UNIVERSITI PUTRA MALAYSIA

# ENHANCEMENT OF BIOCONTROL ACTIVITIES OF *TRICHODERMA HARZIANUM* RIFAI THROUGH PROTOPLAST FUSION

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By

WONG MUI YUN

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Abstract of thesis submitted to the Senate of Universiti Putra Malaysia in fulfillment of the requirements for the degree of Master of Agricultural Science.

**ENHANCEMENT OF BIOCONTROL ACTIVITIES OF *TRICHODERMA HARZIANUM* RIFAI THROUGH PROTOPLAST FUSION**

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**January 1999**

**Chairperson : Professor Dr. Sariah Meon**

**Faculty : Agriculture**

Enhancement of the biocontrol activities of *Trichoderma harzianum* Rifai against two soilborne pathogens, *Sclerotium rolfsii* and *Ganoderma boninense* through protoplast fusion was attempted. Mycelial cultures from three indigenous isolates from the rhizospheres of groundnut (IMI 378843), chilli (IMI 378844) and oil palm (IMI 378841) were used for the protoplast isolation and fusion studies. The result showed that the optimum release of viable protoplasts was obtained when mycelial cultures at the exponential stage was incubated for 4 h in Novozym 234 (Sigma) as the lytic enzyme at concentration of 7 mg/ml dissolved in 0.7 M NaCl and 0.6 M sorbitol. Pretreatment of mycelium with 0.01 M 2-mercaptoethanol gave no significant difference on protoplast yield of the three isolates studied. The protoplast yield was within the range of $10^6$-$10^8$ protoplasts/ml and the average size of the protoplasts was 2.5-10.0 μm.

Chemically induced fusion, using polyethylene glycol (PEG), among the three isolates yielded a total of 12 fusants. The fused protoplasts

germinated 18 h after incubation in liquid Protoplast Regeneration Medium (PRM). When plated on solid PRM, the fusants regenerated into single colonies between 24–48 h after incubation. Of the 12 fusants obtained, five fusants showed non-parental type in isozyme analysis. They were further evaluated based on the cultural and morphological analysis, biomass growth, antagonistic activities against *S. rolfsii* and *G. boninense*, tolerance to commonly used fungicides, and the ability to produce two extracellular lytic enzymes, β-1,3-glucanase and chitinase.

Despite isozyme banding patterns of the five fusants showing non-parental type, there was similarity in colony and microscopic appearance with the parental isolates. Two fusants (D and E), showed significantly ($p<0.01$) better performance in antagonistic activities against both *S. rolfsii* and *G. boninense* than their parental isolates. All the five fusants showed no improvement in biomass growth and tolerance to sublethal doses of Quintozene, Propiconazole and Penconazole. However, these fusants and their parental isolates showed significantly ($p<0.01$) higher tolerance to these fungicides than the target pathogens. The production of β-1,3-glucanase and chitinase using substrate media were not detected in both the fusants and their parental isolates. Regardless of its genetic basis, the diversity of progeny obtained through protoplast fusion in *T. harzianum* can be used as a source of improved strains for biological control.

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# MEMPERBAIKI AKTIVITI-AKTIVITI KAWALAN BIOLOGI OLEH KULAT *TRICHODERMA HARZIANUM* RIFAI MELALUI PENGGABUNGAN PROTOPLAS

**Oleh**

**WONG MUI YUN**

**Januari 1999**

**Pengerusi : Profesor Dr. Sariah Meon**

**Fakulti : Pertanian**

Percubaan untuk memperbaiki aktiviti-aktiviti kawalan biologi oleh kulat *Trichoderma harzianum* Rifai terhadap dua patogen bawaan tanah *Sclerotium rolfsii* dan *Ganoderma boninense* melalui penggabungan protoplas telah dilakukan. Kultur miselium daripada tiga asingan tempatan yang di ambil dari rhizosfera kacang tanah (IMI 378843), cili (IMI 378844) dan kelapa sawit (IMI 378841) telah digunakan dalam kajian pengasingan dan penggabungan protoplas. Hasil kajian menunjukkan bahawa pembebasan optimum protoplas yang berdayasaing telah diperolehi apabila kultur miselium pada peringkat eksponen dieram selama 4 jam dalam Novozym 234 (Sigma) sebagai enzim litik pada kepekatan 7 mg/ml yang dilarutkan dalam 0.7 M NaCl dan 0.6 M sorbitol. Keputusan praperlakuan miselium dengan 0.01 M 2-mercaptoethanol menunjukkan perbezaan tidak ketara bagi hasil pemprotoplasan dari tiga asingan yang dikaji. Hasil pemprotoplasan yang diperolehi adalah di antara $10^6$-$10^8$ protoplas/ml dan purata saiz protoplas adalah di antara 2.5-10.0 μm.

Penggabungan protoplas secara kimia dengan menggunakan 'polyethylene glycol' (PEG) di antara tiga asingan tersebut menghasilkan sejumlah 12 'fusant'. Protoplas yang telah bergabung itu bercambah dan menghasilkan hifa 18 jam selepas pengeraman dalam media cecair 'Protoplast Regeneration Medium' (PRM). Di atas media pepejal PRM, 'fusant' mengalami regenerasi dan membentuk koloni tunggal di antara 24-48 jam selepas pengeraman. Daripada 12 'fusant' yang diperolehi, lima 'fusant' menunjukkan corak isozim yang berbeza dari induk mereka. Kelima-lima 'fusant' ini kemudian dikaji lebih lanjut dalam aspek kultur dan morfologi, pertumbuhan 'biomass', aktiviti-aktiviti kawalan biologi terhadap *S. rolfsii* and *G. boninense*, toleransi terhadap racun kulat yang biasa digunakan dan keupayaan untuk menghasilkan dua enzim litik, 'β-1,3-glucanase' dan 'chitinase'.

Walaupun corak isozim kelima-lima 'fusant' tersebut menunjukkan perbezaan dengan induk mereka tetapi aspek kultur dan morfologi adalah sama. Dua 'fusant' (D dan E), menunjukkan keupayaan kawalan biologi yang ketara ($p<0.01$) lebih baik terhadap kedua-dua *S. rolfsii* dan *G. boninense* daripada induk mereka. Kelima-lima 'fusant' menunjukkan tiada peningkatan dalam pertumbuhan 'biomass' dan toleransi terhadap Quintozene, Propiconazole dan Penconazole berbanding dengan induk mereka. Walau bagaimanapun, 'fusant' dan induk menunjukkan toleransi yang ketara ($p<0.01$) lebih baik terhadap racun-racun kulat ini berbanding dengan kedua-dua patogen tersebut. Penghasilan enzim litik 'β-1,3-glucanase' dan 'chitinase'dengan induksi menggunakan media substrat tidak dapat dikesan bagi 'fusant' dan induk mereka. Tanpa mengira asas genetik, kepelbagaian progeni yang diperolehi melalui penggabungan protoplas dalam *T. harzianum* boleh digunakan sebagai satu sumber untuk memperbaiki kulat ini bagi tujuan kawalan biologi.

# CHAPTER 1

# INTRODUCTION

Modern agriculture is highly dependent on chemical pesticides. The repeated use of such chemicals has polluted the environment and encouraged the development of resistance among the target organisms. This has resulted in the use of ever-increasing amounts of pesticides. The exposure of human populations and natural habitats to increasing levels of pesticides are becoming unacceptable, and have prompted the search for new strategies for pest and disease control that reduce or possibly eliminate the dose of pesticide required. Biological control has proven to be a potential alternative to the use of chemicals for the management of plant diseases although a small amount of chemicals is needed in certain biocontrol measures, for example, the use of chemicals for induced resistance in plants (Kuc, 1995).

One approach to biological control has been the use of antagonistic microorganisms that compete with, or directly attack, the pathogen. However, with the exception of *Bacillus thuringiensis*, biological control has not found widespread use in commercial agriculture mainly because, to date, control of plant diseases with microbial agents has been less effective and reliable than synthetic fungicides. This is probably due to the less superior performance of the microbial agents. Thus, the increase use of biological control of plant diseases requires identification of highly effective strains and genetic improvement of these strains, as well as improved production and delivery methods.

Fungi in the genus *Trichoderma* has been identified as a potential biocontrol agent against many phytopathogenic fungi. The antagonistic ability of *Trichoderma* was discovered more than 50 years ago (Weindling, 1932). The potential of the fungus to serve as a biocontrol agent was already suggested at that early stage. However, only during the last two decades has a world wide effort been carried out to develop the fungus as a commercial preparation. Today, there is accumulating evidence that *Trichoderma* species which are easily isolated from soil and readily grown, are among the most promising biocontrol agents in terms of large-scale applications.

*Trichoderma harzianum* Rifai is the most studied of the *Trichoderma* species identified for biological control, and is the most effective in disease suppression. *T. harzianum* is known to produce a wide array of extracellular lytic enzymes that are involved in the process of antagonism against pathogenic mycelia and sclerotia (Benhamou and Chet, 1996; Benhamou and Chet, 1993). Chitinolytic enzymes such as endochitinase and chitobiosidase (Lorito *et al.*, 1993a) from *T. harzianum* are active against the broadest range of pathogens and show the highest degree of synergy with other enzymes, or with biological and chemical control agents (Goldman *et al.*, 1994).

A number of *Trichoderma* species have been shown to effectively controlled the following soilborne fungi : *Sclerotium rolfsii* (Jinantana, 1995; Henis *et al.*, 1984), *Rhizoctonia solani*, *Pythium* spp., *Fusarium* spp., *Aspergillus niger* (Lynch, 1987; Chet and Henis, 1985; Elad *et al.*, 1983), *Sclerotium cepivorum* (De Oliveira *et al.*, 1984) and others. *Trichoderma* has also been successfully sprayed against *Botrytis* spp. on strawberries (Tronsmo and Dennis, 1977) and apples (Tronsmo and Ystaas, 1980) in above-ground control.

Besides biocontrol activities, *Trichoderma* enhances germination of seeds and plant growth as measured by increases in weight, height and branch, and flower production (Baker *et al.*, 1984; Harman, 1982). This serves as a valuable factor in using *Trichoderma* as a biocontrol agent. Also, *Trichoderma* can be a very useful and efficient component in the integrated pest and disease management where the integration of isolates of *Trichoderma* resistant to low doses of pesticide can lead to a synergistic effect resulting from suppression of competitive soil microflora. *Trichoderma* was shown to tolerate fungicides such as methyl bromide, PCNB, benomyl, captan, maneb and prothiocarb (Sivan *et al.*, 1984; Ruppel *et al.*, 1983; Papavizas *et al.*, 1982; Hadar *et al.*, 1979; Munnecke *et al.*, 1973).

*Trichoderma* species possess great genetic variability. Some strains have a wide spectrum of biological activity, other strains may control only specific pathogens, while still others may have little or no biocontrol efficacy. Some strains may grow poorly under some environmental conditions, while others grow well under these same adverse conditions. Therefore, an alternate target for future research is on genetic manipulation to enhance the ability of the antagonist to control a wide range of diseases, to adapt to various environmental conditions, to be rhizosphere competent, to tolerate low doses of pesticides, and to be commercially viable.

There are several different processes available for producing improved bioprotectants, namely mutagenesis, the use of recombinant DNA and protoplast fusion. Protoplast fusion is a method of choice for fungi lacking sexual stage such as *Trichoderma* where the occurrence of conventional sexual recombination is either too low or none. Protoplast fusion is a method which efficiently induces heterokaryosis where it allows the recombination in the progeny of different characteristics from two or more parental strains

following the removal of cell wall and exposing the protoplast membrane, processes that are less achievable or impossible with intact cells.

Protoplast fusion has been successfully carried out using *T. reesei* (Toyama *et al.*, 1984), *T. koningii* (Hong *et al.*, 1984) and *T. harzianum* (Sivan and Harman, 1991; Stasz *et al.*, 1988). However, it gave rise to great variability in biocontrol and mycoparasitic ability of the fusants (Migheli *et al.*, 1995; Stasz and Harman, 1990). Therefore, there is still a need to produce superior *Trichoderma* strain which could be used as an effective biocontrol agent, particularly strains which are rhizosphere competent in the local environmental conditions.

Research on indigenous isolates of *T. harzianum* and their potentials is still lacking in Malaysia. Recently, Jinantana (1995) had isolated a few isolates of *T. harzianum* from different rhizospheres at different locations in West Malaysia. Two isolates were identified to be potential antagonists against *Sclerotium rolfsii* based on *in vitro* screening methods but the proliferation rate of these isolates in the soil was poor. Moreover, the performance of these isolates in their tolerance to fungicides is not known.

In this study, an attempt was made to enhance the biocontrol activities of the indigenous isolates of *T. harzianum* Rifai through protoplast fusion. Thus, the objectives of this study are as the following:

1. To isolate and fuse protoplasts from isolates of *Trichoderma harzianum.*
2. To characterize fusants through isozyme analysis, cultural and morphological analysis, biomass growth, tolerance to fungicides and the ability to produce β-1,3-glucanase and chitinase.
3. To evaluate fusants for their biocontrol activities against *Sclerotium rolfsii* and *Ganoderma boninense.*

# CHAPTER 2

# LITERATURE REVIEW

## Biology of *Trichoderma*

In 1969, Rifai distinguished nine species aggregates of *Trichoderma* based on microscopic characters. They are *T. piluliferum*, *T. polysporum*, *T. hamatum*, *T. koningii*, *T. aureoviride*, *T. harzianum*, *T. longibrachiatum*, *T. pseudokoningii*, and *T. viride*.

*Trichoderma* species are saprophytic soil fungi. Most species of *Trichoderma* are photosensitive, sporulating readily on many natural and artificial substrates in a concentric pattern of alternating rings in response to diurnal alternation of light and darkness, with conidia being produced during the light period. Exposure of agar cultures for 20 to 30 seconds to light of 85 to 90 lux intensity is sufficient to induce sporulation. Maximum photoinduction activity occurred at around 380 and 440 nm, with sporulation not occuring below 254 nm or above 1,100 nm (Gressel and Hartmann, 1968). The photoinduced conidiation in *Trichoderma* can be inhibited by chemicals such as azaguanine, 5-fluorouracil, actinomycin D, cycloheximide, phenethyl alcohol and ethidium bromide (Betina and Spisiakova, 1976).

An important aspect of sporulation almost completely disregarded in the last 50 years is the ability of *Trichoderma* to produce chlamydospores (Papavizas, 1985). Although chlamydospores were routinely mentioned in taxonomy papers (Domsch *et al.*, 1980; Rifai, 1969), very little has been

reported on the formation and ecological importance of these structures or their potential role in biological control. Previous reports (Lewis and Papavizas, 1984; Lewis and Papavizas, 1983) have demonstrated the formation of chlamydospores by *T. hamatum*, *T. harzianum*, and *T. viride* in both liquid and solid fermentation media, in sterile soil and soil extracts, and in natural plant debris and amended natural soil. Clamydospores are important structures which enable soil-inhabiting fungi to survive especially when under adverse conditions not conducive to the survival of the smaller, ephemeral, single-walled conidia.

Molecular and biochemical processes involved in germination have largely been ignored and this is perhaps due to the ease with which conidia of *Trichoderma* germinate on many substrates (Papavizas, 1985). The conidia require an external source of nutrients for germination *in vitro* and the response of conidia to nutrients is affected by the H-ion concentration, with germination being greater under acidic conditions than under neutral conditions (Danielson and Davey, 1973).

Even less is known about the germination of chlamydospores *in vitro*. Although fresh chlamydospores germinate well (approximately 75% on nutrient agar (Lewis and Papavizas, 1983), only 13 to 31% of chlamydospores from air-dried preparations germinate. This suggests that the dried chlamydospores (expected to be found in biocontrol preparations) may be dormant but become germinable under appropriate conditions.

*Trichoderma* species produce toxic metabolites which act as fungistatic antibiotic on pathogenic fungi. These toxic metabolites are gliotoxin by *T. lignorum*, later stated to be *G. fibriatum* (Weindling, 1941), viridin by *T. viride* (Brian and McGowan, 1945), trichodermin by *T. viride* and *T. polysorum* and other peptide antibiotics by *T. hamatum* (Dennis and Webster, 1971a, b).

Papavizas *et al.* (1982) found that several UV-induced mutants of *T. harzanium* produce two unidentified metabolites, one heat-liable, the other heat-stable.

*Trichoderma* species are not only good sources of various toxic metabolites and antibiotics, but are also good sources of various lytic extracellular enzymes such as exo- and endoglucanases, cellobiase, and chitinase (Papavizas, 1985).

## Ecology of *Trichoderma*

*Trichoderma* are widely distributed all over the world (Domsch *et al.*, 1980) and they occur in nearly all soils and other natural habitats, especially in those containing or consisting of organic matter. Individual species aggregates may be restricted in their geographic distribution and *Trichoderma* seems to be a secondary colonizer, as its frequent isolation from well-decomposed organic matter indicates (Danielson and Davey, 1973). *Trichoderma* is also found on root surfaces of various plants (Parkinson *et al.*, 1963), on decaying bark especially when it is damaged by other fungi (Danielson and Davey, 1973), and on sclerotia or other propagules of other fungi (Wells *et al.*, 1972).

Certain strains of *T. hamatum* and *T. pseudokoningii* are adapted to conditions of excessive soil moisture, and that *T. viride* and *T. polysporum* are restricted to areas where low temperatures prevail, whereas *T. harzianum* is most commonly found in warm climatic regions, and *T. hamatum* and *T. koningii* are widely distributed in areas of diverse climatic conditions (Danielson and Davey, 1973).

Soil samples taken from agricultural regions show that the natural population of *Trichoderma* is rather low, usually not exceeding $10^2$ CFU/g soil (Chet, 1987). Liu and Baker (1980) reported that soil suppressiveness is accompanied by a significant increase in *T. harzianum* propagule density. Chet and Baker (1981) reported that low pH apparently enhances the propagation of fungi in general, and *Trichoderma* in particular, and as a consequence, it is favorable for the development of suppressiveness. These findings confirmed a former study indicating that acidification of soil could induce suppressiveness by *Trichoderma*, which survives longer in moist soil than in dry soil (Liu and Baker, 1980).

Caldwell (1958) was among the first to observe that chlamydospores survive in soil better than conidia. Lewis and Papavizas (1984) demonstrated the potential of various *Trichoderma* species aggregates to form chlamydospores readily and in great numbers in natural soil or in fragments of organic matter after the introduction of the fungus to the soil as conidia.

## Potential for Biological Control

The potentials of the *Trichoderma* species for biological control include their ability to act as mycoparasite of hyphae and resting structures of plant pathogens (Cook and Baker, 1983; Hubbard *et al.*, 1983), their ability to bring about suppressiveness of soil to soilborne plant pathogens (Cook and Baker, 1983; Baker and Chet, 1982), and their ability to act as a strong rhizosphere competent (Sivan and Harman, 1991; Ahmad and Baker, 1988a). The fungi also demonstrated their potential to be incorporated into seed treatment system as an alternative approach to introducing them into soil (Harman *et al.*, 1981), and their potential to control above-ground plant diseases (Tronsmo and Ystaas, 1980; Tronsmo and Dennis, 1977).

The ability of *Trichoderma* to act as mycoparasites of hyphae and resting structures of plant pathogens has been demonstrated not only *in vitro* (Cook and Baker, 1983) but also in natural soil (Hubbard *et al.*, 1983). *Trichoderma* species effectively control the following soilborne fungi: *Sclerotium rolfsii* (Jinantana, 1995; Henis *et al.*, 1984), *Rhizoctonia solani*, *Pythium* spp., *Fusarium* spp., *Aspergillus niger* (Lynch, 1987; Chet and Henis, 1985; Elad *et al.*, 1983), *Sclerotium cepivorum* (De Oliveira *et al.*, 1984) and others. *Trichoderma* has also been successfully sprayed against *Botrytis* spp. on strawberries (Tronsmo and Dennis, 1977) and apples (Tronsmo and Ystaas, 1980) in above-ground control. Since 1993, two *Trichoderma* species have been registered in the United States for use against plant diseases. They are *Trichoderma harzianum* registered as F-Stop and *Trichoderma harzianum/polysporum* as BINAB T.

Soil suppressiveness is another important evidence of the importance of *Trichoderma* in the biological control of plant diseases. Their ability to bring about suppressiveness to soilborne plant pathogens has been studied extensively (Cook and Baker, 1983; Baker and Chet, 1982). *T. hamatum* and *T. harzianum*, isolated from composed hardwood bark were among the fungi most effective in inducing suppressiveness and capable of restoring suppressiveness to heat-treated media amended with composted hardwood bark (Nelson *et al.*, 1983).

Mutation of wildtype *T. harzianum* isolates is successful in inducing rhizosphere competence (Ahmad and Baker, 1987). This attribute of rhizosphere competence has also been induced by protoplast fusion (Sivan and Harman, 1991). Rhizosphere competence of beneficial microorganisms applied to seeds results in secondary deployment of these bioprotectants along the root (Harman, 1990). Thus, proliferation and colonization of the developing roots by *T. harzianum* may prevent root infection by root-rot and wilt pathogens (Sivan and Chet, 1989; Sivan *et al.*, 1987). Rhizosphere

competence also ensures that relatively high population densities of the biocontrol agents persist in the rhizosphere (Ahmad and Baker, 1987).

The attribute of rhizosphere competence of *T. harzianum* not only contributes to the biological control of plant diseases, but also induces plant growth in terms of weight, height and yield, and significant increases in incidence of emergence of seedlings (Ahmad and Baker, 1988b; Chang *et al.*, 1986). These studies suggest that the attribute of rhizosphere competence in a biocontrol agent potentially contributes to the enhancement of biocontrol efficiency, plant growth, and increased yield (Ahmad and Baker, 1988b).

Harman *et al.* (1981) suggested applications of *Trichoderma* to seed as an alternative approach to introducing them into soil which requires smaller amounts of biological material than in-furrow or broadcast applications. Seed treatment is an attractive delivery system for either fungal or bacterial bioprotectants. Bioprotectants applied to seeds not only protect seeds (Sivan and Chet, 1986; Hadar *et al.*, 1984) but also colonize and protect roots (Ahmad and Baker, 1987; Chao *et al.*, 1986), and increase plant growth (Chet, 1987; Chang *et al.*, 1986).

Studies have been carried out on the use of *Trichoderma* to control above-ground plant diseases either through wound applications or spraying plants with conidia. Results of the efficacy of *Trichoderma* against diseases vary depending on the temperature, the inoculum concentration of conidia, and the timing of conidia application. Successful examples were demonstrated by Tronsmo and Dennis (1977) on the effectiveness of *T. viride* and *T. polysporum* against storage rot (*Botrytis cinerea* and *Mucor mucedo*) on strawberry, and by Tronsmo and Ystaas (1980) on the effectiveness of *T. harzianum* against eyespot disease (*B. cinerea*) on apple. Furthermore, products containing two psychrophilic species aggregates, *T. viride* and *T.*

*polysporum*, for the control of silver leaf disease on trees and *Verticillium* wilt on mushrooms have been registered for commercial use in France and the United Kingdom.

Since *Trichoderma*, when applied with a foodbase or as a seed coating, can survive for long periods of time and even propagate in soil (Harman *et al.*, 1980), its combination with chemical, cultural or physical methods (Chet *et al.*, 1982; Katan *et al.*, 1976) can achieve a long-term controlling effect on soilborne plant pathogenic fungi.

## Mechanisms of Biological Control

Possible mechanisms involved in *Trichoderma* antagonism are: (a) antibiosis, whereby the fungi produce volatile or non-volatile antibiotics (Dennis and Webster, 1971a,b); (b) competition, when space or nutrients (i.e. carbon, nitrogen, microelements) are limiting factors (Weller, 1988; Schippers *et al.*, 1987); and (c) mycoparasitism, whereby *Trichoderma* attack another fungus by excreting lytic enzymes (such as proteases, glucanases and chitinases) that enable them to degrade the latter's cell walls and utilize its nutrients (Geremia *et al.*, 1993; Chet, 1990; Ridout *et al.*, 1988). Parasitism by *Trichoderma* spp. is destructive, causing the death of the host fungus (Barnett and Binder, 1973). Via these mechanism, *Trichoderma* antagonise other fungi, thereby serving as a potential biological control agent of plant diseases (Chet, 1987, 1990; Baker, 1987).

Many fungi produce fungistatic or fungicidal metabolites (antibiotics) which diffuse from hyphae and slow or stop the growth of competitors from some distance away. Inhibition by antibiosis is often species-specific and a response only occurs when appropriate species meet. Antibiosis phenomena