Transmission electron microscopic study of the cytological changes in Sclerotium rolfsii parasitized by a biocontrol fungus Trichoderma sp.

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There is a recent trend towards the use of eco-friendly biological control agents for protecting crops from pest and disease, especially soil-borne plant pathogens, as an alternative to existing chemical methods. Among various biocontrol agents, Trichoderma, which has multiple mechanisms for the biocontrol of phytopathogens, is used widely. The present study explores the role of Trichoderma sp. in inducing cytological changes in fungal plant pathogens during parasitization. In dual culture plates, all the fungal isolates (SE6, KT6, KT28, and BRT11) along with a standard culture of T. harzianum were able to antagonize and mycoparasitize two soil-borne fungal phytopathogens (Sclerotium rolfsii and Rhizoctonia solani) of chickpea wilt complex. The suppression of S. rolfsii was slower than that of R. solani. The interaction between T. harzianum and sclerotia of S. rolfsii was studied by light microscopy and transmission electron microscopy (TEM). Ultra-structural examinations revealed that growth and development of Trichoderma resulted in extensive host cell alterations, such as retraction, aggregation and disintegration of cytoplasmic contents. Lysis and deformation of hyphal cell wall, degradation and disappearance of cytoplasmic contents and loss of cellular integrity in sclerotia of S. rolfsii parasitized by T. harzianum is clearly apparent from transmission electron micrographs.

Keywords: biocontrol; Trichoderma sp.; fungal phytopathogens; TEM

Introduction

Soil-borne phytopathogens cause more than 50% of crop loss in India, with fungi being the most aggressive and causing considerable detriment to crop of economic importance. Several fungal genera have been recognized as playing a major role in root disease complexes of various crops. Among them, wilt-causing fungal pathogens, viz. Sclerotium rolfsii, Rhizoctonia solani and Fusarium oxysporum, are considered the most important, devastating and challenging, being responsible for seed rot, seedling blight, root rot and mature plant wilt, culminating in a 60–70% yield loss of several economic crops (Gupta et al. 1987). Sclerotia produced by these phytopathogens can survive in soil for 3 years and, because they do not all germinate or die at the same time, inocula builds up over time and can render an agricultural area less productive (Suleman et al. 2008). Biological control by antagonistic micro-organisms is a potential, non-chemical and eco-friendly tool for crop protection against phytopathogenic fungi and the management of several plant diseases (Papavizas 1985). Considering the cost of chemical pesticides and hazards involved, biological control of plant diseases is now increasingly being practiced all over the world. The use of microorganisms to antagonize plant pathogens is risk-free when it results in enhancement of resident antagonists (Monte 2001).

Antagonists of phytopathogenic fungi have been used to control plant diseases and 90% of such applications have been carried out with different strains of the fungus Trichoderma. They colonize roots, attack, parasitize and gain nutrition from the parasitized pathogenic fungi, thus enhancing root growth (Korolov et al. 2008). Trichoderma species have developed rhizosphere competence through evolving numerous mechanisms for both attacking other fungi and for enhancing plant and root growth (Segerra et al. 2009). These properties include mycoparasitism, antibiosis, competition for nutrients or space, tolerance to stress through enhanced root and plant development, solubilization and sequestration of organic nutrients, induced resistance and production of cell wall hydrolytic enzymes (Howell et al. 2003). Trichoderma species display the ability to attack the mycelium and sclerotia of the pathogen but very few reports are available on ultrastructural changes in the pathogens. The extraordinary ability of Trichoderma to attack resistant, resting structures is related to the synergistic action of lytic enzymes, including proteases, lipases, glucanases and chitinases (Nicole and Chet 1996).

The present investigation was undertaken to study the interaction between the biocontrol agent and the fungal phytopathogens in dual culture plates and the cytology of parasitism using transmission electron microscopy of the parasitized sclerotia of S. rolfsii.
Materials and methods

Fungal isolates and growth conditions

Four fungal isolates, viz. *Trichoderma virens* (SE6, KT28), *T. viridae* (KT6) and *Aspergillus flavus* (BRT11), were evaluated for their antagonistic activities against two root rot/wilt-causing fungal phytopathogens of chick pea, viz. *Rhizoctonia solani* and *Sclerotium rolfsii*. The fungal cultures were compared for their efficiencies with the standard culture of *Trichoderma harzianum* Rifai, MTCC792 (Th-std). All fungal isolates were obtained from the departmental culture collection and maintained routinely on potato dextrose agar (PDA) medium at 4°C.

Dual culture tests

The interaction of all fungal cultures with two root rot/wilt-causing fungal phytopathogens (*S. rolfsii* and *R. solani*) was tested on PDA by the dual culture plate technique (Dennis and Webster 1971). Mycelial plugs (5 mm in diameter), collected from actively growing colonies of fungal isolate and phytopathogens, were placed 3 cm apart on the surface of the agar and allowed to grow at 28°C. The antagonist and its host grew towards each other. Two weeks later, macroscopic observations of the plates showed that the antagonist multiplied abundantly and established close contact with the sclerotia of the pathogen. Sclerotia of *S. rolfsii* parasitized by *T. harzianum* were carefully removed and processed for TEM.

Light microscopic studies

Sclerotia (dormant structures) of *S. rolfsii* collected either from pure culture or dual culture plates were cut in ultrathin sections (5 μm) using an ultracutter microtome. The sections were stained with methylene blue and observed under light microscope at 400×.

Ultrastructural (TEM) studies

Parasitized and non-parasitized sclerotia of *S. rolfsii*, collected either from pure culture or dual culture plates, were cut into two halves and immediately fixed by immersion in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 12 h at room temperature. The samples were cut into several blocks of 1–2 mm, given three washes of 0.1 M buffer (pH 7.4) for 15 min and again fixed in osmium tetrachloride (secondary fixative) for 2 h. The blocks were dehydrated in three changes of 30, 50, 70, 90 and 100% acetone followed by two changes of 100% toluene for 30 min each, before being transferred to a mixture of equal parts of araldite and toluene overnight and impregnation was carried out for 2 days in each araldite. Blocks were finally embedded in another change of fresh araldite and polymerized for 2 h. Thin sections (70–80 nm) were cut on an ultra-microtome and placed onto uncoated copper grids. The sections were stained in a saturated solution of uranyl acetate and lead citrate for 15 min and examined in a 100-kV JEOL electron microscope.

Results

Antagonistic potential against fungal phytopathogens

All fungal isolates, when evaluated for their in vitro antagonistic potential against *S. rolfsii* and *R. solani*, successfully inhibited the growth of both pathogens (Figure 1). Isolate SE6 was superior, as it completely parasitized both pathogens (*R. solani* and *S. rolfsii*) in the least time (120 and 168 h, respectively). Isolates KT6, KT28 and the standard culture of *T. harzianum* (Th-std) were almost equally effective, as they showed an almost equal period of mycoparasitization for both pathogens. Isolate BRT11 took the longest time for complete parasitism of *R. solani* (176 h) and *S. rolfsii* (224 h) in comparison to other isolates or the standard culture of *T. harzianum* (Table 1). Thus, it was apparent that *R. solani* is more susceptible to fungal antagonists (Figure 2).

Histological changes under light microscopy

Examination of cross-sections of infected and healthy sclerotia of *S. rolfsii* under light microscopy revealed several changes. The healthy sclerotium is composed of two main layers, the outer rind (OR) and the medulla (M);
the outer rind is composed of darkly pigmented, tightly arranged cells, while the medulla consists of multishaped, loosely organized hyphal cells. The outermost rind cells were usually empty, collapsed and sometimes broken, while the cells in the medulla are regular in shape and filled with intensely stained granules and are surrounded by a thick wall that is further surrounded by an amorphous matrix. The matrix forms a bridge between the medullary cells and several hyphal cells are seen embedded in the matrix. The sclerotium parasitized by *T. harzianum* reveals clear cut changes in the medulla. Several deformed and decaying medullary hyphal cells can be visualized in the photomicrograph. Moreover, changes in cellular organization in parasitized sclerotia are also visible. The healthy sections of sclerotium comprise more compact and regular cytoplasmic granular inner content compared to the parasitized sclerotium (Figure 3).

**Ultra structural (TEM) changes in parasitized pathogen**

Observations of ultrathin sections via TEM provide a more elaborate picture of the structural organization of healthy unparasitized sclerotia and cytological alterations caused by biocontrol agent *T. harzianum* during parasitization. The healthy sections under transmission electron microscopy revealed that unparasitized sclerotia comprise healthy hyphal cells surrounded by a thick regular, electron-dense cell wall. The hyphal cells contain regular cytoplasm in which compact and regular granulate intracytoplasmic inclusions are visible. The walls of contiguous cells are separated by an electron-opaque middle lamella. Cell walls are connected to fibrillar matrix through the middle lamella. Thus, this matrix acts as a bridge between neighbouring cells. A close examination of the interface between the wall and the fibrillar matrix revealed that both structures were separated by a thin, electron-opaque fibrillar layer.

Examination of numerous sections of infected or parasitized sclerotia of *S. rolfsii* showed that *Trichoderma harzianum* caused extensive cell alterations, such as retraction and aggregation of the cytoplasm. The cell wall invaded by the antagonistic fungus led to a break in the outer shell of sclerotia causing its destruction, as judged by cell wall disruption. It is clearly visible in the photomicrograph that cellular integrity of the infected hyphal cell was completely lost and there was a deformed structure showing loosening of the cell wall. It is also observed that the compact cytoplasmic granules are gradually degraded and disappear, as indicated by the reduced number of these condensed granules in the hypha of parasitized sclerotia. The highly dense and compact structural organization

### Table 1. Time period required for complete colonization of fungal phytopathogens (*S. rolfsii* and *R. solani*) by biocontrol fungal isolates.

| Cultures | *Sclerotium rolfsii* | *Rhizoctonia solani* |
|----------|----------------------|----------------------|
| SE₆      | 168                  | 120                  |
| KT₆      | 184                  | 136                  |
| KT₂₈     | 184                  | 136                  |
| BRT₁₁    | 224                  | 176                  |
| *T. harzianum* | 176    | 128                  |

Figure 3. Light microscopic photograph of sclerotia of *Sclerotium rolfsii* (a) non-parasitized and (b) parasitized by *Trichoderma harzianum* at 400×. OR, outer rind; M, medulla; MC, medullary cells; I, intracellular matrix; D, cell decay; d, cell deformation.
While investigating for antagonistic potential against the two soil-borne fungal phytopathogens (S. rolfsii and R. solani), a variation in their effectiveness was observed. The magnitude of mycoparasitism was found in the order of T. virens (SE₆) > T. harzianum (Th-std) > T. viridae (KT₆) > Aspergillus flavus (BRT₁₁). Wide variations in the antagonism of a population of T. harzianum have also been reported by Davet (1986). Thus, all the isolates have the capacity to parasitize the pathogens, although the time taken to overgrow the pathogen was different. R. solani was more susceptible to infection by all cultures. Our results are in agreement with a previous investigation (Mishra 1998), which also reported R. solani to be more susceptible to inhibition by the isolates of Trichoderma compared to S. rolfsii and Fusarium oxysporum. On the basis of the observations, it may be concluded that isolate SE₆ has maximum potential for reduction of root rot/wilt disease of plants and is better in comparison to the standard culture of T. harzianum. This potential may be due to higher mycoparasitism of pathogen, antibiosis or synthesis of lytic enzymes by this isolate. Trichoderma is also known to produce several cell wall-degrading enzymes, such as chitinase, β-(1-3) glucanase (Di Pietro et al., 1993), and secondary metabolites, which are involved in antagonisms (Maheshwari et al. 2001).

Transmission electron micrographs of parasitized sclerotium of S. rolfsii showed clear deformation and lysis of the fungal cell wall. Compact cytoplasmic granules also gradually degraded and disappeared, as indicated by the reduced number of these condensed granules in the hypha of parasitized sclerotium. Infected sclerotia become flattened, soft and disintegrated. The present findings are in harmony with those of Sayed and Embaby (2006), who also reported several histological changes, such as malformation, lysis and decay of the outer wall and inner content in infected sclerotia of the pathogenic fungi. Deformation and degradation of cell walls of the pathogen, as observed in the present investigation, may be due to synthesis and excretion of cell wall-degrading enzymes such as chitinase, glucanase and proteinase, by the fungal antagonist T. harzianum (Elad and Monte 2000). However, other mechanisms may also be involved, because most Trichoderma strains produce volatile and non-volatile toxic metabolites and impede colonization by antagonized micro-organisms. Among these metabolites, the production of harzianic acid, almenticins, tricholin, peptaibols, 6-pentyl-α pyrone, viridian and others have been described. Good inhibition of pathogens may also be achieved as some fungal cultures were also equipped with the ability to produce HCN and siderophore, properties reported to antagonize fungal pathogens (Benitez et al. 2004). According to our observations, host cytoplasmic disorganization in the medullar region often occurred in advance of physical contact with T. harzianum, suggesting that diffusible compounds were responsible for the disintegration of infected sclerotia.

Discussion

Biological control of plant pathogens, especially soil-borne plant pathogens, by microorganisms has been considered a more natural and environmentally acceptable alternative to the existing chemical treatment methods. In soil, plant disease suppression by the biocontrol agent Trichoderma sp. has been widely documented. Their dominance in soil may be attributed to their diverse metabolic capability and aggressive competitive nature. Several mechanisms, such as production of a variety of antibiotics, chitinolytic enzymes, growth-promoting hormones, siderophores, HCN and catalase, for plant growth promotion and suppression of phyto-pathogens, by this fungus have been reported. Synthesis of cell wall-degrading (lytic) enzymes is one of common biocontrol mechanisms of this fungus for suppression of fungal pathogens of plant.

Figure 4. Transmission electron micrograph of sclerotia of Sclerotium rolfsii. (a) and (c) Non-parasitized sclerotia at 12,000× and 15,000×, respectively, showing intact hyphal wall. (b) and (d) Parasitized sclerotia at 10,000× and 15,000×, respectively, showing deformation and lysis of hyphal wall and gradual degradation of condensed cytoplasmic content. I, intracellular matrix; ML, middle lamella; CW, cell wall; C, cytoplasm; CC, condensed cytoplasmic contents; i, cell wall lysis; ii, degradation and disappearance of cytoplasmic contents (malformation).
observed disturbances, as also reported by Nicole and Chet (1996).

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