Pak. J. Bot., 46(1): 357-361, 2014.

LOW TEMPERATURE SCANNING ELECTRON MICROSCOPIC STUDIES ON THE INTERACTION OF *GLOBODERA ROSTOCHIENSIS* WOLL. AND *TRICHODERMA HARZIANUM* RIFAI

SAIFULLAH[1] AND NAQIB ULLAH KHAN[2]

[1]*Department of Plant Pathology, The University of Agriculture, Peshawar, Pakistan*
[2]*Department of Plant Breeding and Genetics, The University of Agriculture, Peshawar, Pakistan*
Corresponding author's e-mail:abdulkafi.saifullah@gmail.com

Abstract

Low temperature scanning electron microscopic (LTSEM) studies revealed that *Trichoderma harzianum* infected mature potato cysts nematode eggs by penetrating directly the cyst wall or via natural opening of mouth. Mycelial penetration on cyst wall or egg surface has been seen. The penetration of cyst wall or egg surface was either chemical or mechanical (directly or with appresorium) or both. Freeze fractionation showed the presence of mycelia inside the eggs.

Introduction

Golden nematode of potatoes (PCN) has been recorded since 1881 as an important and cosmopolitan pest of potatoes (Evans & Brodie, 1980). The damage being very serious and worldwide (Evans & Stone, 1977). Chemical applications has been one of the most reliable methods in pest management, however, resistance to most of the conventional chemicals has developed (Delp, 1980, Jatala, 1986; Ahmad & Leather, 1994). Control of deep infestation of PCN in the soil is far more difficult with nematicides. Further more, use of resistant or tolerant cultivars can favor growth of numbers of nematodes that can selectively grow on that cultivar and this is the case with *G. rostochiensis* and *G. pallida* pathotypes. Use of Biological control agents have been used as alternative to chemicals. Among various groups of antagonists, fungi have received much attention because many can be easily cultured and grown on large scale, and stored before use. The start of infection by *T. harzianum* on the cyst wall to the killing of larvae inside the egg observed with LTSEM is reported in this paper.

Materials and Methods

Trichoderma harzianum was isolated on the surface of a mature cyst of PCN with a novel method reported earlier (Saifullah, 1996).

Obtaining clean brown females of PCN

Potato tubers were sown in autoclaved commercial compost in 11 cm diameter plastic pots. Two weeks after sprouting, each plant was inoculated with 35-40 cysts. Vermiculite was washed in water 2-3 times to remove small particles and autoclaved at 121^{o}C for 20 minutes. After three weeks, the plants were carefully removed, roots washed with a water jet and transferred to the vermiculite in pots. The plants were fed with nutrients to keep them green. After about 2 months, clean cysts were obtained by floatation in water. The cysts were collected on a 300 micron mesh sieve. A selection of cysts was checked for any infection on the eggs. The eggs were found healthy and uninfected.

Low Temperature Scanning Electron Microscopy

Penetrating structures on the cyst wall: Cysts were surface-sterilized with 0.1% Na-hypochlorite for one minute and washed ten times with sterile distilled water. These cysts were put on a 10-days old culture of *T. harzianum* for three days. A piece of agar containing cysts on its surface was placed onto a copper stub using carbon dag (Leit-C, Agar Scientific, UK). The stub was inserted into a holder precooled in liquid N_2 at 190^{o}C and the assembly transferred to the cold stage (-190^{o}C) of an Emscope SP200A sputter-cryo system. The cysts were viewed in a JEOL JSM 840A scanning electron microscope with a cold stage at -160^{o}C. Any surface ice present was sublimated by raising the stage temperature to –70^{o}C for 1-2 minutes, as necessary. The specimens were transferred to the SP2000A and sputter-coated with gold before detailed viewing by LTSEM with 5kv electron accelerating voltage.

Penetrating structures on the immature eggs: Fertile young females of *G. rostochiensis* were plucked from the sides of a pot culture under a stereoscopic microscope, 35 days after inoculation. A few cysts were crushed on a slide in a drop of water under a cover slip and the eggs observed at high magnification (X40) with a Dilux 22 (Leitz Germany) microscope. The larvae inside the eggs were at an early stage of embryonic development. The eggs were surface sterilized, washed and incubated on fungal culture for one week. Eggs on a block of agar were processed and viewed by LTSEM as above.

Freeze fracturing: Fifty cysts were placed in groups of 3-5 on a 10 day old culture of the fungus and incubated for 28 days in the dark. A piece of agar containing cysts on its surface was placed onto a recessed copper stub using carbon dag (Leit-C, Agar Scientific, UK.). The stub was inserted into a holder precooled in liquid N2 at -196^{0}C and transferred to a cryostat (Bright Instruments Ltd.), cooled to -30^{0}C and the frozen hydrated specimen trimmed with a cold glass knife on a microtome (Williamson & Duncan, 1989). The stub was then returned to liquid nitrogen, rapidly mounted in a pre-cooled copper holder at -190^{0}C and introduced into an Emscope SP2000A sputter-cryo system. The holder was transferred under vacuum to the cold stage of the SEM and the specimens partially freeze-dried by raising the stage temperature to -70^{o}C for 1-2 minutes. Specimens were sputter coated with gold and viewed by LTSEM as before.

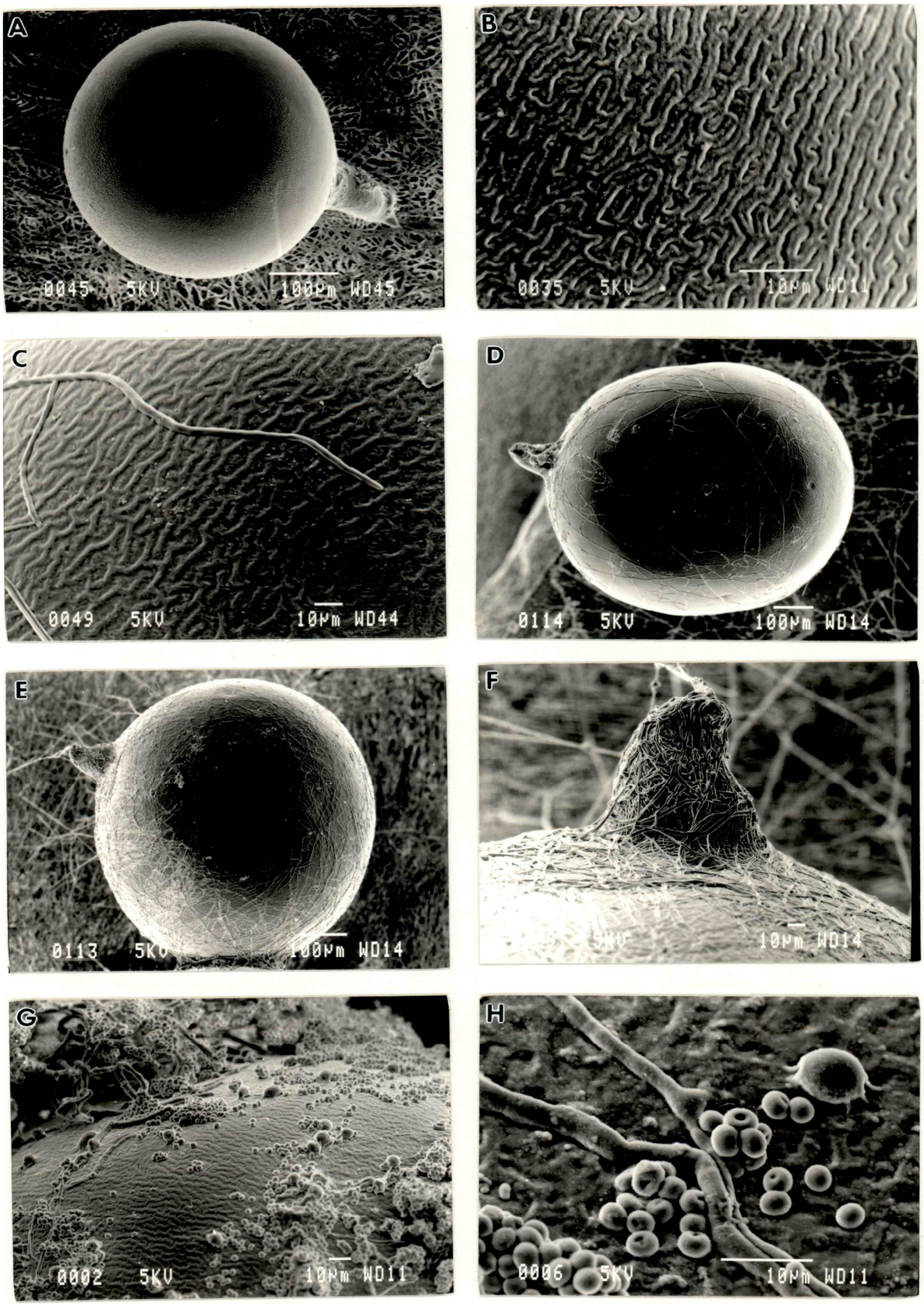

Fig. 1(A-H). Low temperature scanning electron micrographs of *Trichoderma harzianum* on the surface of *Globodera rostochiensis* brown cyst.

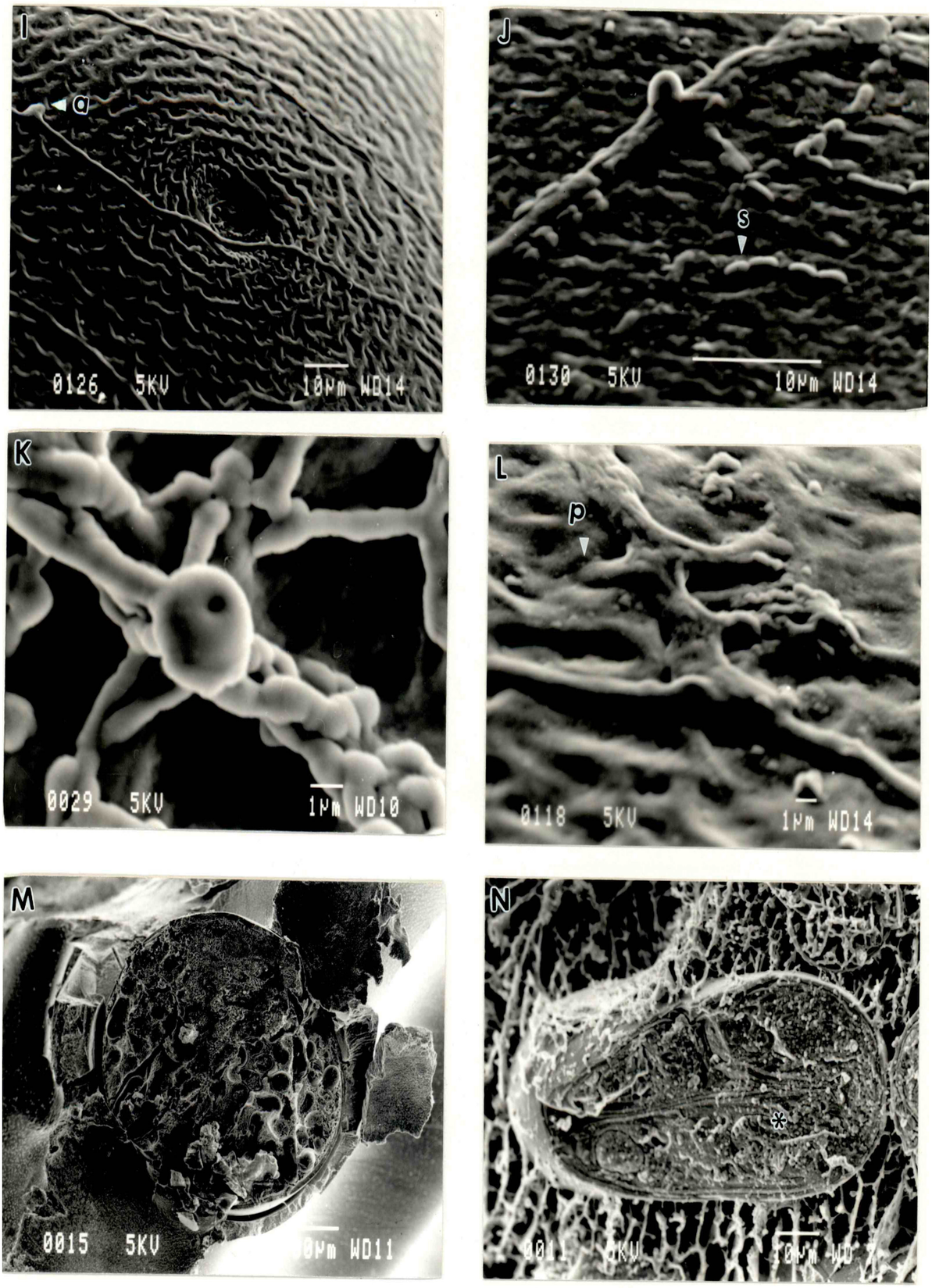

Fig. 2(I-N). Low temperature scanning electron micrographs of *Trichoderma harzianum* on the surface of *Globodera rostochiensis* brown cyst and freeze fractionation.

Results and Discussion

The surfaces of healthy and infected cyst and egg surfaces of *G. rostochiensis* were observed with LTSEM. The infection increased with time. More hyphae accumulated the neck region of the nematode. Hyphae of the fungus were seen penetrating the cyst surface directly (probably) with the help of some enzymes or mechanically with appressorium or both (Fig. 1 A-H, Fig. 2 I-L). Freeze fractionation of the cyst revealed the presence of fungus within the eggs (Fig. 2, M & N). The fungus was found to have chemically digested area on the cyst surface on both sides of hyphae (Fig 2J, K & L). This showed that the fungus destroyed the cyst surface forming a channel in which it was growing. Hyphae of the fungus were seen stitching the cyst or egg surfaces (Fig. 3A-D).

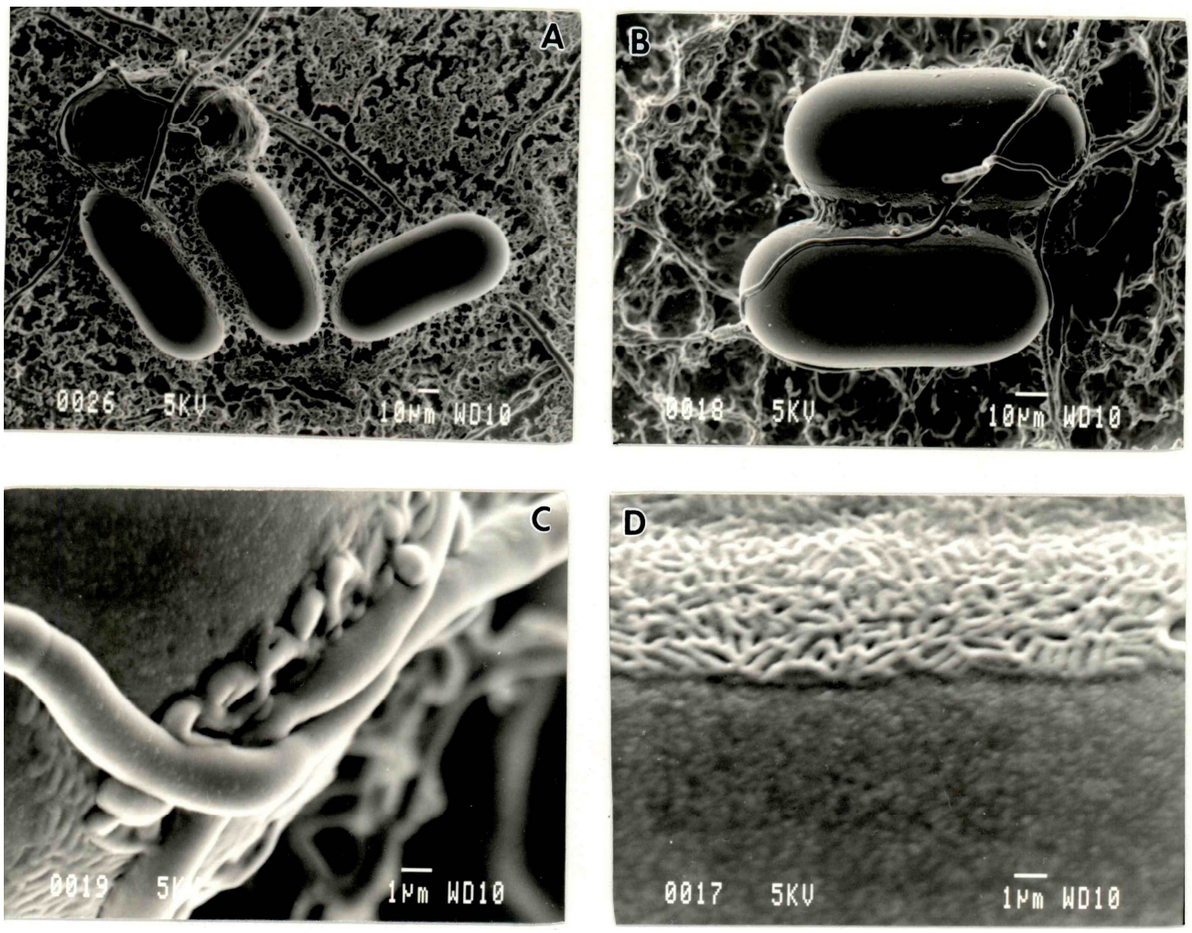

Fig. 3 (A-D). Low temperature scanning electron micrographs of *T. harzianum* growing on surface of immature eggs of *Globodera rostochiensis*.

Species of *Trichoderma* are known as the most promising biocontrol agents against a range of plant pathogenic fungi (Chet and Baker, 1980; Elad *et al*., 1980; Lifshitz *et al*., 1986; Sivan *et al*., 1987). The mechanism by which these fungi kill other pathogenic fungi is probably mycoparasitism which presumably requires the production of enzymes for the digestion of fungal cell walls (Chet, 1987). *Trichoderma* species are known to produce different lytic enzymes such as polysaccharide lyases, proteases and lipases (Cherif & Benhamou, 1990; Harman *et al*., 1993). However, the interaction between the *Trichoderma* species and nematodes have been little explored (Windham *et al*., 1989; Dos Santos *et al*., 1992). Dos Santos *et al*., (1992) reported that *T. harzianum* was a good egg parasite of *Meloidogyne incognita*. Previously, Saifullah and Thomas (1996) found that *T. harzianum* has been found a potent biocontrol agent of potato cyst nematodes. Direct penetration on the surface of a mature cyst and its accumulation at the mouth region was very interesting. This shows that the fungus is able to mechanically penetrate (directly) or indirectly through the mouth region and destroy the eggs.

The technique of low temperature scanning electron microscopy is superior to the conventional SEM which involves critical point drying and dehydration by passing through a series of ethanol of different grades. A small error during dehydration can lead to shrinkage or braking of specimens (Jones & McHardy, 1985). There is also danger of losing important specimens or important structures during dehydration. The LTSEM technique is quick, cheap, time saving and is not laborious.

References

Ahmad, S.I. and S.R. Leather. 1994. Suitability and potential of entomopathogenic microorganisms for frest pest management – some points for consideration. *Int. J. Pest Manag*., 40: 287-292.

Cherif, M. and N. Benhamou. 1990. Cytochemical aspects of chitin breakdown during the parasitic action of *Trichoderma spp*. on *Fusarium oxysporum* f sp. *radicis-lycopersici*. *Phytopathol*., 80: 1406-1414.

Chet, I. 1987. *Trichoderma*-Application, mode of action , and potential as a biocontrol agent of soil borne plant pathogenic fungi. Pp 147-160. *In* Innovative Approaches to Plant Disease Control. (Ed.): I. Chet, John Wiley & Sons, New York.

Chet, I. and R. Baker. 1980. Induction of suppressiveness to *Rhizoctonia solani* in soil. *Phytopathol*., 70: 994-998.

Delp, C.J. 1980. Coping with resistance to Plant Disease control agents. *Plant Dis*., 64: 652-657.

Dos Santos, M.A., S. Ferraz and J.J. Muchov. 1992. Evaluation of 20 species of fungi from Brazil for biocontrol of *Meloidogyne incognita* race-3. *Nematropica*, 22: 183-192.

Elad, Y., I. Chet and J. Katan. 1980. *Trichoderma harzianum*, a biocontrol agent effective agaaisnt *Sclerotium rolfsii* and *Rhizoctonia solani*. *Phytopathol*., 70: 119-121.

Evans, K. and B.B. Brodie. 1980. The origin and distribution of the golden nematode in the USA. *Am. Potato J*., 57: 79-89.

Evans, K. and A.R. Stone. 1977. A review of the distribution and biology of the potato cyst-nematodes *Globodera rostochiensis* and *G. Pallida*. Pest Articles and New Summaries, 23: 178-189.

Harman, G.E., C.K. Hayes, M. Lorito, R.M. Broadway, A. Dipierro, C. Peterbauer and A. Tronsmo. 1993. Chitionlytic enzymes of *Trichoderm harzianum*, purification chitobiosidase and endochitinae. *Phytopathol*., 83: 313-318.

Jatala, P. 1986. Biological control of plant-parasitic nematodes. *Ann. review Phytopathol*., 24: 453-89.

Jones, D. and W.J. McHardy. 1985. Cryofixation techniques with special reference to soil fungi. *Microbiol. Sci*., 2:225

Lifshitz, R., M.T. Windham and R. Baker. 1986. Mechanism of biological control of preemergence damping-off of pea by seed treatment with Trichoderma spp. *Phytopathol*., 76: 720-725.

Saifullah and B.J. Thomas. 1996. Studies on the parasitism of *Globodera rostochiensis* by *Trichoderma harzianum* using Low Temperature Scanning Electron Microscopy. *Afro-Asian J. Nematol*., 6: 117-122.

Saifullah. 1996. A method to isolate nematode destroying fungi from the soil. *Afro-Asian J. Nematol*., 6: 166-167.

Sivan, A., O. Ucko and I. Chet. 1987. Biological control of Fusarium crown rot of tomato by *Trichoderma harzianum* under field conditions. *Plant Dis*., 71: 587-592.

Williamson, B. and G.H. Duncan. 1989. Use of cryo-techniques with scanning electron microscopy to study infection of mature red raspberry fruits by *Botrytis cinera*. *New Phytologist*, 111: 81-88.

Windham, G.L., M.T. Windham and W.P. Williams. 1989. Effect of *Trichoderma spp*. on maize growth and *Meloidogyne arenaria* reproduction. *Plant Dis*., 73:493-495.

(Received for publication 8 February 2012)