Journal of Invertebrate Pathology 122 (2014) 1–5

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Confirmation of *Neozygites floridana* azygospore formation in two-spotted spider mite (*Tetranychus urticae*) in strains from tropical and temperate regions

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ARTICLE INFO

Article history:
Received 9 April 2014
Accepted 30 July 2014
Available online 6 August 2014

Keywords:
Azygospore formation
Zygospore formation
Neozygites floridana
Tetranychus urticae
Two-spotted spider mite

ABSTRACT

Neozygites floridana is an obligate fungal pathogen of mites in the family Tetranychidae and is an important natural enemy of the two-spotted spider mite (*Tetranychus urticae*). Until now, information about the formation of azygospores remained to be fully confirmed. In this study, we document the formation of azygospores by a Brazilian *N. floridana* strain and the formation of azygospores and zygospores by a Norwegian *N. floridana* strain, both in the host *T. urticae*. Evidence of both zygosporogenesis and azygosporogenesis was also found in the same individual in the Norwegian stains. Further we report the presence of immature azygospores with 1–3 nuclei for the Norwegian strains, immature resting spores (probably azygospores) with 1–8 nuclei for the Brazilian strain, and mature resting spores with 2 nuclei for both the Norwegian and the Brazilian strains (azygo- or zygospores). Our observations suggest that the immature resting spore (prespore) of both strains begins in a multinucleate condition but that the nuclear number is reduced during maturation until mature resting spore is binucleate regardless of its origin as a zygospore or azygospore.

1. Introduction

The entomopathogenic fungal genus *Neozygites* belongs to the order *Neozygitales* in the class *Neozygitomycetes* in the phylum Entomophthoromycota (Humber, 2012). Fungi in this genus attack small arthropods such as mealy bugs, aphids, thrips and mites (Keller, 1991). According to Humber (2012), an extensive amount of data is still needed to reveal important information about the classification and biology of *Neozygites*. The species *Neozygites floridana* (Entomophthoromycota: Neozygitales: Neozygitaceae) belongs to this genus and is pathogenic to several species of mites in the family Tetranychidae (Keller, 1997). For *N. floridana,* important information about fungal structures, especially the formation of azygospores, still remains to be fully confirmed.

According to Keller (1991, 1997) and Keller and Petrini (2005) *Neozygites* resting spores are dark brown to black, spherical or ellipsoid, smooth or ornamented and binucleate, while resting spores of many other Entomophthoromycota are multinucleate (Keller and Petrini, 2005). Keller (1997, 2007) further suggests that a zygospore is developed by budding from a conjugation bridge after a conjugation of two hyphal bodies (Fig. 1). During the early development of the young zygospore it receives one nucleus from each hyphal body (Keller, 1997; Humber, 1989). Subsequently a thick wall is formed and the substantially emptied walls of the hyphal bodies with the remaining nuclei collapse and disintegrate (Keller, 1997, 2007). Further, Keller (1991) suggests that all species in the genus *Neozygites* form zygospores only, while in most other genera in the Entomophthoromycota zygospore and azygospore formation occurs. Weiser (1968), however, reported azygospore formation by *Triplosporium tetranychi* sp. n. (Phycomycetes, Entomophthoraceae), a species close to *N. floridana* (Bałazy, 1993), in its host *Tetranychus althaeae* (syn. *Tetranychus urticae* (Acari: Tetranychidae)) but Keller (1997, 2007) suggested that this finding needs confirmation. Further, Nemoto and Aoki (1975) report observations of *Entomophthora floridana* (syn. *N. floridana*) azygospores in the host *Oligonychus hondoensis* (Acari: Tetranychidae), and Ishikawa (2010) reports of formation of azygospores of *Neozygites* sp. in the host *Tetranychus kanzawai* (Acari: Tetranychidae).

In this paper we describe and confirm the formation of azygospores and zygospores by *N. floridana* in the host *T. urticae* in strains from Brazil and Norway.

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http://dx.doi.org/10.1016/j.jip.2014.07.008
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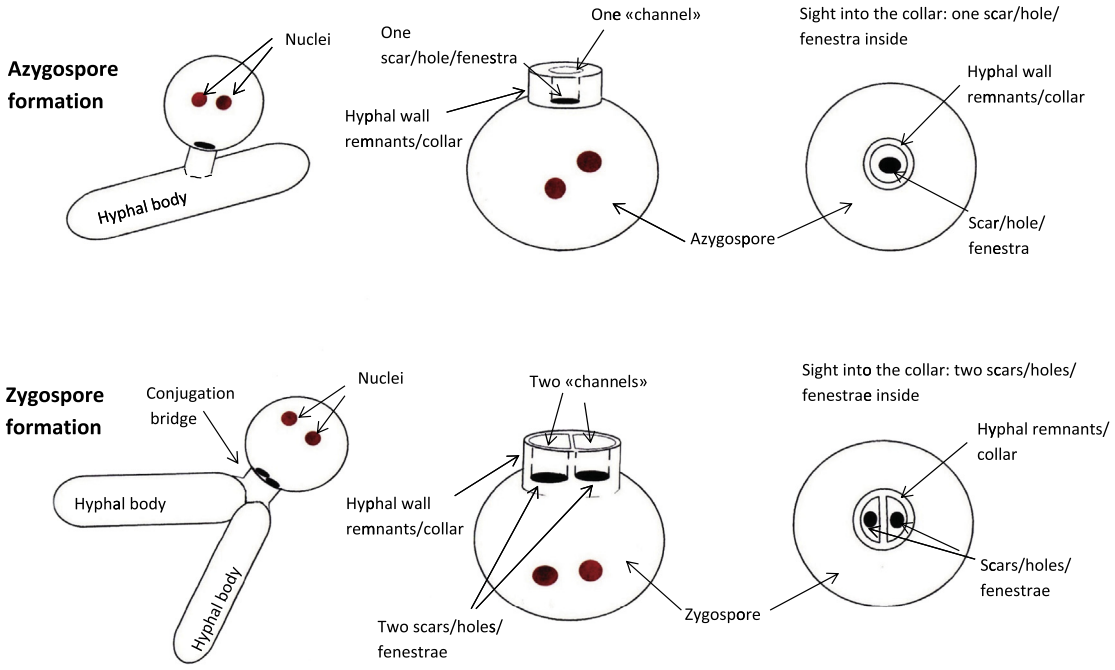

Fig. 1. Schematic illustration and terms used in *N. floridana* azygospore and zygospore formation.

2. Materials and methods

To investigate the possible formation of azygo- and zygospores, preserved slides of *T. urticae* infected with *N. floridana* of a Brazilian strain (ESALQ 1420) and a Norwegian strain (NCRI 271/04) were obtained from a laboratory experiment on induction of resting spore formation at 11 °C and 15 °C (Duarte et al., 2013). Further, 15 preserved slides containing cadavers with resting spores collected from different locations in Norwegian strawberry fields (Lier in Buskerud (59°47′N, 10°16′E) and Kise in Hedmark (60°46′N, 10°48′E) were used in this study. A total of 229 Norwegian and 209 Brazilian slides were observed for resting spores. Out of these, only 17 Brazilian and 18 Norwegian slides where further studied to observe for zygo- or azogospore formation. Obtained slides with *T. urticae* infected with *N. floridana* had been squash-mounted in 0.0075% cotton blue in 50% lactic acid and were observed under a phase contrast microscope at 100–600×. The following structures mentioned below and in Fig. 1 were of special interest to be able to investigate the possible formation of azygo- or zygospores: (1) Budding of hyphal bodies. (2) Number of nuclei inside budding hypal bodies. (3) Number of nuclei inside immature (prespores) and mature resting spores. (4) Numbers (one or two) of fenestrae inside emptied hyphal wall remnants (collars) of the resting spores. Top-down view into the collar is necessary to observe this. (5) Another way to determine if the resting spore is an azygo- or zygospore would be to look at the emptied hyphal wall remnants, which according to Humber (1981) provide the only temporary evidence for the mode of formation of mature resting spores in Entomophthoromycota by determining the "pedigree" of these resting spores. The observations reported in this study were found in three or more mites unless other is stated in the text.

3. Results

3.1. Brazilian strain ESALQ 1420

Only azygospore formation was observed in the Brazilian isolate in this study. In *N. floridana*-infected *T. urticae* (squash-mounted while still living) we found that young azygospores developed by budding from terminal or lateral positions on the hyphal bodies (Fig. 2A and B). Most of the time only one azygospore was seen budding from each hyphal body (Fig. 2A) but we also observed rarely that two buds were formed from the same hyphal body (Fig. 2B), although the fate of these dual azygosporogenesis is unknown.

In most of the squash-mounts of *N. floridana*-killed *T. urticae* cadavers, the fungus had completed the budding stage and was seen as immature resting spores. Hence, it was not possible to observe conjugation of hyphal bodies (zygospore formation) or budding from a single hypha (azygospore formation). The hyphal bodies normally had four nuclei prior to budding, and in some of the observations of buddings it seemed like only one nucleus was transferred from the hyphal body and into the budding azygospore (Fig. 2C). A variety of number of nuclei (from 1 to 8) were observed in the immature resting spores. Some of these immature spores seemed to contain only a single large nucleus (Fig. 2D), and some displayed the nuclei in a diffuse state while others clearly had two or more distinctly delimited nuclei (Fig. 2E). In older but still immature (almost mature) resting spores and in mature resting spores, two nuclei were most often seen (Fig. 2F and G). Immature resting spores from the Brazilian strain varied in size and shape (Fig. 2D and H) while the almost mature and mature resting spores were more uniformly subglobose to obovoid (Fig. 2F and G). The mature resting spores have a dark brown melanized episporium (outer wall) that was smooth (Fig. 2G). Immature resting spores appeared in swollen cadavers with a light gray to a light brown color, and mature resting spores were found in dark brown to black cadavers that were totally filled with resting spores (Fig. 2H).

3.2. Norwegian strains

The Norwegian strain, NCRI 271/04, produced both azygo- and zygospores in *T. urticae*. Both azygosporogenesis and zygosporogenesis could be seen in the same individuals (Fig. 3A). Zygospores formed at the conjugation point between two hyphal bodies (Fig. 3A and B), and azygospores bud from any position on the hyphal body (not shown). We had few observations of nuclei in

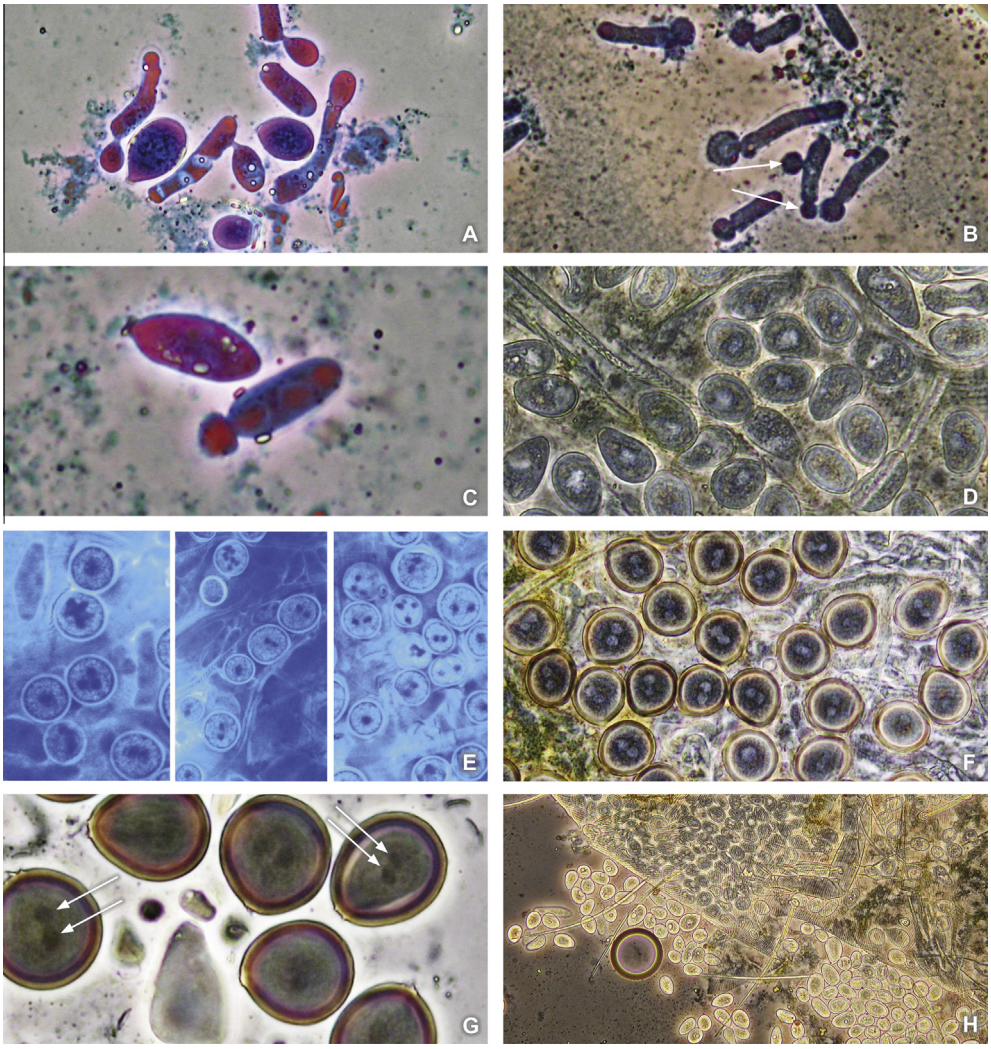

Fig. 2. Formation of resting spores of *Neozygites floridana* in *Tetranyhus urticae* in the Brazilian strain ESALQ 1420. (A and B) Azygospores budding from hyphal bodies. Arrows shows two buds from the same hyphal body. (C) An azygospore budding from a hyphal body, the nuclei are seen inside the spore and hyphal body. (D) Immature resting spores with one large nucleus. (E) Immature resting spores (prespores) with different number of nuclei. (F) Almost mature resting spores with two nuclei. (G) Mature resting spores with two nuclei (arrows). (H) Squash mounted cadaver of *T. urticae* totally filled with immature resting spores.

this strain due to few mites with resting spores, but in one mite, 1–3 nuclei were observed in immature azygospores (not shown). Mature resting spores displayed two nuclei (not shown) but whether these were azygo- or zygospores could not be confirmed.

Both azygo- and zygosporogenesis were also found in *N. floridana*-killed *T. urticae* cadavers collected from the two different strawberry locations (Lier and Kise) in Norway (Fig. 3C and D). Immature resting spores were seen with 1–3 nuclei but mostly two nuclei were observed (Fig. 3C). Mature resting spores in some cadavers displayed two nuclei (Fig. 3E) but whether these were azygo- or zygospores were not possible to confirm.

We were not able to observe resting spore in top-down-views and were therefore not able to see the fenestrae, but we were able to indicate azygosporogenesis by observing the remnants from the attachment of one hyphal body (gametangium) (Fig. 3F) and to indicate zygosporogenesis by observing the remnants from attachment of two gametangia (Fig. 3G). Further, Fig. 3H indicates azygo- and zygosporogenesis in the same mite given that both the two structures shown in Fig. 3F and G is present in the same individual. Most mature resting spores had distinct remnants from the attachment to the hyphal body/bodies (Fig. 3F–H).

The mature resting spores in the Norwegian strains were usually globose to subglobose, and they were surrounded by a dark brown melanized rough episporium (Fig. 3E–H) but in some cadavers resting spores had an ellipsoidal shape and smooth episporium (not shown). The cadavers were not totally filled with resting spores (Fig. 3I) as with the Brazilian strain. Formation of both conidia and resting spores in the same mite was commonly seen (Fig. 3J).

4. Discussion

In this study we have documented the formation of azygospores in the Brazilian strain and both azygo- and zygospore formation in Norwegian strains of *N. floridana*-infected *T. urticae*. This is the first full confirmation of the formation of azygospores in *N. floridana*-infected *T. urticae*. Weiser (1968) was, however, the first to report azygospore formation in *N.* (=*Triplosporium*) *tetranychi* in *T. althaeae* in Czechoslovakia, and his illustration of azygospore formation and the shape of the resting spores is comparable with our observations for the Brazilian *N. floridana* strain. Weiser (1968) did not observe any conjugation of hyphal bodies and hence no zygospore formation. In earlier unpublished studies, only zygosporogenesis was, however, observed for Brazilian strains of *N. floridana* and *Neozygites tanajoae*. Mietkiewski et al. (1993) observed

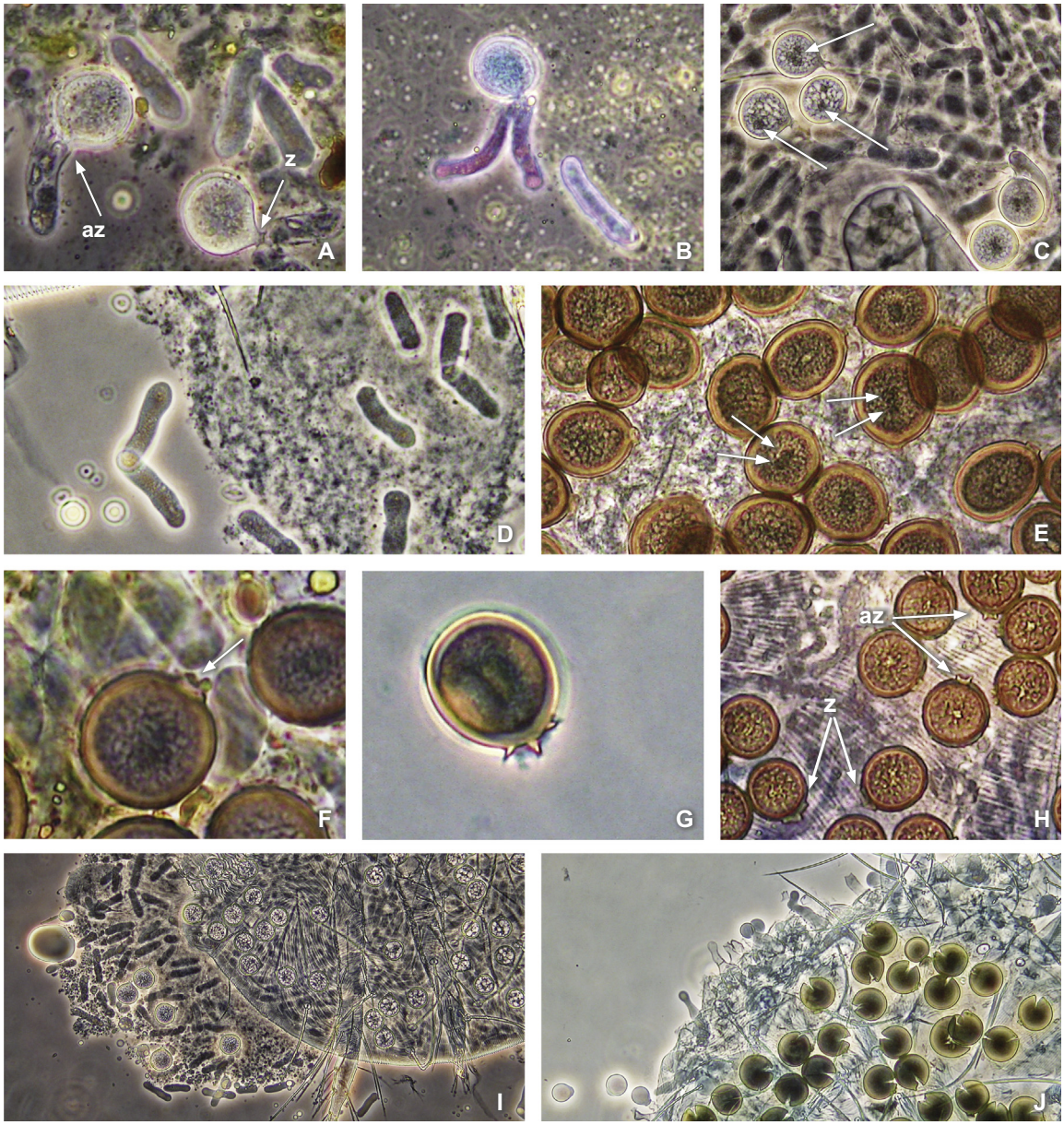

Fig. 3. Formation of resting spores of *Neozygites floridana* in *Tetranyhus urticae* in Norwegian strain NCRI 271/04 (A, B, F and G) and different cadavers found in the field (C–E and H–J). (A) Zygospore (z) and azygospore (az) formation in the same mite. (B) Zygospore formation. (C) Immature resting spore, azygospore formation. Arrows show one, two and three nuclei inside the spores. (D) Hyphal bodies conjugating before zygosporogenesis. (E) Mature resting spores with two nuclei (arrows). (F) Mature resting spores (azygospore) with narrow remains from attachment to a gametangium. (G) Mature resting spore (zygospore) with wide remains from two gametangia. (H) Mature resting spores with narrow and wide remains from attachment to gametangia, both zygospores (z) and azygospores (az) in the same individual mite. (I) Squash mounted cadaver of *T. urticae* with immature resting spores (azygo- or zygospore not known). (J) Cadaver of *T. urticae* containing resting spores and producing primary conidia simultaneously.

no conjugation of hyphal bodies in Polish material of *N. floridana* in *T. urticae* despite numerous careful analyses of hyphal bodies in different developmental phases and could therefore not confirm zygospore formation. Further, they do not report whether azygospore formation was observed. Nemoto and Aoki (1975) report of azygospores budding from clavate hyphal bodies of *E. floridana* in the spider mite *O. hondoensis* and they could not find binucleate zygospores. Ishikawa (2010) observed formation of azygospores by *Neozygites* sp. (*N. tetranychi* or *N. floridana*) in the spider mite host *T. kanzawai*.

Humber (2012) states that in *Neozygitomycetes* mature resting spores (zygospores) may have two adjacent round fenestrae ('holes' in the episporium) that raise a ridge of gametangial wall remnant between them. This supports our findings of remnants from the attachment of hyphal body/bodies to the resting spore both for the Norwegian and the Brazilian strains, in both immature and mature resting spores. Generally less distinct hyphal remnants were observed for the Brazilian strain than for the Norwegian strain (Figs. 2D and F–G and 3F–H). For some of the remnants on the resting spore of the Norwegian strains it looks like only one hyphal body might have been attached to the spore, and we therefore suggest that these might be azygospores (Fig. 3F), while, as mentioned in Humber (1981) and earlier in this paper, the doubled gametangial remnants on other spores suggest that two hyphal bodies were attached to the spore and that these spores are probably zygospores (Fig. 3G and H). Weiser (1968) describes that in

some cases there were a collar of remnants of the hypha around the round suture of the scar (azygospores) of *T. tetranychi* in the spider mite host *T. athaeae*. His illustrations look similar to the Brazilian strain with rather indistinct remnants.

We further document immature azygospores with 1–3 nuclei (Norwegian strains), immature resting spores (probably azygospores) with 1–8 nuclei (Brazilian strain) and mature resting spores with two nuclei (Norwegian and Brazilian strains, azygo- or zygospores). Weiser (1968) describes two nuclei inside mature azygospores of the fungus *T. tetranychi,* which is close to *N. floridana,* in *T. althaeae*. Also according to Humber (1989), Keller (1991, 1997) and Keller and Petrini (2005), zygospores in *Neozygites* are binucleate. We observed that hyphal bodies in the mites normally had four nuclei and that one nucleus might be transferred to the budding azygospore (Fig. 2C). Keller (1997) described that the cells of neozygitoid fungi exert strong control over nuclear number and, perhaps most significantly, a round of mitosis in gametangia immediately preceding conjugation and zygosporogenesis. However, Delalibera et al. (2004) observed that zygosporogenesis in *N. tanajoae* is preceded by reduction in nuclei number from the usual 3–4 to only two nuclei in gametangial cells. Our observations seems to correspond well with the results found by McCabe et al. (1984) for the fungi *Entomophaga grylli*, *Conidiobolus thromboides* and *Erynia radicans* since they report that the immature resting spore (prespore) begins in a multinucleate condition but that the nuclei are lost during maturation until the mature resting spore is binucleate regardless of their origins as zygospores or azygospores.

By comparing the pictures of immature and mature resting spores in the Norwegian and the Brazilian *N. floridana* strains we observed that resting spores produced by the Norwegian strains are more uniform in size and shape and are more globose to subglobose (Fig. 3) than the Brazilian strain that is subglobose to obovoid (Fig. 2). Further, *T. urticae* killed by the Brazilian strain were totally filled with resting spores (Fig. 2H) while *T. urticae* killed by the Norwegian strains contained fewer resting spores (Fig. 3I). We also observed that *T. urticae* killed by Norwegian strains usually produced primary conidia, capilliconidia and resting spores in the same cadaver while this was not observed for the Brazilian strain. Nemoto and Aoki (1975) observed, however, both conidial formation and resting spores in some individuals of *N.* (=*Entomophthora*) *floridana*-infected *O. hondoensis*. This was also the case for *Neozygites tetranychi*-killed *T. althaeae* and *T. urticae* from Czechoslovakia (Keller, 1997).

More detailed studies are necessary to clarify what happens with the nuclei in the gametangia before formation of resting spores and also with the nuclei inside the immature resting spores during formation of mature azygo- and zygospores for the Brazilian and Norwegian strains.

Acknowledgments

This research was funded by the Norwegian Foundation for Research Levy on Agricultural Products (FFL) and the Agricultural Agreement Research Funds (JA) through the BERRYSYS project www.bioforsk.no/berrysys (Project number 190407/199) and from The National Council for Scientific and Technological Development (CNPq) in Brazil. We thank Dr. Erling Fløistad at Bioforsk for help with editing the figures.

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