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Survival of the Teliosporogenous Mycelia of *Neovossia indica* in Infected Wheat Grains

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Abstract: In this investigation, survival of the teliosporogenous mycelia in 6, 7, 8 and 9 months old bunted kernels which had been infected artificially in the laboratory was examined. The sorus of each treatment was crashed in sterile distilled water and the suspension was placed on the surface of sterile distilled water in a completely randomized design with 5 replicates per treatment. Ten to 15 days later, floccose erected mycelia showing characteristics of the teliosporogenous mycelia appeared in the plates containing suspension that had been prepared from 6-8 months old infected kernels. Fifteen days after inoculation, colony numbers of teliosporogenous mycelia per plate were counted. Two- three weeks after transferring the mycelia to fresh YPDA, teliospores formed on this medium. After 4 weeks, mean teliospore formation on YPDA at 20°C and under laboratory conditions were 591.5 and 832 per plate, respectively. Also the teliosporogenous mycelial growth rate on YPDA at 20°C and room temperature were 2.31 and 1.85 mm day^{-1}, respectively. Fungus teliosporogenesis was studied *in vitro* and *in situ* by light, fluorescent and scanning electron microscope. During the teliosporogenesis, teliospore initials were formed in apical position in a lateral right-angled outgrowth of the teliosporogenous hyphae. The dikaryotic plasma was concentrated in the apical portion of the hypha. Fusion of nuclei occurred during the early enlargement of teliospore initial. The swelling, pyriform to spherical protoplast of the teliospore initial was delimited from the empty part of teliosporogenous hypha by a sheath, which was hyaline as observed by LM. The empty part of the hypha may form appendages. Underneath the sheath, the exosporium with ornamented surface and the smooth endosporium was deposited as seen in the mature teliospores. This is the first report of survival of the teliosporogenous mycelia in the Karnal bunt infected kernels after several months of storage. This may be of great importance in pathogen survival and disease epidemiology.

Key words: Teliosporogenous, teliosporogenesis, dikaryotic, mycelia, karnal bunt, *Neovossia indica*

INTRODUCTION

Neovossia indica Mundkur (Mitra), the causal agent of Karnal bunt of wheat, was added to the list of smut fungi parasitizing wheat in India over 75 years ago (Mitra, 1931). Since then the disease has been reported from Pakistan (Munjal, 1975), Syria and Afghanistan (Locke and Watson, 1955; Fuentes-Davila, 1996), Mexico (Duran, 1972), Nepal (Singh *et al*.,1989), USA (Ykama *et al*., 1996; Bonde *et al*., 1997; Bonde and smilanick, 2000; Marshall and Work, 2003; Pascoe and Priest, 2005; Rush and Riemenschneider, 2005) and South Africa (Crous *et al*., 2001).

Wheat contamination with *N. indica* is subject to the international regulation by 78 countries (Dowell and Boratynski, 2002). In spite of the fact that Karnal bunt was considered as a quarantine disease in Iran, it was reported from Jiroft, in Kerman province in 1996 (Torabi *et al*., 1996). Further investigations revealed the presence of disease in some other provinces like Fars and Hormozgan (Behroozin, 1997; Alizadeh and Saeedi, 1999). Since then, disease has affected almost one hundred thousands of ha. of the fields in the mentioned provinces. The amount of infection varied from 2 to 28% based on the sampling of intercepted wheat and wheat field (Alizadeh and Saeedi, 1999).

Many studies have been conducted to assess the effect of soil type and irrespective of weather on the survival and longevity of *N. indica* teliospores in soil (Babadoost and Mathre, 2004; Bonde and Berner, 2004; Bonde and Nester, 2004). The results of the above studies have revealed that the teliospores could remain viable in soil for more than 32 months in Montana. Bonde and Nester (2004) have shown that neither field site nor soil depth have any effect on the total number of viable teliospores or on the teliospore germination percentages. In the previous studies teliosporogenous mycelia were isolated from artificially inoculated young caryopses (Fuentes-Davila and Duran, 1986; Moosawi-Jorf and Alizadeh, 2000) and observed by light and electron

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microscope using preparation of thin sections from bunted grains (Bauer *et al*., 1997), However the survival of this viable mycelia in the stored infected kernels have not been investigated.

In this study, for the first time, we showed that the viable dikaryotic (teliosporogenous) mycelia can survive in infected kernels for 8 months, then the successive stages in teliospore formation process were observed by light and fluorescent microscope. More ever, teliosporogenesis was investigated using scanning electron microscopy.

MATERIALS AND METHODS

Preparation of inoculim and infected kernels: A wheat cultivar, WL711, highly susceptible to Karnal bunt (Aujla *et al*.,1990; Alizadeh and Saeedi, 1999) was selected for this study. Plants were grown in pots in greenhouse at 17-25°C under natural light (about 13 h day^{-1}) in cereal pathological lab of Seed and Plant Institute, Karaj, Iran.

Bunted kernels were collected from Dorz-o-Sayeban village, Lar, in Fars province, Iran and the teliospores were harvested. The teliospores were germinated on 2% water agar at 20°C. A culture of germinated teliospores was inverted over a petri dish containing Potato-Dextrose Agar amended with 0.1% Yeast extract (YPDA) for 24 h at 20°C. During this time period, allantoid secondary sporidia showered onto YPDA. The YPDA plates then were incubated at 20°C. Within 4-5 days a white, floccose colony was formed and actively discharged abundant secondary sporidia (Aujla *et al*.,1990).

To prepare infected Kernels, plants were injected with a suspension of 2×10^5 sporidia per ml by a hypodermic syringe (Fuentes-Davila, 1996). Inoculated spikes were sprayed with distilled water and covered with transparent plastic bags. The treated plants were incubated for 24 h. at 20°C under continuous light from two 40W fluorescent bulbs and kept in this condition until maturity. After that, grains were harvested and stored for 6, 7, 8 and 9 months in the laboratory conditions. Then the infected kernels were examined for survival of the teliosporogenous mycelia in Plant Protection Lab, Shahid Chamran University, Ahwaz, Iran.

Isolation of teliosporogenous mycelia from infected grains: From each set of the 6, 7, 8 and 9 months old artificially infected wheat kernels, one infected kernel with infection coefficient number 3 (CI = 3) was selected. The infected kernel which had relatively large and closed sorus, was disinfected in 0.5% sodium hypochlorite for 2 min and rinsed with sterile distilled water three times. Then the sorus was crashed by a scalpel in 10 mL sterilized distilled water. One milliliter of the prepared suspension was placed over the surface of sterile distilled water in each plate. The rest of the suspension was treated with a solution containing 500ppm lactic acid for a night then placed in the plates in the same manner as explained above as negative control (Krishna and Singh, 1983). The experiment was conducted in a completely randomized design with 5 replicates per treatment. The petri plates were incubated at 20°C and 12 h. light period. After 15 days, the mean numbers of colonies of the teliosporogenous mycelia were counted in each treatment and then, the mycelia were transferred to the PDA amended with 0.1% yeast extract (YPDA).

Growth rate of the teliosporogenous mycelia: Seven mm blocks of the colony margin of the teliosporogenous mycelia were cut out and put in the center of the plates containing YPDA. A series of plates were maintained in an incubator at 20°C and 12 h. photoperiod and another series were kept in room temperature. The experiment was conducted in a Completely Randomized Design (CRD) with four replicates per treatment. Two days later, the margin of colonies were marked and considered as acclimation period. After 4 weeks, increments in colony diameter were measured (mm day^{-1}).

Teliosporogenesis in axenic culture: Teliosporogenous mycelia were cultured on the YPDA and incubated in the conditions as mentioned above. Plates were arranged in a CRD with four replications. After 4 weeks, for counting the teliospores that have been formed from teliosporogenous mycelia, the containing of each petri dish was mixed in a blender with a small amount of water, filtered using two layers of cheese cloth and the results were passed through a 500-mesh sieve. The remaining residue on the sieve was resuspended in 50 mL water. Then 1 mL of this suspension was placed in a small quarter glass. The number of teliospores were counted by means of a binocular (x50). The teliospore counting was repeated 5 times.

Light microscopy of teliosporogenesis: In different stages of growth, the mycelial cultures of teliosporogenous hyphae were fixed by flooding cultures with a solution of 4% formaldehyde in 0.25 M sucrose, at pH of 7.0 for a minimum of 18 h at 5°C. Then, specimens were post fixed in 70% ethanol for at least 24 h at 5°C. After that, the solution was removed and the specimens were washed twice in sterile distilled water (Therrien *et al*., 1988). Then the teliosporogenous hyphae and different stages of teliospore formation were stained with HCl-Giemsa, ethidium bromide (1.0 μg mL^{-1}, in 0.1 M phosphate buffer, pH 7.2) and acridine orange (0.1 mg mL^{-1} in 0.1 M phosphate buffer, pH 6.0) (Kiernan, 1990; Thinggaard and Leth, 2003). The specimens that stained with HCl-Giemsa were observed by light microscope and those stained with ethidium bromide and acridine orange were observed by an Olympus microscope, model BX50 equipped with

epifluorescent condenser using WG (520-545 nm excitation wavelength) and WB (490-500 nm excitation wavelength) fluorescence cubes, respectively.

Scanning electron microscopy (SEM) of teliosporogenesis: The sori of infected grains were sectioned transversely and longitudinally. The sections were immersed in 2% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 24 h, at room temperature, then washed in buffer. Post fixation of the specimens was done using 1% aqueous OsO_4 for at least 2 h and washed again with the buffer (Cashion *et al*., 1988; Bauer *et al*., 1997). The specimens were dehydrated using ethanol series and coated with gold in a sputter coater model SCD005. The samples were observed using Philips scanning electron microscope model XL30 at 25 Kv.

RESULTS

Isolation of teliosporogenous mycelia: Ten to 15 days after incubation, floccose erected mycelia with characteristics of the teliosporogenous mycelia of *N. indica* appeared on the surface of sterile distilled water in 6-8 months old grains. These mycelia were transferred to the fresh YPDA. After 15-20 days, teliospores were formed on this media (Fig. 1). No teliosporogenous mycelia were grown in the control plates, indicating that the mycelia were not originated directly or indirectly from teliospores.

Colony numbers of the teliosporogenous mycelia in each treatment were counted 15 days after inoculation (Table 1). Data analysis using SAS program indicated that there was a significant difference between the means of the colony numbers of teliosporogenous mycelia in each treatment. Based on mean comparison of these differences using LSD test (p = 0.05) four groups were identified in the treatments. No teliosporogenous mycelia were observed in negative control (Table 1). According to the dilution factor (X10) the mycelial units capable of forming colony was determined in each sorus (cfu/sorus) with infection coefficient of number 3 (Table 1). The investigated mycelia were isolated and transferred to the YPDA. Teliosporogenesis was started 15-20 days after transforming (Fig. 1). In this process, the teliospores were formed only apically (Fig. 1) and they became mature after 3-4 weeks (Fig. 1).

Growth rate of the teliosporogenous mycelia: The average of the growth rate of teliosporogenous mycelia on the YPDA in incubator (20°C and 12 h photoperiod) and laboratory condition was 2.31 and 1.85 mm day^{-1} respectively (Table 2). Data analysis using, respectively (Table 2). Data analysis using SAS program showed that there was a significant difference between the means of the growth rates in incubator and room temperature conditions. Using OSD test (p = 0.05), indicated the significant difference between these treatments (Table 2).

Table 1: Colony number means of the teliosporogenous mycelia of *N. indica* treated with and without acid, isolated from infected grain which were stored for several months after harvesting

	Months after harvesting			
Treatments	6	7	8	9
sterile distilled water	10.2a* (102 cfu/sorus)	4.6b (46 cfu/sorus)	0.6c (6 cfu/sorus)	0c
Lactic acid (negative check)	0	0	0	0

*Data not followed by the same latter are significantly (p = 0.05) different according to LSD test

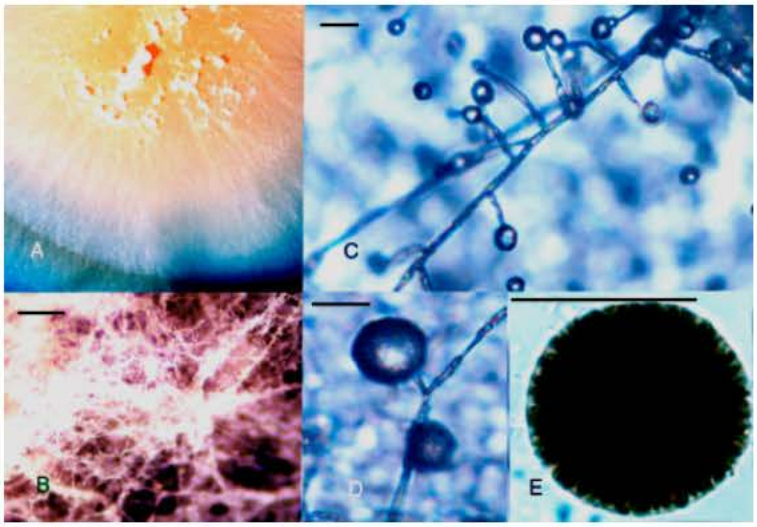

Fig. 1: A,B: Isolated the teliosporogenous mycelia of *Neovossia indica* in the medium, (B: bar = 100 μm), C, D-Teliospores formed on the tips of short lateral outgrowths of the teliosporogenous mycelia, (bar = 40 and 20 μm, respectively), E-Mature teliospore with developed exosporium wall (bar = 20 μm)

Teliosporogenesis in axenic culture: The mean number of the teliospores formed on the YPDA at 20°C (incubator) and room temperature was 591.5 and 832 in each petri plate, respectively (Table 2). Data analysis using SAS program indicated that there was a significant difference between the means of the teliospores number formed in both conditions. According to the LSD test (p = 0.05), the mean number of the teliospores that formed on medium were categorized into two groups (Table 2).

Table 2: Mean comparison of growth rate of teliosporogenous mycelia and teliospore formation rate in the YPDA medium, at 20°C and room temperature

	Growth rate of telio sporogenous mycelia		Teliospore formation rate	
Incubation	Mean (mm day^{-1})	Groups (p = 0.05)*	Mean (No.)	Groups (p = 0.05)*
Incubator (20°C)	2.3	A	591.5	A
Room temperature	1.8	B	832	B

*Data not followed by the same latter are significantly (p = 0.05) different according to LSD test

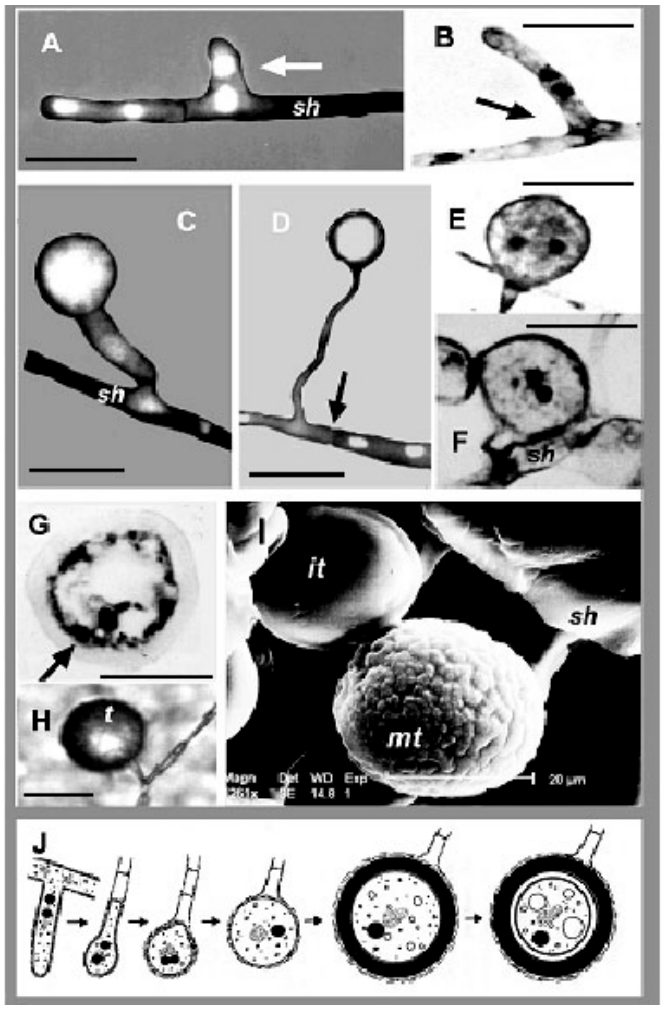

Fig. 2: The teliosporogenous mycelia and teliospore formation of *Neovossia indica, in vitro* and *in situ*. A) right-angled outgrowth (arrow) of the teliosporogenous hypha (sh), bar = 20 µm, B) dikaryotic probasidial initial with Y-shaped septum (arrow), bar = 20 µm, C,D) elongated probasidia with terminal enlargement, sh = teliosporogenous hypha, bar = 20 µm, E, F) stages during which conjugately associated nuclei come progressively closer together, prior to karyogamy, bar = 20 µm, G) immature teliospore, with diploid nucleus (arrow) after karyogamy, bar = 20 µm, H) terminal teliospore formation on the teliosporogenous hypha (sh) in culture, bar = 20 µm, I) scanning electron microscopy of teliospore formation in apical position in an outgrowth of the teliosporogenous hypha (sh), mt = mature teliospore, it = immature teliospore, bar = 20 µm, J) Schematic illustration of teliospore development of *N. indica*. The haploid nuclei of dikaryotic teliosporogenous cells fuse and form diploid nuclei during the early enlargement of teliospore initial. Fig. A and C: stained with ethidium bromide, Fig. D: with acridine orange, Fig. B and E-G with HCl-Giemsa, Fig. H: unstained)

Microscopic studies of the teliosporogenesis: The teliosporogenous hyphae and different stages of the teliospore development were observed by light and fluorescent microscopy. The results indicated that teliospores developed individually by enlargement of the terminal parts of the lateral right-angled outgrowths of the teliosporogenous hyphae known as probasidial initials (Fig. 2A-D). In this process, two associated nuclei migrated into the developing initials (Fig. 2 A and B) and often, Y-shaped septa were formed at the base of the probasidial initials (Fig. 2B). As the initials elongate, the apices enlarged (Fig. 2C and D). The dikaryotic plasma concentrated in the terminal cell of the hypha (Fig. 2 C and D) with the subapical part of the hyphal cell being emptied and eventually segmented by a septum (Fig. 2E and F). Fusion of nuclei occurred during the early enlargement of the teliospore initial (Fig. 2G). In the growth media, teliospores formed terminally on the lateral outgrowths of the teliosporogenous hyphae (Fig. 2H). Studies by scanning electron microscopy revealed that locating and observation the teliospore initials on the teliosporogenous hyphae, in sori, in the infected grains was difficult. The cause of this difficulty was coiling and forming a hymenium-like layer by teliosporogenous hyphae. However, teliospore initials were born on the tips of short lateral outgrowths of teliosporogenous hyphae (Fig. 2I). Underneath the sheath (Fig. 2G) the exosporium, with ornamented surface and the endosporium were deposited (Fig. 2I).

DISCUSSION

This is the first report of the existence of viable teliosporogenous mycelia in the infected wheat kernels several months after harvesting. The existence of dikaryotic mycelia in the grains make it possible for the fungus to continue its sporogenesis in suitable environment after harvesting. Dikaryotic mycelia had been previously isolated from young caryopsis by Fuentes-Davila and Duran (1985) and Moosawi-Jorf and Alizadeh (2000) and growth and sporogenesis of these mycelia were described by the same authors. In their studies, despite repeated subculturing, the fungus continued to form teliospores for 12-18 months. The results of the present study confirmed the result of mentioned above studies. It should be mentioned that in previous studies (Fuentes- Davila and Duran, 1985; Moosawi-Jorf and Alizadeh, 2000) authors investigated teliosporogenesis in the mycelia that had been isolated from young caryopsis, but in the present study, dikaryotic mycelia were isolated from up to 8 months stored infected grains. The treated crashed sori with lactic acid formed no sporogenous mycelia. This indicated that the sporogenous mycelia isolated in this study, were not originated from preformed teliospores in sori, but originated from dikaryotic teliosporogenous mycelia itself. These results clarified that sporogenous mycelia at least could survive 8 months after harvest and continue to produce functional teliospores in suitable condition. Therefore this kind of mycelia could support survival of the fungus to the next growing season. This approves that initial inoculum of wheat Karnal bunt could be initiated from teliospores directly and/or dikaryotic sporogenous mycelia indirectly. However the role of infected kernels in epidemiology of Karnal bunt in the fields remained to be determined (Stein and Maples, 2005).

Besides of the isolation of teliosporogenous mycelia, in this study, teliospore development and its nuclear condition were investigated simultaneously. Type of teliospore formation and its cytology in this study was in full agreement with findings of Krishna and Singh (1983); Fuentes-Davila and Duran (1986) and Robbinson *et al.* (1987).

Krishna and Singh (1983) in their cytological study of teliospore germination showed that mature teliospores contain a single diploid nucleus which divides meiotically as the promycelium emerges. Nuclear stages of the fungus in the culture and host tissue was studied by Fuentes-Davila and Duran (1986). *In vitro*, during the formation of teliospores, closely associated nuclei of teliosporogenous mycelia migrated to probasidial initials which had formed at right-angle to the parental hyphae. Karyogamy was not seen in their investigation but circumstantial evidence suggested that it occurred during early stages of the probasidial development (Fuentes-Davila and Duran, 1986).

In this study, the process of teliospore formation and the concomitant changes in nuclear condition were easily observed in culture media and confirmed the works of Krishna and Singh (1983), Fuentes-Davila and Duran (1986) and Robbinson *et al.* (1987).

Aujla *et al.* (1988) has claimed that when infected florets were directly stained with trypan blue, rounding up of the terminal and intercalary cells of teliosporogenous hyphae would be observed. Aujla *et al.* (1988) suggested that such structures which develops just like chlamydospore and also forms in chains, are teliospore initials. In this study, based on the teliospore development in culture and scanning electron microscopy of infected grains, it was proven that teliospores of *N. indica* forms only in apical position on outgrowths of teliosporogenous hyphae, not as the forms that Aujla *et al.* (1988) has suggest. This kind of teliosporogenesis, that was observed in our study, has

been known as the Tilletia type" (Piepenbring *et al*., 1998). In the *Tilletia type* of spore development, only in certain parts of the teliosporogenous hyphae, terminal, subterminal or intercalary position, teliospores will develop and delimited by a hyaline sheath. This kind of teliospore development occurs in the species such as *Conidiosporomyces*, *Ingoldiomyces*, *Neovossia*, *Oberwinkleria* and *Tilletia* with dusty teliospore mass, as well as in those of *Doassinga*, *Entyloma*, *Erratomyces* and *Rhamphospora* with teliospores permanently embedded in host tissue (Piepenbring *et al*., 1998).

Ultrastructure of teliospore ontogeny has been described by Robbinson *et al.* (1987). Their SEM and TEM indicated that the teliospores originated as a swelling cut off at the tip of a teliosporogenous hypha by a septum. The wall of the parental teliosporogenous cell forms the primary wall of the spore initial, that could fused with the secondary wall which is deposited underneath of the primary layer to form a sheath. Other layers were formed by centripetal deposition of secondary wall material against the primary wall (Robbinson *et al*., 1987). Our SEM observations are confirmed the findings of Robbinson *et al.* (1987).

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