozygites floridana* (Entomophthorales: Neozytaceae) and cotton aphid (Homoptera: Aphididae) suppression. **Journal Economic Entomology**, Lanham, v. 93, p. 1118-1126, 1986.

WEKESA, V.W.; KNAPP, M.; DELALIBERA JR., I. Side-effects of pesticides on the cycle of the mite pathogenic fungus *Neozygites floridana*. **Experimental and Applied Acarology**, Amsterdam, v.46, p. 287–297, 2008.

WHITE, M.M.; JAMES, T.Y.; O'DONNELL, K.; TANABE, Y.; SUGIYAMA, J. Phylogeny of the Zygomycota based on nuclear ribosomal sequence data. **Mycologia**, New York, v. 96, p. 872-884, 2006.

WIEN, H.C. Microenvironmental variations within the high tunnel. **HortScience**, Alexandria, v. 44, n. 2, p. 235-238, 2009.

XIAO, C.L.; CHANDLER, C.K.; PRICE, J.F.; DUVAL, J.R.; MERTELY, J.C.; LEGARD, D.E. Comparison of epidemics of Botrytis fruit rot and powdery mildew of strawberry in large plastic tunnel and field production systems. **Plant Disease**, Saint Paul, v. 85, p. 901–909, 2001.

ZAMBOLIM, L.; COSTA, H. Manejo integrado de doenças do morangueiro. In: CARVALHO, S.P. (Ed.) **Boletim do morango: cultivo convencional, segurança alimentar, cultivo orgânico**. Belo Horizonte: FAEMG, 2006. p. 55-80.

ZHOU, X.; FENG; M. Biotic and abiotic regulation of resting spore formation *in vivo* of obligate aphid pathogen *Pandora nouryi*: Modeling analysis and biological implication. **Journal of Invertebrate Pathology**, San Diego, v. 103, p. 83-88, 2010.

ZHOU, X.; FENG, M.; ZHANG, L. The role of temperature on *in vivo* resting spore formation of the aphid-specific pathogen *Pandora nouryi* (Zygomycota: Entomophthorales) under winter field conditions. **Biocontrol Science and Technology**, Oxford, v. 22, p. 93-100, 2012.



## 2 EFFECTS OF CROPPING SYSTEM AND PESTICIDE REGIME ON PLANT PATHOGENS, TWO-SPOTTED SPIDER MITE AND THEIR NATURAL ENEMIES IN STRAWBERRIES

### Abstract

The area of strawberry production in protect environment in Brazil, has been increasing in the recent years and the main production system is low tunnel. However, when not managed properly, the use of plastic tunnels may severely increase problems with pest insects and mites and some plant pathogenic fungi; this may result in increased use of acaricides, insecticides and fungicides. The interactions among pest insect and mites, plant pathogenic fungi and natural enemies are numerous and complex, and often studied separately. In a two years study, the effect of strawberry production systems (low tunnel and open field) and pesticide application on population dynamics of two-spotted spider mite *Tetranychus urticae* (TSSM), its natural enemies (*Neozygites floridana* and predatory mites), and plant diseases were evaluated. The experiment was conducted in Inconfidentes-MG in Brazil. Two strawberry cropping systems were tested, open field and low tunnel, and within each system five different pesticide regimes were tested: 1) Chemical pesticides for the control of pests insects and mites and plant diseases 2) Inoculative release of *N. floridana* to control TSSM and chemical fungicides for the control of plant diseases 3) Inoculative release of *N. floridana* to control TSSM and inundative release of antagonistic microorganisms to control plant diseases 4) Inundative release of *Metarhizium anisopliae* and *Beauveria bassiana* to control TSSM and the use of chemical fungicides for the control of plant diseases 5) Chemical acaricides for the control of TSSM and inundative release of antagonistic microorganisms to control plant diseases. The population of *T. urticae* attained the highest level in treatments with chemical control of pest, independently of the crop system, low tunnel and open field. The fungus *N. floridana* was observed late in the crop cycle in both years, on treatments with and without inoculative releases of it, and the higher prevalences were associated with high host densities. The predatory mites, specially the species *Neoseiulus anonymus*, occurred naturally during the two crop seasons. The incidence of fungal plant diseases was greater in the open field than in low tunnel, no pattern of incidence was observed for the new disease “Vermelhão”. The highest temperature was found in most part of the time in low tunnel, and the highest relative humidity in open field.

Keywords: *Neozygites floridana*; *Tetranychus urticae*; Fungal plant diseases; Predatory mites; Microclimate conditions; Pesticides; Crop systems

### 2.1 Introduction

South America accounts for approximately 4% of the total world strawberry (*Fragaria × ananassa* Duch) production, and the crop plays important social and economic roles in the region, since farmers employ nearly 50,000 full- or part-time workers in strawberry production (KIRSCHBAUM; HANCOCK, 2000). Brazil is the largest strawberry producer in South America and strawberries, have been cultivated in Brazil since 1960

(CASTRO, 2004). The production has increased in recent years and there is an estimated annual production of 105,000 tons of strawberries in Brazil which occupies approximately 3,500 ha of land (ANTUNES, 2006). The largest strawberry producing states in Brazil are Minas Gerais, São Paulo and Rio Grande do Sul respectively (RIGON, 2005).

Production of strawberry in high or low plastic tunnels is becoming increasingly common in both warm areas, such as California, Florida and the Mediterranean region and in the colder regions such as North Europe (FREEMAN; GNAYEM, 2004, WITTEW; CASTILLA, 1995). Currently, about 50% of the European strawberry production is carried out in protected environments (HUKKANEN et al., 2007), and the main strawberry production system in Brazil is low tunnel (ANTUNES; PERES, 2012). Plastic tunnels are used to provide protection from unsuitable weather (heavy rains, sunburn, etc) and some important diseases. Further, the use of plastic tunnels results in an expanded strawberry growing season and high yield and quality (XIAO et al., 2001, ÖZDEMİR, 2003, ÖZDEMİR; GÜNDÜZ, 2004).

Plastic tunnels, create favorable conditions for the two-spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae) (SVENSSON, 2006) and powdery mildew (XIAO et al., 2001) caused by *Sphaerotheca macularis* (Wallr. Ex Fries) Jacz f. sp. *fragariae* (Peries). Both of these organisms can lead to major economic losses for strawberry producers, and reports have indicated yield reductions of up to 25% due to spider mites (WALSH; ZALOW; SHAW, 1998) and 60% due to powdery mildew (HORN; BURNSIDE; CARVER, 1972).

*T. urticae* is one of the most important pests of strawberries (CHIAVEGATO; MISCHAN, 1981) and may cause significant reduction in strawberry fruit production. In Brazil the control of *T. urticae* has been almost done exclusively based on the use of chemical pesticides (MORAES; FLECHTMANN, 2008). Despite the extensive use of these chemicals *T. urticae*, control on strawberry is often not satisfactory due to many factors. Problems with acaricide resistance have been reported for *T. urticae* in many countries including Brazil (SATO et al., 2004, 2005). Another problem associated with the indiscriminate use of pesticides is the resurgence of pests caused by the elimination of natural enemies (VAN de VRIE; McMURTY; HUFFAKER, 1972). Natural enemies, especially predatory mites, have been used more often to manage spider mites, however (CRANHAM; HELLE, 1985), and has reduced the dependence on acaricides in strawberry production (RHODES; LIBURD, 2006; FITZGERALD et al., 2007; FRAULO; LIBURD, 2007).

Several plant pathogenic fungi are also cause of serious problems in the Brazilian strawberry production (ZAMBOLIM; COSTA, 2006). Although plastic tunnels reduce most fungal diseases in strawberry, powdery mildew is a serious pathogen in strawberries produced in plastic tunnels (XIAO et al, 2001, COSTA; VENTURA, 2006). Plant pathogenic fungi in strawberry in Brazil are mainly managed with chemical fungicides and between 30 and 40 fungicide applications are performed during a crop season (ZAMBOLIM; COSTA, 2006). This often results in strawberries with high levels of pesticide residues (PARA, 2011).

The interactions among pest insect and mites, plant pathogenic fungi and natural enemies are numerous and complex, and often studied separately. In this study we therefore aim for a system approach on how to reduce the use of pesticides and problems with pests and plant pathogenic fungi in low tunnel and open field in Brazil. We specifically studied the effect of low tunnel and different (chemical and biological) pest and disease management on the population development of *T. urticae*, its natural enemies, *Neozygites floridana* (Weiser and Muma) Remaudière and Keller and predatory mites, and plant pathogenic fungi.

## **2.2 Development**

### **2.2.1 Material and methods**

#### **2.2.1.1 Experimental design**

The experiment was organized as a randomized block design in a split-plot arrangement with six blocks. Each block consisted of two cropping systems (open field and low tunnel) and each cropping system in each block consisted of five pest management regimes (Appendix A). The strawberry field had 15 beds that were 100 cm wide and 48 m long; half of the bed have the treatment in low tunnel and the other half in open field. Each bed was planted with 3 rows of plants and 25 cm within and between rows. The distance between beds was 2 m and 4 m between blocks. There were six blocks of five treatments in each system (low tunnel and open field). The experiment was conducted over a period of two years, 2010 and in 2011, in the experimental field of “Instituto Federal de Educação, Ciência e Tecnologia do Sul de Minas Gerais”, located in Inconfidentes, Brazil (22° 19' 2" S and 46° 19' 42" W), at an altitude of 864 m. The strawberry variety Oso Grande was used and new strawberry plants were planted every year. In 2010 seedlings were transplanted in the middle

of May 2010 and in the beginning of April in 2011. Plant beds were covered by black polyethylene film before planting and plants were irrigated by drip irrigation.

Adult *T. urticae* females were introduced at low density in all plots seven (July 1) and 14 (August 10) weeks after planting in 2010, to standardize and ensure a *T. urticae* infestation of the experimental field. Two leaf discs of jack bean plants (*Canavalia ensiformis*) with ca 20 *T. urticae* were used to infest each experimental plot. *T. urticae* used for infesting the experimental field was from a colony established from mites collected in Piracicaba, São Paulo, Brazil, and maintained on jack bean plants in a screenhouse at the Department of Entomology and Acarology at ESALQ-USP (Piracicaba-SP). Before *T. urticae* was added to the plants, two mites per leaf from the colony were mounted to confirm that the *T. urticae* were not infected by *N. floridana*.

### **2.2.1.2 Pest management regimes**

The different pest management regimes tested is presented in the Table 2.1. Strawberry plants were periodically sprayed with the following chemical pesticides that are used in integrated production of strawberries in Brazil (Table 2.2.). The chemical pesticides and fungicides were applied twice a month throughout the experiment. During the chemical pesticides applications, plastic barriers were used between beds to reduce drift of residues between treatments. The fungus *N. floridana* was inoculated in July 26 and September 28 in 2010, and in June 25 and August 27 in 2011, in the treatments 2,3,7 and 8.

During the first crop cycle 2010, a smaller number of spreads of pesticides was needed than in the second crop, 2011, because in the first year we had fewer problems with pests and plant diseases in the beginning of the crop cycle, so were done three applications of acaricides/insecticides and one application of fungicide.

Table 2.1 - List of treatments used in the experiment

<b>Treatment</b>	<b><i>T. urticae</i> control</b>	<b>Plant diseases control</b>
<b>1 (low tunnel) and 6 (open field)</b>	Chemical acaricide/insecticide	Chemical fungicide
<b>2 (low tunnel) and 7 (open field)</b>	Inoculative releases of <i>N. floridana</i>	Chemical fungicide
<b>3 (low tunnel) and 8 (open field)</b>	Inoculative releases of <i>N. floridana</i>	Biological fungicide
<b>4 (low tunnel) and 9 (open field)</b>	<i>Metarhizium anisopliae</i> and <i>Beauveria bassiana</i>	Chemical fungicide
<b>5 (low tunnel) and 10 (open field)</b>	Chemical acaricide/insecticide	Biological fungicide

Table 2.2 - Pesticides used in the field study

Trade name	Active ingredient	Concentration (CR)	Type of pesticide	N° of application		Treatment
				2010	2011	
Boveril WP <sup>1</sup>	<i>Beauveria bassiana</i>	500g/100l	Acaricide/insecticide	0	16	4 and 9
Dipel	<i>Bacillus thuringiensis</i>	75ml/100l	Insecticide	1	1	3, 4, 8 and 9
Metarril SC <sup>1</sup>	<i>Metarhizium anisopliae</i>	200ml/100l	Acaricide/insecticide	0	16	4 and 9
Vertimec 18 EC	Abamectin	75ml/100l	Acaricide/insecticide	0	3	1,5,6 and 10
Ortus 50 SC	Fenpyroximate	100ml/100l	Acaricide	0	1	1,5,6 and 10
Danimen 300 EC	Fenpropathrine	65ml/100l	Acaricide/insecticide	0	2	1,5,6 and 10
Omite 720 CE	Propargite	30ml/100l	Acaricide	0	3	1,5,6 and 10
Actara 250WG	Thiametoxam	10g/100l	Insectide	1	1	1,5,6 and 10
KarateZeon 50	Lambda-cyhalothrin	80ml/100l	Insecticide	2	0	1,5,6 and 10
Trichodermil WP <sup>2</sup>	<i>Trichoderma harzianum</i>	780g/100l	Fungicide	1	10	3,5,8 and 10
BioSafe	<i>Bacillus subtilis</i>	72ml/100l	Fungicide	0	10	3,5,8 and 10
Kamoi	<i>Clonostachys rosea</i>	100ml/100l	Fungicide	0	1	3,5, 8 and 10
Amistar	Azoxystrobin	13g/100l	Fungicide	1	2	1,2,4,6,7 and 9
Cercobin 700 WP	Thiophanatemethyl	70g/100l	Fungicide	0	1	1,2,4,6,7 and 9
Rovral SC	Imibenconazole	150ml/100l	Fungicide	0	3	1,2,4,6,7 and 9
Sialex 500 WP	Procimidone	50g/100l	Fungicide	0	2	1,2,4,6,7 and 9

CR = Mean recommended concentration of the formulated product

1= products used together for control of *T. urticae* twice a month throughout the experiment

2= Product used for control of rot diseases applied directed to the base of each plant every 20 days throughout the experiment

### 2.2.1.3 Monitoring of fungal plant diseases, *T. urticae* and their natural enemies

The prevalence of fungal plant diseases, *T. urticae* and their natural enemies (*N. floridana* and predatory mites) were evaluated twice a month from July 20 to November 30 in 2010 and from June 20 to December 5 in 2011. The observation of fungal plant diseases was carried out by visual evaluations of plants with signs or/and symptoms of fungal diseases. Prevalence of *Botrytis* was evaluated in the field and complemented with evaluation post-harvest (considering that many fruits did not show symptoms in the field). Identifications of plant pathogenic fungi were confirmed by the laboratory “Clínica Hiroshi Kimati”, ESALQ/USP).

Prevalence of *T. urticae* and their natural enemies (*N. floridana* and predatory mites) were observed by collecting approximately 15 strawberry leaves (compound of three leaflet) in the middle row of each plot starting with the second plant of the row and collecting a leaf at approximately every other plant. Leaves were placed in paper bags and then transported to the Laboratory of Patology and Microbial Control at ESALQ/USP (Piracicaba-SP) where numbers of *T. urticae* and predatory mites were counted under a compound microscope (40-80x). Predatory mites were picked out and stored in microcentrifuge tubes (1.5mL) with alcohol 70% for subsequent identification down to species level. Further, two adult *T. urticae* females were randomly selected from each leaf and mounted in Hoyer’s medium with cotton blue on a microscope slide to observe for *N. floridana* prevalence (observed as capiliconidia attached to the mite body and/or presence of hyphal bodies inside the mite) by the use of a phase contrast microscope (400x).

### 2.2.1.4 Macro- and microclimatic data collection

Macroclimatic data (temperature, relative humidity and precipitation) at 2m above the ground were continuously monitored by a weather station (La Cross Technology - Heavy Weather Pro HW-2812) located in the experimental field. Microclimatic data (temperature and RH) were collected at 15 mm below the strawberry leaf surface by the use of MSR145 USB loggers. One was placed in a low tunnel plot and one in an open field plot.

### 2.2.1.5 Statistical analysis

The effect of crop system and pesticides applications on accumulated number of *T. urticae*, predatory mites, and *T. urticae* with *N. floridana* per leaf were analyzed by ANOVA, after log transformation of the data. The effect of crop system and pesticides applications on accumulated number of fungal plant diseases and of the disease “vermelhão” (english translation = redness – unknown agent), were all analyzed by the use of ANOVA. When significant effects were found, post hoc comparisons using the Tukey’s HSD test were conducted to evaluate differences between means ( $p < 0.05$ ). Pearson correlation analysis was also considered between the variables, *T. urticae* with predatory mites, and from *T. urticae* and *T. urticae* with *N. floridana* ( $p < 0.05$ ). All statistical analyses were carried out using SAS (SAS Institute Inc., 1999).

## 2.2.2 Results

### 2.2.2.1 Effect of crop system and pesticide application in strawberry on *T. urticae*, *N. floridana*, predatory mites and fungal plant diseases in 2010

In 2010, there was a late infestation of *T. urticae*. The treatment with chemical control of pests and plant diseases in low tunnel (T1-t-c-c) presented a significantly ( $F= 5.19$ ,  $fd= 43$ ,  $p < 0.0001$ ) higher number of *T. urticae* compared to all treatments. The second groups of treatments with higher *T. urticae* populations were T5-t-c-bc, T6-f-c-c and T10-f-c-c that have in common the chemical control of pests. The treatments T2, T3, T4, T7, T8, and T9 presented the accumulated during the evaluation of and less than 20 mites per leaf and they all consisted of biological control of pests (Figure 2.1. and Table 2.3.).

The fungus *N. floridana* was observed only in the three last sample dates, starting in October, 12, 2010. The highest number of *T. urticae* with *N. floridana* was found in the treatment with chemical control of pests and biocontrol of fungal plant pathogens in low tunnel (T5-t-c-bc) with a cumulated number of *T. urticae* with *N. floridana* per leaf equal to 11.05 (approximately 1/3 of the cumulative number of mites), and this treatment was significantly higher than any of the other treatments ( $F= 1.10$ ,  $fd= 43$ ,  $p= 0.0006$ ). The treatments that presented more than one *T. urticae* with *N. floridana* per leaf were T1, T5, T6 and T10 all of them presented the above 30 *T. urticae* per leaf. The highest prevalence

of the *N. floridana* was in fact found in the treatment where we did not inoculated it (Figure 2.1. and Table 2.3.). In 2010, the variable number of *T. urticae* with *N. floridana* was positive and significantly (Pearson's correlation coefficient= 0.4060116; p= 0.001198) correlated to number of *T. urticae*.

Predatory mites were found naturally in all treatments starting in August, 5. The population of predatory mites increased substantially in the treatment T3-t-n-bc in September, 10, at least 18 days before the increase in any other treatment. In this treatment, the population of *T. urticae* remained low throughout the crop season. Except for the treatment T3, the treatments with cumulated number of predatory mites greater than 1 per leaf also presented high density of *T. urticae*. However, no statistically significant difference of predatory mites densities were found (F= 1.09, *fd*= 43, p= 0.3903) (Figure 2.1. and Table 2.3.). The variable number of *T. urticae* was positive and significantly (Pearson's correlation coefficient= 0.4150101; p= 0.001566) correlated to number of predatory mites.

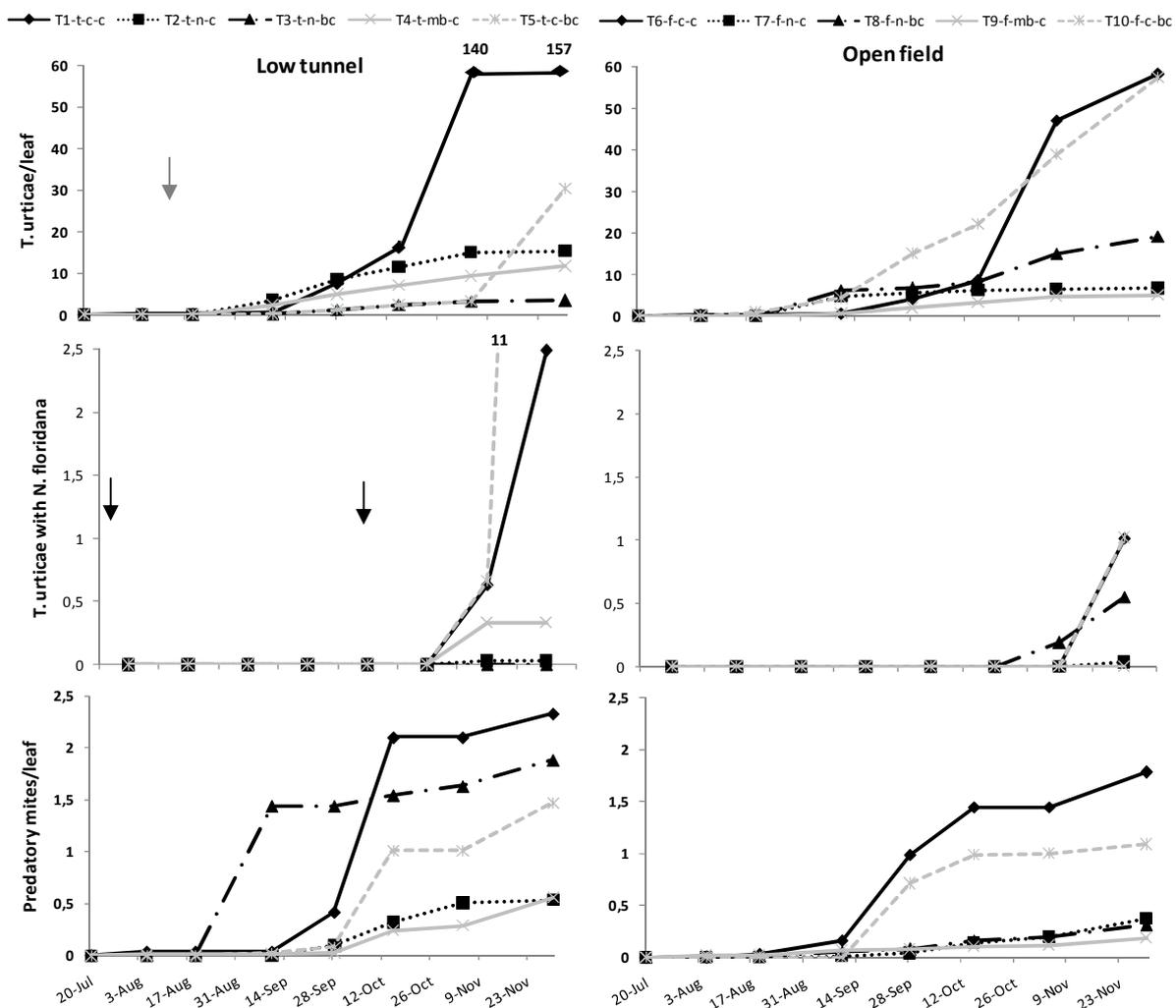


Figure 2.1 - Effect of crop system and pesticide application in strawberry on the cumulative number of *T. urticae*, predatory mites and *T. urticae* with *N. floridana* per leaf in 2010. Treatments (T1-T10): First letters in treatments: t= low tunnel, f= open field; Second letters in treatments: c= chemical acaricide control of *T. urticae*, n= inoculative release of *Neozygites* to control *T. urticae*, mb= inundative release of *Metarhizium* and *Beauveria* to control *T. urticae*; Third letter in treatments: c= chemical fungicide to control fungal plant diseases, bc= microbiological control agents to control fungal plant diseases. Example: T1 t-c-c =low tunnel (t) chemical acaricide control of *T. urticae* (c) and chemical fungicide control of fungal plant diseases (c). (↓) Grey arrow- data of infestation of *T. urticae*, black arrow- data of inoculative releases of *N. floridana*.

Table 2.3 - Effect of crop system and pesticide application in strawberry on cumulative number of *T. urticae*, *T. urticae* with *N. floridana*, predatory mites per leaf, and mean incidence of fungal plant diseases (%) and “vermelhão” (%) in 2010

2010					
Treatment	<i>T. urticae</i>	<i>N. floridana</i>	Predatory mites*	Fungal plant diseases	“Vermelhão”*
<b>T1-t-c-c</b>	156.4 ± 56.3 <b>A</b>	2.48 ± 1.15 <b>B</b>	2.33 ± 1.63	8.6 ± 4.3 <b>BC</b>	1.5 ± 1.0
<b>T2-t-n-c</b>	15.4 ± 7.5 <b>C</b>	0.03 ± 0.03 <b>B</b>	0.53 ± 0.16	7.9 ± 4.7 <b>BC</b>	2.3 ± 2.3
<b>T3-t-n-bc</b>	3.5 ± 1.2 <b>C</b>	0.0 <b>B</b>	2.03 ± 1.66	6.6 ± 5.0 <b>C</b>	1.3 ± 1.3
<b>T4-t-mb-c</b>	11.6 ± 5.1 <b>C</b>	0.33 ± 0.33 <b>B</b>	0.56 ± 0.30	4.9 ± 2.0 <b>C</b>	2.0 ± 2.0
<b>T5-t-c-bc</b>	30.4 ± 12.2 <b>B</b>	11.05 ± 8.20 <b>A</b>	1.74 ± 0.93	5.0 ± 2.1 <b>C</b>	4.2 ± 2.8
<b>T6-f-c-c</b>	58.1 ± 24.4 <b>B</b>	1.01. ± 0.53 <b>B</b>	1.78 ± 0.86	17.4 ± 6.8 <b>A</b>	4.1 ± 2.8
<b>T7-f-n-c</b>	6.7 ± 4.8 <b>C</b>	0.03 ± 0.03 <b>B</b>	0.37 ± 0.21	16.72 ± 9.6 <b>A</b>	3.6 ± 2.0
<b>T8-f-n-bc</b>	19.2 ± 9.6 <b>C</b>	0.55 ± 0.53 <b>B</b>	0.31 ± 0.12	14.11 ± 6.5 <b>AB</b>	1.8 ± 1.8
<b>T9-f-mb-c</b>	4.9 ± 1.9 <b>C</b>	0.0 <b>B</b>	0.19 ± 0.06	13.8 ± 5.2 <b>AB</b>	3.4 ± 2.8
<b>T10-f-c-bc</b>	57.3 ± 18.9 <b>B</b>	1.02 ± 0.8 <b>3B</b>	1.09 ± 0.51	15.9 ± 3.7 <b>A</b>	2.7 ± 1.8

\*= Means not significantly different ( $p > 0.05$ ). Different letters between rows denote significant differences using Tukey’s HDS tests ( $p > 0.05$ ). Treatments (T1-T10): First letters in treatments: t= low tunnel, f= open field; Second letters in treatments: c= chemical acaricide control of *T. urticae*, n= inoculative release of *Neozygites* to control *T. urticae*, mb= inundation release of *Metarhizium* and *Beauveria* to control *T. urticae*; Third letter in treatments: c= chemical fungicide to control fungal plant diseases, bc= microbiological control agents to control fungal plant diseases. Example: T1 t-c-c =low tunnel (t) chemical acaricide control of *T. urticae* (c) and chemical fungicide control of fungal plant diseases (c)

The prevalence of fungal plant diseases was low in 2010. Three leaf diseases were found, the strawberry leaf spot caused by *Mycosphaerella fragariae* (Tul.) Lindau, Pestalotiopsis leaf spot caused by *Pestalotiopsis longisetula* Guba and “vermelhão” (unknown agent). Despite many similarities of “vermelhão” with plant diseases, the causal agent is still unknown. Significantly higher prevalence of fungal plant diseases were found in the open field in the treatments T6-f-c-c and T7-f-n-c ( $F= 4.15$ ,  $fd= 43$ ,  $p= 0.0007$ ) and the treatments with the lowest fungal plant diseases incidence were T3-t-n-bc, T4-t-mb-c and T5-c-bc. The prevalence found for *M. fragariae* was 9.95% in the treatment T10-f-c-bc, and 9.50% for *P. longisetula* in the treatment T6-f-c-c. No significant differences between treatments were found for the disease “vermelhão” (Figure 2.2. and Table 2.3.).

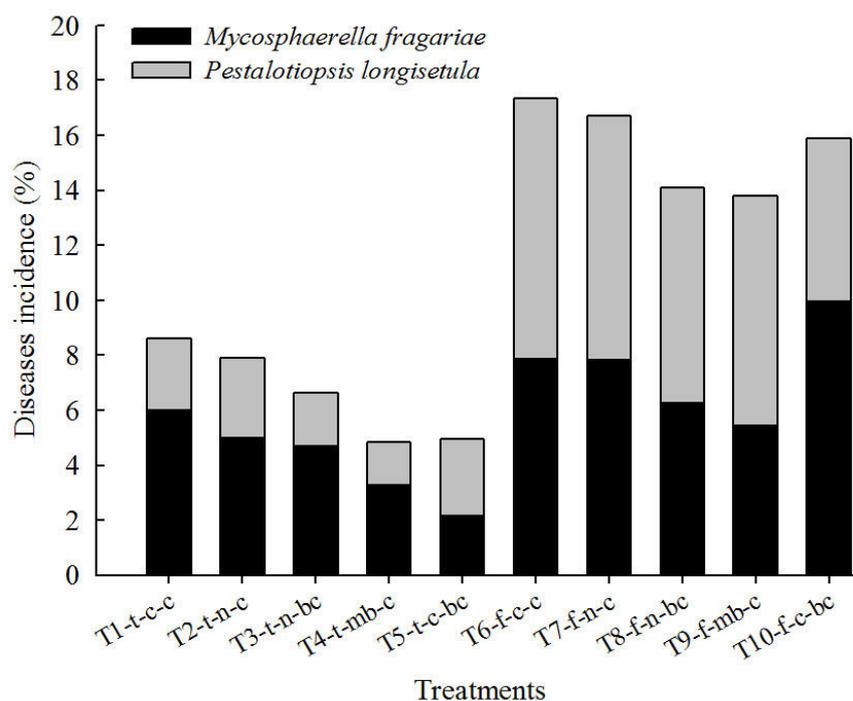


Figure2.2 - Effect of crop system and pesticides application in strawberry on the fungal plant diseases incidence per treatment in 2010. Treatments (T1-T10): First letters in treatments: t= low tunnel, f= open field; Second letters in treatments: c= chemical acaricide control of *T. urticae*, n= inoculative release of *Neozygites* to control *T. urticae*, mb=inundation release of *Metarhizium* and *Beauveria* to control *T. urticae*; Third letter in treatments: c= chemical fungicide to control fungal plant diseases, bc= microbiological control agents to control fungal plant diseases. Example: T1 t-c-c =low tunnel (t) chemical acaricide control of *T. urticae* (c) and chemical fungicide control of fungal plant diseases (c)

### 2.2.2.2 Micro- and macroclimatic data in low tunnel and open field in 2010

In 2010, the mean microclimatic temperatures were almost the same in low tunnel and open field and varied between 13.1 and 26.4 °C in low tunnel, and from 12.8 to 25.3 °C in open field. Overall, higher maximum temperature and lower minimum temperatures were found in low tunnel compared to open field. The lowest microclimatic temperatures were found in low tunnel during winter (August) 4.1°C and the highest temperatures were also found in low tunnel during spring (end of October) at 43.8°C. The microclimatic mean temperature in the tunnel was similar or higher than in the open field. The maximum microclimatic relative humidity (RH) was close to 100% from the end of July to the beginning of October in the open field. In the low tunnel, the microclimatic RH was quite similar to the open field until September, when the maximum RH fell to around 90%. In the low tunnel, the mean microclimatic RH was higher than 65% throughout most of the growing season but at the end of the season the RH was as low as 23.4 % RH. The microclimatic minimum RH was quite similar to the mean RH in July, but it started to decrease after this time until the end of the season, and the lowest microclimatic RH was found in open field (Figure 2.3). Because of equipment failure, microclimate data from open field were not available for the end of the crop season. In the macroclimate data, the mean temperature was lower than to the microclimate data from open field and low tunnel, except in the beginning of the experiment when they were similar. The macroclimate RH varied with the seasons, in the beginning it was very low compared to the microclimate data, but from the middle of September to middle of October, some periods it was lower, and others periods it was higher than the microclimate values.

A precipitation of 440 mm was recorded for the whole growing season, with the highest precipitation from the end of September when the density of mites increases in most part of the treatments in open field. Further, the period of rainfall coincided with the period with the highest incidence of fungal plant diseases in open field.

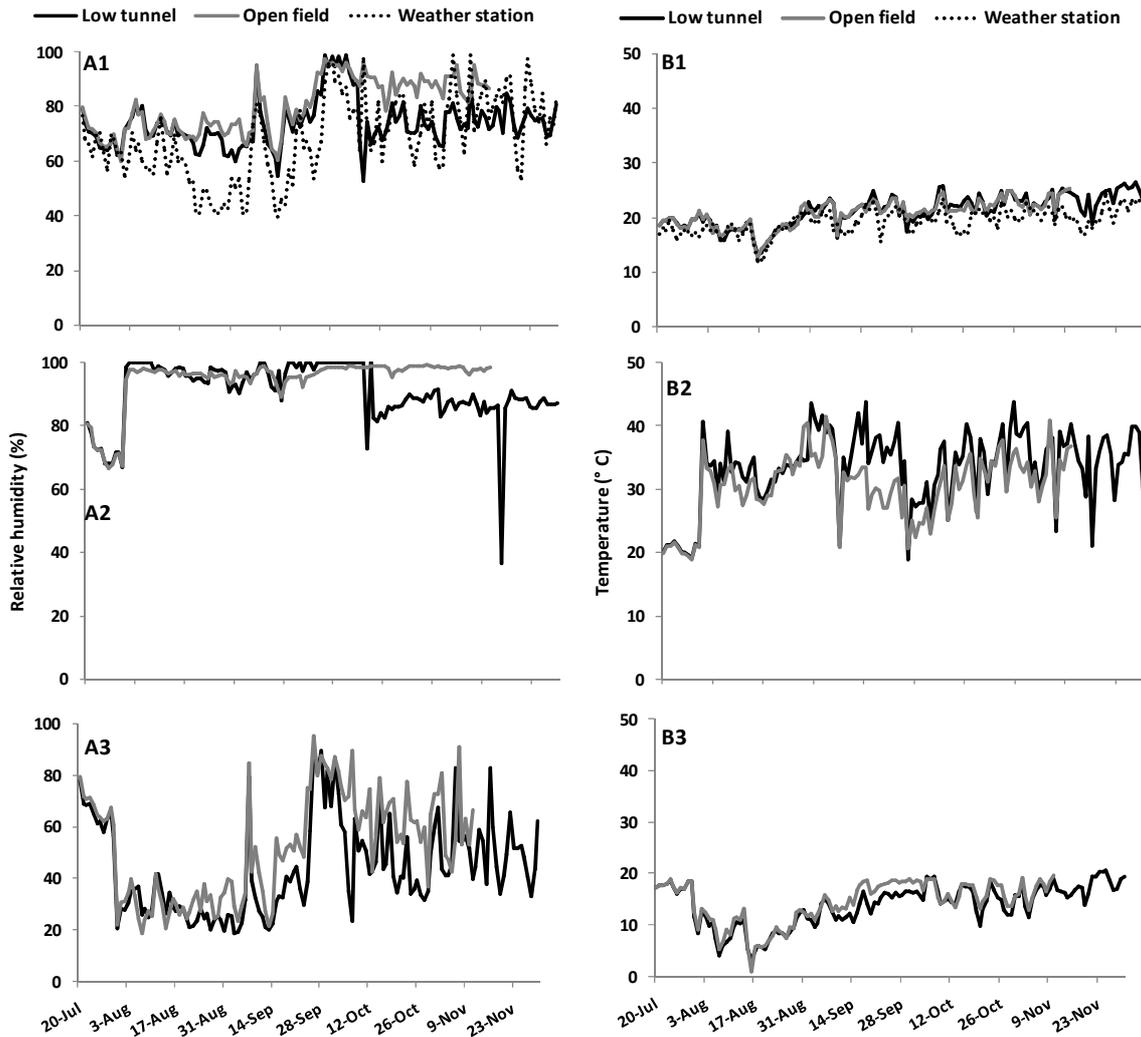


Figure 2.3 - Microclimatic temperature and relative humidity in open field and low tunnel (15 mm from leaf surface) and macroclimatic temperature and relative humidity at a weather station (2 m above ground) in 2010. A1 and B1 = Mean relative humidity and temperature; A2 and B2 = Maximum relative humidity and temperature; A3 and B3 = Minimum relative humidity and temperature

### 2.2.2.3 Effect of crop system and pesticide application in strawberry on *T. urticae*, *N. floridana*, predatory mites and fungal plant diseases in 2011

In 2011, a significantly higher number of *T. urticae* were found in four treatments: T1-t-c-c, T5-t-c-bc, T6-f-c-c and T10-f-c-bc ( $F= 5.79$ ,  $df= 45$ ,  $p< 0.0001$ ). In common these treatments had chemical control of pests in both systems, low tunnel and open field (Figure 2.4. and Table 2.3.).

*N. floridana* was observed first not only in plots where it had been released, both also in plots where it had not been released (Figure 2.4. and Table 2.3.). The significantly higher number of *T. urticae* with *N. floridana* per leaf was found in treatments with chemical control of pests and fungal plant diseases in low tunnel, T1-t-c-c (8.44 *T. urticae*

with *N. floridana* /leaf) ( $F= 2.96$ ,  $df= 45$ ,  $p= 0.0075$ ). The second highest cumulative population of *N. floridana* was observed in the treatments T5-t-c-bc, T6-f-c-c and T10-f-c-bc. The four treatments with highest prevalence of the *N. floridana* consisted in fact of treatments where we did not release it and attained high densities of *T. urticae*. The variable number of *T. urticae* was positive and significantly (Pearson's correlation coefficient= 0.6783967;  $p< 0.0001$ ) correlated to number of *T. urticae* with *N. floridana*.

Predatory mites were detected naturally from the beginning of July. No significant effect of tunnel or pesticides application were found in the number of predatory mites per leaf ( $F= 0.0637$ ,  $df= 45$ ,  $p= 0.0889$ ) (Table 2.3.). Figure 2.4 shows that the treatments with cumulative number of predatory mites greater than 0.4 mites/ leaf was observed in treatments in low tunnel without chemical control of pests; T2-t-n-c, T3-t-n-bc, and T4-t-mb-c. No correlation between the variables *T. urticae* and predatory mites was observed ( $p= 0.138$ ).

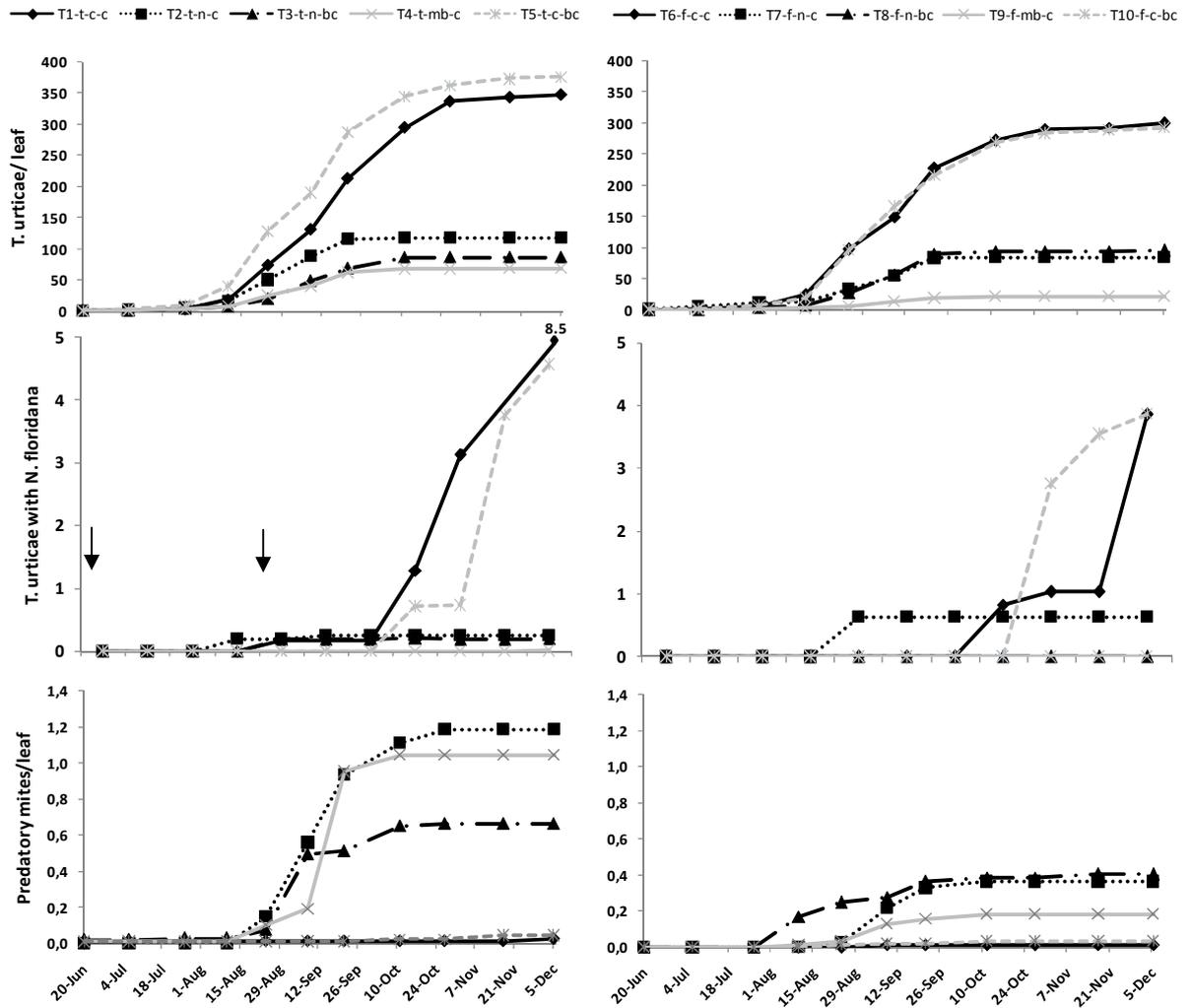


Figure 2.4 - Effect of crop system and pesticide application in strawberry on the cumulative number of *T. urticae*, predatory mites and *T. urticae* with *N. floridana* per leaf in 2011. Treatments (T1-T10): First letters in treatments: t= low tunnel, f= open field; Second letters in treatments: c= chemical acaricide control of *T. urticae*, n= inoculative release of *Neozygites* to control *T. urticae*, mb= inundative release of *Metarhizium* and *Beauveria* to control *T. urticae*. Third letter in treatments: c= chemical fungicide to control fungal plant diseases, bc= microbiological control agents to control fungal plant diseases. Example: T1 t-c-c =low tunnel (t) chemical acaricide control of *T. urticae* (c) and chemical fungicide control of fungal plant diseases (c). (↓) Black arrow- data of inoculative releases of *N. floridana*

Table 2.3 - Effect of crop system and pesticides application in strawberry on cumulative number of *T. urticae*, *T. urticae* with *N. floridana*, predatory mites per leaf, and fungal plant diseases incidence (%) and “vermelhão” incidence (%) in 2011

2011					
Treatment	<i>T. urticae</i> <sup>1</sup>	<i>N. floridana</i> <sup>1</sup>	Predatory mite*	Fungal plant diseases	“Vermelhão”
<b>T1-t-c-c</b>	347.1 ± 23.8 <b>A</b>	8.44 ± 3.58 <b>A</b>	0.10 ± 0.13	2.1 ± 1.6 <b>C</b>	5.4 ± 4.2 <b>AB</b>
<b>T2-t-n-c</b>	118.1 ± 51.7 <b>B</b>	0.25 ± 0.19 <b>C</b>	1.14 ± 0.31	1.7 ± 0.6 <b>C</b>	3.9 ± 2.6 <b>AB</b>
<b>T3-t-n-bc</b>	87.3 ± 50.0 <b>B</b>	0.20 ± 0.19 <b>C</b>	1.26 ± 1.01	1.5 ± 0.8 <b>C</b>	3.5 ± 3.0 <b>AB</b>
<b>T4-t-mb-c</b>	69.0 ± 45.4 <b>B</b>	0.01 ± 0.01 <b>C</b>	0.42 ± 0.22	2.6 ± 1.2 <b>C</b>	4.6 ± 3.1 <b>AB</b>
<b>T5-t-c-bc</b>	375.3 ± 114.3 <b>A</b>	2.76 ± 1.10 <b>B</b>	0.05 ± 0.02	2.3 ± 1.1 <b>C</b>	1.9 ± 1.7 <b>B</b>
<b>T6-f-c-c</b>	298.9 ± 64.5 <b>A</b>	3.50 ± 1.21 <b>B</b>	0.01 ± 0.01	8.5 ± 3.2 <b>B</b>	4.8 ± 2.8 <b>AB</b>
<b>T7-f-n-c</b>	85.7 ± 21.6 <b>B</b>	0.63 ± 0.56 <b>C</b>	0.25 ± 0.18	13.2 ± 7.0 <b>A</b>	5.9 ± 2.9 <b>A</b>
<b>T8-f-n-bc</b>	95.9 ± 19.1 <b>B</b>	0.00 <b>C</b>	0.18 ± 0.07	7.2 ± 3.2 <b>BC</b>	3.4 ± 2.0 <b>AB</b>
<b>T9-f-mb-c</b>	22.9 ± 3.9 <b>B</b>	0.00 <b>C</b>	0.34 ± 0.23	7.7 ± 3.7 <b>B</b>	4.7 ± 3.5 <b>AB</b>
<b>T10-f-c-bc</b>	292.2 ± 87.5 <b>A</b>	3.9 ± 2.05 <b>B</b>	0.03 ± 0.02	7.7 ± 3.7 <b>B</b>	2.4 ± 1.0 <b>B</b>

\*=Means not significantly different ( $p > 0.05$ ). Different letters between rows denote significant differences using Tukey's HSD tests ( $p > 0.05$ ). Treatments (T1-T10): First letters in treatments: t= low tunnel, f= open field; Second letters in treatments: c= chemical acaricide control of *T. urticae*, n= inoculative release of *Neozygites* to control *T. urticae*, mb=inundation release of *Metarhizium* and *Beauveria* to control *T. urticae*; Third letter in treatments: c= chemical fungicide to control fungal plant diseases, bc= microbiological control agents to control fungal plant diseases. Example: T1 t-c-c =low tunnel (t) chemical acaricide control of *T. urticae* (c) and chemical fungicide control of fungal plant diseases (c)

Highest prevalence of fungal plant diseases were found in treatment with inoculative release of *N. floridana* and chemical control of fungal plant diseases in open field (T7-f-n-c), and they was significantly ( $F= 11.49$ ,  $fd= 45$ ,  $p< 0.0001$ ) higher than all other treatments. The treatments presenting the second highest prevalence of fungal diseases were also all in open field (T6-f-c-c, T8-f-n-bc, T9-f-mb-c and T10-f-c-bc). No clear pattern was observed for “vermelhão” (Figure 2.5. and Table 2.3.) ( $F= 3.2$ ,  $fd=45$ ,  $p< 0.0001$ ). In 2011, six fungal plant diseases were observed: strawberry black flower rot caused by *Colletotricum acutatum* Simmonds, lead blight caused by *Dendrophoma obscurans* (Ell. & Ev.) H. W. Anderson, strawberry leaf spot caused by *M. fragariae*, Pestalotiopsis leaf spot caused by *P. longisetula*, powdery mildew caused by *S. macularis* and “vermelhão” (unknown agent). The fungal plant diseases described below occurred in all of the treatments, except the fungal disease *M. fragariae* that do not occur in the treatments T3-t-n-bc and T3-f-n-bc. Powdery mildew was observed only in one treatment in the low tunnel (T1-t-c-c) during one evaluation in the beginning of July (Figure 2.5).

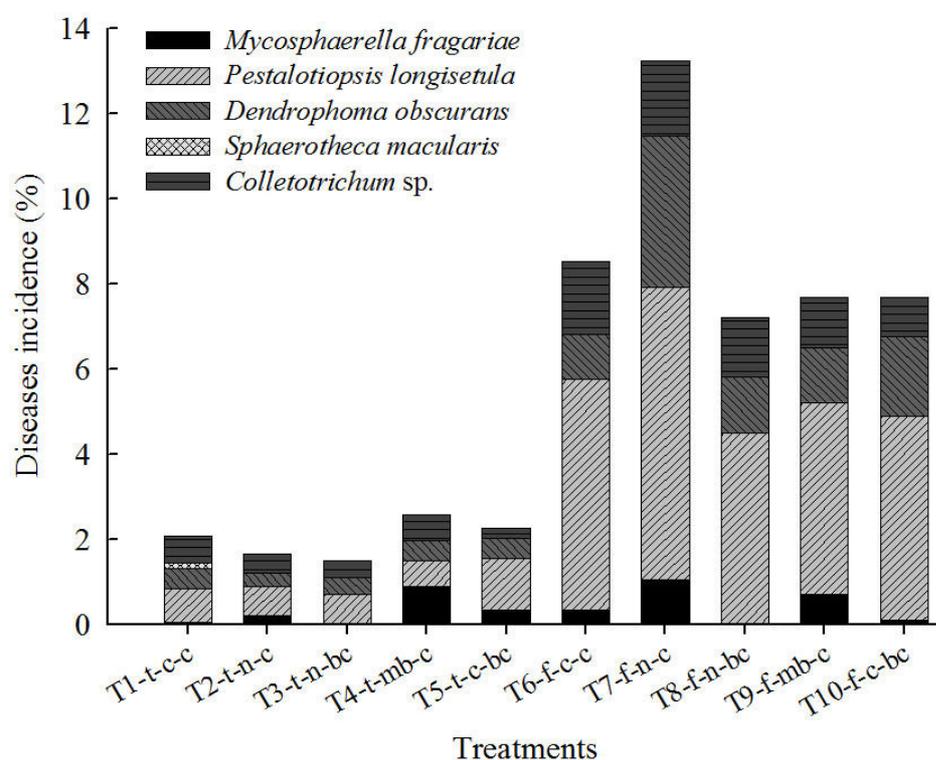


Figure 2.5 - Effect of crop system and pesticide application in strawberry on the fungal plant diseases incidence per treatment in 2011. Treatments (T1-T10): First letters in treatments: t= low tunnel, f= open field; Second letters: c= chemical acaricide control of *T. urticae*, n= inoculative release of *Neozygites* to control *T. urticae*, mb=inundative release of *Metarhizium* and *Beauveria* to control *T. urticae*; Third letter: c= chemical fungicide to control fungal plant diseases, bc= microbiological control agents to control fungal plant diseases. Example: T1 t-c-c =low tunnel (t) chemical acaricide control of *T. urticae* (c) and chemical fungicide control of fungal plant diseases (c)

#### **2.2.2.4 Micro- and macroclimatic data in low tunnel and open field in 2011**

In 2011, the mean microclimate temperatures were almost the same in low tunnel and open field and varied between 10.5 and 28.1 °C in low tunnel and from 11 to 29 °C in open field. The maximum temperature was higher in open field notably from July, 4 to August, 15 and the highest temperature was observed in low tunnel in middle of October in low tunnel. The maximum relative humidity (RH) attained was close to 100% all of the time in open field, and in low tunnel the RH attained this percentage only after October. During winter, RH was very low especially in open field. The mean RH was different in the beginning of July and in October with smaller value in open field than in low tunnel (Figure 2.6). Concerning the macroclimate data, the mean and maximum RH from August to October, presented some peaks with much lower value compared with the microclimate data from low tunnel and open field. Moreover for the temperature the differences observed between microclimate and macro climate were not so significant.

Rainfalls of approximately 510 mm was recorded during the whole growing season, and the rain started to be more constant from September on, and the highest precipitation was observed on 17/October/2011 (data non shown).

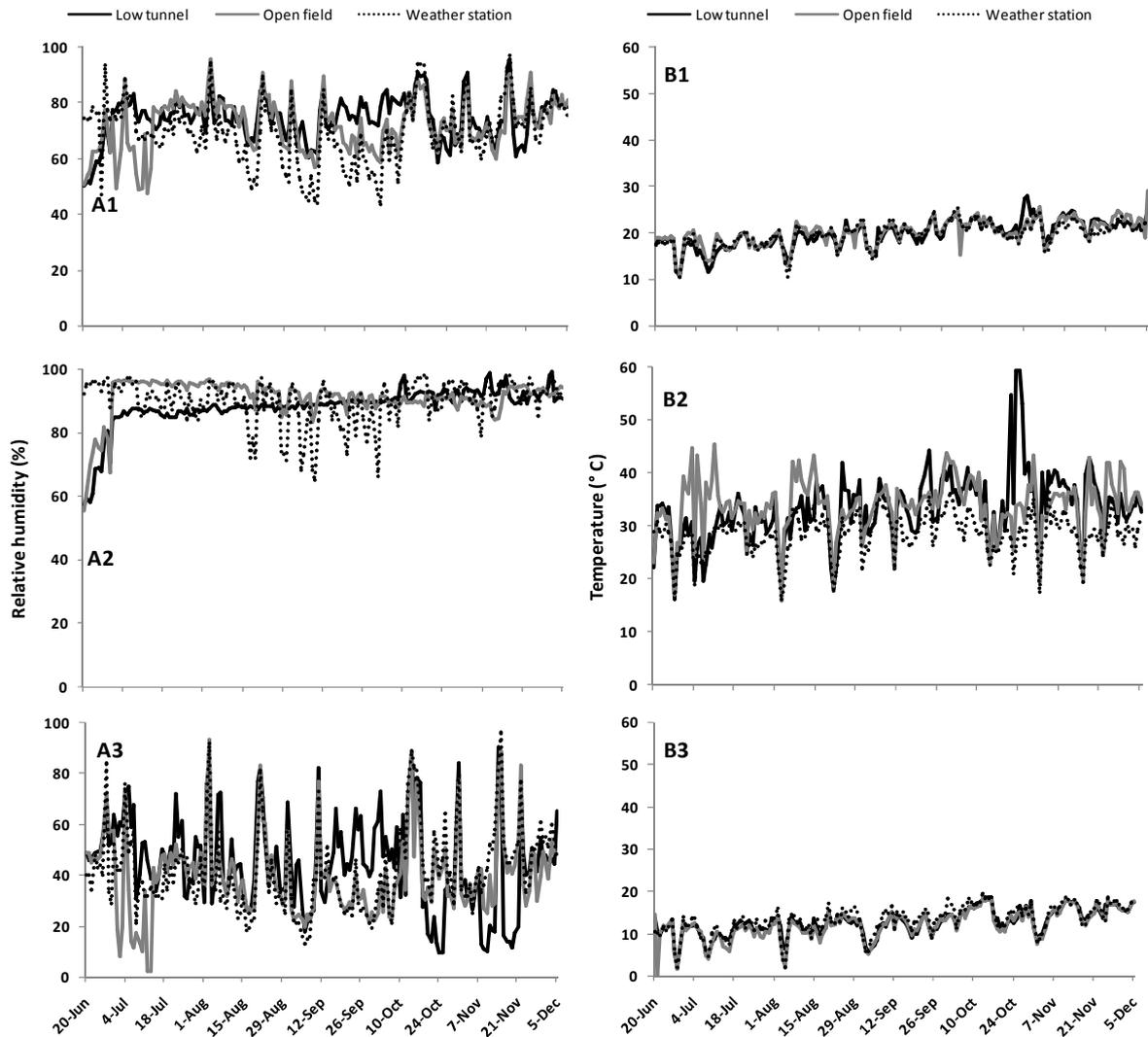


Figure 2.6 - Microclimatic temperature and relative humidity in open field and low tunnel (15 mm from leaf surface) and macro climate temperature and relative humidity at weather station (2 m above ground) in 2011. A1 and B1= Mean relative humidity and temperature; A2 and B2 =Maximum relative humidity and temperature; A3 and B3= Minimum relative humidity and temperature

Considering both seasons, from the 466 predatory mites identified to species level, the most abundant species found on strawberry leaves were *Neoseiulus anonymus* (Chant) (57.51%) and *Phytoseiulus macropilis* (Banks) (25.75%); the other four species found [*Phytoseiulus fragariae* Denmark and Schicha, *Proprioseiopsis cannaensis* Muma, *Iphiseiodes zuluagai* Denmark and Muma, and *Amblydromalus limonicus* Garman and McGregor] were represented by few specimens (16.74%).

### 2.2.3 Discussion

In this study we investigated the effect of the crops system and the regime of pesticides application on strawberry, in *T. urticae*, its natural enemies (*N. floridana* and predatory mites) and plant pathogenic fungi. The use of low tunnel could not be associated with higher densities of *T. urticae* in strawberry, but it seems that, the major factor affecting positively the increase of this spider mite was the application of chemical acaricides and insecticides. In both crop seasons, the highest levels of *T. urticae* were found in treatments with chemical control of pests, and not necessary with chemical control of plant diseases, especially in the second crop season.

The pesticides, especially the insecticides/acaricides used in our study, could have a stimulatory effect on *T. urticae* populations. Among the pesticides used, one is neonicotinoid (Thiamethoxam) and two are pyrethroids (Fenpropathrine and Cyhalothrin-lambda), which are recognized for stimulated their reproduction and, consequently, allowed the population density of this mites to increase in the field, a phenomenon known as hormoligosis (GERSON; COHEN, 1989; TRICHILO; WILSON, 1993; AKO et al., 2004; SZCZEPANIEC et al., 2011). It is also possible that the *T. urticae* population in the experimental field presented some levels of resistance to the acaricides/insecticides used. It has been reported the resistance of *T. urticae* to 92 active ingredients in the world, from this, 18 reports were in Brazil (<http://www.pesticideresistance.org/>). From the four chemical acaricides used in the experiment, two presents report of *T. urticae* resistance in Brazil, Abamectin (SATO et al., 2005) and Fenpiroximate (SATO et al., 2004).

The fungus *N. floridana* occurred later in the season in treatments with and without inoculative releases in both years indicating that the experimental releases were not successful and the fungus occurred naturally in the field. The treatments that presented the highest prevalence of the fungus in both years were in treatments with high densities of *T. urticae*; where we did not release it, in both systems. In 2010, the treatment T5-t-c-bc, which presents biocontrol of fungal plant diseases, presented high level of *T. urticae* with *N. floridana* per leaf, and the population of *T. urticae* did not attained very high level in this treatment. This could suggest that the early occurrence of the fungus prevented the increase of the spider mite population. In other treatments, the fungus appeared only after the increase in the host population. In 2011, the fungus attained high level in the treatments with chemical control of pests and fungal plant diseases, in both systems. These treatments also presented a

very high level of hosts *T. urticae*. The correlation analysis suggests that the fungus *N. floridana* was positively associated with the number of their host *T. urticae* a host density dependent response. Host population density is cited by several authors as a factor that limits occurrence of epizootics (TANADA; KAYA, 1992; WATANABE, 1987) as it affects the rate of transmission and consequently the rate of infection by the pathogen. The crop system and the pesticides applications might not affect the population of this fungus. Studies of populations dynamics of fungus *N. floridana* and their host *Tetranychus evansi*, in protect and unprotect environment, and different pesticides application regimes showed that the host population density is the factor responsible for the epizooties caused by the fungus in protected environment, and the pesticides regimes did not affect the population dynamics of the fungus (DUARTE et al., 2009). *Neozygites* spp. are considered by many authors to be a major factor causing decline in populations of *T. urticae* in different crops when microclimatic conditions are suitable (CARNER; CARNEDAY, 1970; BOYKIN et al., 1984; SMITLEY; KENNEDY; BROOKS, 1986, KLUBERTANZ; PEDIGO; CARLSON, 1991).

The predatory mites were found in both crop seasons in most of the treatments in open field and low tunnel. The population densities of predatory mites did not differ among the crops systems and pesticides regimes tested. *Neoseiulus anonymus* was the most abundant phytoseiid species found in the field experiment. This predator has a relatively restricted world distribution. It has only been reported in Central and South America and on some Caribbean island (DEMITE et al., 2012). The second most abundant species was *Phytoseiulus macropilis*, and has been reported in strawberry fields in Brazil (FERLA, MARCHETTI; GONÇALVES, 2007; OLIVEIRA et al., 2009) most commonly reported, from southern US to Argentina, including many Caribbean islands, but it has also been reported from tropical islands of other parts of the world, especially in the Pacific Ocean. Scanty reports of this species from other parts of the world should be confirmed (DEMITE et al., 2012). The distribution of both *N. anonymus* and *P. macropilis* extends to regions where temperatures are relatively low in the winter and both seem unable to develop well in areas with reduced rainfall and low RH (G.J. Moraes, personal observation). No previous study was conducted in southern Minas Gerais specifically to determine the phytoseiids on strawberry.

The fungal plant diseases prevalence in strawberry was lower in treatments in low tunnel than in open field. The greater prevalence of fungal plant diseases in open field are probably associated with higher relative humidities and the effect of rain on splash dispersal of plant diseases that include detachment of pathogens by the impact of water drops and

transportation of spore-bearing splash droplets to new locations (ALLEN, 1988, YANG; MADDEN; BRAZEE, 1991, HUBER; FITT; McCARTNEY, 1996). The reduction of fungal plant diseases in low tunnel can be attributed to a few possible mechanisms. Inside tunnels, plants were protected from rain, wind and splash dispersal of inoculums, important factors in spore production and the dispersal of spores of many fungal pathogens (MAAS, 1998). Xiao et al. (2001) also suggested that the reduction of fungal plant diseases could be due to the shorter periods, of leaf wetness, that it was over 60% less in tunnel than in open field. The disease “Vermelhão” occurs in all of the treatments in both crop seasons, and the prevalence was not high and there were no patterns on the incidence of this disease. This disease is important in strawberry field in Brazil, and due of their severity this disease can cause a significant reduction of production because in most of the cases the plants die. This disease has been observed for at least five years, occurring randomly in the beds, in isolated plants in strawberry field (HENZ; REIS, 2009). The powdery mildew was observed in only one evaluation in 2011. Xiao et al. (2001) suggested that the development of powdery mildew is favored by low light intensity, moderate to high relative humidity without free moisture, and temperatures of approximately 15 to 27 °C, but it was not possible to observe some pattern in the incidence of the powdery mildew in this experiment.

The highest temperature was found in 2010 in low tunnel, and the highest relative humidity in open field. In 2011 the same results was found for the relative humidity, that was high in open field in most part of the time, but in this year were observed highest temperature in open field, especially in the winter season. Studies about environmental conditions in tunnels indicate that the temperature has moderate variations in high tunnels (KADIR; CAREY, 2006, WIEN, 2009). Studies on microclimate changes in plasticulture, including high and low tunnel, demonstrate that the temperature becomes higher in plastic tunnel despite the relative humidity decrease in this system when compare with open field (FREEMAN; GNAYEM, 2004). The problem of *T. urticae* in strawberry field in Brazil in low tunnel could be because most of the farmers do not open and close the low tunnel daily, and this might increase the temperature inside the tunnels. In this experiment, the low tunnel were opened during the early morning and closed at the end of the afternoon. High temperatures were observed in low tunnel, but it seems that this is not the major factor associated with high infestation by *T. urticae*.

To the best of our knowledge, no field experiments have been conducted to determine the effect of the crop systems and pesticides on the pests, their natural enemies and fungal plant diseases on strawberries field. And our findings showed that: 1) The highest number of

*T. urticae* was found in treatments with chemical control of pests, and not necessarily associated with low tunnel; 2) *N. floridana* was found late and it was associated with high host densities; 3) Predatory mites occurred in most of the conditions tested; 4) Fungal plant diseases incidence was higher in treatments in open field, and no pattern was observed for the disease “Vermelhão”; 5) The highest temperature was found in most of the time in low tunnel, and the highest relative humidity was in open field.

## References

- AKO, M.; BORGEMEISTER, C.; POEHLING, H.; ELBERT, A.; NAUEN, R. Effects of Neonicotinoid Insecticides on the Bionomics of Twospotted Spider Mite (Acari: Tetranychidae). **Ecotoxicology**, New York, v. 97, n. 5, p. 1587-1594, 2004.
- ALLEN, R.C. The mechanics of splashing. **Journal of Colloid and Interface Science**, New York, v.124, p.309-316, 1988.
- ANTUNES, L.E.C.; PERES, N.A. Strawberry Production in Brazil and South America. **International Journal of Fruit Science**, Philadelphia, v.13, p. 156-161, 2013.
- ANTUNES, L.E.C. Situação da produção integrada de morango (PIMo). In: ENCONTRO NACIONAL DO MORANGO, 3.; ENCONTRO SOBRE PEQUENAS FRUTAS E FRUTAS NATIVAS DO MERCOSUL, 2., 2006, Pelotas. Pelotas: Embrapa Clima Temperado, 2006. v. 1, p. 101-104. (Documentos, 167).
- BOYKIN, L. S, CAMPBELL, W.V, BEUTE, M. K. Effect of pesticides on *Neozygites floridana* (Entomophthorales: Entomophthoraceae) and arthropod predators attacking the two spotted spider mite (Acari: Tetranychidae) in North Carolina peanut fields. **Journal of Economic Entomology**, Lanham, v. 77, p. 969-975, 1984.
- CARNER, G.R.; CANERDAY, T.D. Field and laboratory investigations with *Entomophthora fresenii*, a pathogen of *Tetranychus* spp. **Journal of Economic Entomology**, Lanham, v. 61, p. 956-959, 1968.
- CASTRO, R.L. Melhoramento genético do morangueiro: avanços no Brasil, In: SIMPÓSIO NACIONAL DO MORANGO, 2.; ENCONTRO DE PEQUENAS FRUTAS E FRUTAS NATIVAS, 1., 2004, Pelotas. Pelotas: Embrapa Clima Temperado, 2004. p. 21–36.
- CHIAVEGATO, L.G.; MISCHAN, M.M. Efeito do ácaro *Tetranychus urticae* (KOCH, 1836) BOUDREAUX; DOSSE, 1963 (Acari, Tetranychidae) na produção do morangueiro (Fragaria spp.). **Cientifica**, São Paulo, v.9, p.257-266, 1981.
- COSTA, H.; VENTURA, J.A. Manejo Integrado de Doenças do Morangueiro. In: ENCONTRO NACIONAL DO MORANGO, 3.; ENCONTRO SOBRE PEQUENAS FRUTAS E FRUTAS NATIVAS DO MERCOSUL, 2., 2006, Pelotas. Pelotas: Embrapa Clima Temperado, 2006. v. 1, p. 17-28. (Documentos, 167).

- CRANHAM, J.E.; HELLE, W. Pesticide resistance in Tetranychidae. In: HELLE, W.; SABELIS, M.W. (Ed.). **Spider mites: their biology, natural enemies and control**. Amsterdam: Elsevier, 1985. v. 1B, p. 405–421.
- DEMITE, P.R.; MORAES, G.J.; MCMURTRY, J.A.; DENMARK, H.A.; CASTILHO, R.C. (2012) *Phytoseiidae Database*. Available in: [www.lea.esalq.usp.br/phytoseiidae](http://www.lea.esalq.usp.br/phytoseiidae). Accessed on 28/XI/2012.
- DUARTE, V.S.; SILVA, R.A.; WEKESA, V.W.; RIZZATO, F.B.; DIAS, C.T.S.; DELALIBERA JR., I. Impact of natural epizootics of the fungal pathogen *Neozygites floridana* (Zygomycetes: Entomophthorales) on population dynamics of *Tetranychus evansi* (Acari: Tetranychidae) in tomato and nightshade. **Biological Control**, Orlando, v. 51, p. 81–90, 2009.
- FERLA, N.J.; MARCHETTI, M.M.; GONÇALVES, D. Ácaros predadores (Acari) associados à cultura do morango (*Fragaria* sp, Rosaceae) e plantas próximas no Estado do Rio Grande do Sul. **Biota Neotropica**, v.7, n. 2, p. 1–8, 2007.
- FITZGERALD, J.; PEPPER, N.; EASTERBROOK, M.; POPE, T.; SOLOMON, M. Interactions among phytophagous mites, and introduced and naturally occurring predatory mites, on strawberry in the UK. **Experimental and Applied Acarology**, Amsterdam, v. 43, p. 33–47, 2007.
- FRAULO, A.; LIBURD, O. Biological control of twospotted spider mite, *Tetranychus urticae*, with predatory mite, *Neoseiulus californicus*, in strawberries. **Experimental and Applied Acarology**, Amsterdam, v.43, p. 109–119, 2007.
- FREEMAN, S.; GNAYEM, N. Use of plasticulture for strawberry plant production. **Small Fruit Review**, Philadelphia, v.4, n. 1, p. 21–32, 2004.
- GERSON, U.; COHEN, E. Resurgences of Spider Mites (Acari: Tetranychidae) Induced by Synthetic Pyrethroids. **Experimental and Applied Acarology**. Amsterdam, v. 6, p. 29–46, 1989.
- HENZ, G. P.; REIS, A. Alerta vermelho: “vermelhão” do morango do morangueiro. **Revista Cultivar HF**, Pelotas, v.42, p.20–22, 2009.
- HORN, N.L.; BURNSIDE, K.R.; CARVER, R.B. Powdery mildew of strawberry. **Plant Disease Reporter**, Washington, v.56, p.368, 1972.
- HUBER, L.; FITT, B.D.L.; McCARTNEY, H.A. The incorporation of pathogen spores into rain-splash droplets: a modeling approach. **Plant Pathology**, Oxford, v. 45, p. 506–517, 1996.
- HUKKANEN, A.T.; KOKKO, H.I.; BUCHALA, A.J.; McDOUGALL, G.J.; STEWART, D.; KÄRENlampi, S.O.; KARJALAINEN, R.O. Benzothiadiazole induces the accumulation of phenolics and improves resistance to powdery mildew in strawberries. **Journal of Agricultural and Food Chemistry**, Easton, v.55, p.1862–1870, 2007.

KADIR, S.; CAREY, E.; ENNAHLI, S. Influence of high tunnel and field conditions on strawberry growth and development. **HortScience**, Alexandria, v. 41, p. 329–335, 2006.

KLUBERTANZ, T.H.; PEDIGO, L.P.; CARLSON, R.E. Impact of fungal epizootics on the biology and management of the two spotted spider mite (Acari: Tetranychidae) in soybean. **Environmental Entomology**, College Park, v. 20, p. 731-735, 1991.

KIRSCHBAUM, D.S., HANCOCK, J.F. The strawberry industry in South America. **HortScience**, Alexandria, v. 35, n. 5, p.807-811, 2000.

MAAS, J.L. **Compendium of strawberry diseases**. 3<sup>rd</sup>ed. St. Paul: American Phytopathological Society, 1998. 138 p.

MORAES, G.J.; FLECHTMANN, C.H.W. Ácaros encontrados em diferentes espécies vegetais de importância econômica. In: \_\_\_\_\_. **Manual de acarologia: acarologia básica e ácaros de plantas cultivadas no Brasil**. Ribeirão Preto: Holos, 2008. cap. 9, p. 106-208.

ÖZDEMİR, E. Early production of strawberry cultivars grown under plastic houses on sand dunes. **Small Fruit Review**, Philadelphia, v. 2, p. 81-86, 2003.

ÖZDEMİR, E.; GÜNDÜZ, K. Comparison of bag raised bed treatments for strawberry production under unheated greenhouse conditions. **Journal of the American Pomological Society**, University Park, v.58, p. 118-122, 2004.

PROGRAMA DE ANÁLISE DE RESÍDUOS DE AGROTÓXICOS EM ALIMENTOS. **Nota técnica para divulgação dos resultados do PARA de 2010: relatório geral de atividades 2010**. Brasília: Agência Nacional de Vigilância Sanitária, 2011. 26p.

RIGON, L.; CORRÊA, S.; REETZ, E.; VENCATO, A.; ROSA, G.R.; BELING, R.R. Pequenas frutas. **Anuário Brasileiro da Fruticultura**, Santa Cruz do Sul, v. 1, n. 1, p. 90-97, 2005.

RHODES, E.M.; LIBURD, O.E. Evaluation of predatory mites and Acramite for control of twospotted spider mites in strawberries in North Central Florida. **Journal of Economic Entomology**, Lanham, v.99, p. 1291–1298, 2006.

SAS INSTITUTE. **SAS/STAT user's guide: version 8**. Cary, 1999. v. 13.

SATO, M.E.; DA SILVA, M.Z.; RAGA, A.; SOUZA FILHO, M.F. Abamectin resistance in *Tetranychus urticae* Koch (Acari: Tetranychidae): selection, cross-resistance and stability of resistance. **Neotropical Entomology**, Londrina, v. 34, p. 991–998, 2005.

SATO, M.E.; MIYATA, T.; DA SILVA, M.Z.; RAGA, A.; SOUZA FILHO, M.F. Selections for fenpyroximate resistance and susceptibility, and inheritance, cross-resistance and stability of fenpyroximate resistance in *Tetranychus urticae* Koch (Acari: Tetranychidae). **Applied Entomology and Zoology**, Tokyo, v. 39, p. 293–302, 2004.

SMITLEY, D.R.; KENNEDY, G.G.; BROOKS, W.M. Role of the entomogenous fungus, *Neozygites floridana*, in population declines of two spotted spider mite *Tetranychus urticae*,

on field corn. **Entomologia Experimentalis et Applicata**, Dordrecht, v. 41, p. 255-264, 1986.

SVENSSON, B. Organic strawberries in tunnels with control of powdery mildew and mites. In: NJF SEMINAR 389. PEST, DISEASE AND WEED MANAGEMENT IN STRAWBERRY Ð PROGRESS AND CHALLENGES FOR THE NORDIC PRODUCTIONNJF, 2006, Helsinki.**Abstract...** Helsinki: NJF Report, 2006. Abstract 17.

SZCZEPANIEC, A.; CREARY, S.F.; LASKOWSKI, K.L.; NYROP, J.P.; RAUPP, M.J. Neonicotinoid Insecticide Imidacloprid Causes Outbreaks of Spider Mites on Elm Trees in Urban Landscapes. **PLoS ONE**, San Francisco, v. 6, n. 5, e20018.doi:10.1371/journal.pone.0020018, 2011.

TANADA, Y; KAYA, H.K. Epizootiology, p.595-633. In: \_\_\_\_\_**Insect Patology**. California: Academic Press, chap.16, 633p. 1992.

TRICHILO, P.J.; WILSON, L.T. An ecosystem analysis of spider mite outbreaks: physiological stimulation or natural enemy suppression. **Experimental and Applied Acarology**. Amsterdam, v. 17, p. 291-314, 1993.

VAN de VRIE, M.; McMURTRY, J.A.; HUFFAKER, C.B. Ecology of tetranychid mites and their natural enemies: A review. III. Biology, ecology, and pest status, and hostplantrelations of tetranychids. **Hilgardia**, Oakland, v. 41, p. 387-403, 1972.

WALSH, D.B.; ZALOM, F.G.; SHAW, D.V. Interaction of the two spotted spider mite (Acari: Tetranychidae) with yield of day-neutral strawberries in California. **Journal of Economic Entomology**, Lanham, v.91, p.678–685, 1998.

WATANABE, H. The host population, p. 71-112.In: Fuxa, J.R.; Tanada, Y (EdS.). **Epizootiology of insect diseases**. New York, Wiley, 555p. 1987.

WIEN, H.C. Floral crop production in high tunnels. **HortTechnology**, Alexandria, v. 19, p.56-60, 2009.

WITWER, S.; CASTILLA, N. Protected cultivation of horticultural crops worldwide. **HortTechnology**, Alexandria, v.5, p.6–23, 1995.

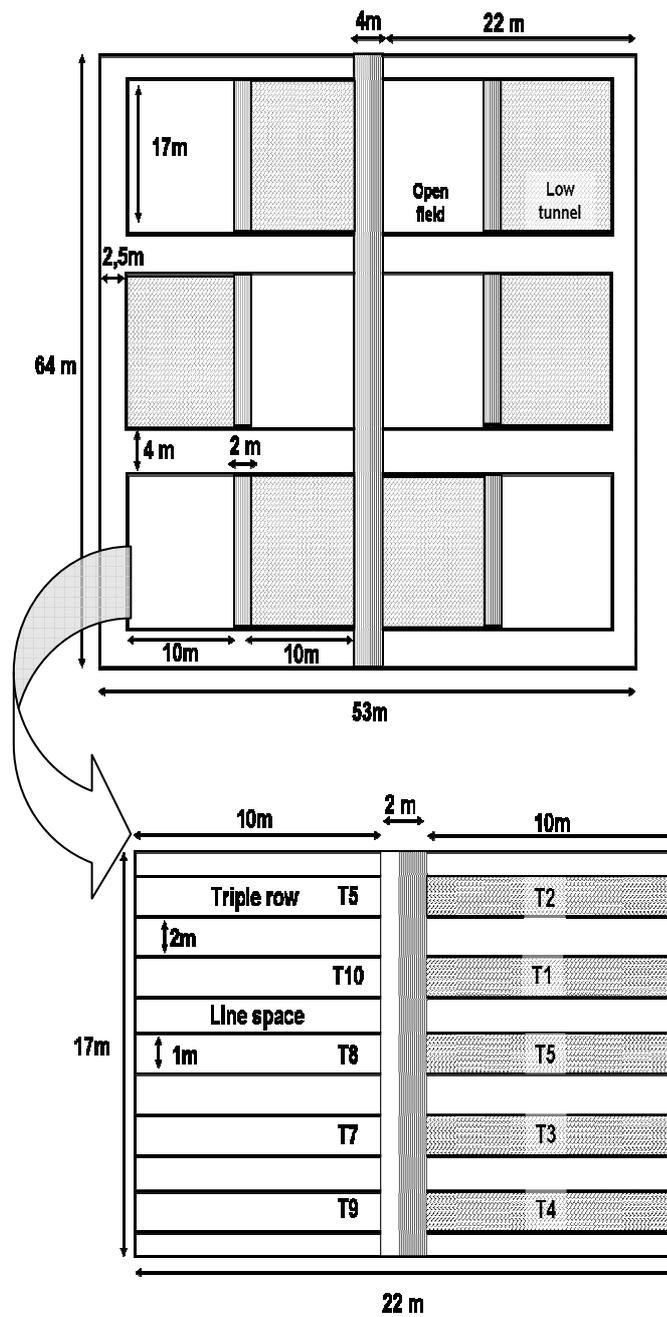
XIAO, C.L.; CHANDLER, C.K.; PRICE, J.F.; DUVAL, J.R.; MERTELY, J.C.;LEGARD, D.E. Comparison of epidemics of Botrytis fruit rot andpowdery mildew of strawberry in large plastic tunnel and fieldproduction systems. **Plant Disease**, Saint Paul, v. 85, p. 901–909, 2001.

YANG, X.; MADDEN, L. V.; BRAZEE, R. D. Application of the diffusion equation for modeling splash dispersal of point source pathogens. **New Phytologist**, Cambridge, v.118, p.295-301, 1991.

YOSHIDA, Y. Strawberry production in Japan: history and progress in production technology and cultivar development. **International Journal of Fruit Science**, Philadelphia, v.13, p. 103-113, 2013.

ZAMBOLIM, L.; COSTA, H. Manejo integrado das doenças do morangueiro. In: CARVALHO, S.P. de. (Ed.). **Boletim do morango**: cultivo convencional, segurança alimentar, cultivo orgânico. Belo Horizonte: FAEMG, 2006. p. 55-80.

## **Appendix**



Appendix A -Lay-out of the experimental field with the 6 blocks in the upper map. Lower map describe the 10 treatments for each block (subplots)

### 3 ABIOTIC AND BIOTIC FACTORS AFFECTING RESTING SPORE FORMATION IN THE MITE PATHOGEN *Neozygites floridana*

#### Abstract

*Neozygites floridana* is an obligate mite pathogenic fungus in the Entomophthoromycota that is known to produce different types of spores: primary conidia, capilliconidia, and two types of resting spore: azygospore and zygozspore. It has been suggested that resting spores are produced as a strategy to survive adverse conditions (e.g., winter, dry season, host absence). Many studies have examined the factors associated to resting spores formation in other Entomophthoromycota, but this topic has not been studied with *N. floridana*. In this study, possible mechanisms involved in the regulation of resting spores formation are investigated in the hosts *Tetranychus urticae* and *Tetranychus evansi*. Abiotic and biotic factors mimicking adverse conditions in temperate and tropical regions were tested with isolates from Norway and Brazil to induce resting spore production. A total, 42 combinations of abiotic and biotic conditions were tested, but only one condition stimulated the formation of a high number of resting spores, and this occurred in only one isolate. The Brazilian isolate ESALQ1420 produced a large number of resting spores (51.54%) in *T. urticae* at a temperature 11 °C, photoperiod of 10L:14D, light intensity of 42-46 ( $\mu\text{mol m}^{-2}\text{s}^{-1}$ ), on non-senescent plants (non-diapausing females). Small percentages of mites with resting spores (0-5 %) were found for the Norwegian isolate NCRI271/04 under the conditions tested, and very low percentages of resting spores (up to 1%) were observed in *T. evansi* infected by the Brazilian isolate ESALQ1421. The percentages of resting spores found for the Norwegian isolate in our laboratory studies are similar to the prevalence reported in earlier field studies. These findings support the hypothesis that the main overwintering strategy for *N. floridana* in temperate regions is to form hyphal bodies inside live hibernating *T. urticae* females and not as resting spores. It also seems unlikely that resting spores formation is the major strategy for surviving adverse conditions in tropical climates, given that the conditions that best induced resting spore formation by the Brazilian isolate are not common in the tropics. It was also documented in this study the formation of azygospore (asexual) resting spores in the Norwegian isolate and the Brazilian isolate ESALQ1420.

Keywords: *Neozygites floridana*; Resting spores; *Tetranychus evansi*; *Tetranychus urticae*; Biotic and abiotic factor; Azygospores; Zygozspores

#### 3.1 Introduction

The entomopathogenic fungal genus *Neozygites* belongs to the order *Neozygiales* in the class *Neozygitymycetes* in the phylum Entomophthoromycota (GRYGANSKYI et al., 2012). Fungi in this genus attack small arthropods such as mealybugs, aphids, thrips and mites (KELLER, 1991). *Neozygites floridana* (Weiser and Muma) Remaudière and Keller is

pathogenic to several species plant feeding spider mites (KELLER, 1997) and it is an important natural enemy of the two-spotted spider mite, *Tetranychus urticae* Koch and the red tomato spider mite *Tetranychus evansi* Baker and Pritchard (Acari: Tetranychidae) (KLUBERTANZ; PEDIGO; CARLSON, 1991; HUMBER; MORAES; SANTOS, 1981; DUARTE et al., 2009).

For many of the fungal species within Entomophthoromycota, zygospores and azygospores are considered important for the fungal survival during periods with adverse conditions (e.g., winter, dry season, host absence), and they are therefore called resting spores (HAJEK, 1997). *N. floridana* is an obligate pathogen, and this fungal species may also form resting spores to survive adverse conditions (HAJEK, 1997; DELALIBERA JR. et al., 2000; ELLIOT; MUMFORD; MORAES, 2002; KLINGEN; WAERSTED; WESTRUM, 2008). Resting spores of *N. floridana* have been reported in the field in temperate regions in *T. urticae* populations in late summer, fall and winter (KLUBERTANZ; PEDIGO; CARLSON, 1991; MIETKIEWSKI; BALAZY; VAN DER GEEST, 1993), and *N. floridana* resting spore prevalences of up to 13.8% were found in *T. urticae* in Norway (KLINGEN; WAERSTED; WESTRUM, 2008). Carner (1976) suggested that *Neozygites* resting spores were restricted to northern/ temperate regions, where the weather is often below freezing during the fall and winter. No field studies on prevalence of resting spores of *N. floridana* under tropical conditions have been performed, but field studies with *Neozygites tanajoae* Delalibera Jr.; Humber; Hajek showed that resting spores of *N. tanajoae* in *Mononychelus tanajoa* Bondar populations were found under tropical condition in Brazil. Low prevalences of resting spores of *N. tanajoae* in *M. tanajoa* (up to 3.75%) was detected in Brazil by Delalibera Jr. et al. (2000), whereas higher prevalences (34-38%) were found by Elliot, Mumford and Moraes (2002). However resting spores of *Neozygites* have not been found in other studies in tropical regions (ALVAREZ et al., 1993; YANINEK et al., 1996, RIBEIRO et al, 2012).

Several factors, such as photoperiod, temperature, host age, inoculum density, and the fungal isolate, may be important for the induction of resting spores in fungi in the Entomophthoromycota (GLARE; MILNER; CHILVERS, 1989; HAJEK; SHIMAZU, 1996; THOMSEN; EILENBERG, 2000; HUANG; FENG, 2008; ZHOU; FENG, 2010; ZHOU; FENG; ZHANG, 2012). For *Zoophthora radicans* (Brefeld) Batko, the resting spore production was negatively correlated with temperature and positively correlated with the relative humidity (RH) and inoculum density (GLARE; MILNER; CHILVERS, 1989). Hajek and Shimazu (1996) tested the effect of temperature, photoperiod, host molting status on resting spore formation in *Entomophaga maimaiga* Humber, Shimazu and Soper, they

found that the factor with greatest impact on the type of spore produced was host age. Resting spore formation was negatively associated with the larval molting status, the cadavers of those larvae that molted or exhibited pre-molt characters during the period between infections and death contained fewer resting spores. High levels of fungal inoculums also increased the resting spore formation. In a field study, Thomsen and Eilenberg (2000) found that *Entomophthora muscae* (Cohn) Fresenius form resting spores only in females of *Delia radicum* L. and that the proportion of female with resting spores was negatively correlated with the day length. Further, Huang and Feng (2008) hypothesize that resting spore formation of the aphid pathogenic fungus *Pandora nouryi* (Remaudière and Hennebert) Humber depends on the inoculum concentration. Later, Zhou and Feng (2010) tested the effect of three parameters on resting spore formation of *P. nouryi*. They suggest that the most important factor for resting spore production is spore density but that temperature and photoperiod are also important. In an even later study Zhou, Feng and Zhang (2012) suggest that temperature is the most important factor for resting spore production of *P. nouryi* in *Myzus persicae* Sulzer under winter field conductions. To our knowledge, no controlled experiments have been conducted to determine which factors are most important for the induction of resting spores in *N. floridana* isolates from temperate or tropical regions. One laboratory study with a Brazilian strain of *N. tanajoae* report resting spores in 24 % of *M. tanajoa* individuals under conditions mimicking field conditions at which high prevalences of resting spores were found (ELLIOT; MUMFORD; MORAES, 2002).

Resting spores within Entomophthoromycota are thick-walled structures believed to survive adverse conditions, formed either asexually from a hyphal body (azygospores) or sexually by conjugation of two hyphal bodies (zygospores) (KELLER, 2007). According to Keller, (1997), *Neozygites* is the only genus within the Entomophthoromycota that produce only zygospores. Their resting spores are dark-brown to black, spherical or ellipsoid, smooth or ornamented and binucleate (KELLER, 1997). All other resting spores are multinucleate. The sexual life cycle of *N. floridana* starts with the conjugation of two rod-shaped hyphal bodies which induces doubling of the number of nuclei. The conjugation bridge is formed laterally in the terminal part of the hyphal bodies. The zygote develops by budding from the conjugation bridge. The mature zygospore is spherical to slightly subspherical and surrounded with a brown to black, slightly ornamented episporium. The two degenerated conjugating hyphal bodies leave two scars after detachment (KELLER 1997).

Therefore, in the present study, we conducted controlled experiments to identify factors that might be important for the induction of resting spores of *N. floridana* isolates

from spider mites from temperate (Norway) and tropical (Brazil) regions. The conditions tested mimic the field conditions under which resting spores have been observed in temperate and tropical regions. Thus, we tested conditions found in the beginning of the dry seasons in tropical regions and conditions found during the fall and winter in temperate regions.

## **3.2 Development**

### **3.2.1 Material and methods**

#### **3.2.1.1 Experiments mimicking temperate regions conditions**

##### **3.2.1.1.1 *T. urticae* culture reared on non-senescent and senescent plants**

The *T. urticae* used in this culture were collected on the strawberry, *Fragaria x ananassa*, in Ås, in southeastern Norway (59°42' N, 10° 44' E). *T. urticae* were reared on non-senescent bean plants, *Phaseolus vulgaris* L, in an acclimatized room at 21 °C, 60% RH, and L16:D8. The plants were watered three times per week. Old and weak plants were replaced as needed, usually once a week.

Senescent bean plants were used to induce diapause (red mites) in *T. urticae*. The two-spotted spider mite *T. urticae* females are known to hibernate during the winter (VEERMAN, 1985), and the diapause is induced by short day length (VEERMAN, 1977), but temperature and a lack of nutrition from host plant may also contribute to the induction of this stage (VEERMAN, 1985). We hypothesized that infection in diapausing mites might induce resting spore production in *N. floridana*; therefore, we aimed to produce diapausing *T. urticae* as one of the variables in the temperate region treatments. Diapausing *T. urticae* were obtained from old plants by maintaining the old plants in a Plexiglas cage in the climatized room as described above, but these plants were watered only once a week to stress them and accelerate the process of plants senescence.

##### **3.2.1.1.2 *N. floridana* isolate**

Norwegian and Brazilian *N. floridana* isolates were used in the experiments mimicking the temperate region conditions. The Norwegian isolate (NCRI 271/04) was collected from *T. urticae* on the strawberry in Ås, in southeastern Norway (59°42' N, 10° 44'

E) and the Brazilian isolate (ESALQ1420) was collected *T. urticae* on the jack bean, *Canavalia ensiformis*, in Piracicaba, São Paulo, Brazil (22° 42' 30" S, 47° 38' 00" W).

#### **3.2.1.1.3 *N. floridana* cadaver production**

Leaf discs (1.5 cm diameter) from bean plants were placed with the underside up on 1.5% water Agar in a Petri dish (5 cm in diameter and 2 cm high) and three *N. floridana*-killed *T. urticae* cadavers were placed with their dorsal side up on the leaf disc. Petri dishes with cadavers on leaf discs were then placed in a plastic box (22x16x7cm), covered with aluminum foil to ensure darkness, and incubated at 20 °C and 90% RH in a climatic chamber. Cadavers were checked under a compound microscope (80x) after 24 h of incubation, and only the cadavers with good sporulation and capilliconidia production were used. Thirty uninfected adult *T. urticae* females were then placed on each leaf disc with cadavers for *N. floridana* inoculation. Water was added to the water agar surrounding the leaf disc in the Petri dish to prevent the mites from escaping the leaf disc. The *T. urticae* were then incubated for 24 h under the conditions described above. The leaf discs containing *N. floridana*-inoculated *T. urticae* were then transferred to uninfested bean plants after 24 h. The mites then walked from the leaf disc onto the bean plant and remained there until they died and mummified. Pods and tendrils were removed to prevent the plant from dangling and allowing the *T. urticae* to crawl off the plant. Leaves that overlapped or grew close together were also cut off to ensure a dry microclimate keeping the newly mummified cadavers dry and preventing them from sporulating. Plants with *N. floridana*-inoculated *T. urticae* were kept under ambient laboratory conditions at 22-25 °C, 20-30 % RH and 24 h light. The dry, non-sporulating cadavers produced on the plant were collected after 7-10 days and kept in small, unbleached cotton cloth pieces in 1.8 ml NUNC Cryo Tubes™ and stored at 5 °C until used in the experiments.

#### **3.2.1.1.4 Experimental setup for abiotic (light, temperature) and biotic (*T. urticae* “diapause” condition) factors**

To inoculate *T. urticae* with the fungus *N. floridana* we used the protocol as described above. Inoculated mites were then transferred with a fine paint brush onto a bean leaf disc (1.5 cm diameter) placed with the underside up on 1.5% water agar in 30 ml vials with lids.

Twelve holes were made in the lid of the vials with n° 2 insect pin for aeration. At least 60 individual mites were included in each treatment for each isolate. Vials with *T. urticae* were kept under the treatment conditions described in Table 3.1. until they died of *N. floridana* infection. The two light qualities tested were provided by 1) warm white fluorescent lamps (Philips –Master TL-D 90, referred to as “Light quality 1” in this chapter), and 2) cool white fluorescent lamps (Mitsubishi – 40SW (Ra61), referred to as “Light quality 2” in this chapter). The effects of a decrease in temperature were also tested. The treatments were maintained for 4 h at -10, -5, 0, or 5 °C during the light period.

*T. urticae* were evaluated daily during the light period, and dead mites were checked for symptoms associated with the presence of hyphal bodies and resting spores. Dead mites were then mounted in 0.075% Cotton Blue in 50% lactic acid to permit the observation of hyphal bodies and resting spores under a compound microscope (400×). The time of infection lethality (the time from infection to mite death) was calculated for mites with hyphal bodies and for mites with resting spores.

#### **3.2.1.1.5 Statistical analysis**

The effects of different abiotic and biotic factors on the percentage of *T. urticae* with resting spores were analyzed with ANOVA after the arcsine transformation of the data. When significant effects were found, post hoc comparisons using Tukey’s HSD test were conducted to evaluate the pair-wise differences between means ( $P < 0.05$ ). All statistical analyses were carried out in the SAS package (SAS Institute Inc., Cary North Carolina).

### 3.2.1.2 Experiments mimicking tropical regions conditions <sup>1</sup>

## 3.2.2 Results

### 3.2.2.1 Experiments mimicking temperate regions conditions

A total of 3,106 mites (not including a series of pilot experiments) were tested at 26 different combinations of conditions. However, a significantly higher rate of resting spores, 51.54 % ( $F=20.54$ ,  $p<0.0001$ ), was found for only one condition: the Brazilian *N. floridana* isolate at 11 °C (no temperature drop), with a photoperiod of 10L:14D, light intensity of 42-46 ( $\mu\text{mol m}^{-2}\text{s}^{-1}$ ), and light quality 1 in non-diapausing *T. urticae* females from non-senescent plants (Table 3.1). No significant difference in resting spore production was observed for any of the other combinations of conditions for any of the isolates tested. One combination of conditions resulted in a low level resting spore production (1.4%) for the Brazilian *N. floridana* isolate in *T. urticae* females; several combinations of conditions also resulted in resting spore production for the Norwegian *N. floridana* isolate in *T. urticae* females, but only at low levels (1.4-5%). The majority of the spore forming conditions (8 out of 9 combinations) included at a 10L:14D light regime.

The time to lethality in *T. urticae* females varied from 10.0-18.0 days for the Norwegian isolate and from 20.0-21.85 days for the Brazilian isolate. The mites containing resting spores survived longer than the mites with hyphal bodies.

*T. urticae* cadavers containing resting spores from the Norwegian *N. floridana* isolate (NCRI 271/04) were quite different from *T. urticae* cadavers containing resting spores from the Brazilian *N. floridana* isolate (ESALQ 1420). Swollen fungus-killed cadavers filled with hyphal bodies, referred to as mummies, were opaque orange/light brown for the Brazilian isolate (Fig 3.1 B1) but dark-brown/black for the Norwegian isolate (Fig 3.1 A1). When *N. floridana* produces resting spores cadavers, *T. urticae* first turn gray/ light brown and then shiny dark brown/black and a slightly swollen (Fig 3.1 A2 and B2). When resting spores reach maturity, the cuticle of the mites becomes fragile. *T. urticae* cadavers with immature Norwegian *N. floridana* resting spores were of equal size and shape, whereas *T. urticae*

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<sup>1</sup> This part was performed by Ana Elizabete Lopes Ribeiro (RIBEIRO, 2009) and it was included considering that it will be published in a single paper and the discussion is relevant for the thesis.<sup>1</sup>

cadavers with immature Brazilian *N. floridana* resting spores varied in size and shape (Fig 3.1 A3 and B3). The majority of the *T. urticae* cadavers with resting spores also contained hyphal bodies (Fig 3.1 A3 and B3).

Only one collar from the attachment to the hyphal body, indicating its azygospore nature, was observed in the resting spores produced by the Brazilian isolate ESALQ1420, at 11 °C (no temperature drop) with a photoperiod of 10L:14D, light intensity of 42-46 ( $\mu\text{mol m}^{-2}\text{s}^{-1}$ ), and light quality of 1, in non-diapausing *T. urticae* females from non-senescent plants, and also at 15 °C (no temperature drop), with photoperiod of 10L:14D, light intensity of 247-280 ( $\mu\text{mol m}^{-2}\text{s}^{-1}$ ), and light quality of 1, in non-diapausing *T. urticae* females from non-senescent plants. However resting spores of the Norwegian presented one or two collar from the attachment of the hyphal bodies. Based on this, it seems that azygospores (asexual) were observed in the Brazilian isolates and for the Norwegian isolates were observed both zygosporos (sexual) and azygospores (asexual).

Table 3.1 - Effect of different combinations of photoperiod, mean temperature, temperature drop, light intensity and light quality on resting spores produced in *N. floridana*-killed *T. urticae*. Isolates from Norway (NCRI 271/04) and from Brazil (ESALQ 1420)

Photoperiod	Mean temperature (temperature drop <sup>1</sup> ) °C	Host plant conditions	Light intensity (light quality) <sup>2</sup>	Isolate Esalq 1420		Isolate NCRI271/04			
				N° of mites	Hyphal bodies (%)	Resting spores (%)	N° of mites	Hyphal bodies (%)	Resting spores (%)
12L:12D	25 (-10)	Non senescent	165-243 (2)	40	22.5	0	47	17.0	0
	25 (-5)			42	35.7	0	47	25.5	0
	25 (0)			40	30.0	0	46	26.1	0
	25 (5)			40	20.0	0	46	15.2	0
	15			42-46 (1)	60	78.3	0	59	79.7
10L:14D	15 (-10)	Non senescent	165-243 (2)	72	50.0	0	69	73.9	0
			247-280 (1)	72	55.6	0	69	66.7	0
			30-35(2)	72	54.2	0	72	38.9	0
			42-46 (1)	69	68.1	0	72	56.9	1.4
			165-243 (2)	72	55.6	0	72	59.7	0
	15	Senescent	247-280 (1)	71	73.2	0	72	52.8	2.8
			30-35(2)	69	68.1	0	60	56.7	5.0
			42-46 (1)	60	81.7	0	63	68.3	3.2
			165-243 (2)	72	51.4	0	69	65.2	0
			247-280 (1)	72	68.1	1.4	64	75.0	0
	13	Non senescent	30-35(2)	72	75.0	0	72	63.9	0
			42-46 (1)	72	55.6	0	72	50.0	4.2
165-243 (2)			72	86.1	0	72	58.3	1.4	
247-280 (1)			72	66.7	0	72	69.4	0	
30-35(2)			69	78.3	0	60	70.0	1.7	
11	Non senescent	42-46 (1)	72	68.1	0	60	58.3	0	
60			91.7	0	60	70.0	0		
111			73.0	51.5	102	93.6	4.7		
60			83.3	0	58	62.1	0		
6			59	67.8	0	60	78.3	0	
14L:10D	15	Non senescent	42-46 (1)	58	93.1	0	59	75.7	1.7
16L:08D				58	93.1	0	59	75.7	1.7

<sup>1</sup>Temperature drop= fall of the temperature for 4h during the light period. <sup>2</sup>Light intensity ( $\mu\text{mols}^{-2}\text{m}^{-1}$ ) and light quality (1= white fluorescent lamps Philips –Master TL-D 90 and 2= cool white fluorescent lamps Mitsubishi – 40SW (Ra61))

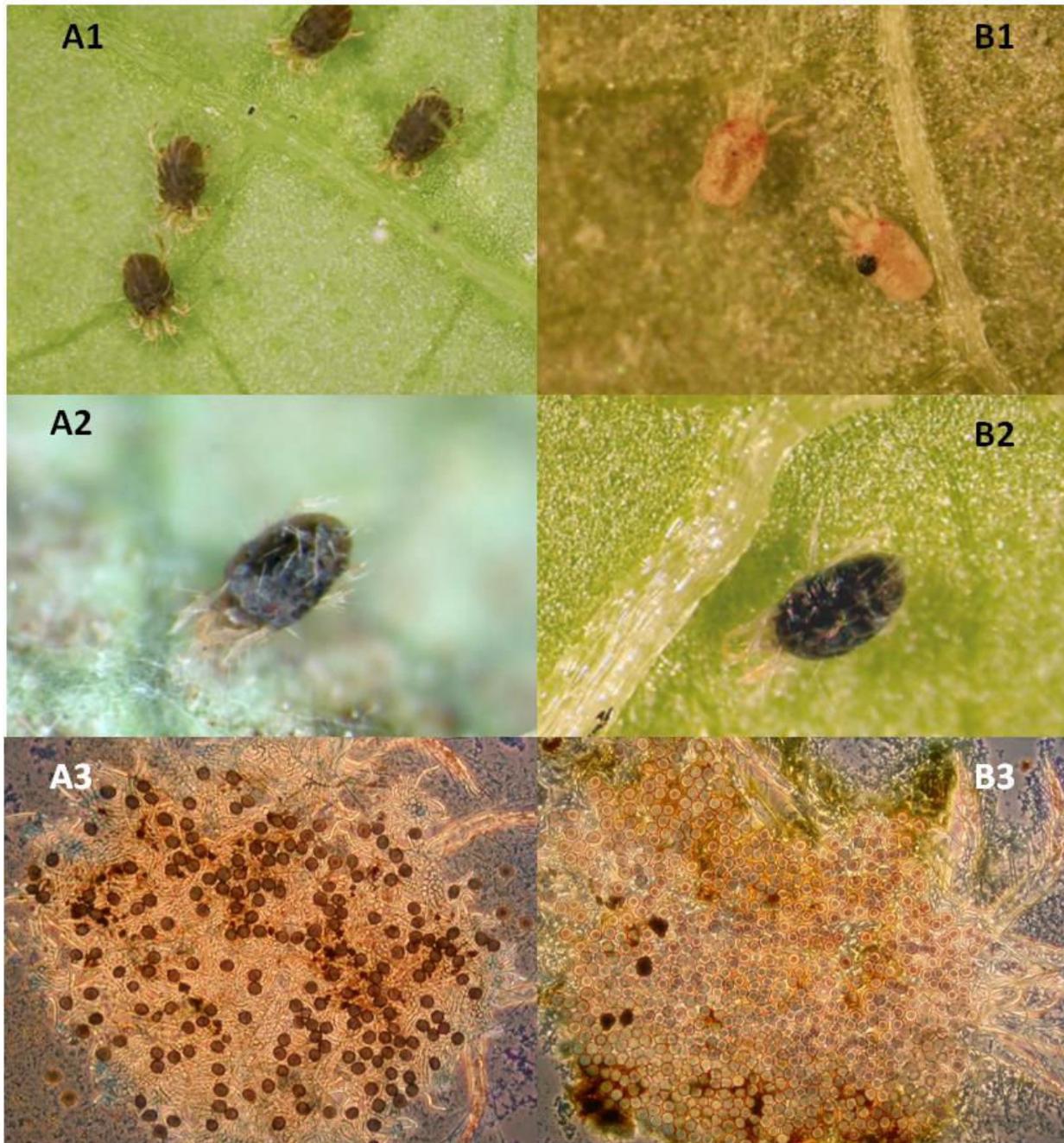


Figure 3.1 - *T. urticae*-killed by the fungus *N. floridana*. A (1-3) = Norwegian isolate (NCRI271/04), A1 = Dark brown cadavers containing hyphal bodies; A2 = Black/dark brown cadavers with resting spore, A3 = Matures resting spores in squash mount B (1-3) = Brazilian isolate (ESALQ1420), B1 = Light brown/orange cadavers containing hyphal bodies; B2 = Black/dark brown resting spores cadavers, B3 = Almost matures resting spores in squash mount

### 3.2.2.2 Experiments mimicking tropical regions conditions

Even though 13,516 *T. urticae* and *T. evansi* (including a pilot experiment, data not shown) were tested under 13 different conditions, no *T. urticae* and a very low percentage of *N. floridana* (ESALQ 1421)-killed *T. evansi* adult females (up to 1.0%) produced resting

spores under the following conditions: 32 °C, RH: 70%, 12L:12D and unfested leaves. Further, 0.5 % of *N. floridana* (ESALQ 1421)-killed *T. evansi* adult females produced resting spores under the following conditions: 35 °C, RH: 60%, 12L:12D, unfested leaves. A third condition that resulted in 0.5% of resting spores in *N. floridana* (ESALQ 1421)-killed *T. evansi* adult females: 25 °C (light period) and 15 °C (dark period), RH: 60%, 11L:13D, leaves with chlorosis.

*T. evansi* cadavers containing *N. floridana* ESALQ1421 resting spores were shiny dark brown/black and retaining their original mite shape. When resting spores were mature, the *T. evansi* cuticle became fragile. Further, the mature resting spores of *N. floridana* (ESALQ1421)-killed *T. evansi* were equal in size and shape. *N. floridana* (ESALQ1421)-killed *T. evansi* cadavers with hyphal bodies were distinct from cadavers with resting spores and became swollen and with light brown/orange in color.

### 3.2.3 Discussion

In this study, a high percentage (51.5%) of *T. urticae* with *N. floridana* resting spores was only found for the Brazilian isolate ESALQ1420 in the experiments mimicking temperate regions conditions (11 °C, 10L:14D, and a light intensity of 42-46  $\mu\text{mol m}^{-2}\text{s}^{-1}$ ). However, a small percentage (1.4-5 %) of *T. urticae* with resting spores was found for the Norwegian isolate NCRI 271/04 under certain temperate regions mimicking conditions. Most of the resting spores produced by the Norwegian isolate (8 out of 9 occasions) were produced under 10L:14D light regime, and the remaining spores were produced under a 16L:08D light regime. At Ås, in the Southeastern, days with 10 h of light occur in fall (17 October) and winter (24 February) and days with 16 h of light occur at the end of the summer (10 August) and in the spring (1 May) (<http://www.timeanddate.no>). Our results are therefore consistent with earlier field studies in temperate regions that indicated that resting spores of local *N. floridana* isolates in *T. urticae* seem to be induced in fall when the hibernation of *T. urticae* females is also induced (KLINGEN; WAERSTED; WESTRUM, 2008). In São Paulo, Brazil, 14 h of darkness never occurs; the shortest day (10 h 40 min) occurs on the winter solstice (21 June). In the experiments mimicking tropical conditions, resting spores were found at very low levels (up to 1.0%) and only on *T. evansi* infected by the Brazilian *N. floridana* isolate ESALQ1421 at high temperatures (32 and 35 °C) and a 12L:12D light regime. In São Paulo, Brazil, days with 12 h of light occur during spring (17 September) and fall (24 March).

Between-strain differences in the ability to form resting spores have been observed for *Z. radicans* (GLARE; MILNER; CHILVERS, 1989) and *E. maimaiga* (KOGAN; HAJEK, 2000), but this phenomenon has never been investigated in species of *Neozygites* affecting tetranychid mites. The low percentages of resting spores found for the Norwegian isolate in our laboratory studies are similar to the prevalences found in earlier field studies (KLINGEN; WAERSTED; WESTRUM, 2008). This observation may further confirm, as suggested by Klingen, Waersted and Westrum (2008), that the major overwintering strategy of *N. floridana* in temperate regions is to exist as hyphal bodies inside live hibernating *T. urticae* females and that resting spores are produced mainly for sexual recombination. Other reports have described *N. floridana* resting spores in temperate regions during the autumn and winter, but most of these spores are found at low levels. Klubertanz, Pedigo, and Carlson (1991) found resting spores of *Neozygites* sp. in overwintering *T. urticae* in soybeans at a level of approximately 8% of mites sampled. Brandenburg and Kennedy (1981) investigated the overwintering strategy of *Entomophthora floridana* (syn. *N. floridana*) in *T. urticae* for two years and observed resting spores in only one sample, collected in autumn (28% of spider mites with resting spores). *T. urticae* with resting spores of *Entomophthora* sp. (syn. *N. floridana*) were observed at some locations in the USA (Clemson, Alabama, Blackville), but no resting spores were found (CARNER, 1976). In temperate regions, *T. urticae* hibernate as adult females (VAN DE BUND; HELLE, 1960; HELLE, 1962; VEERMAN, 1985), but this has never been observed in tropical regions. *T. urticae* hibernation is induced by short day length, low temperature, and a lack of nutrition from its host plant (VEERMAN, 1985). The Brazilian isolate ESALQ 1420 produced a high level of resting spores under conditions that are common in temperate regions but rare in most tropical sites. It is also unclear whether resting spore formation is the major strategy for survival under adverse conditions in tropical climates considering that the conditions that best induce resting spore formation for the Brazilian isolate are not common in the tropics.

Resting spores of *Neozygites* spp. have rarely been found in tropical regions. During nearly 20 years duration of the cassava green mite project investigating *N. tanajoae*, resting spores were found only occasionally in laboratory and field studies. Resting spores of *Neozygites* sp. (= *N. tanajoae*) were observed in the northeastern of Brazil during the winter (DELALIBERA JR. et al., 2000; ELLIOT; MUMFORD; MORAES, 2002). In a field study with *M. tanajoa* and its natural enemy, *N. tanajoae* Hountondji et al. (2002) found only four mites with resting spores in an examination of over 460,000 mites. In more recent studies, resting spores were found in the southeast and south of Brazil (DUARTE et al., 2009;

DUARTE et al unpublished data; ROGGIA et al., unpublished data). These regions have colder winter conditions than are found in the northeast, but the mites with resting spores were found not in summer, when the plants become senescent. The factors associated with the induction of resting spores production in tropical regions are still unclear, but it seems that resting spores production might occur during different seasons of the year and that the highest resting spore production levels are found during the inhospitable conditions or in the absence of potential hosts. Differences among *N. floridana* strains from different regions with regard to their ability to form resting spores might have evolved as *Neozygites* adapted to different climatic conditions, resulting in poorly adapted physiology to other conditions (ELLIOT; MUMFORD; MORAES, 2002; KLINGEN, WAERSTED; WESTRUM, 2008).

The *T. urticae* containing resting spores normally did not die as quickly as *T. urticae* with hyphal bodies. The time of lethality was negatively correlated with temperature. This finding is in accordance with Smitley, Brooks and Kennedy (1986), who found that the main time to lethality of *T. urticae* infected with *N. floridana* was 15, 5, 4 and 7 days after inoculation when maintained at 10, 20, 30 and 37 °C respectively. Normally, hosts infected by the Brazilian isolate ESALQ 1420 die at five days after inoculation at 25 °C, and those infected with the Norwegian isolate die at seven days after inoculation at 20 °C (DELALIBERA JR personal information; KLINGEN personal information).

Zygospor (sexual) and azygospor (asexual) resting spores formation were observed in the Norwegian isolate. In the Brazilian isolates ESALQ1421 and ESALQ1420 was observed zygospor and azygospor, respectively. The fact that, zygospor was observed in ESALQ1421 and only azygospor was found ESALQ 1420, does not mean that they do not produce the other type of resting spore. It possible that, azygospor are only produced at lower temperatures and we did not test lower temperature for the Brazilian isolate ESALQ1421. KELLER (1991) suggested that among the Entomomophthoromycota, the sexual or asexual nature of resting spore formation seems to be constant only in the genus *Neozygites*, where all species form zygospor. In all other genera zygospor and azygospor formation occurs. Although Weiser (1968) reported azygospor from *Neozygites tetranynchi* (Weiser) Remaudière and Keller which was the only case in the genus but Keller (2007) suggested that this needs confirmation. *N. tetranynchi* was only known from a single collection and it is probably a synonym of *N. floridana* (BALAZY, 1993).

Although we were not able to answer many of our initial questions about resting spore formation and the role of this type of spore, we identified a set of conditions that can consistently produce resting spores, which will be useful for further investigations.

## References

- ALVAREZ, J.M.; ACOSTA, A.; BELLOTTI, A.C.; BRAUN, A.R. Estudios de patogenicidad de un hongo asociado a *Mononychellus tanajoa* (Bondar), ácaro plaga de la yuca (*Manihot esculenta* Crantz). **Revista Colombiana de Entomología**, Bogotá, v. 19, p. 3-5, 1993.
- BALAZY, S. **Flora of Poland, fungi (Mycota)**. Krakow: Institute of Botany, 1993. v. 24: Entomophthorales, 356 p.
- BRANDENBURG, R.L.; KENNEDY, G.G. Overwintering of the pathogen *Entomophthora floridana* and its host, the two-spotted spider mite. **Journal of Economic Entomology**, Lanham, v. 74, p. 428-431, 1981.
- CARNER, G.R. A description of the life cycle of *Entomophthora* sp. in the two-spotted spider mite. **Journal of Invertebrate Pathology**, San Diego, v. 28, p. 245-254, 1976.
- DELALIBERA JR., I.; MORAES, G.J.; LAPOINTE, S.T., SILVA, C.A.D.; TAMAI, M.A. Temporal variability and progression of *Neozygites* sp. (Zygomycetes: Entomophthorales) in populations of *Mononychellus tanajoa* (Bondar) (Acari: Tetranychidae). **Anais Sociedade Entomologica do Brasil**, Jaboticabal, v. 29, p. 523-535, 2000.
- DUARTE, V.S.; SILVA, R.A.; WEKESA, V.W.; RIZZATO, F.B.; DIAS, C.T.S.; DELALIBERA JR., I. Impact of natural epizootics of the fungal pathogen *Neozygites floridana* (Zygomycetes: Entomophthorales) on population dynamics of *Tetranychus evansi* (Acari: Tetranychidae) in tomato and nightshade. **Biological Control**, Orlando, v. 51, p. 81-90, 2009.
- ELLIOT, S.L.; MUMFORD, J.D.; MORAES, J.G. The role of resting spores in the survival of mite-pathogenic fungus *Neozygites floridana* from *Mononychellus tanajoa* during dry periods in Brazil. **Journal of Invertebrate Pathology**, San Diego, v. 81, p. 148-157, 2002.
- GLARE, T.R.; MILNER, R.J.; CHILVERS, G.A. Factors affecting the production of resting spores by *Zoophthora radicans* in the spotted alfalfa aphid, *Therioaphis trifolii* f. *Maculata*. **Canadian Journal of Botany**, Ottawa, v. 67, p. 848-855, 1989.
- GRYGANSKYI, G.P.; HUMBER, R.A.; SMITH, M.E.; MIADLIKOVSKA, J.; WU, S.; VOIGT, K.; WALTHER, G.; ANISHCHENKO, I.M.; VILGALYS, R. Molecular phylogeny of the Entomophthoromycota. **Molecular Phylogenetics and Evolution**, Orlando, v. 65, p. 682-694, 2012.
- HAJEK, A.E. Ecology of terrestrial fungal pathogens. In: JONES, J.G. (Ed.). **Advances in microbial ecology**. New York: Plenum Press, 1997. v.15, p. 193-249.
- HAJEK, A.E.; SHIMAZU, M. Types of spores produced by *Entomophaga maimaiga* infecting the gypsy moth *Lymantria dispar*. **Canadian Journal of Botany**, Ottawa, v. 74, p. 708-715, 1996.

HELLE, W. Genetic of resistance to orgaphosphorus compounds and its relation to diapause in *Tetranychus urticae* Koch (Acari). **Tijdschrift Over Plantenziekten**, Dordrecht, v. 68, p. 155-195, 1962.

HOUNTONDI, F.C.C.; YANINEK, J.S.; MORAES, G.J.; ODUOR, G.I. Host specificity of the cassava green mite pathogen *Neozygites floridana*. **BioControl**, Dordrecht, v. 47, p. 61-66, 2002.

HUANG, Z.; FENG, M. Resting spore formation of aphid-pathogenic fungus *Pandora nouryi* depends on the concentration of infective inoculums. **Environmental Microbiology**, College Park, v.10, n.7, p. 1912–1916, 2008.

HUMBER, R.A.; MORAES, G.J.; SANTOS, J.M. Natural infection of *Tetranychus evansi* (Acarina: Tetranychidae) by a *Triplosporium* sp. (Zygomycetes: Entomophthorales) in northeastern Brazil. **Entomophaga**, Paris, v. 26, n. 4, p. 421-425, 1981.

KELLER, S. Arthropod-patogenic Entomophthorales of Switzerland. II. *Erynia*, *Eyniopsis*, *Neozygites*, *Zoophthora* and *Tarichium*. **Sydowia**, Horn, v. 43, p. 39-122, 1991.

\_\_\_\_\_. The genus *Neozygites* (Zygomycetes, Entomophthorales) with special reference to species found in tropical regions. **Sydowia**, Horn, v. 49, n.2, p. 118-146, 1997.

\_\_\_\_\_. Fungal structures and biology. In: \_\_\_\_\_. (Ed.) **Arthropod-patogenic Entomophthorales: biology, ecology, identification**. Luxemborg: CostOffice, 2007. p. 27-54.

KLINGEN, I.; WAERSTED, G.; WESTRUM, K. Overwintering and prevalence of *Neozygites floridana* (Zygomycetes: Neozygitaceae) in hibernating females of *Tetranychus urticae* (Acari: Tetranychidae) under cold climatic conditions in strawberries. **Experimental and Applied Acarology**, Amsterdam, v. 46, p.231-245, 2008.

KLUBERTANZ, T.H.; PEDIGO, L.P.; CARLSON, R.E. Impact of fungal epizootics on the biology and management of the two spotted spider mite (Acari: Tetranychidae) in soybean. **Environmental Entomology**, College Park, v. 20. p. 731-735, 1991.

KOGAN, P.H.; HAJEK, A.E. In vitro formation of resting spores by the insect pathogenic fungus *Entomophaga maimaiga*. **Journal of Invertebrate Pathology**, San Diego, v. 75, p. 193-201, 2000.

MIETKIEWSKI, R.; BALAZY, S.; VAN DER GEEST, L.P.S. Observations on a mycosis of spider mites (Acari: Teranychidae) caused by *Neozygites floridana* in Poland. **Journal of Invertebrate Pathology**, San Diego, v. 61, p.317–319, 1993.

RIBEIRO, A.E.L. **Aspectos biológicos de *Neozygites* (Weiser & Muma) (Zygomycetes: Entomophthorales) e dinâmica populacional de *Tetranychus evansi* Baker & Pritchard (Acari: Tetranychidae) e seus inimigos naturais em *Solanum americanum* Mill.** 2009. 85 p. Tese (Doutoradoem Entomologia Agrícola)– Universidade Federal Rural de Pernambuco, Recife, 2009.

RIBEIRO, A.E.L.; GONDIM JR., M.G.C.; MELO, J.W.S.; DELALIBERA JR., I. *Solanum americanum* as a reservoir of natural enemies of the tomato red spider mite, *Tetranychus evansi* (Acari: Tetranychidae). **International Journal of Acarology**, Oak Park, v. 38, n. 8, p. 692-698, 2012.

SAS INSTITUTE. **SAS/STAT user's guide**: version 8. Cary, 1999. v. 13.

SMITLEY, D.R.; KENNEDY, G.G.; BROOKS, W.M. Role of the entomogenous fungus, *Neozygites floridana*, in population declines of two spotted spider mite *Tetranychus urticae*, on field corn. **Entomologia Experimentalis et Applicata**, Dordrecht, v. 41, p. 255-264, 1986.

THOMSEN, L.; EILENBERG, J. *Entomophthora muscae* resting spore formation in vivo in the host *Delia radicum*. **Journal of Invertebrate Pathology**, San Diego, v. 76, p. 127-130, 2000.

VAN DE BUND, C.F.; HELLE, W. Investigation of the *Tetranychus urticae* complex in North West Europe (Acari: Tetranychidae). **Entomologia Experimentalis et Applicata**, Dordrecht, v. 3, p. 142-156, 1960.

VEERMAN, A. Aspects of the induction of diapause in a laboratory strain of the mite *Tetranychus urticae*. **Journal of Insect Physiology**, New York, v. 23, p. 703-711, 1977.

VEERMAN, A. Diapause. In: HELLE, W.; SABELIS, M.W. (Ed.). **Spider mites their biology, natural enemies and control**. Amsterdam: Elsevier, 1985. v. 1A, p. 279-316.

WEISER, J. *Triplosporium tetranychii* sp. n. (Phycomycetes, Entomophthoraceae), a fungi infecting the red mite *Tetranychus althaeae* Hanst. **Folia Parasitologica**, Praha, v. 15, p. 115-122, 1968.

YANINEK, J.S.; SAIZONOU, S.; ONZO, A.; ZANNOU, I.; GNANVOSSOU, D. Seasonal and habitat variability in the fungal pathogens, *Neozygites* cf. *Floridana* and *Hirsutella thompsonii*, associated with cassava mites in Benin, West Africa. **Biocontrol Science and Technology**, Oxford, v. 6, p. 23-33, 1996.

ZHOU, X.; FENG, M. Biotic and abiotic regulation of resting spore formation *in vivo* of obligate aphid pathogen *Pandora nouryi*: modeling analysis and biological implication. **Journal of Invertebrate Pathology**, San Diego, v. 103, p. 83-88, 2010.

ZHOU, X.; FENG, M.; ZHANG, L. The role of temperature on *in vivo* resting spore formation of the aphid-specific pathogen *Pandora nouryi* (Zygomycota: Entomophthorales) under winter field conditions. **Biocontrol Science and Technology**, Oxford, v.22, p. 93-100, 2012.

#### 4 PHYLOGENY AND DEVELOPMENT OF MOLECULAR PROBES FOR THE MITE PATHOGENIC FUNGI *Neozygites* spp.

##### Abstract

*Neozygites floridana* is a fungus pathogenic to mites in the family Tetranychidae and is highly specific to its hosts. Because of uncertainties concerning the delimitation of species within the genus *Neozygites*, all pathogens of this group associated with spider mites are identified as *N. floridana*, except the pathogen of the cassava green mite recently named *Neozygites tanajoae*. They can probably survive harsh conditions (e.g., winter, dry seasons) as resting spores on plant residues or in the soil or as hyphal bodies inside live hibernating mites. The development of molecular probes for quantification of *Neozygites* in soil by real-time PCR will be important to understand the population dynamics of this fungus in the field. The objectives of the present study were therefore to sequence genes to conduct the phylogenetic analysis of this group of mite pathogenic fungi and develop efficient real-time PCR-based analysis for detection and quantification of *N. floridana* propagules from the soil. The SSU rDNA primer generated approximately 1,400 bases pair of four isolates. The phylogenetic three showed that *Neozygites* is a fungal group far from other Entomophthoromycota and that they present high intra-specific variability between isolates within *N. floridana*. In the cluster of *Neozygites*, the isolates splitted from the host which they were obtained; one cluster with host of Arachnida and other of Hexapoda. The isolates obtained from Arachnida were splitted in hosts of the genera *Tetranychus* and *Mononychelus*. The last split was in the genus *Tetranychus*, where they separated according to the geographic origin of the host, above or below the latitude 45° N. This data indicates that the isolates from different *Tetranychus* species may be in fact different *Neozygites* species, not described yet. We developed a real-time PCR probe for detection and quantification of *N. floridana* in the soil. The qPCR probe was successful in quantifying *N. floridana* artificially seeded into the soil, but it was not possible to detect the fungus in soils samples from the field where it was found, this can be due the fungus was not viable in those particular samples examined.

Keywords: *Neozygites floridana*; Spider mites; Phylogenetic analysis; SSU rDNA; Real-time PCR; Entomophthoromycota

##### 4.1 Introduction

The genus *Neozygites* belongs to the order *Neozygiales* in the class *Neozygitomycetes* in the phylum Entomophthoromycota (HUMBER, 2012). Fungi in this genus attack small arthropods such as mealybugs, aphids, thrips and mites (KELLER, 1991). The species *Neozygites floridana* (Weiser and Muma) Remaudière and Keller (Entomophthoromycota: Neozygiales) is pathogenic to several species of spider mites (KELLER, 1997). *N. floridana* has been reported to cause natural epizootics, which can substantially reduce spider mite populations and thereby reduce or prevent serious crop damage (HUMBER; MORAES; SANTOS, 1981; BRANDENBURG; KENNEDY, 1982, DUARTE et al., 2009). Fungi in the

genus *Neozygites* has a restrict host range and cause high infection levels in their host populations (PELL et al., 2001). The host specificity of this fungus and its ability to cause epizootics makes it an interesting candidate for biological control and integrated pest management since it might be used in combination with other biological control agents (HOUTONDJI et al., 2002).

Even though *N. floridana* presents a high potential for the use in biological control of spider mites, the taxonomic classification of this fungus is complex, and few taxonomic studies have been conducted concerning this fungus compared with other entomopathogens (HUMBER, 2008). To understand the relationships of these fungi with their hosts and the environment is important for the planning future biocontrol strategies (HAJEK, 1997). Because of uncertainties concerning the delimitation of species within this genus, the pathogens in this group associated with spider mites, except the cassava green mite, have been referred to as *N. floridana*. In 2004, however, Delalibera Jr., Hajek and Humber (2004) confirmed that a fungus pathogenic to the cassava green mite, *Mononychellus tanajoa* Bondar, earlier referred to as *N. floridana*, was in fact a new species, named *Neozygites tanajoe*. *N. tanajoe* was distinguished from *N. floridana* based on 18S small-subunit (SSU) sequences, host ranges, nutritional requirements for growth *in vitro*, tolerance to cold (4 °C) and abilities to withstand specific cryopreservation techniques.

Molecular techniques have shown intra-specific variations in the following entomopathogens in the fungal phylum Entomophthoromycota: *Pandora*, *Zoophthora*, *Entomophaga* (HODGE; SAWYER; HUMBER, 1995, HAJEK et al., 1996, JENSEN; EILENBERG, 2001, NIELSEN et al., 2001; TYMON; SHAH; PELL, 2004). Few members of the genus *Neozygites* have been characterized molecularly and only eight isolates of the species *N. tanajoe*, *N. floridana* and *Neozygites parvispora* (McLeod and Carl) Remaudière and Keller had the genes of the SSU rDNA region sequenced (DELALIBERA Jr.; HAJEK; HUMBER, 2004). Isolates of *N. floridana* from different species in the family Tetranychidae are morphologically similar, but have high degree of specificity to the host from which they are collected (DELALIBERA Jr.; HAJEK; HUMBER, 2004; RIBEIRO et al., 2009).

Recent studies on phylogeny of entomopathogenic fungi in *Metharizium* and *Beauveria*, have been done using other genes such as  $\beta$ -tubulin (Bt), elongation factor (EF1- $\alpha$ ), intergenic spacer region (IGS), RNA polymerase II largest subunit (RPB1), RNA polymerase second largest subunit (RPB2) (REHNER; BUCKLEY, 2005; BISCHOFF; REHNER; HUMBER, 2009). The concatenation of different genes allows significant increases in the power of discrimination and robustness of the phylogenetic analysis. The

sequential and/or combined use of sequences of several genes makes it possible to refine the phylogenetic approach and provides molecular basis for accurate species identification. For *Neozygites*, there are only sequences of the SSU region available. By increasing the number of *Neozygites* sequences available would facilitate the phylogenetic analysis of the group.

Although *in vitro* production of *N. floridana* is difficult, *in vivo* production using mummified cadavers is the most viable method for production of this fungus. The fungus is normally “isolated” from spider mite cadavers in the field. This is possible mainly when the disease is in the epizootic phase what last only a few days and sometimes during the enzootic levels. It is difficult and time consuming to observe *N. floridana* in the field (NORDENGEN; KLINGEN, 2006), this can be approached by observation of cadavers in the field, collection of live mites that are incubated or squash mounted to observe for mortality and/ or fungal structures. *N. floridana* can survive the winter in northern locations as hyphal bodies inside hibernating females and probably as resting spores on plant residues or in the soil from one year to another (KLINGEN; WAERSTED; WESTRUM, 2008). Non-molecular methods are available for the observation of *N. floridana* resting spores and other fungal structures on plant residues, in the soil or other parts of the spider mite habitat. However, it is difficult and work consuming to assess the prevalence as well as to follow the development of this pathogen in host populations and in its habitat throughout the year.

One alternative for quantification of fungi in the field is through DNA quantification in the soil by the use of PCR and this has been carried out with several species of the phylum Entomophthoromycota (CASTRILLO et al., 2007; FOURNIER; WIDMER; ENKERLI, 2010). The development of molecular probes for quantification of *Neozygites* in soil by real-time PCR will be important to understand the population dynamics of this fungus in the field. The objectives of the present study were therefore: 1) to sequence genes to conduct a phylogenetic analysis of this class of mite pathogenic fungi 2) to develop an efficient real-time PCR-based method for detection and quantification of *N. floridana* propagules from the soil.

## 4.2 Development

### 4.2.1 Material and Methods

#### 4.2.1.1 Fungal isolates

For the sequencing and phylogenetic analysis study three *Neozygites* spp. isolates from Brazil, and one isolate from Norway were used (Table 4.1.). The isolates were collected as mummified mites killed by the fungus and stored at -10 °C using the method described by Delalibera Jr. et al. (2006) until used in the experiment. To develop specific real-time PCR primer pairs for the fungus *Neozygites* using SSU rDNA two isolates, one from Brazil (ESALQ 1420 – *in vivo* culture) and one from the United States (ARSEF662 – *in vitro* culture) were used. The isolate ARSEF662 was kept in *in vitro* cultures (Nt-1 medium = IPL-41 +5% fetal bovine serum + 0.3% yeastolate (DELALIBERA Jr.; HAJEK; HUMBER, 2003) in 25 cm<sup>2</sup> culture flasks containing 3 ml of medium and incubated at 25 ±2 °C in the dark until used.

Table 4.1 - Origin of isolates of *Neozygites* spp. used in this study

Isolate	Host specie	Host plant	Location (coordinates)
Esalq1419 <sup>1</sup>	<i>Tetranychus evansi</i> Baker and Prichard	Tomato – <i>Solanum sculentum</i> L.	Piracicaba/SP/Brazil (22°42'30"S,47°38'00"W)
Esalq1420 <sup>1</sup>	<i>Tetranychus urticae</i> Koch	Jack bean – <i>Canavalia ensiformis</i> (L.) D.C.	Piracicaba/SP/Brazil (22°42'30"S,47°38'00"W)
Esalq1439 <sup>1</sup>	<i>Mononychellus planki</i> McGregor	Soybean - <i>Glycine Max</i> (L.) Merr.	Londrina/PR/Brazil (23°08'47"S,51°19'11"W)
ARSEF 662 <sup>2</sup>	<i>Tetranychus urticae</i> Koch	-	Wilmington/North Carolina/USA (34°13'32"N,77° 56'42"W)
NCRI271/04 <sup>3</sup>	<i>Tetranychus urticae</i> Koch	Strawberry - <i>Fragaria x ananassa</i> Duch	Ås/Akersus/ Norway (59°42" N, 10°44" E)

<sup>1</sup>ESALQ= Identification code from the collection of isolates of the Laboratory of Pathology and Microbial Control of Insects (ESALQ /USP-Brazil).

<sup>2</sup>ARSEF= Agricultural Research Service Collection of Entomopathogenic Fungal Cultures-USA.

<sup>3</sup>NCRI= from Norwegian Institute for Agricultural and Environmental Research (BIOFORSK).

#### **4.2.1.2 Sequencing and phylogenetic analysis of *Neozygites* spp.**

##### **4.2.1.2.1 DNA extraction**

Two mummified mites from each isolate were transferred to a microcentrifuge tube (0.5 mL) with 10.0  $\mu$ L of sterilized water. To release the hyphal bodies, the mummies were macerated using a sterile micropipette tip (20.0  $\mu$ L) and DNA was extracted by using InstaGene<sup>TM</sup>Matrix (Bio-Rad Inc.) according to manufacturer's instructions.

##### **4.2.1.2.2 PCR amplification**

Genes used in this study were: translation elongation factor 1-alpha (EF-1 $\alpha$ ), RNA polymerase II largest subunit (RPB1), RNA polymerase second largest subunit (RPB2),  $\beta$ -tubulin (Bt), the nuclear ribosomal intergenic spacer region (IGS) and 18S small-subunit (SSU).

Table 4.2 - Primer pairs used in this study

<b>DNA Region</b>	<b>Primer pairs sequence (5'-3')</b>	<b>Reference</b>
EF-1 $\alpha$ intron	EF1T (5'-ATGGGTAAGGARGACAAGAC-3') and EF2T (5'-GGAAGTACCAGTGATCATGTT-3')/	REHNER; BUCKLEY, 2005
EF-1 $\alpha$ exon	983F (5'-GCYCCYGGHCAYCGTGAYTTYAT-3') and 2218R (5'-ATGACACCRACRGCACRGTGTG-3')	REHNER; BUCKLEY, 2005
RPB1	RPB1Af (5'-GARTGYCCDGGDCAYTTYGG-3') and RPB1C (5'-CCNGCDATNTCRTRTCCATRTA-3')	STILLER; HALL, 1997
RPB2a	fRPB2-5F (5'-GAYGAYMGWGATCAYTTYGG-3') and RPB2-7cR (5'-CCCATRGCTTGYYTTRCCCAT-3')	LIU; WHELLEN; HALL, 1999
RPB2b	fRPB2-7cf (5'-ATGGGYAARCAAGCYATGGG-3') and RPB2-3053R (5'-TGRATYTTRTCRTCSACCATRTG-3')	LIU; WHELLEN; HALL, 1999 and REEB; LUTZONI; ROUX, 2004
$\beta$ -tubulin	T1 (5'-AACATGCGTGAGATTGTAAGT-3') and T22 (5'-TCTGGATGTTGTTGGGAATCC-3')	O'DONNELL; CIGELNIK, 1997
IGS	LSU4 (5'-CCGTYCTTCGCCYCGATTTCC -3') and Ma-IGS1 (5'-CGTCACTTGTATTGGCAC - 3')/ NS2 (5'-GGCTGCTGGCACCAGACTTGC - 3') and 630U (5'-CTTAGGGTAGGCTGCTTGTT)	PANTOU; MAVRIDOU; TYPAS, 2003 and WHITE et al., 1990
SSU	nu-SSU-0021-5' (5'-CTGGTTGATTCTGCCAGT-3') and nu-SSU-1780-3' (5'-AATGATCCTTCCGCAGGT-3')	GARGAS; DePRIEST, 1996 and DePRIEST, 1993

The PCR reactions of the translation elongation factor (EF-1 $\alpha$ ) were conducted with an initial denaturation for 2 min at 94.0 °C. The annealing temperature for the first amplification cycle was 66.0 °C, and was subsequently incrementally reduced by 1.0 °C per cycle over the next 9 cycles. An additional 36 amplification cycles were then performed, each consisting of 30 s denaturation at 94.0 °C, annealing for 30 s at 56.0 °C, extension for 1 min at 72.0 °C and final extension for 10 min at 72.0 °C.

The PCR reactions of the RNA polymerase II largest subunit (RPB1), RNA polymerase second largest subunit (RPB2) were conducted with an initial denaturation for 1 min at 95.0 °C, followed by 25 cycles with denaturation for 45 s at 95.0 °C, annealing for 40 s at 52.0 °C, extension for 2 min at 72.0 °C and final extension for 10 min at 72.0 °C.

The PCR reactions of the  $\beta$ -tubulin (Bt) region were conducted with an initial denaturation for 3 min at 94.0 °C, followed by 35 cycles with denaturation for 35 s at 94.0 °C, annealing for 55 s at 52.0 °C, extension for 2 min at 72.0 °C and final extension for 10 min at 72.0 °C.

The PCR reactions of the nuclear ribosomal intergenic spacer region (IGS) were conducted with initial denaturation for 3min at 94.0 °C followed by 30 cycles of denaturation for 1 min at 94.0 °C; annealing for 1 min at 54.0 °C; extension for 2 min at 72.0 °C; and final extension for 5 min at 72.0 °C.

The PCR reactions of the 18S small-subunit (SSU) region were conducted with an initial denaturation for 3 min at 94.0 °C, followed by 35 cycles with denaturation for 1 min at 95.0 °C, annealing for 1 min at 54.0 °C, extension for 2.5 min at 72.0 °C and final extension for 10 min at 72.0 °C.

All PCR reactions were carried out following the company's recommendations: 200.0  $\mu$ M of each dNTP, 15.0 mM MgCl<sub>2</sub>, 2.5 units Taq DNA polymerase (Invitrogen Inc.), 1x Taq Buffer, 0.3  $\mu$ M of each primer and 10.0–100.0 ng DNA template. PCR-amplified products were gel-purified in 1% agarose gel and the products were visualized with SYBR Green (Life Technologies Inc.) and Low Range ladder (Fermentas Inc.) was included as DNA size marker.

PCR purification was carried out with QIAquick PCR Purification Kit (QUIAGEN Inc.). Sequencing was conducted at the Universidade de São Paulo (Centro de Biotecnologia Agrícola – ESALQ/USP).

#### 4.2.1.2.3 Phylogenetic analysis

The sequences obtained were aligned using the program Clustal X2 (LARKIN et al., 2007). The phylogenetic analyses were performed with MEGA v. 5.10 (TAMURA et al., 2011), using Maximum Likelihood (ML) and Neighbor-Joining (NJ) with substitution model GTR+G (NEI; KUMAR, 2000). The purpose of conducting ML and NJ analyses was to investigate the effect of more restrictive assumptions of substitution models on the results. For all analyses, *Rozella allomycis* (GenBank no.AY635838) was used as out-group to root the tree. The robustness of each branch was determined using non-parametric bootstrap test (FELSESTEIN, 1985), with 1,000 replicates. The pairwise distance ( $\rho$ ) between and within groups was calculated with a standard error obtained by a bootstrap test with 1,000 replicates. These analyses were conducted using the same model described above carried out in the program MEGA5.10.

#### 4.2.1.3 Development of specific real-time PCR primer pairs to identify *Neozygites floridana* in soil by using SSU rDNA

##### 4.2.1.3.1 PCR primers

The SSU rDNA sequences of *N. floridana* were aligned using the program ClustalX2 (LARKIN et al., 2007). The sequences obtained were compared with SSU rDNA sequences of *Neozygites* spp. available at GenBank database (National Resource for Molecular Biology Information). The sequences of the primers selected were evaluated for the possibility of formation of dimmers and hairpins and compatibility of annealing temperature with the program "OligoAnalyser" (<http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/>). The specificity of each primer was confirmed by absence of significant homology with other known DNA sequence using the Basic Local Alignment Search Tool (BLAST) (ALTSCHUL et al., 1990) of GenBank database (National Resource for Molecular Biology Information). Alternative species specific primers were designed by manual sequences inspection. The primers developed were: Neoz-398F (5'-GCAGCATCCCGGTAGTAAAA-3'), Neoz-1060F (5'-ACGGGAGCCTTATGTTAAACTTAAGG-3'), Neoz-1182R (5'-TTCGGTTTGCTAAAACCAAAGTAGGCTT-3'), Neoz-1066F (5'-GCCTTATGTTAAACTTAAGGGACTAGTG-3'), Neoz-1199R (5'-

CACCTCTTGTTTATTCGGTTTGC-3'), and Neoz-513R) (5'-ATTCTAATAGACCTGTTCGGCTACG-3').

As a positive control, the primer pairs were used with DNA extracted from the fungus *N. floridana* (isolate Esalq 1420), and the DNA extracted from pure cultures of other fungus generally found in soil as *Fusarium* sp. The primers were also evaluated for their ability of amplification of metagenomic DNA from soil, extracted with the kit DNA Isolation Kit<sup>®</sup>PowerSoil (Mobio Inc.). Quantification of DNA was performed with a spectrophotometer using absorbance (A) 260.0 nm and the DNA concentration of all samples was adjusted to 50.0 ng/ $\mu$ L.

Real-time PCR amplifications were performed in 20.0  $\mu$ L thin-walled tubes in Rotor-Gene 6000 HRM (Corbett Research). Each 20.0  $\mu$ L reaction mixture contained 10.0  $\mu$ L of SYBR Green 2X, 5.2  $\mu$ L of sterile distilled water, 0.4  $\mu$ L of each primer (10.0  $\mu$ M), and 4.0  $\mu$ L of DNA template (5 ng/ $\mu$ L). Negative control reactions contained the same mixtures with 4.0  $\mu$ L of sterile water replacing the DNA template. The thermal cycling conditions were an initial denaturation for 3 min at 95.0  $^{\circ}$ C followed by 35 cycles at 94.0  $^{\circ}$ C for 1 min, at different annealing temperatures (55, 57.8, 60.0 and 63.5  $^{\circ}$ C) for 30 s and at 72.0  $^{\circ}$ C for 40 s, with a final step of extension for 10 min at 72.0  $^{\circ}$ C. PCR-amplified products were gel-purified in 1% agarose gel and the products were visualized with SYBR Green (Life Technologies Inc.) and Low Range ladder (Fermentas Inc.) was included as DNA size marker.

#### 4.2.1.3.2 Standard curves

Two methods were used to establish the standard curve for *Neozygites*. One using the *in vitro* culture of *N. floridana* isolate ARSEF662 and one *in vivo* culture (*N. floridana*-killed *T. urticae* cadaver) of the isolate ESALQ1420. To obtain the standard curve the best primer pairs tested were used.

Method 1: The *in vitro* culture of the isolate ARSEF662 was concentrated by consecutive centrifugation and quantified in Neubauer chamber. Standard curves based on threshold cycles ( $C_t$ ) with the dilution series of fungal genomic DNA ( $1.5 \times 10^7$ ,  $1.5 \times 10^6$ ,  $1.5 \times 10^5$ ,  $1.5 \times 10^4$ ,  $1.5 \times 10^3$ ,  $1.5 \times 10^2$  hyphal bodies) were then constructed. These concentrations of hyphal bodies were seeded in 0.5g of soil and the DNA extraction were performed with the DNA Isolation Kit<sup>®</sup>PowerSoil (Mobio Inc.) following the manufacturer's recommendation.

Method 2: To increase accuracy of the standard curve for qPCR of the *in vivo* culture (*N. floridana*-killed *T. urticae* cadaver) of the isolate ESALQ1420, the amplified fragment of *N. floridana* was cloned in the plasmid pGEM. A PCR with the best specific primer pairs selected using metagenomic DNA from *N. floridana*-killed *T. urticae* cadavers was then conducted. The PCR reactions were run with an initial denaturation for 3 min at 95.0 °C followed by 35 cycles at 94.0 °C for 1 min, 60.0 °C for 30 s, 72.0 °C for 30 s and final extension for 10 min at 72.0 °C. The PCR reactions were carried out following the company's recommendations: 5.0 µl of 10x PCR Buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 1.5 µl of 50 mM MgCl<sub>2</sub>, 1.0 µL of 10 mM dNTP, 0.5 µL de 5 U µL<sup>-1</sup>Taq DNA Polymerase (recombinant) (Invitrogen Inc.), 1.5 µL of each primer (10 pmoles µL<sup>-1</sup>), 5.0 µL of metagenomic DNA, and 5.0 µL of sterile water.

The amplified fragment was purified in 1.0% agarose gel in 1x TAE buffer and the products were visualized with SYBR ® Green (Life Technologies Inc.). The picture of agarose gel was performed using a densitometer StormTm (GE Healthcare) and analyzed with the software Image Quant TL (GE Healthcare). Target fragments were cut from the gel with a scalpel and purified using the Invisorb DNA Fragment Cleanup kit (Invitek Inc.). The purified fragments were then cloned into plasmid pGEM-T, with the kit pGEM-T Easy Vector System (Promega Inc.). The binding reaction was performed according to the manufacturer's original protocol. The plasmids linked were transformed in chemically competent *Escherichia coli* cell strain DH5-α by thermal shock incubating the bacteria in contact with plasmids for 20 min on ice followed by 2 min at 42.0 °C and 3 min on ice. Bacteria containing the recombinant plasmid were selected on selective LB culture medium containing 100.0 mg L<sup>-1</sup> of ampicillin and 20.0 mg L<sup>-1</sup> of X-GAL and their plasmids were extracted by alkaline lyses (SAMBROOK et al. 2001). Plasmids were quantified by fluorometer Qubit (Life Technologies) and QubitdsBR Assay Kit and to obtain accurate plasmids quantification was diluted obtaining the following concentrations: 1, 1x10<sup>-1</sup>, 1x10<sup>-2</sup>, 1x10<sup>-3</sup>, 1x10<sup>-4</sup>, 1x10<sup>-5</sup> ng/µL.

Real-time PCR amplifications for both DNA samples (*in vivo* and *in vitro* culture) were performed in 20.0 µL thin-walled tubes in Rotor-Gene 6000 HRM (Corbett Research). Each 20.0 µL reaction mixture contained 10.0 µL of SYBR Green 2X, 5.2 µL of sterile distilled water, 0.4 µL of each primer (10 µM), and 4.0 µL of DNA template. Negative control reactions contained the same mixtures with 4.0 µL of sterile water replacing the DNA template. The thermal cycling conditions were performed under the following conditions: one cycle at 50.0 °C for 2 min, and 95.0 °C for 2 min, followed by 40 cycles at 95.0 °C for 15 s

and 60.0 °C for 30 s. A melting curve temperature profile was obtained for one cycle at 60.0 °C for 1 min, followed by increasing 1.0 °C s<sup>-1</sup> until attaining 95.0 °C, and 95.0 °C for 15 s.

C<sub>t</sub> values were calculated by the Rotor-Gene™ 6000 software program to indicate significant fluorescence signals rising above background during the early cycles of the exponentially growing phase of the PCR amplification process. A standard curve was obtained by plotting the C<sub>t</sub> value, which is defined by crossing cycle number or crossing point, versus the logarithm of the concentration of each dilution series of fungal genomic DNA.

#### 4.2.1.3.3 Testing *N. floridana* DNA extraction and real-time PCR in soils

To determine the efficacy of the *N. floridana* DNA extraction method and sensitivity of the qPCR assays on soils, the samples were seeded with different concentrations of *N. floridana*. This was done by adding 5 and 100 *N. floridana*-killed *T. urticae* isolate ESALQ1420 cadavers to 0.5 g of soil. The soil with *N. floridana*-killed *T. urticae* cadavers were then macerated with liquid nitrogen. The process was repeated twice for each quantity of fungal inoculums. The molecular probes were also tested on soil samples collected from a field where *N. floridana*-killed mites were observed (see field experiment in Chapter 2). The process was repeated twice for each genomic DNA soil concentrations. The DNA extraction of the soil seeded with the fungus, and the test with the environmental soil samples, were performed with the DNA Isolation Kit<sup>®</sup> PowerSoil (Mobio Inc.) following the manufacturer's recommendation.

## 4.2.2 Results

### 4.2.2.1 Sequencing and phylogenetic analysis of *Neozygites* spp.

Most of the primer pairs tested in this study did not amplified *N. floridana*. The primer used to amplify the genes IGS from the ribosomal DNA amplified only the positive control (DNA from *Metarhizium*), no amplification was found in the DNA of *Neozygites*. The same results were found for the genes from the nuclear DNA: RPB1, RPB2,  $\beta$ -tubulin and EF1- $\alpha$  (exon and intron region). These primer tested were developed for a different group of fungi and works for entomopathogenic fungi such as *Beauveria* and *Metarhizium* and may not bind *N. floridana* template DNA. The quality of the DNA extracted from *N. floridana*

was confirmed by positive amplification of the SSU rDNA region. The quality of DNA extracted from *Metarhizium* sp., which was used as a positive control for the primer pairs tested, was confirmed by positive amplifications.

Only the primer pairs of the region SSU amplified *Neozygites* DNA. Approximately 1,400 base pairs PCR products were sequenced on both strands using PCR primers and the internal primers COMPSSU5' (5'-TGAGACTACAACGGTATTCTAATC-3') (DELALIBERA JR.; HAJEK; HUMBER, 2004) and NFREV (5'-ATTAAACCGCACGCTCCA-3') and NFFWD (5'-AGCGCTACACTGCATGCAGCAA'-3') developed in this study.

Partial sequences from the SSU rDNA were obtained from four isolates of *N. floridana*. Sequences obtained in this study were aligned with other SSU rDNA sequences available at the GenBank database (7 sequences of *Neozygites* and 21 sequences of entomopathogenic fungi from phylum Entomophthoromycota) using ClustalX2 (LARKIN et al., 2007).

The topologies obtained with the Maximum Likelihood (ML) and Neighbor-Joining (NJ) analyses were mostly similar, and both analyses suggested that the phylum Entomophthoromycota was a monophyletic group and the class Neozygitomycetes creates a monophyletic group. The Neozygitomycetes was the most divergent among the Entomophthoromycota (Bootstrap value 81). In the cluster of *Neozygites*, the isolates splitted from the host which they were obtained. These divisions happened first by Class into Arachnida (Acari) and Hexapoda (Insecta). The genus *Neozygites* originated from spider mites present a monophyletic cluster separated from *N. parvispora*, an isolate collected in Switzerland from *Thrips tabaci* (Insecta: Thripidae). This mite pathogenic fungi presents three distinct groups: Group 1 contains *N. floridana* isolates from the same host *T. urticae* collected in Europe in latitudes above 45 °N (Norway (NCRI271/04) and Switzerland (AF296758), Group 2 contains isolates from the genus *Tetranychus* collected in South and North America (*T. evansi* from Brazil (ESALQ1419), *T. urticae* from Brazil (ESALQ1420) *T. urticae* from USA (AY233985), *T. urticae* from Colombia (AY 233984)). The pairwise distance was used to analyze the divergence between isolates in the same group in the phylogenetic analysis. The value found to the number of base substitutions per site was less than 0.2 and this means that the substitution model is accurate for this data (RUSSO; MIYAKI; PEREIRA, 2001). The greatest distance between groups was found in Group 1, where isolates were collected from the same host species (*T. urticae*) in Norway and Switzerland. Group 3 contains isolates only from a host of genus *Mononychellus* collected in

South America (*M. planki* (ESALQ1439), *M. tanajoa* (AY233982) and *M. tanajoa* (AY233981) from Brazil) and in Africa (*M. tanajoa* (AY233983)) (Figure 4.1.).

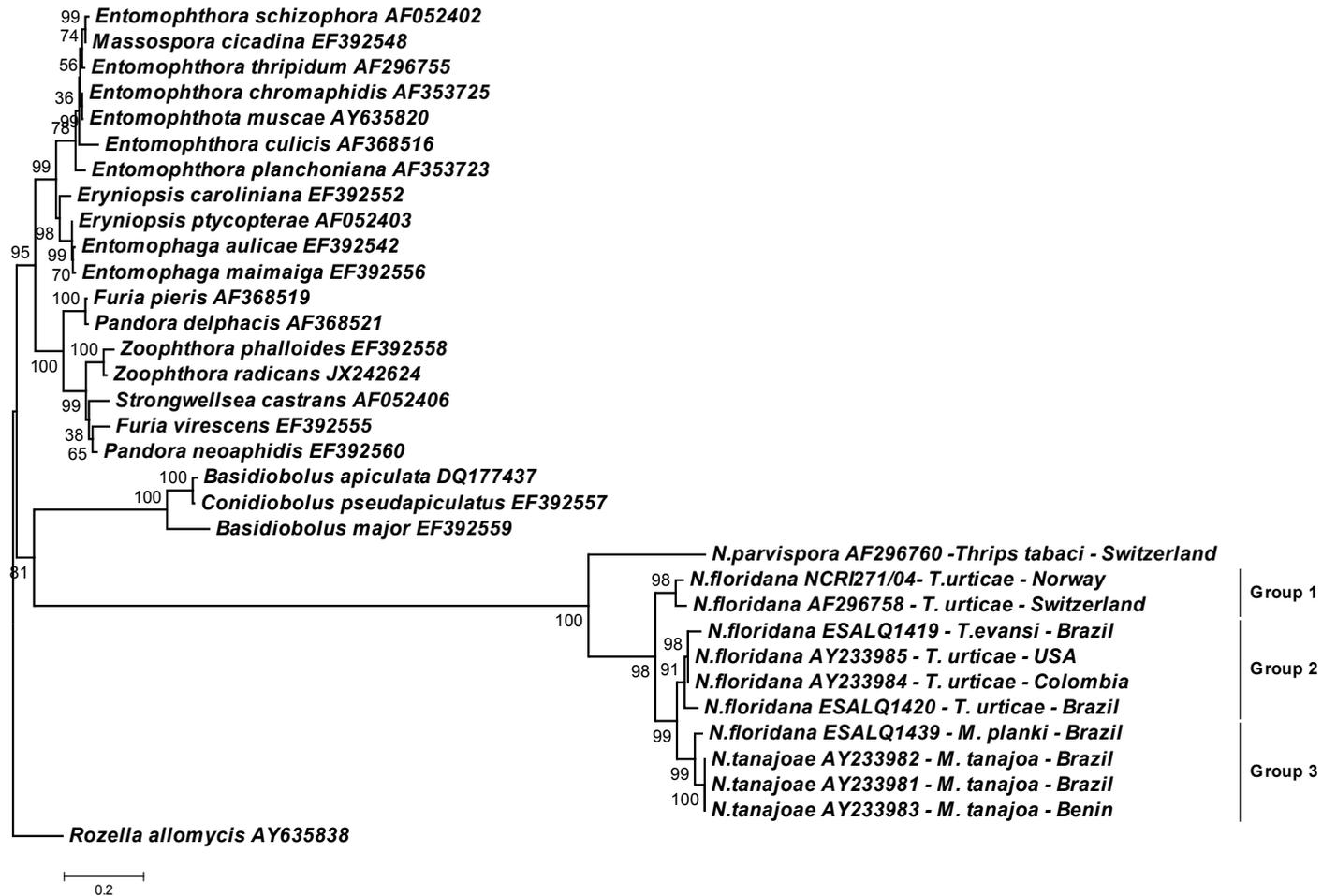


Figure 4.1 - Maximum Likelihood tree obtained based on the analysis of SSU rDNA sequences under substitution model GTR+G. Values on the branches indicate bootstrap values, obtained with 1,000 replicates. The scale bar corresponds to 20 changes. Fungal denomination followed by the GeneBank accession number or isolate number from the isolates sequencing in this study. Abbreviation of genera: N. = genus *Neozygites*; T. = *Tetranychus*; M. = *Mononychellus*. Group 1: *N. floridana* isolates from the same host *T. urticae* collected in Europe. Group 2: isolates from the host of genus *Tetranychus* collected in South and North America. Group 3: isolates from hosts of the same genus *Mononychellus* collected in South America and Africa

The pairwise distance between groups from the phylogenetic analyses with high value in the branches (bootstrap value>90) (FELSENSTEIN, 2004) ranged from 0.015 to 0.032 (Table 4.3).

Table 4.3 - Pairwise distance and standard error within groups of phylogenetic analysis of SSU rDNA sequences of *Neozygites* with bootstrap value up to 90

<b>Group *</b>	<b>Distance</b>	<b>Standard error</b>
<b>1</b>	<b>0.032</b>	<b>0.005</b>
<b>2</b>	<b>0.022</b>	<b>0.003</b>
<b>3</b>	<b>0.015</b>	<b>0.002</b>

\*Groups obtained in the phylogenetic analysis using ML and NJ models (Figure 4.1.)

#### **4.2.2.2 Development of real-time PCR primer pairs specific for the fungus *Neozygites floridana* using SSU rDNA**

Different annealing temperatures, 55, 57.8, 60.5 and 63.5 °C were tested in this study because this parameter could be an important factor affecting PCR specificity (DIACO, 1995; ROBINSON; MONIS; DOBSON, 2006). In conventional PCR the primer pairs Neoz-398F/Neoz-513R (P1) and Neoz-1066F/Neoz-1182R (P4) amplified the target DNA of *Neozygites* in all tested temperatures and no unspecific amplification was observed. The primer pairs Neoz-1066F/Neoz-1199R (P2) was tested previously with all temperatures, and resulted in amplification of *Neozygites* in all temperatures, but at 55.0 and 57.8 °C, no target amplification of *Fusarium* sp. and metagenomic DNA from soil were found (data not shown). Therefore, only PCR with 60.5 °C alignment temperature was performed to amplify *Neozygites* DNA (Figure 4.2). Primers Neoz-1060F and Neoz-1199R (P3) also amplified DNA from *Fusarium* sp. and metagenomic DNA from the soil (Figure 4.2), therefore, they were excluded from the next step with real-time PCR.

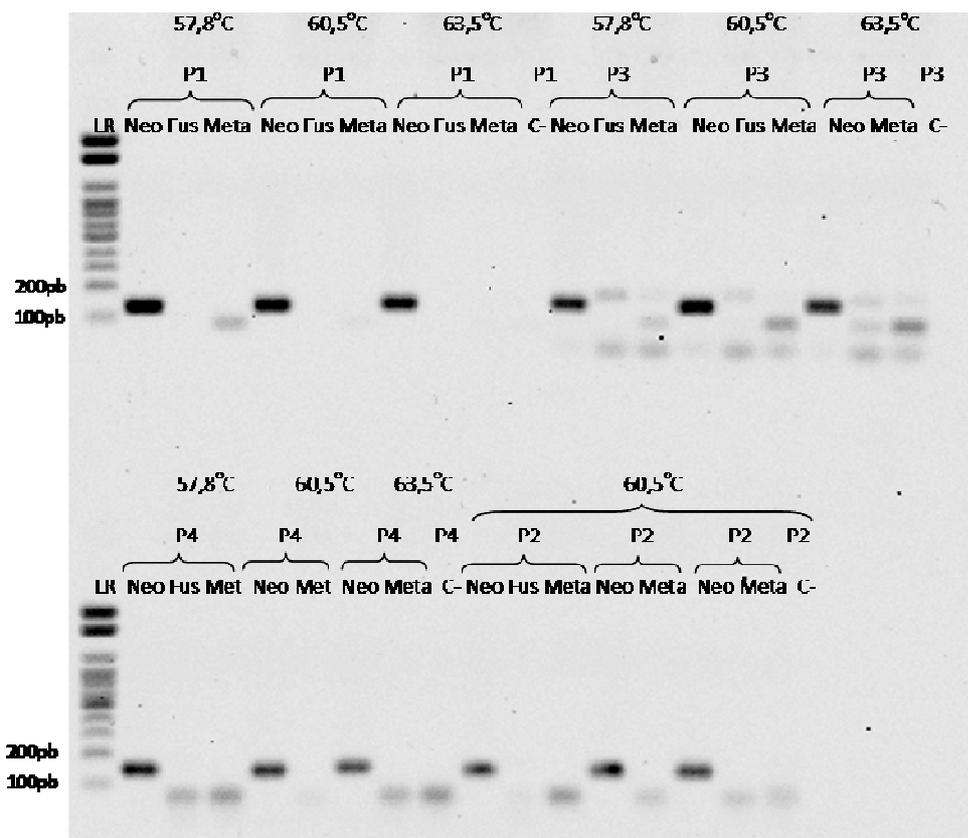


Figure 4.2 - Agarose gel (1%) electrophoresis of PCR-amplified products from DNA of *N. floridana* (Neo), *Fusarium* sp. (Fus), and metagenomic DNA from soil (Met).LR= ladder, P1= Neoz-398F/Neoz-513R; P2= Neoz-1066F/Neoz-1199R; P3= Neoz1060F/Neoz-1199R and, P4= Neoz-1066F/Neoz1182R. C= Negative control

Three primer pairs were used in this study, and based on the correlation coefficient of the standard curve, the best primer pair was Neoz-1066F/Neoz-1182R (P4) (Table 4.4.).

Table 4.4 - Cycle threshold average value, standard deviation (SD) obtained based on real-time PCR of SSU rDNA region of *N. floridana* using a serial dilution of cloned DNA

DNA concentration (pg/ $\mu$ L)	Cycle threshold ( $C_t$ ) $\pm$ Standard deviation (SD)		
	Neoz-388F and Neoz-513R	Neoz-1060F and Neoz-1199R	Neoz-1066F and Neoz-1182R
1000	20.63 $\pm$ 0.06	18.55 $\pm$ 0.16	18.71 $\pm$ 0.04
750	20.18 $\pm$ 0.06	18.64 $\pm$ 0.02	19.17 $\pm$ 0.12
500	23.50 $\pm$ 4.38	19.12 $\pm$ 0.00	19.93 $\pm$ 0.99
250	20.80 $\pm$ 0.10	19.75 $\pm$ 0.02	20.41 $\pm$ 0.17
NTC*	—	—	—

\*non-template control

The standard curve constructed using dilution series of hyphal bodies of *N. floridana* resulted in the threshold value 0.1. The  $C_t$  values increased with the dilution of the hyphal bodies, and the best  $C_t$  values were approximately 19 and 23 cycles, obtained with

concentration of  $1.5 \times 10^6$  and  $1.5 \times 10^5$  hyphal bodies/g of soil respectively. The lowest dilution  $1.5 \times 10^3$  and  $1.5 \times 10^2$ /g of soil, resulted in  $C_t$  values at around 30 cycles that is similar to the negative control (Figure 4.3.A). The linear correlation coefficient was 0.988, and the slope was -3.475 (Figure 4.3.B).

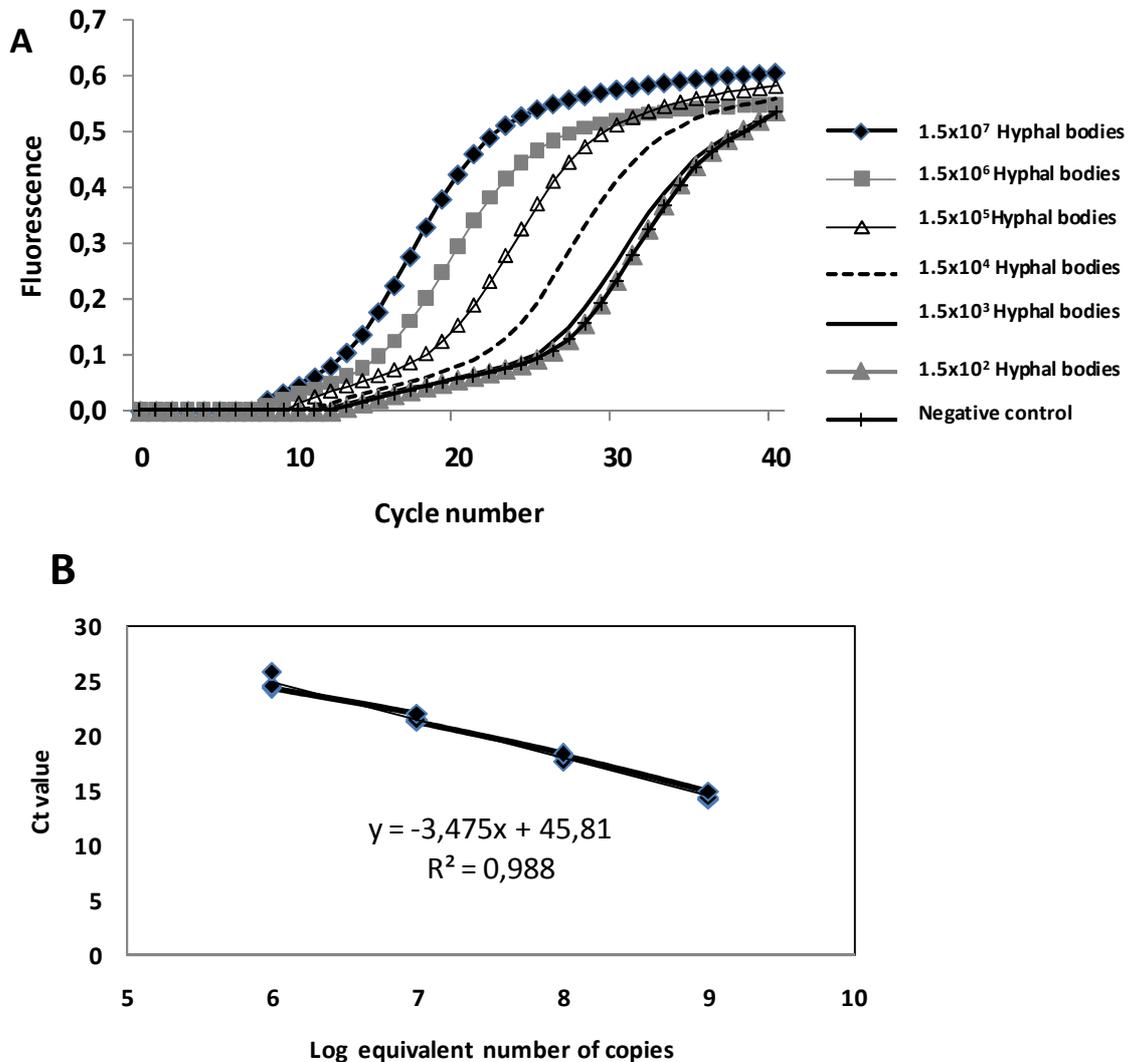


Figure 4.3 - Quantification of hyphal bodies of *N. floridana* per gram of soil. (A) Real-time PCR fluorescence kinetics versus cycle number of dilution series of target DNA (SSU rDNA). (B) Standard curve with the correlation coefficient ( $R^2$ ) obtained by plotting the concentration of DNA versus the cycle number required to elevate the fluorescence signal above the threshold (0.1)

The standard curve constructed by the use of dilution series of *N. floridana* DNA multiplied in plasmid vector resulted in the threshold value 0.15. The  $C_t$  values increased with the dilution of the DNA (Figure 4.4.A). The linear correlation coefficient was 0.975, and the slope was -2.8 (Figure 4.4.B).

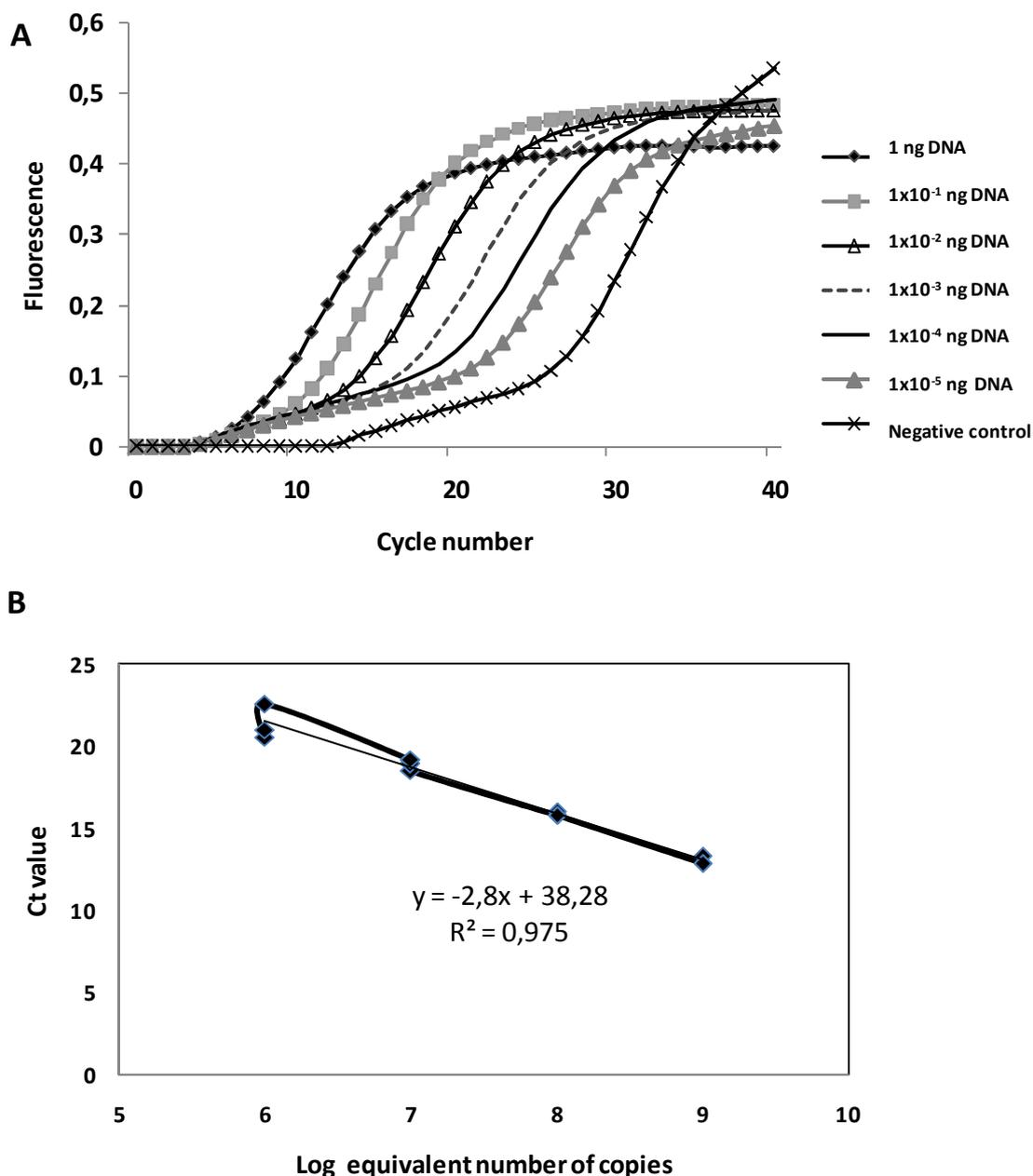


Figure 4.4 - Quantification of *N. floridana* recombinant DNA replicated into plasmid vector. (A) Real-time PCR fluorescence kinetics versus cycle number of dilute series of target DNA (SSU rDNA). (B) Standard curve with the correlation coefficient ( $R^2$ ) obtained by plotting the concentration of DNA versus the cycle number required to elevate the fluorescence signal above the threshold (0.15)

Analysis of Ct values of two amounts of fungus *N. floridana* isolate ESALQ1420 added to soil samples showed that in the soil inoculated with 5 and with 100 *N. floridana*-killed *T. urticae* cadavers, the Ct values obtained for all of the four concentrations tested were 21.72-22.83 and 16.72-18.24, respectively. The Ct values obtained from the soil samples collected in the field where *N. floridana* has been observed were similar to the Ct values of the no template control (Table 4.5).

Table 4.5 - DNA amplification by real-time PCR using soils seeded with *N. floridana*. Ct values  $\pm$  standard deviation (SD) of the samples. Threshold value 0.2

Samples	Ct value $\pm$ SD
Field soil sample (1ng/ $\mu$ L)	31.74 $\pm$ 0.26
Field soil sample (2.5ng/ $\mu$ L)	33.00 $\pm$ 0.84
Field soil sample (5ng/ $\mu$ L)	31.66 $\pm$ 0.73
Field soil sample (10ng/ $\mu$ L)	31.74 $\pm$ 0.03
5 <i>N. floridana</i> * (1ng/ $\mu$ L)	22.87 $\pm$ 0.06
5 <i>N. floridana</i> * (2.5ng/ $\mu$ L)	21.98 $\pm$ 0.13
5 <i>N. floridana</i> (5ng/ $\mu$ L)	21.72 $\pm$ 0.13
5 <i>N. floridana</i> * (10ng/ $\mu$ L)	22.35 $\pm$ 0.53
100 <i>N. floridana</i> * (1ng/ $\mu$ L)	18.24 $\pm$ 0.16
100 <i>N. floridana</i> * (2.5ng/ $\mu$ L)	17.15 $\pm$ 0.19
100 <i>N. floridana</i> * (5ng/ $\mu$ L)	17.03 $\pm$ 0.38
100 <i>N. floridana</i> * (10ng/ $\mu$ L)	16.72 $\pm$ 0.02
Non-templateControl	30.84 $\pm$ 1.89

\* Numbers of *N. floridana*-killed *T. urticae* cadavers added to the soil sample

### 4.2.3 Discussion

In this study, a phylogenetic analysis of SSU rDNA of Entomophthoromycota is presented. This analysis includes 4 sequences of isolates of *N. floridana* from Norway and Brazil, and showed that *Neozygites* is a group of fungi very far from other Entomophthoromycota as it has been suggested by White et al. (2006) and Gryganskyi et al. (2012). Very few samples of *Neozygites* spp. has been sequenced and so far only SSU rRNA and  $\beta$ -tubulin sequences are available. This may be due to difficulties to produce *N. floridana* *in vitro* and to purify DNA from *in vivo* cultures of this fungus. When DNA from a fungus is extracted this is normally done from an *in vivo* culture free of DNA from other organisms, and this has not been possible with most species of *Neozygites*. Difficulties of amplification *N. floridana* DNA may be due to the polymorphism of the template sequences in relation to the primer pairs used, which were developed for different group of entomopathogenic fungus.

It also showed a high intra-specific variability between isolates of *N. floridana*. The cluster formed by isolates from hosts of the genus *Mononychelus* suggested that there is an evolutionary history between pathogen and host. The isolates originated from *Tetranychus* presented high divergence especially between the two isolates from Norway and Switzerland compared to the other isolates from different host species and places. This may indicate that different species are involved. In a study conducted by Ribeiro et al. (2009) on the host range of *N. floridana* isolates, all isolates tested were pathogenic to all spider mite species tested,

*M. tanajoa*, *Schizotetranychus sacharum* Flechtmann and Baker, *Tetranychus abacae* Baker and Pritchard, *T. evansi*, *Tetranychus ludeni* Zacher and *T. urticae*. However, the isolates caused higher levels of infection and significant mummification only to the tetranychid species from which they were collected.

Keller (1997) examined several isolates of *Neozygites* associated with tetranychid mites, and refers to all the isolates examined as *N. floridana* based on the data of fungal structures, as primary conidia, secondary conidia and resting spores. Delalibera Jr., Hajek and Humber (2004) suggested, however, that, the morphological analysis of fungal structures of fungi in the *Neozygites* can not be used to distinguish different species because of the relative small number of useful morphologic characters. Further they showed that ribosomal analyses of mite pathogenic species of *Neozygites* could be a very good tool to identify different species of *Neozygites*. Beside the phylogenetic analysis of this fungus some characters of the spider mite cadavers corroborate our theory that *N. floridana* is not a single species, but a complex of species. Cadavers of *M. planki*, *T. evansi* and *T. urticae* containing hyphal body of *Neozygites* from Brazil are orange to light brown while *T. urticae* cadavers from Norway are dark brown. *M. tanajoa* cadavers with hyphal bodies of *N. tanajae* are also light brown to orange (Delalibera Jr. personal communication).

The difficulty concerning the classification of species from the genus *Neozygites* is due to the structural simplicity of these fungi and some important parts of the life cycle are very rarely found, such as resting spores. Although morphologically similar *Neozygites* displays various physiological and molecular characteristics between isolates. It may be possible that *N. floridana* is a species complex which *N. tanajae* was the first to be separately.

The use of standard curve based on known concentrations of genomic DNA makes possible the quantify DNA from this fungus from any source. In this study standard curves with  $r^2$  values of 0.975 and 0.988 were constructed after real-time PCR amplifications of different genomic DNA concentrations, from cloning and hyphal bodies, confirming the linearity of the quantification process between exponential increases of DNA concentrations and real-time PCR threshold cycles.

Environmental sampling to monitor entomopathogen titers in the soil, a known reservoir of insect pathogenic fungi (HAJEK, 1999), is important in the evaluation of conditions that could trigger epizootics and in the development of strategies for insect pest management. Real-time PCR using the SYBR enabled the quantification of fungal genomic DNA from known amount of propagules of *N. floridana*. This is the first reported study on

this fungus using quantitative real-time PCR. The method can detect products at higher and lower range of DNA concentration, and in the test with soil seeded with known amount, 10 mummified mites per gram of soil, were possible to detect the fungus in all of the concentrations tested, thus indicating good detection sensitivity of qPCR assay.

The primer pairs selected specific to *N. floridana* amplified under optimized PCR conditions single fragments of appropriate size and did not amplify non-target DNA. However, PCR-based sampling of microorganisms in the soil is dependent upon the efficient lysis of these organisms and the removal of contaminants co-extracted with rDNA. Humic acids, in particular, are known to inhibit Taq polymerase in PCR and to interfere with detection assays (STEFFAN et al., 1988; TEBBE; VAHJEN, 1993; TSAI; OLSON, 1992). Comparing the  $C_t$  values of standard curves constructed with hyphal bodies inoculated to the soil and with *N. floridana* recombinant DNA replicated into plasmid vector, it seems that the soil do not reduce the detection limit for this fungus.

The fungus was detected in soil seeding samples with  $1.5 \times 10^4$  hyphal bodies and also 10 mummified mites/g of soil. However, when these molecular probes were tested with soil from field experiments where *Neozygites* has been found, the fungus was not detected. This can be due the fungus was not viable in those particular samples examined. Further tests with other soils known for *Neozygites* prevalence are needed. The detection and quantification of genomic DNA of this fungus using real-time PCR approach described in this study holds promise for the quantification of fungal DNA directly extracted from unknown soil samples.

## References

- ALTSCHUL, S.F.; GISH, W.; MILLER, W.; MYERS, E.W.; LIPMAN, D.J. Basic local alignment search tool. **Journal of Molecular Biology**, London, v. 215, p. 403-410, 1990.
- BISCHOFF, J.F.; REHNER, S.A.; HUMBER, R.A. A multilocus phylogeny of the *Metarhizium anisopliae* lineage. **Mycologia**, New York, v. 101, n. 4, p. 512-530, 2009.
- BRANDENBURG, R.L.; KENNEDY, G.G. Relationship of *Neozygites floridana* (Entomophthorales: Entomophthoraceae) to two-spotted spider mite (Acari: Tetranychidae) populations in field corn. **Journal of Economic Entomology**, Lanham, v. 75, p. 691-694, 1982.
- CASTRILLO, L.A.; THOMSEN, L.; JUNEJA, P.; HAJEL, A.E. Detection and quantification of *Entomophaga maimaiga* resting spores in forest soil using real-time PCR. **Mycological Research**, Cambridge, v. 111, p. 324-331, 2007.

DELALIBERA JR., I.; HAJEK, A.E.; HUMBER, R.A. Use of cell culture media for cultivation of the mite pathogenic fungi *Neozygites tanajoae* and *N. floridana*. **Journal of Invertebrate Pathology**, San Diego, v. 84, p. 119-127, 2003.

\_\_\_\_\_. *Neozygites tanajoae* sp. nov., a pathogen of the cassava green mite. **Mycologia**, New York, v. 96 n. 5, p. 1002–1009, 2004.

DELALIBERA JR., I.; DEMETRIO, C.G.B.; MANLY, B.F.J.; HAJEK, A.E. Effect of relative humidity and origin of isolates of *Neozygites tanajoae* (Zygomycetes: Entomophthorales) on production of conidia from cassava green mite, *Mononychellus tanajoa* (Acari: Tetranychidae) cadavers. **Biological Control**, Orlando, v. 39, p. 489-496, 2006.

DePRIEST, P. Small subunit rDNA variation in a population of lichen fungi due to optimal group I introns. **Gene**, Amsterdam, v. 134, p. 67–74, 1993.

DIACO, R. Practical considerations for the design of quantitative PCR assays. In: INNIS, M.A.; GELFAND, D.H.; SINSKY, J.J.(Ed.). **PCR strategies**. New York: Academic Press, 1995. p. 85–108.

DUARTE, V.S.; SILVA, R.A.; WEKESA, V.W.; RIZZATO, F.B.; DIAS, C.T.S.; DELALIBERA JR., I. Impact of natural epizootics of the fungal pathogen *Neozygites floridana* (Zygomycetes: Entomophthorales) on population dynamics of *Tetranychus evansi* (Acari: Tetranychidae) in tomato and nightshade. **Biological Control**, Orlando, v. 51, p. 81-90, 2009.

FELSENSTEIN, J. Confidence-limits on phylogenies: an approach using the bootstrap. **Evolution**, New York, v. 39, p. 783–791, 1985.

FELSENSTEIN, J. **Inferring phylogenies**. Sunderland: Sinauer Associates, 2004. 664 p.

FOURNIER, A.; WIDMER, F.; ENKERLI, J. Assessing winter-survival of *Pandora neoaphidis* in soil with bioassays and molecular approaches. **Biological Control**, Orlando, v.54, p. 126-134, 2010.

GARGAS, A.; DePRIEST, P.T. A nomenclature for fungal PCR primers with examples from intron containing SSU rDNA. **Mycologia**, New York, v. 88, p. 745-748, 1996.

GRYGANSKYI, G.P.; HUMBER, R.A.; SMITH, M.E.; MIADLIKOVSKA, J.; WU, S.; VOIGT, K.; WALTHER, G.; ANISHCHENKO, I.M.; VILGALYS, R. Molecular phylogeny of the Entomophthoromycota. **Molecular Phylogenetics and Evolution**, Orlando, v. 65, p.682-694, 2012.

HAJEK, A. E.; HODGE, K. T.; LIEBHERR, J. K.; DAY, W. H.; VANDENBERG, J. D. Use of RAPD analysis to trace the origin of the weevil pathogen *Zoophthora phytonomi* in North America. **Mycological Research**, Cambridge, v. 100, p. 349–355, 1996.

HAJEK, A.E. Ecology of terrestrial fungal pathogens. In: JONES, J.G. (Ed.). **Advances in microbial ecology**. New York: Plenum Press, 1997.v.15, p. 193-249.

\_\_\_\_\_. Pathology and epizootiology of *Entomophaga maimaiga* infections in forest Lepidoptera. **Microbiology and Molecular Biology Reviews**, New York, v. 63, p. 814–835, 1999.

HODGE, K.T.; SAWYER, A.J.; HUMBER, R.A. RAPD-PCR for identification of *Zoophthora radicans* isolates in biological control of potato leafhopper. **Journal of Invertebrate Pathology**, San Diego, v. 65, p. 1-9, 1995.

HOUNTONDI, F.C.C.; YANINEK, J.S.; MORAES, G.J.; ODUOR, G.I. Host specificity of the cassava green mite pathogen *Neozygites floridana*. **BioControl**, Dordrecht, v.47, p. 61-66, 2002.

HUMBER, R.A. Evolution of entomopathogenicity in fungi. **Journal of Invertebrate Pathology**, San Diego, v. 98, p. 262-266, 2008.

\_\_\_\_\_. *Entomophthoromycota*: a new phylum and reclassification for entomophthoroid fungi. **Mycotaxon**, Ithaca, v.120, p. 477-492, 2012.

HUMBER, R.A.; MORAES, G.J.; SANTOS, J.M. Natural infection of *Tetranychus evansi* (Acarina: Tetranychidae) by a *Triplosporium* sp. (Zygomycetes: Entomophthorales) in Northeastern Brazil. **Entomophaga**, Paris, v.26, n. 4, p. 421–425, 1981.

JENSEN, A.B.; EILENBERG, J. Genetic variation within the insect-pathogenic genus *Entomophthora*, focusing on the *E. muscae* complex, using PCR-RFLP of ITS II and LSU rDNA. **Mycological Research**, Cambridge, v. 105, p. 307–312, 2001.

KELLER, S. Arthropod-patogenic Entomophthorales of Switzerland. II. *Erynia*, *Eyniopsis*, *Neozygites*, *Zoophthora* and *Tarichium*. **Sydowia**, Horn, v. 43, p. 39-122, 1991.

\_\_\_\_\_. The genus *Neozygites* (Zygomycetes, Entomophthorales) with special reference to species found in tropical regions. **Sydowia**, Horn, v. 49, n.2, p. 118-146, 1997.

KLINGEN, I.; WAERSTED, G.; WESTRUM, K. Overwintering and prevalence of *Neozygites floridana* (Zygomycetes: Neozygitaceae) in hibernating females of *Tetranychus urticae* (Acari: Tetranychidae) under cold climatic conditions in strawberries. **Experimental and Applied Acarology**, Amsterdam, v. 46, p.231-245, 2008.

LARKIN, M.A.; BLACKSHIELDS, G.; BROWN, N.P.; CHENNA, R.; MCGETTIGAN, P.A.; MCWILLIAM, H.; VALENTIN, F.; WALLACE, I.M.; WILM, A.; LOPEZ, R.; THOMPSON, J.D.; GIBSON, T.J.; HIGGINS, D.G. Clustal W and Clustal X version 2.0. **Bioinformatics**, Oxford, v. 23, p. 2947-2948, 2007.

LIU, Y.J.; WHELEN, S.; HALL, B.D. Phylogenetic relationships among ascomycetes: evidence from an RNA polymerase II subunit. **Molecular Biology and Evolution**, Oxford, v. 16, n. 12, p. 1799-1808, 1999.

NEI, M.; KUMAR, S. **Molecular evolution and phylogenetics**. New York: Oxford University Press, 2000. 339p.

NIELSEN, C.; SOMMER, C.; EILENBERG, J.; HANSEN, K.; HUMBER, R. Characterisation of aphid pathogenic species in the genus *Pandora* by PCR techniques and digital image analysis. **Mycologia**, New York, v. 93, p. 864–874, 2001.

NORDENGEN, I.; KLINGEN, I. Comparison of methods for estimating the prevalence of *Neozygites floridana* in *Tetranychus urticae* populations infesting strawberries. **Journal of Invertebrate Pathology**, San Diego, v. 92, p. 1-6, 2006.

O'DONNELL, K.; CIGELNIK, E. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. **Molecular Phylogenetics and Evolution**, Orlando, v. 7, p. 103–116, 1997.

PANTOU, M.P.; MAVRIDOU, A.; TYPAS, M.A. IGS sequence variation, group-I introns and the complete nuclear ribosomal DNA of the entomopathogenic fungus *Metarhizium*: excellent tools for isolate detection and phylogenetic analysis. **Fungal Genetics and Biology**, Orlando, v. 38, p. 159–174, 2003.

PELL, J.K.; EILENBERG, A.E.; HAJEK, A.; STEINKRAUS, D.C. Biology, ecology and pest management potential of Entomophthorales. In: BUTT, T.M.; JACKSON, C.; MAGAN, N. (Eds.). **Fungi as biocontrol agents: progress, problems and potential**. New York: CABI Publ., 2001. chap. 4, p.71-154.

REEB, V.; LUTZONI, F.; ROUX, C. Contribution of RPB2 to multilocus phylogenetic studies of the euascomycetes (Pezizomycotina, Fungi) with special emphasis on the lichen-forming Acarosporaceae and evolution of polyspory. **Molecular Phylogenetics and Evolution**, Orlando, v. 32, p. 1036–1060, 2004.

REHNER, S.A.; BUCKLEY, E. A *Beauveria* phylogeny inferred from nuclear ITS and EF-1 sequences: evidence for cryptic diversification and links to *Cordyceps* teleomorphs. **Mycologia**, New York, v. 97, p.84–98, 2005.

REMONSELLEZ, F.; GALLEGUILLOS, F.; Van RENSBURG, S.J.; RAUTENBACH, G.F.; GALLEGUILLOS, P.; CASTILLO, D.; DEMERGASSO, C. Monitoring the microbial community inhabiting a low-grade copper sulphide ore by Quantitative Real Time PCR analysis of 16S rRNA genes. **Advanced Materials Research**, Zurich, v.20/21, p. 539-542, 2007.

RIBEIRO, A.E.L.; GONDIM JR.; M.G.C.; CALDERAN, E.; DELALIBERA JR., I. Host range of *Neozygites floridana* isolates (Zygomycetes: Entomophthorales) to spider mites. **Journal of Invertebrate Pathology**, San Diego, v. 102, p. 196-202, 2009.

ROBINSON, B.S.; MONIS, P.T.; DOBSON, P.J. Rapid, sensitive, and discriminating identification of *Naegleria* spp. by real-time PCR and melting-curve analysis. **Applied and Environmental Microbiology**, Washington, v.72, p.5857–5863, 2006.

RUSSO, C.A.B.; MIYAKI, C.Y.; PEREIRA, S.L. Reconstrução filogenética: métodos geométricos. In: MATIOLI, R.S. (Ed.). **Biologia molecular e evolução**. Ribeirão Preto: Hollos, 2001. p. 108-116.

STEFFAN, R.J.; GOKSOYR, J.; BEJ, A.K.; ATLAS, R.M. Recovery of DNA from soils and sediments. **Applied and Environmental Microbiology**, Washington, v. 54, p. 2908–2915, 1988.

STILLER, J.W.B.; HALL, D. The origin of red algae: implications for plastid evolution. **Proceedings of the National Academy of Science of the United States of America**, Washington, v. 94, p. 4520–4525. 1997.

TAMURA, K.; PETERSON, D.; PETERSON, N.; STECHER, G.; NEI, M.; KUMAR, S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. **Molecular Biology and Evolution**, Chicago, v. 28, p. 2731–2739, 2011.

TEBBE, C.C.; VAHJEN, W. Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and yeast. **Applied and Environmental Microbiology**, Washington, v. 59, p. 2657–2665, 1993.

TSAI, Y.L.; OLSON, B.H. Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. **Applied and Environmental Microbiology**, Washington, v. 58, p. 2292–2295, 1992.

TYMON, A.M.; SHAH, P.A.; PELL, J.K. PCR-based molecular discrimination of *Pandora neoaphidis* isolates from related entomopathogenic fungi and development of species-specific diagnostic primers. **Mycological Research**, Cambridge, v. 108, n. 4, p. 419–433, 2004.

WHITE, M.M.; JAMES, T.Y.; O'DONNELL, K.; CAFARO, M.J.; TANABE, Y.; SUGIYAMA, J. Phylogeny of the Zygomycota based on nuclear ribosomal sequence data. **Mycologia**, New York, v. 98, p. 872–884, 2006.

WHITE, T.J.; BRUNS, T.; LEE, S.; TAYLOR, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: INNES, M.A.; GELFAND, D.H.; SNINSKY, J.J.; WHITE, T.J. (Ed.). **PCR protocols: a guide to methods and applications**. San Diego: Academic Press, 1990. p. 315–322.