ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

INVESTIGATIONS ON THE EFFECT OF *TRICHODERMA VIRIDE* AND *PSEUDOMONAS FLUORESCENS* AGAINST LATE BLIGHT OF POTATO *(SOLANUM TUBEROSUM)* UNDER GREENHOUSE CONDITIONS.

BY

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Dedicated to my beloved parents

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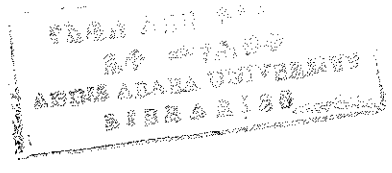

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LIST OF ABBREVIATIONS

AUDPC: Area under disease progress curve

DAP: Di-ammonium phosphate

DMSO: Dimethyl Sulfoxide

EPA: Environmental Protection Agency (U.S.A)

EARO: Ethiopian Agricultural Research Organization

HARC: Holeta Agricultural Research Center.

IDM: Integrated disease management

KBA: King's B agar

O.D: Optical density

PDA: Potato dextrose agar

R.H: Relative humidity

R.p.m: Revolution per minute

SPSS: Statistical Package for the Social Sciences

TANU: Tamil Nadu Agricultural University

V_8 agar: Vegetable juice agar

ABSTRACT

Late blight caused by the oomycete pathogen, *Phytophthora infestans* (Mont.) de Bary, is the most devastating disease of potato throughout the world including Ethiopia. The disease is usually controlled by repeated applications of chemical fungicides. The serious pollution problems associated with the use of chemicals and the development of resistance to the available fungicides by new and more aggressive *P. infestans* strains urge us to have alternative control strategies such as biological control. *Trichoderma viride*-TNAU and *Pseudomonas fluorescens*-Bak150 were tested for their efficacy in controlling the pathogen *in vitro* and in greenhouse. *In vitro* antagonism test between *T. viride* and *P. infestans* showed a radial growth inhibition of the pathogen by 36.7% followed by a complete overgrowth of *T. viride* on *P. infestans*. *P. fluorescens* inhibited the radial growth of the pathogen by 88%. Foliar spray method used in the greenhouse trial involved foliar spraying of the plants with suspensions of (1) *T. viride*; (2) *P. fluorescens*, (3) mixed culture of the two antagonists and (4) chemical fungicide mancozeb followed by spraying the pathogen three days later. Percentage leaf area infection was measured every week and area under the disease progress curve (AUDPC) was calculated and compared among the treatments. The result showed that *T. viride* (AUDPC=260) and *P. fluorescens* (AUDPC=765.1) significantly ($P<0.05$) reduced the disease compared to the untreated check (AUDPC=1045). *T. viride* was found to be more efficient than *P. fluorescens* and the mixed culture of the two. No significant difference was observed between the mixed culture and the inoculated/untreated check. On the other hand, seed treatment method of the greenhouse trial involved dipping artificially infected seed tubers in to the suspensions of the antagonists followed by planting the tubers. The result showed no significant difference ($P>0.05$) in the disease severity (AUDPC) among each of the treated ones and the inoculated/untreated controls. Thus, neither the bioagents nor the chemical fungicide was able to control or suppress the transmission of the pathogen from the infected tubers to the emerging sprouts and the subsequent progress of the disease.

I. INTRODUCTION

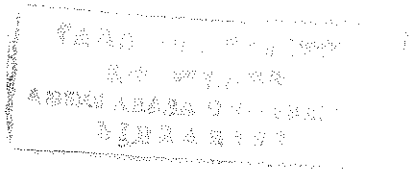
Potato (*Solanum tuberosum*) is the most important non-cereal crop in the world (Struik and Wiersema, 1999). The geographic distribution of potato is almost worldwide. It is grown as a major source of food in most countries with a temperate climate (Rich, 1983).

Late blight, caused by the oomycete pathogen, *Phytophthora infestans*, is probably the single most important disease of potatoes worldwide. It is destructive wherever potatoes are grown without the use of fungicides, except in hot, dry, irrigated areas (Thurston and Schultz, 1981).

Historically, the disease has often been destructive (Fernandez-Northcote *et al.*, 2000). This disease was responsible for the Irish famine in the 1840s, a humanitarian disaster with very long-term socio-economic consequences, some of which is still apparent today (Talbot, 2004). Late blight is one of the main limiting factors for potato production in the world. If the disease is not controlled, losses can reach 100% under optimal conditions (Fernandez-Northcote *et al.*, 2000). The disease causes losses of billions of dollars annually and is a threat to potato production all over the world (Latijnhouwers *et al.*, 2004).

Modern day crop protection including late blight disease relies heavily on the use of chemical fungicides. On a worldwide basis, control of late blight of potato utilizes almost one-fourth of the annual money output for fungicides (Erwin and Ribeiro, 1996). Unfortunately, potential hazards of chemical pesticides to the environment and to the public health are increasing. The increased awareness and concerns have led to the cancelled registration of several agriculturally important pesticides (Mazzola, 1998). Moreover, chemical control of late blight in particular is becoming more difficult due to the appearance of new and more aggressive *P. infestans* strains (Fernandez-Northcote *et al.*, 2000). Thus, alternative control strategies should be sought (Ellis *et al.*, 1999).

Biological control of crop diseases using microbial inoculants is receiving increased attention as an environmentally friendly alternative to the use of chemical pesticides



(Moenne-Loccoz *et al.*, 2001). Studies on biological control of late blight of potato are lacking and hence the aim of this study is to evaluate the effectiveness of *Trichoderma viride* and *Pseudomonas fluorescens* against late blight of potato under greenhouse conditions.

II. OBJECTIVES

2.1. General objective

- To test the potential of *Trichoderma viride*-TNAU and *Pseudomonas fluorescens*-Bak 150 (a local isolate) in controlling late blight of potato both individually as well as in combination under greenhouse conditions.

2.2. Specific objectives

- To test *in vitro* antagonism of *T. viride*-TNAU and *P. fluorescens*-Bak 150 against *P. infestans* and to determine *in vitro* compatibility between the two antagonists.

- To determine the effectiveness of spraying individual and mixed cultures of *T. viride*-TNAU and *P. fluorescens*-Bak 150 in reducing the severity of leaf infection resulting from airborne inocula of *P. infestans*.

- To examine the effects of seed treatments in controlling or suppressing the transmission of the pathogen from infected seed tubers to emerging sprouts and/or the subsequent progress of the disease using individual and mixed cultures of *T. viride*-TNAU and *P. fluorescens*-Bak 150.

- To examine the effects of the seed treatments in affecting the number of leaves, number of sprouts and height of plants developing from infected potato tubers.

III. LITERATURE REVIEW

3.1. POTATO (SOLANUM TUBEROSUM)

Potato (*Solanum tuberosum*) is without doubt the most important of all vegetables, and is the fourth most important food crop in the world after wheat, maize and rice (Hawkes, 1990; Phillips and Rix, 1993). It is cultivated as a major crop in countries with very large populations, in different climatological zones; including temperate regions, the subtropics and the tropics, under very different agro-ecological conditions; lowlands as well as highlands, and in very different socio-economic environments. Potato is grown at a significant scale in more than 130 countries, and covers about 18 million hectares worldwide annually. Potato tubers are consumed by 1 billion people worldwide (Struik and Wiersema, 1999).

The potato that is known as an important world crop is a single species, *Solanum tuberosum*, belonging to the family Solanaceae (Hawkes, 1990). *Solanum tuberosum* apparently originated in the Andes mountains of South America, from northern Chile to Venezuela, and in the Central America, from the northern parts of Mexico to the Southwest of the U.S.A (Struik and Wiersema, 1999).

Kidane-Mariam (1980) credits the German botanist Shimper with introducing the potato to Ethiopia in 1858. There are five major potato production regions in Ethiopia: Central Ethiopia, Eastern Harerge, Northwest Ethiopia, South Ethiopia and Western Ethiopia (GILB, 2004). These regions are shown in Fig.1. The area under potato in Ethiopia is estimated more than 100,000 ha.

Solanum tuberosum is a herbaceous perennial cultivated as an annual. The commercially significant portion of the plant is the tuber, which is a swollen underground stem. The swelling of the tuber is due to the translocation and storage of photosynthates (carbohydrates) which occurs as the aerial portion of the plant reaches maturity.

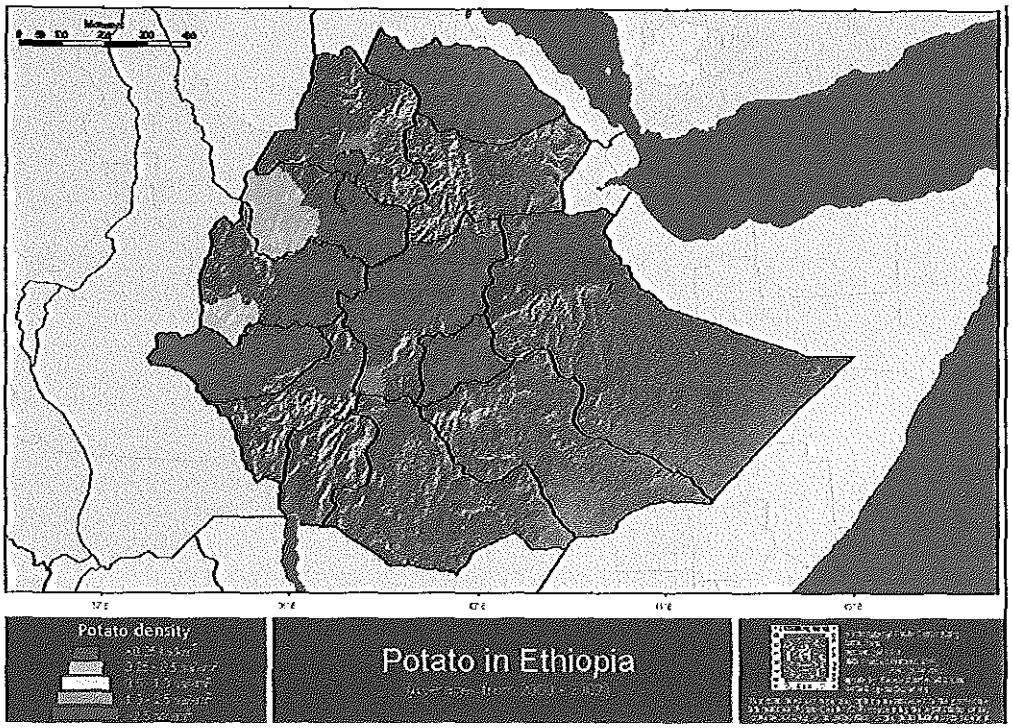


Figure 1. Potato production regions in Ethiopia. Adapted from GILB (2004).

3.2. POTATO DISEASES

Potato is susceptible to several diseases, some of which are widespread and others are localized. The causal agents of these diseases include bacteria, fungi, viruses, mycoplasmas, viroids, nematodes and adverse environment. The "Index of plant diseases in the United States" lists approximately 160 diseases and disorders of *Solanum tuberosum* (Rich, 1983). About 50 are caused by fungi, 30 by viruses, 10 by bacteria, and another 50 or so are either non-parasitic or due to unknown causes. Several others are due to nematodes or insects.

Some examples of common potato diseases and their causative agents include: Early blight (*Alternaria solani*), Late blight (*Phytophthora infestans*), fungal wilt (*Verticillium* spp. and *Fusarium* spp.), Powdery scab (*Spongospora subterranea*), Black scurf (*Rhizoctonia solani*), Dry rot (*Fusarium* spp.), Silver scurf (*Helminthosporium solani*), Leak (*Pythium* spp.), Black dot (*Colletotrichum coccodes*), Black wart (*Synchytrium endobioticum*), Common scab (*Streptomyces scabies*), Soft rot (*Erwinia carotovora*), Bacterial wilt (*Ralstonia solancearum*), Ring rot (*Corynebacterium sepedonicum*), Gangrene (*Phoma exigua*), Potato leafroll

(potato leafroll virus), and Rugose mosaic (potato virus Y) (Agrios, 1978; Peters and Jones, 1981; Meijers, 1987). Of all these and other diseases of potato, late blight is the most devastating disease of potato worldwide and here emphasis is given to its control.

3.3. LATE BLIGHT OF POTATO

Phytophthora infestans, the cause of late blight of potato, is probably the most notorious of all plant pathogens being responsible for the Irish potato famine in the 1840's (Griffiths *et al.*, 2003) that led to the death of over a million people and the emigration of many more (Beyer *et al.*, 2002). Late blight is also known as potato murrain, black blight, winter blight or potato blight (Rich, 1983; Roberts and Royd, 1984).

In the nineteenth century, potatoes formed the foundation of the daily diet of Irish farmers. The typical Irishman consumed over 3kg of potato each day. Late blight was rampant, the potato crop of 1846 failed and consequently many Irish starved. Late blight was not limited to Ireland in the 1840s. The disease was widespread at that time in the northern United States and northern Europe. The differences in Ireland that made late blight so devastating were the nearly absolute reliance of the Irish people on potatoes as a food source. As a consequence, Ireland's population had dropped by 3 million- 1 million dead because of starvation and associated maladies and 2 million who had immigrated to the United States, Canada and other countries (Campbell and Madden, 1990). Thus, with the possible exception of the ergot disease of rye, late blight of potato has caused more human suffering than any other disease of plants (Roberts and Royd, 1984).

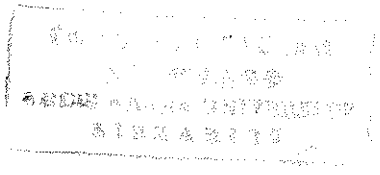
The disease is not simply a historical footnote, it is still widespread throughout potato growing regions of the world, and it is virtually impossible to grow potatoes effectively in temperate climates without some form of late blight disease control (Talbot, 2004). In the past two decades, the frequency and severity of the disease have increased in many parts of the world and have been a serious threat to potato production (Bakonyi *et al.*, 2002).

Late blight is also the major devastating disease of potatoes in Ethiopia and occurs throughout the major potato production areas. In the western part of the country, although the crop used to be important in the cropping system, due to this disease farmers have almost stopped cultivating potato. Studies have shown yield losses ranging from 5.9 to 61.7% depending on the level of susceptibility of the varieties (Bekele Kassa and Yaynu Hiskias, 1996). Under favourable environmental conditions and in the absence of any control measures, this disease is capable of destroying virtually 100% of the above ground parts of susceptible cultivars (Fahim *et al.*, 2003). In Ethiopia the disease reportedly caused approximately 100% crop loss on unimproved local cultivar (Bekele Kassa and Yaynu Hiskias, 1996). Worldwide losses due to late blight and control measures are estimated to exceed $5 billion annually. *P. infestans* is thus regarded as a threat to global food security (Birch and Whisson, 2001; Kamoun, 2003).

3.3.1. Taxonomy of *Phytophthora infestans*

Phytophthora infestans (Mont.) de Bary is classified under the Kingdom Chromista, Phylum Oomycota, Class Oomycetes, Order Peronosporales, Family Peronosporaceae and Genus *Phytophthora,* of which it is the type species (Birch and Whisson, 2001). Previously, because of their filamentous growth, taxonomists classified oomycetes with fungi. However, contemporary studies of metabolism, rRNA sequence, and cell wall composition have shown that they are taxonomically distinct from the fungi and are more closely related to chrysophytes, diatoms and brown algae (Judelson, 1997; Birch and Whisson, 2001; Shattock, 2002).

The genus *Phytophthora* (Greek "plant destroyer") is a large genus of over 60 species and comprises some of the world's most devastating plant pathogen species. Among these are the causes of important diseases of soybeans, oil palms, cocoa, numerous tree species, as well as cucumbers and strawberries (Talbot, 2004). Of all the *Phytophthora* species, however, the most notable and best studied is *Phytophthora infestans* (Birch and Whisson, 2001). Dr. Montagne first described the pathogen in 1845, calling it *Botrytis infestans*. In 1876, Anton de Bary renamed it *Phytophthora infestans* (Mont.) de Bary (Rich, 1983).



3.3.2. Morphology of *Phytophthora infestans*

P. infestans is a coenocytic oomycete. The mycelium produces branched sporangiophores distinguishable from the somatic hyphae. Asexual reproduction is via sporangia that are borne at the tips of sporangiophore branches. The sporangia are hyaline, thin-walled, papillate, ellipsoid to lemon shaped, ranging in length from 21-38 µm and in width from 12-23 µm (Thurston and Schultz, 1981). Sporangia may germinate by means of a germ tube, but most commonly they form about 8 uninucleate zoospores that swim freely in water. The zoospores are biflagellated, one tinsel type that is directed forward and the second is whiplash type which is directed backward.

The cell walls of oomycetes are mainly composed of β-1,3-glucan polymers and cellulose. Within the oomycetes, *Phytophthora* species are both sterol and thiamine auxotroph and typically require exogenous sources of β-hydroxy sterols for sporulation and thiamine for growth (Kamoun, 2003).

3.3.3. Mating types in *Phytophthora infestans*

P. infestans is a heterothallic oomycete with two compatibility mating types designated as A1 and A2 (Ann *et al.*, 1998). The A1 and A2 mating types are believed to represent compatibility types that differ in hormone production and response rather than dimorphic sexual forms (Judelson, 1997). Self-fertile *P. infestans* strains forming oospores (sexual spores) are sometimes present in the population, but usually to a much smaller extent (Stromberg *et al.*, 2001). Previously, the two mating types (A1 and A2) were found only in the highlands of central Mexico, the possible origin of *P. infestans* (Fry *et al.*, 1993). In Mexico, both mating types are found at a frequency of approximately 1:1(O'Sullivan *et al.*, 1995). The A1 mating type of the pathogen was introduced to Europe in the 1840s from Mexico. From Europe, it probably spread to the rest of the world by international trade of seed potatoes. On the other hand, the A2 mating type was confined to Mexico until the late 1970s (Birch and Whisson, 2001); elsewhere, only A1 isolates were found. A second migration probably occurred from Mexico in the late 1970s that included both mating types A1 and A2 (Fry *et al.*, 1993). This provided the pathogen with the opportunity

for sexual reproduction for the first time in more than a hundred years in countries other than Mexico (Shattock, 2002).

A report on mating types of 42 Ethiopian isolates collected from different areas showed only A1 mating type. However, the study indicated that self-fertility and oospore production was much higher in Ethiopian isolates compared to Kenyan isolates (Schiessendoppler *et al.*, 2003).

3.3.4. Sexual reproduction in *Phytophthora infestans* and its implications

When the two mating types grow adjacently, hormones within a mating zone stimulate the development of male (antheridia) and female (oogonia) gamentangia (Birch and Whisson, 2001). The first visible response is a swelling of hyphal tips, which develop in to antheridia and oogonia. A swollen antheridial initial becomes penetrated by the oogonial initial, which subsequently expands to form an oogonium. Meiosis occurs in the gametangia, generating haploid nuclei (Fabritius *et al.*, 2002). Then the antheridial nucleus migrates and fertilizes the oogonial nucleus, which finally develops into a thick-walled and hardy oospore (Agrios, 1997).

Oospores can withstand unfavourable conditions and survive in the soil for at least one year, but most likely for much longer (Struik and Wiersema, 1999; Bakonyi *et al.*, 2002). Oospores germinate by means of a germ tube that produces a sporangium, although occasionally the germ tube grows directly into mycelium (Agrios, 1997). Germination of the oospore releases progeny of either A1 or A2 mating type.

There are at least two major implications from the possibility of sexual reproduction in a region. First, sexual reproduction increases the genetic variability of the organism, and may result in an increased virulence and/or fungicide resistance (Bakonyi *et al.*, 2002). Second, the products of sexual recombination, oospores withstand harsh environments and survive in the soil, thus affecting the epidemiology of the disease (Fry *et al.*, 1989).

3.3.5. Biology and disease cycle

Late blight disease of potato is characterized by production of many cycles of inoculum within one crop year and is a classic example of a polycyclic disease (Erwin and Ribeiro, 1996). Foliage infection results in yield reductions due to the premature killing of the haulm (Cooke and Little, 2001). The late blight pathogen as an asexual organism is essentially an obligate parasite and can therefore only survive between seasons in infected tubers (Stromberg *et al.*, 2001). A study in the Netherlands showed that as few as one blighted tuber per square kilometre of land is sufficient to initiate an epidemic (Erwin and Ribeiro, 1996).

The mycelium spreads in the tissues of blighted tubers and eventually reaches a few of the host shoots produced from infected tubers used as seed. When the mycelium reaches the aerial parts of the plants, it produces sporangiophores which emerge through the stomata of the stems and leaves and project into the air (Agrios, 1978). Secondary infections initiate from wind or splash-borne sporangia. Consequently, sporangia will land on the surface of leaf.

Under damp conditions and temperatures below 12°C sporangia release motile zoospores. The movement of zoospores towards host cells involve negative geotaxis and may also require chemotaxis or electrotaxis (Kamoun, 2003; Van West *et al.*, 2003). Once in contact with the host, zoospores rapidly encyst, a process that involves the development of a cell wall, attachment to the host cell surface and loss of mobility. The cyst then germinates, producing a short germ tube, which differentiates in to a swollen infection structure called an appressorium (Talbot, 2004). The appressorium, a flattened hyphal pressing organ, in turn produces an infection peg that is used to breach the host cuticle.

Following penetration of the leaf, the germ tube develops into a profusely branched mycelium which grows between the cells and sends long curled haustoria into the cells (Agrios, 1978) forming biotrophic feeding relationship with plant cells. The host cells eventually die, and hence *P. infestans* is considered a hemibiotroph (Judelson, 1997). A few days after infection, under favourable weather conditions, numerous

(Alexopoulos and Mims, 1979). Twelve degrees Centigrade appears to be a point of differentiation in which sporangia germinate almost entirely by releasing 3 to 8 zoospores at temperatures up to 12°C, whereas above 12°C sporangia may germinate directly by producing a germ tube (Agrios,1997; Latijnhouwers *et al.*, 2004).

With regard to relative humidity, 100% is the optimum and 91% minimum for sporangial production (Alexopoulos and Mims, 1979). Sporangia lose their viability in 3-6 hours at relative humidities below 80%. Germination of sporangia takes place only when free water or dew is present on the leaves (Agrios, 1978).

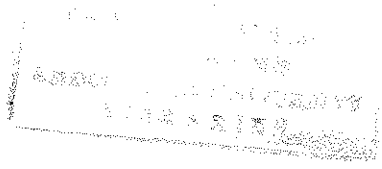
3.3.7. Disease symptoms

The symptoms of late blight in the foliage of potato begin as water-soaked spots. usually at the tips or on the margins of the lower leaflets (Roberts and Royd, 1984). In cool moist weather conditions, the spots enlarge rapidly and form brown blighted areas with indefinite borders. A zone of white downy growth appears at the border of the lesions on the underside of the leaves. Soon entire leaves are infected, die and become limp (Agrios, 1997). Fields severely infested by late blight have a distinctive odour resulting from the rapid break down of potato leaf tissue (Thurston and Schultz, 1981). In dry weather, the activities of the pathogen are checked and hence the lesions stop enlarging, turn black, curl, and wither (Agrios, 1997).

Infected tubers initially show slightly brown or purple blotches consisting of water soaked, dark, somewhat reddish brown tissue that extends 5 to 15mm into the flesh of the tuber. Later the infected regions become firm, dry and sunken. In damp soils, the tuber decays rapidly before harvest. In harvested tubers rotting continues during storage. Eventually infection is followed by secondary fungal or bacterial infection known as wet rot (Birch and Whisson, 2001).

3.3.8. Host range of *Phytophthora infestans*

Some *Phytophthora* species attack only one or two species of host plants, but others may cause diseases on many different kinds of host plants (Agrios, 1997). *P. infestans* in particular infects a wide range of solanaceous species. However,



economically important hosts are potato, tomato, egg plant and some other South American hosts (tree tomato and pear melon) on which it causes late blight (Birch and Whisson, 2001).

3.4. POTATO LATE BLIGHT CONTROL

Effective control of potato late blight requires implementing an integrated management approach. Integrated disease management (IDM) is an approach that employs the use of all available options for controlling a particular disease (Kankwatsa *et al.*, 2002). Thus, IDM of late blight may include: cultural control, control by host resistance, chemical control, and biological control.

Cultural control

Cultural practices are the first line of defence that involve the use of disease free tubers (preferably certified seeds), destroying all cull and volunteer potatoes, and solanaceous weeds, careful scheduling of sprinkler irrigation (as leaf wetness is critical factor for late blight development) and deep planting of tubers (to prevent infection of tubers from sporangia or zoospores that wash downward from infected leaves during rain storms).

Control by host resistance

Control by host resistance involves using potato varieties which are resistant to late blight. Two types of resistance to *P. infestans* in potatoes are recognized: (1) Specific resistance (also called vertical, race specific or monogenic resistance) and (2) General resistance (also called horizontal, field, race non-specific, polygenic, or rate limiting resistance) (Thurston and Schultz, 1981).

Vertical resistance is characterized by single gene interaction between host and pathogen genotypes. The level of host resistance developed is near immunity. However, since it is usually controlled by a single gene, mutation or sexual recombination allows the development of a new race of *P. infestans* capable of infecting leaves of the host with single-gene resistance (Erwin and Ribeiro, 1996).

On the other hand, horizontal resistance is not race specific, and is most often conditioned by multiple genes. This type of resistance does not induce immunity or prevent infection but acts against all pathologic races by slowing the rate of infection and reducing the size of lesions. Horizontal resistance has been durable and stable over the years, although it can be overcome by environmental conditions favourable to the pathogen and/ or an increase in inoculum (Erwin and Ribeiro, 1996).

Chemical control

The control of oomycetes, such as *P. infestans*, has always been a major aim in the chemical protection. Indeed a significant proportion of the worldwide pesticide expense has been devoted to the control of oomycetes, of which the specific control of *P. infestans* represents about one-fourth (Griffiths *et al.*, 2003). The biochemistry of oomycetes is different from that of the true fungi and thus, they are often tolerant of fungicides that can control the latter (Erwin and Ribeiro, 1996). Two types of fungicides are used for the chemical control of late blight: contact fungicides and systemic fungicides.

Contact fungicides (also called non-systemic, protective or preventive fungicides) affect the pathogen structures on the plant surface area acting during germination and penetration phases. Once the pathogen is inside the plant, these fungicides are useless (Fernandez-Northcote *et al.*, 2000). Contact fungicides for the control of late blight include copper compounds (Bordeaux mixture, Copper oxychloride, and Cuprous oxide), organic tin compounds (Fentin acetate or Fentin hydroxide), dithiocarbamates (Zineb, Maneb, and Mancozeb), Chlorothalonil, and phthalimide (Folpet). Dithiocarbamates are currently the most commonly used fungicides for late blight control. They act mainly during sporangium and zoospore germination inactivating amino acids or important biochemical processes that involve enzymes with thiol- groups (Fernandez-Northcote *et al.*, 2000).

Systemic fungicides (also known as curative fungicides) are taken up passively or actively through roots, stems, leaves, or flowers and translocated to another area in the plant. Translocation can be across the leaf (translaminar), upward to new growth (apoplastic), or downward (symplastic) (Erwin and Ribeiro, 1996). Some examples of

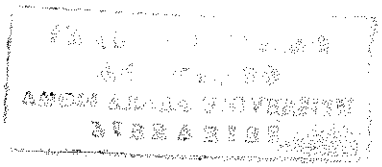
reasons related to health and/or environment in Sweden (Fernandez-Northcote *et al.*, 2000). In addition to the hazardous effects of pesticides to human health, non-target organisms (non-pathogens) become victims resulting in the disruption of the biological balance (Baker and Cook, 1974; Ragunathan and Divakar, 1996).

Another reason for the need to have alternative control measures is the development of resistance by plant pathogens to chemical pesticides. The introduction of metalaxyl, the most effective fungicide against late blight, was a breakthrough in the control of foliar diseases caused by *Phytophthora* species. Unfortunately, soon after its introduction for commercial use in Ireland, resistance to it became so dominant in the population of *P. infestans* that metalaxyl had to be withdrawn from the market (Erwin and Ribeiro, 1996). Since then, resistance has been reported in Israel, the United States, Canada, Scotland, Netherlands and other European countries (Collier and Le Boutillier, 1995; Erwin and Ribeiro, 1996). Metalaxyl sensitivity test in 42 Ethiopian *P. infestans* isolates revealed that 75% were sensitive, 10% intermediate and 15% resistant (Schiessendoppler *et al.*, 2003).

The resistance to metalaxyl can be expressed not only as total resistance, but also by a gradual increase in tolerance by *Phytophthora* species. Moreover, resistance to metalaxyl extend to all fungicides of similar phenylamide composition (Erwin and Ribeiro, 1996). Mutation has been the primary source of resistance in the US-1 lineage of *P. infestans* (old lineage). In addition to mutation, sexual reproduction, resulting from the dissemination of sexual mating type A2, increased the appearance of resistant populations of *P. infestans* (Fernandez-Northcote *et al.*, 2000). The clonal immigrant lineages detected in North America in the early 1990s, namely, US-6, US-7 and US-8 are all resistant to phenylamides (Shattock, 2002).

There is a great need for biological control of pathogens that go uncontrolled or only partially controlled. For instance, no practical or economically feasible chemical control was known to replace *Agrobacterium radiobacter* strain k-84, which is an effective biocontrol agent against the crown gall pathogen (Estrella and Chet, 1998).

All the reasons mentioned above i.e., environmental pollution and the associated health risks to humans and the ecological disturbance, the declining efficacy of



fungicides due to the development of resistant pathogens, the absence of effective chemical or cultural control measures in some diseases, and the phytotoxicity of some chemical fungicides heightened the scientific interest in biological control of plant pathogens.

Trichoderma species and fluorescent *Pseudomonas* species are among the major microorganisms which have shown great potential in biological control of several plant pathogens.

3.6. TRICHODERMA SPECIES AS BIOCONTROL AGENTS

Trichoderma species have been investigated for over eighty years and have shown to have biocontrol potential against many plant pathogens (Kucuk and Kivanc, 2003). A study by Jones and Stewart (1997) showed that *T. harzianum* (C52) isolate has the potential to control lettuce drop caused by *Sclerotinia minor* in glasshouse systems. Kexiang *et al.* (2002) on the other hand, tested *T. harzianum* T88 and *T. atroviride* T95 against apple ring pathogen (*Botryosphaeria berengeriana* f.sp.*piricola*) both *in vitro* and in greenhouse and found that the test strains have great biocontrol potential on the pathogen. Barbosa *et al.* (2001) also worked on the antagonism of *T. polysporum*, *T. koningii*, *T. viride*, and *T. harzianum* against *Cladosporium herbarum* (pathogen of passion fruit causing verrucose) and found that all except *T. Koningii* had a considerable potential for the biocontrol of *C. herbarum*. Another strain, *T. viride* Pers.ex. Gray, greatly suppressed post-harvest rot of yams caused by *Dioscorea* spp. (Okigbo and Ikediugwu, 2000). Moreover, Naseby *et al.* (2000) tested the antagonistic activities of five strains of *Trichoderma* species against large *Pythium ultimum* inocula and found that the test strains significantly reduced the number of lesions on pea caused by the pathogen.

In addition to the above mentioned pathogens, *Trichoderma* spp. have shown efficiency on biocontrol of *Rhizoctonia solani*, *Pythium aphanidermatium*, *Fusarium oxysporum*, *Fusarium culmorum*, *Gaeumannomyces graminis* var.*tritici*, *Sclerotium rolfsii*, *Phytophthora cactorum*, *Botrytis cinerea* and *Alternaria* spp. (Kexiang *et al.*, 2002; Kucuk and Kivanc, 2003).

As a result of extensive studies on *Trichoderma* species, some isolates have become commercially available (Kucuk and Kivanc, 2003). For instance, a biofungicide formulated with *T. harzianum*, named Trichodex, is used to control soilborne and phylloplane pathogens and other formulations, such as Binab-T, GlioGard and RootShield are used to control several soilborne pathogens which cause damping-off and root rot (Barbosa *et al.*, 2001).

3.6.1. The genus *Trichoderma*

The genus *Trichoderma* comprises a number of saprophytic fungi commonly found in the soil, rotting wood and on plant debris, and which may easily be recognized in culture by their usually green spores (Dubos, 1987). *Trichoderma* species are filamentous imperfect fungi, with teleomorphs belonging to the ascomycete order Hypocreales (Kredics *et al.*, 2004) or, sometimes, to the Eurotiales, Clavicipiales and Spheriales (Dubos, 1987). A given species of *Hypocrea* may have several different *Trichoderma* as anamorphs (Alexopoulos and Mims, 1979); inversely, the same "species group" of *Trichoderma* may be attached to several different species of *Hypocrea* (Dubos, 1987).

3.6.2. Classification of *Trichoderma* species

Trichoderma species belong to the subdivision Deuteromycotina, form-class Deuteromycetes, form-subclass Hyphomycetidae, form-order Moniliales, and form-family Moniliaceae (Alexopoulos and Mims, 1979). Even though the recognition of a *Trichoderma* is relatively easy, identification of the species is much less simple (Dubos, 1987) and there is a considerable confusion over the application of specific names (Kucuk and Kivanc, 2003).

Rifai distinguished nine species aggregates based on microscopic characters (Kucuk and Kivanc, 2003). Speciation was based on conidiophore morphology, i.e., the thickness of the stipe and the degree of complexity of branching, on phialide shape and size, and the shape, size and texture of conidia (Pitt and Hocking, 1985).

Bissett (1991) revised the genus *Trichoderma*. Consequently, the genus is defined to include anamorphs of *Hypocrea*, previously placed in *Gliocladium* and *Verticillium*,

having elongate phialides and irregularly branched conidiophores. He divided the genus in to five sections: section *Trichoderma*, section *Longibrachiatum*, section *Saturnisporum*, section *Pachybasium*, and section *Hypocreanum*. *T. viride* belongs to the section *Trichoderma*. The species in this section have narrow and flexuous conidiophores with branches and phialides uncrowded, frequently paired, and seldom in verticils of more than three.

Now it is recognized that the morphological characteristics are generally found to be highly variable, making them unreliable for species determination in *Trichoderma* (Kucuk and Kivanc, 2003). Thus, DNA sequence analysis is being employed to improve the description and characterization of *Trichoderma* species (Samuels, 2004). As a result of the molecular approaches, the taxa has recently increased from nine to at least thirty three species.

3.6.3. Mechanism of action of *Trichoderma* species in biological control

The success of *Trichoderma* spp. as a biocontrol agent is believed to involve various modes of action, including antibiotic production, secretion of lytic enzymes, mycoparasitism, competition for space and nutrients, and induction of systemic resistance (Cortes *et al.*, 1998; Rocco and Perez, 2001). A given *Trichoderma*-host interaction may involve any of these mechanisms individually or encompass more than one of them acting simultaneously (Cortes *et al.*, 1998) and in fact it seems advantageous for a biocontrol agent to suppress a plant pathogen using multiple mechanisms (Estrella and Chet, 1998).

Antibiosis

Both volatile and non-volatile antibiotics are known to be produced from *Trichoderma* species (Okigbo and Ikediugwu, 2000). Peptaibols (trichorizianines, trichokindins, trichorzins, trichorozins and harzianins), a class of antibiotics, are produced by most species and strains of *Trichoderma*. They generally exhibit antimicrobial activity against fungi and gram positive bacteria. Peptaibols are thought to act on the membrane of the target fungus to inhibit membrane-associated enzymes involved in cell wall synthesis (Wiest *et al.*, 2002). The antibiotics trichodermin, trichodermol,

harzianum A and harzianolide are also known to be produced from *T. viride* and other species of *Trichoderma* (Barbosa *et al.*, 2001). Moreover, Lin *et al.* (1994) reported a ribosome-inactivating antifungal agent, Tricholin from *T. viride*.

In addition to the non-volatile antibiotics mentioned above, *Trichoderma* species especially some *T. viride* strains, produce known volatile antifungal substance called 6-n-pentyl-2H-pyran-2-one (6PAP), which has a characteristic coconut flavour (Cooney and Lauren, 1998; Kalyani *et al.*, 2000; Barbosa *et al.*, 2001). Isocyanide compounds, produced by *Trichoderma* species have also been grouped in this category of volatile metabolites (Kexiang *et al.*, 2002).

Lytic enzymes

Studies have shown that mycoparasitic strains of *Trichoderma* produce a complex set of extracellular enzymes including β-(1,3)-glucanases, chitinases, lipases and proteases when grown on isolated cell walls of *Rhizoctonia solani* (Cortes *et al.*, 1998; Estrella and Chet, 1998; Sandhya *et al.*, 2004). Besides, Barbosa *et al.* (2001) reported that *T. viride*, *T. harzianum* and *T. polysporum* secrete extracellular cellulase. These lytic enzymes are probably responsible for hyphal lysis through the digestion of major cell wall components (Cortes *et al.*, 1998). It is believed that these enzymes act synergistically with the antibiotics to inhibit the growth of fungal pathogens (Mora and Earle, 2001; Wiest *et al.*, 2002). It appears that the weakening of the host cell wall by the enzymes increases the rate of diffusion of the antibiotics through the cell wall.

Mycoparasitism

Mycoparasitism occurs when one fungus exists in intimate association with another from which it derives some or all of its nutrients while conferring no benefit in return (Estrella and Chet, 1998). Perhaps the best known mycoparasite is the fungus *Trichoderma* (Campbell, 1989). This is because *Trichoderma* spp. attack a great variety of phytopathogenic fungi that are responsible for most important diseases of major economic importance worldwide (Estrella and Chet, 1998).

It appears that mycoparasitism is a complex process involving several steps (Chet, 1987). The mycoparasitic relationship between *Trichoderma* and its potential host

might involve biochemical and physiological interactions that precede the microscopically visible phenomena of hyphal coiling, appressorium formation. penetration and cytoplasmic degradation (Cortes *et al.*, 1998).

The initial detectable interaction shows that the mycoparasite grows directly toward the host by a chemotropic reaction (Flores *et al.*, 1997). The chemotropic growth of *Trichoderma* appears to be due to some stimuli in the host's hyphae or toward a gradient of chemicals excreted by the host (Chet, 1987). Subsequently, the hyphae of *Trichoderma* coil tightly around the host (Cortes *et al.*, 1998).

It was shown that a purified lectin from *Sclerotium rolfsii* induces coiling of hyphae of *T. harzianum* and the formation of mycoparasitism related structures around nylon fibres coated with the lectin. Thus, lectins present on the cell walls of the host are suggested to take part in its recognition (Cortes *et al.*, 1998). Similar lectins have been identified from *Rhizoctonia solani* and other host species. A lectin present in *Rhizoctonia solani* hyphae binds to galactose residues on *Trichoderma* cell walls and allows it to locate its prey (Chet, 1987). Following these interactions, *Trichoderma* penetrates into the host mycelium, by partial degradation of its cell wall (Flores *et al.*, 1997).

Competition

Competition is another mechanism by which *Trichoderma* species exert their biocontrol effect. Competition is an indirect effect whereby pathogens are excluded by depletion of food bases or by physical occupation of sites (Maloy, 1993). The study of Barbosa *et al.* (2001) in the *in vitro* antagonism of *Trichoderma* species on *Cladosporium herbarum* revealed that the colonies of *Trichoderma* species grew always faster than *C. herbarum* in single or mixed culture. In the study of biological control of post-harvest rot in yam using *T. viride*, it was found that *T. viride* compete for the same microsites with the pathogens without utilizing the yam tuber as growth substance (Okigbo and Ikediugwu, 2000). Thus, the rapid growth of *Trichoderma* gives it an important advantage in the competition for space and nutrients with plant pathogenic fungi (Barbosa *et al.*, 2001).

In the rhizosphere competition for space as well as nutrients is of major importance. Thus, an important attribute of a successful rhizosphere biocontrol agent would be the ability to remain at high population density on the root surface, providing protection of the whole root for the duration of its life (Estrella and Chet, 1998).

Induced Systemic Resistance

Plants possess inducible defence mechanisms to restrict or block the ability of microbial pathogens to produce disease (Mazzola, 1998). Induced resistance is a plant response to challenge by microorganisms or abiotic agents such that following the inducing challenge de novo resistance to pathogens is shown in normally susceptible plants (Estrella and Chet, 1998). Induced resistance can be localized, when it can be detected only in the plant region originally attacked, or systemic, when resistance occurs subsequently at sites throughout the plant (Estrella and Chet, 1998; Heil and Bostock, 2002).

Studies showed that some *Trichoderma* strains can induce systemic resistance in plants. This can be exemplified by a recent study of Khan *et al.* (2004). Their study revealed that *Trichoderma hamatum* 282 induced systemic resistance in cucumber against *Phytophthora capsici* crown rot and leaf blight. The same strain was found to be the most active inducer of induced systemic resistance in radish against bacterial leaf spot (Khan *et al.*, 2004).

In addition to their biocontrol effects, the ability of *Trichoderma* species to increase the rate of plant growth and development has been known for many years. It was found that a number of *Trichoderma* strains were simultaneously plant growth promoters in vegetables and various seedlings and biocontrol agents (Chet, 1987; Naseby *et al.*, 2000). The mechanism of this phenomenon has not been thoroughly studied. *Trichoderma* may affect minor pathogens in the soil but it may also directly affect the plant by excreting a regulating hormone which may, in turn, increase the growth rate or the efficiency of nutrient uptake (Chet, 1987). Solublization of inorganic plant nutrients by *Trichoderma* (Paulitz and Belanger, 2001) may also be another reason.

3.7. FLUORESCENT PSEUDOMONAS SPECIES AS BIOCONTROL AGENTS

In recent years, there has been relatively more success in obtaining biological control of plant pathogens by bacterization (inoculation of bacteria onto plant seeds or roots) with fluorescent pseudomonads (Sivamani and Gnanamanickam, 1988; Kumar *et al.*, 2002). So far specific strains of fluorescent *Pseudomonas* species have shown efficacy in controlling a number of fungal diseases, including *Pythium* root and seed rot of many crops (Mazzola, 1998; Ellis *et al.*,1999), *Fusarium* wilt in cotton and tomato (Gamliel and Katan, 1993), *Verticillium* wilt of potato (Leben *et al.*,1987), *Rhizoctonia* stem and root rot of peanut (Savithiry and Gnanamanickam, 1987), banana wilt caused by *Fusarium oxysporum* (Sivamani and Gnanamanickam, 1988), disease caused by *Sclerotium rolfsii* in bean (Gamliel and Katan, 1993), and *Rhizoctonia solani* root infection in tomato (Siddiqui and Shaukat, 2002).

In addition to suppressing plant pathogens, certain biocontrol strains of fluorescent *Pseudomonas* species have shown to enhance plant growth and yields (Burr *et al.*, 1978; Sivamani and Gnanamanickam, 1988; Mazzola, 1998). The direct promotion of plant growth by these bacteria (known as plant growth promoting rhizobacteria) is through the production of plant growth promoting substances or facilitation of uptake of certain nutrients from soil (Kumar *et al.*, 2002).

Fluorescent pseudomonads often predominate among bacteria of plant rhizosphere (Sutra *et al.*, 2000). Their flexible metabolism directed toward the dominating substrates released by young parts of the roots, their short generation times relative to many other rhizosphere bacteria, their mobility and their ability to produce a wide range of antagonistic metabolites, make them primary colonizers of young root parts (Schippers *et al.*, 1987) and hence they have good potential in biocontrol of soilborne pathogens (Sutra *et al.*, 2000; Chin-A- Woeng *et al.*, 2003).

3.7.1. The genus *Pseudomonas*

The genus *Pseudomonas* belongs to the family *Pseudomonadaceae*. It encompasses one of the most complex groups of Gram-negative bacteria, with many phenotypic similarities to many genera (Palleroni, 1984). Members of the genus are

characterized by their ability to grow in simple media at the expense of a great variety of simple organic compounds (Palleroni, 1984).

Morphologically *Pseudomonas* species are gram negative, non-spore forming, rod shaped bacteria. They are typically motile by means of one or more polar flagella. Common physiological properties in the genus include chemoorganotrophic nutrition, oxidative metabolism, positive test for catalase and they are usually oxidase positive (Madigan *et al.*, 1997).

Pseudomonas fluorescens belongs to the fluorescent subgroup. In addition to *P. fluorescens*, the subgroup includes *P. aeruginosa*, *P. putida* and *P. syringae*. Most of the strains in the subgroup produce water soluble, yellow-green fluorescent pigments (Madigan *et al.*, 1997).

Two phenotypic characteristics of *P. fluorescens* that distinguish it from *P. putida* are its ability to grow at 4°C, and its ability to hydrolyse gelatin. On the other hand, pyocyanine production and the ability to grow at 43°C distinguish *P. aeruginosa* from *P. fluorescens*. *P. syringae* lacks arginine dihydolase and it is oxidase negative (Madigan *et al.*, 1997; Kumar *et al.*, 2002).

3.7.2. Mechanism of action of Fluorescent Pseudomonads

Just like *Trichoderma* species, fluorescent pseudomonads employ a number of different mechanisms in the suppression of plant pathogens. These mechanisms include competition for substrates or sites, siderophore production, induced resistance, and production of antifungal metabolites.

Competition

Successful survival and the ability to compete efficiently with the better adapted resident microflora are among the important prerequisites for the optimal performance of biocontrol agents (Chin-A-Woeng *et al.*, 2003; Mascher *et al.*, 2003). Competition for nutrients such as carbon, nitrogen or iron is one of the mechanisms through which biocontrol strains can reduce the ability of fungal pathogens to propagate in the soil. Many *Pseudomonas* species have a short generation time

which partly ensures their successful colonization of the plant surface. For instance, *P. fluorescens* WCS365 was found to form microcolonies in tomato root one day after inoculation (Chin-A-Woeng *et al.*, 2003). Mazzola (1998) stated that competition for substrate or site is probably responsible for disease suppression by most *Pseudomonas* spp., but the importance of this mechanism can vary considerably among biocontrol strains and target pathogens. Since these microorganisms occupy the same sites colonized by pathogens, as potential antagonists of pathogens, they often are the first line of defence for plants against infection.

Siderophore production

Iron is an essential element for most microorganisms, as it is a constituent of enzymes with critical roles in electron transfer, RNA synthesis and resistance to reactive oxygen intermediates (Cornelis and Matthijs, 2002). Even though iron is the fourth most abundant element in the earth's crust, the availability of solublized Fe^{3+} in soils is limiting (Guerinot, 1994; Chin-A-Woeng *et al.*, 2003). This is due to the fact that iron is oxidized to highly insoluble ferric-oxyhydroxide polymers (Schippers *et al.*, 1987). Consequently, free Fe^{3+} in an aerobic, aqueous environment is limited to an equilibrium concentration of approximately 10^{-17}M, a value far below the required for optimal growth of microbes (Guerinot, 1994). Thus, in order to satisfy their iron requirement, aerobic bacteria need to produce and excrete molecules that bind the otherwise poorly soluble iron, termed siderophores (Cornelis and Matthijs, 2002).

Siderophores (Greek 'iron bearers') are defined as low molecular weight (generally less than 1000 Daltons), virtually ferric-specific ligands produced by microorganisms that facilitate the solublization and transport of Fe^{3+} (Neilands and Leong, 1986; Guerinot, 1994). The majority of fluorescent pseudomonads produce fluorescent yellow-green siderophores called pyoverdines or pseudobactins, which are efficient iron scavengers (Cornelis and Matthijs, 2002). Therefore, the ability to scavenge iron under Fe^{3+} limitation provides these biocontrol agents with a selective advantage over pathogens or deleterious organisms that possess less efficient iron binding and uptake systems (Chin-A-woeng *et al.*, 2003). Weller (1988) summarized how pathogens are thought to be sensitive to suppression by siderophores. The reasons include: (a) they may not produce siderophores of their own;(b) they are unable to use siderophores produced by the antagonists or by other microorganisms in their

immediate environment; (c) they produce too little siderophore or a siderophore with a lower affinity for iron than those of the antagonists; or (d) they produce a siderophore that can be used by antagonist, but are unable to use the antagonist's siderophore.

In vitro antagonism test between *P. fluorescens* and *P. infestans* showed that the antagonistic effect of *P. fluorescens* decreased when $FeCl_3$ was present in the medium (Torres-Rubio *et al.*, 2000) suggesting that siderophores play an important role.

Induced Systemic Resistance

The study of Siddiqui and Shaukat (2002) indicated that tomato plants treated with *P. fluorescens* CHA0 had consistently lowered *Rhizoctonia solani* disease severity and colonization in the root. This was not due to direct effect of the bacteria on *R. solani* as the bacteria and the fungus were spatially separated. Similar result was found on tobacco treated with the same strain. In the latter case, the siderophore pyoverdine produced by *P. fluorescens* CHA0 is thought to be responsible for the induction of systemic resistance (Mazzola, 1998). Similarly, the production of the phenazine derivative pyocyanine was shown to be involved in the induction of systemic resistance in tomato and bean against *Botrytis cinerea* (Chin-A-Woeng *et al.*, 2003).

Antifungal metabolites

Several studies have revealed the role of secondary metabolites produced by biocontrol strains of fluorescent pseudomonads in the biological control of plant pathogens (Mazzola, 1998). The suppression of *Pythium ultimum* by *P. fluorescens* CHA0 was due to the production of hydrogen cyanide, pyoluteorin and 2, 4-diacetyl phloroglucinol (Mazzola, 1998). On the other hand, phenazine-1-carboxylic acid was found to be responsible for the suppression of *Gaeumannomyces gramini* var. *tritici* by *P. fluorescens* 2-79 (Chin-A-Woeng *et al.*, 2003). In addition to the above mentioned secondary metabolites, certain *P. fluorescens* strains were found to produce potent antifungal metabolites including oomycin A, pyrrolnitrin, and cyclic lipopeptides (Mazzola, 1998; Johansen *et al.*, 2002).

The contribution of the secondary metabolite phenazine to the ecological competence of strains was shown for the phenazine producing strain *P. fluorescens* 2-79. It was found that phenazine-1-carboxylic acid deficient mutant strains had a reduced survival and a diminished ability to compete with the resident microflora (Chin-A-Woeng *et al.*, 2003).

IV. MATERIALS AND METHODS

4.1. SOURCE OF CULTURES

Trichoderma viride

T. viride-TNAU was obtained form Tamil Nadu Agricultural University, India.

Pseudomonas fluorescens

Local isolate, *P. fluorescens*-Bak 150, obtained from Addis Ababa University (Applied Microbiology laboratory) was used. It was isolated from a potato field in Bako region.

Phytophthora infestans

P. infestans was isolated from infected potato leaves from Holeta Agricultural Research Center potato fields. Potato leaflets showing late blight symptoms were collected, washed in fresh water and placed in a humid chamber (inverted Petri-dish with water agar). The plates were incubated at 18°C for two days to facilitate fresh sporulation. Potato slices were then prepared by dipping clean healthy tubers into 70% ethanol for a few seconds, burnt off and sliced. Small pieces of the infected tissue from the sporulating border of the lesion were cut and placed under the potato slices of 1 cm thick in empty Petri-plates and incubated at 18°C for one week until there is abundant sporulation on the upper side of the slices.

Sporangia from the surface of the slices were carefully transferred to a clean-up agar plate using inoculating needle. The clean-up media was Vegetable juice agar (V8 agar) amended with DMSO mix of 20 mg/l Griseofulvin, 19 mg/l Nystatin, 10 mg/l Benlate (50 %wp), 5 mg/l Methoxypurine, 30 mg/l Rifamycin, 5 mg/l Nalidixic acid, 40 mg/l 8-azaguanine and 30 mg/l Neomycin.

4.2. IN VITRO TESTS

4.2.1. Test of Antagonism

4.2.1.1. Antagonistic test between *T. viride* and *P. infestans*.

Dual culture method (Sivakumar *et al.*, 2000) was employed to analyze whether *T. viride* inhibits the growth of *P. infestans* or not. In order to do so, 1cm diameter

mycelial plug of *P. infestans* (9 days old) was placed on one side of a Petri-plate containing rye agar (60 g/l rye, 20 g/l sucrose, 0.05 g/l β-sitosterol and 15 g/l agar) and pre-incubated at 18°C for two days to initiate growth. Then 1cm diameter disc of *T. viride* (9 days old) was placed 6 cm away from the pathogen on the dual plates; whereas in the control plates, sterile PDA disc was placed instead of *T. viride*. The assay was done in triplicate plates and the radial growth of the pathogen was measured after 4 days of incubation at 18°C. The percent radial growth inhibition was calculated in relation to the growth of the control (Sivakumar *et al.*, 2000).

$$\text{Percent inhibition of radial mycelial growth} = (C-T)/C \times 100$$

where C is radial growth measurement of the pathogen in the control plates and T is radial growth of the pathogen in the dual plates.

4.2.1.2. Antagonistic test between *P. fluorescens* and *P. infestans*

The test was performed on Rye agar. A 20 µl of an overnight culture of *P. fluorescens* grown in King's B broth (20 g/l proteose peptone, 1.5 g/l K_2HPO_4, 1.5 g/l $MgSO_4.7H_2O$ and 15 ml Glycerol) was spotted at the center of a Petri-dish and pre-incubated for 2 days at 18°C as done by Georgakopoulos *et al.* (2002). One centimeter disc of *P. infestans* (9 days old) was then inoculated at either side of the bacterial growth in triplicate plates and incubated at 18°C. In the control plates, 20 µl of sterile King's B broth was spotted at the center and near the periphery 1cm disc of *P. infestans* was inoculated. Radial growth inhibition of *P. infestans* was assessed 10 days later by measuring the radial growth of the pathogen in the dual and control plates.

4.2.2. Antagonist compatibility test

This was done to analyze compatibility between *P. fluorescens* and *T. viride* so that they can be used together in mixed culture. To test *in vitro* compatibility between the two antagonists, the dual culture plate method was employed (Siddiqui and Shaukat, 2003). An over night culture of *P. fluorescens* grown in King's B broth was streaked on one side of a Petri-plate (9 cm diameter) containing King's B agar (KBA). The other side of the Petri-plate was inoculated with 5mm disc of *T. viride* from 14 days

old culture plate. The plates were then incubated at 25°C and zone of inhibition (if any) was measured. The test was performed in triplicate plates.

4.2.3. Observation of mycoparasitism by *T. viride*

Slide culture method was employed to see whether *T. viride* parasitizes *P. infestans*. In order to do so, a technique similar to Sivakumar *et al.* (2000) was followed. Clean slides were placed on a z shaped glass rod in 9 cm Petri-dishes and autoclaved. Then small amount of molten rye agar was poured evenly on the slides. A few ml of sterile water was added to the Petri-dish to prevent drying. Mycelial disc of *P. infestans* was inoculated on one side of the slide and pre-incubated for 2 days at 18°C. Then *T. viride* was inoculated at a distance of 2.5 cm away from *P. infestans*. The presence or absence of coiling by *T. viride* or other hyphal interactions (if any) were observed under microscope 4 days after incubation at 18°C.

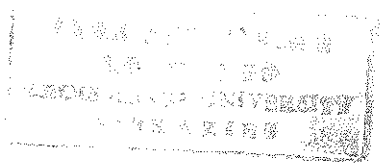
4.3. GREENHOUSE TRIALS

4.3.1. Experimental design

The greenhouse trials were conducted at Holeta Agricultural Research Center (HARC) which is located at 44 km west of Addis Ababa.

In the greenhouse, chambers were made using transparent 0.3 mm thick polythene sheet. The temperature in the chambers typically ranged between 14 and 24°C while conducting the experiment. The relative humidity of the chambers was maintained at above 90% by a humidifier.

Two experiments were carried out in the greenhouse. (1) Foliar spray method and (2) Seed treatment method of the antagonists. In each of the two experiments, six treatments were included. These were treatments with: *T. viride* (TV), *P. fluorescens* (PF), mixed culture of *T. viride* and *P. fluorescens* (MC) and a chemical fungicide Mancozeb (MAN) as a standard check. In addition, negative controls (NC) and positive controls (PC) were included for comparison.



In both experiments nine replicates were used for each treatment (i.e., 9 replicates x 6 treatments= 54 pots) and the pots were arranged in a randomized complete block design method.

4.3.1.1. Soil, pots and fertilizers

Clay soil (pH=5.7) which was obtained from Holeta potato field was used for the greenhouse trial.

Plastic pots of 5L volume (diameter and depth of 20 cm each) were used. The pots were surface sterilized with Clorox and rinsed with sterile tap water. Three kilograms of the soil was added to each pot after being autoclaved at 121°C for 30 minutes. At the bottom of the pots, three holes were made to allow removal of excess water.

Fertilizers, 0.6 g of Di-ammonium phosphate (DAP) and 0.5 g of Urea were added to each pot following the recommended application rates. The recommended application rates of DAP and Urea are 195 and 165 kg/ha respectively.

4.3.1.2. Selection and preparation of potato seed tubers

The potato cultivar (*Solanum tuberosum*) that was used in the greenhouse experiments was Awash (CIP-378501.3). Both the foliage and the tubers are known to be highly susceptible to *P. infestans* in Holeta region. The seed tubers were harvested from HARC and have been stored for 6 months.

Tubers were selected for freedom of any disease, uniformity of sizes (6-8 cm long), and number of sprouts. Tubers were surface sterilized with Clorox (3.5% sodium hypochlorite) for 1 minute and rinsed with sterile distilled water three times (Baxter *et al.*, 1999). Then they were dried in a laminar flow on Napkin sheets.

4.3.1.3. Foliar spray method

This method was employed to evaluate the effectiveness of spraying the antagonists in controlling airborne inocula of the pathogen. The spore and cell suspensions used in the experiment were prepared as follows.

Preparation of *T. viride* suspension

A 250 ml suspension of *T. viride* spores was prepared from nine days old culture plates which were grown on PDA (4 g/l potato extract, 20 g/l glucose and 15 g/l agar) at 25°C. The plates were rinsed with sterile distilled water and the mycelia were carefully scratched off the agar with a bent glass rod. Then the suspension was filtered through 4-layered gauze bandage to separate the spores from the mycelia. The concentration was adjusted to 3.7×10^8 spores/ml (Dubos, 1987) with the help of haemocytometer.

Preparation of *P. fluorescens* suspension

A 250 ml of *P. fluorescens* cell suspension was prepared as follows. *P. fluorescens* was inoculated to King's B broth and placed on shaker (150 r.p.m) for 3 days at room temperature. The concentration of the cells was then adjusted optically to a concentration of 1×10^9 cfu/ml ($O.D_{600}$= 1) (Mulya *et al.*, 1996) with the help of spectrophotometer.

Preparation of Mixed culture

A mixed culture of *T. viride* and *P. fluorescens* was prepared by mixing 125 ml of *T. viride* (3.7×10^8 spores/ml) and 125 ml of *P. fluorescens* (1×10^9 cfu/ml).

Preparation of Mancozeb solution

Mancozeb (Dithane M-45), a contact fungicide, was included for comparison. The application rate is 3 kg/ha. A 250 ml of the solution was prepared by dissolving 1.25 g mancozeb in sterile distilled water.

Preparation of *P. infestans* inoculum

The inoculum was prepared from *P. infestans* cultures grown on V8 agar (100 ml/l V8 juice, 1 g/l $CaCO_3$, 0.05 g/l β-sitosterol and 15 g/l agar) for 9 days in dark at 18°C. Sporangia were harvested from the plates by rinsing the sporangial/mycelial mat with sterile distilled water and scraping the mat using bent glass rod. The suspension was filtered through 4 folds of sterile gauze bandage to separate the sporangia from the mycelia. Then the concentration of the sporangia was adjusted to 1000 sporangia /ml with the help of haemocytometer.

Foliar spraying of antagonists and the pathogen

Healthy potato tubers were planted in the pots containing the sterilized soil. After the seedlings reached the rapid expansion phase (18 days after emergence, 30 cm tall with an average of 10 main leaves on each stem), the control agents were sprayed to run-off on to the plants. For the negative and the positive controls, the plants were sprayed with sterile distilled water.

Three days after the application of the control agents, 300 ml suspension of *P. infestans* (1000 sporangia/ml) (Kirk *et al.*, 1999) was sprayed to all the plants of the different treatments except to those of the positive controls. Before the pathogen was sprayed, the plants were exposed to a high relative humidity (R.H=100%) to wet the surface of the leaves. Following spray of the pathogen, the individual plants were covered with polythene bags to prevent cross infection among adjacent plants. The six treatments applied were:

T1. *T. viride* sprayed and *P. infestans* inoculated (**TV**)

T2. *P. fluorescens* sprayed and *P. infestans* inoculated (**PF**)

T3. A mixed culture of *T. viride* and *P. fluorescens* sprayed and *P. infestans* inoculated (**MC**)

T4. Mancozeb sprayed and *P. infestans* inoculated (**MAN**)

T5. Negative control (Sterile distilled water sprayed and *P. infestans* inoculated) (**NC**)

→ Inoculated/untreated check

T6. Positive control (Sterile distilled water sprayed, *P. infestans* not inoculated) (**PC**)

→ Non-inoculated/untreated check

4.3.1.4. Seed treatment method

Seed tuber infection simulation

To simulate tuberborne infection, healthy tubers were injected with 0.1 ml of sporangial suspension (1000 sporangia/ml) of *P. infestans* using syringe as it is shown in the photograph (Fig.2). This level of inoculum density ensures that the tubers do not rot and they sprout reliably (Appel *et al.*, 2001). Injection was done at a

distance of 1 cm from the main apical sprout at a depth of 2 cm. The wound was covered with a smear of vaseline to prevent desiccation. Then the tubers were left for 36 hours to establish infection. All the tubers except for the positive controls were inoculated with the pathogen.

Figure 3. Potato tuber inoculation with *P. infestans*

Preparation of *T. viride* suspension

A 500 ml suspension of *T. viride* spores was prepared from nine days old culture grown at 25°C in the same way as the foliar spray above. The spore concentration used was 2.4×10^8 spores/ml.

Preparation of *P. fluorescens* suspension

A 500 ml of *P. fluorescens* suspension was prepared as it was done for foliar spray method above. The concentration used was 1×10^9 cfu/ml.

Preparation of Mixed culture

A mixed culture of *T. viride* and *P. fluorescens* was prepared by mixing 250 ml of *T. viride* (2.4×10^8 spores/ml) and 250 ml of *P. fluorescens* (1×10^9 cfu/ml).

Preparation of Mancozeb solution.

The seed application rate of mancozeb is 240 g per100 L (Rohm and Haas, 1964). Thus, 500 ml of the solution was prepared by dissolving 1.2 g in 500 ml of sterile distilled water.

Seed tuber treatment and planting

Thirty six hours after inoculation of the pathogen into the potato seed tubers. the tubers were dipped in to their respective suspensions in 1L beakers; i.e., *T. viride*, *P. fluorescens*, mixed culture of *T. viride* and *P. fluorescens* and mancozeb for one hour. Seed tubers for the negative and the positive controls were dipped in sterile distilled water. After an hour, the tubers were taken out from the solutions and semi-dried in a laminar flow (Horizontal Laminar flow, M.D.H., Limited, UK) for 30 minutes. The six treatments applied were:

T1. Artificially infected tubers dipped into *T. viride* suspension **(TV)**

T2. Artificially infected tubers dipped into *P. fluorescens* suspension **(PF)**

T3. Artificially infected tubers dipped into mixed culture suspension of *T. viride* and *P. fluorescens* **(MC)**

T4. Artificially infected tubers dipped into mancozeb solution **(MAN)**

T5. Artificially infected tubers dipped into sterile distilled water (Negative control) **(NC)**

→Inoculated/ untreated check

T6. Healthy tubers dipped into sterile distilled water (Positive control) **(PC)**

→Non-inoculated/ untreated check

After being semi-dried, the tubers were immediately planted in the pots at a depth of 3 cm below the soil surface. Only one tuber was planted per pot. Water was given liberally once in every two days.

4.3.2. Data collection and Statistical analysis

In both foliar spray and seed treatment methods the individual plants were rated visually on weekly intervals for percentage of leaf area with symptoms of late blight over the disease progress period based on the assessment key shown in Fig.3. The average amount of disease developed over the disease progress period was expressed as the area under the disease progress curve (AUDPC) (Campbell and Madden, 1990) which is used extensively to measure disease progress over a given period (Kirk *et al.*, 1999).

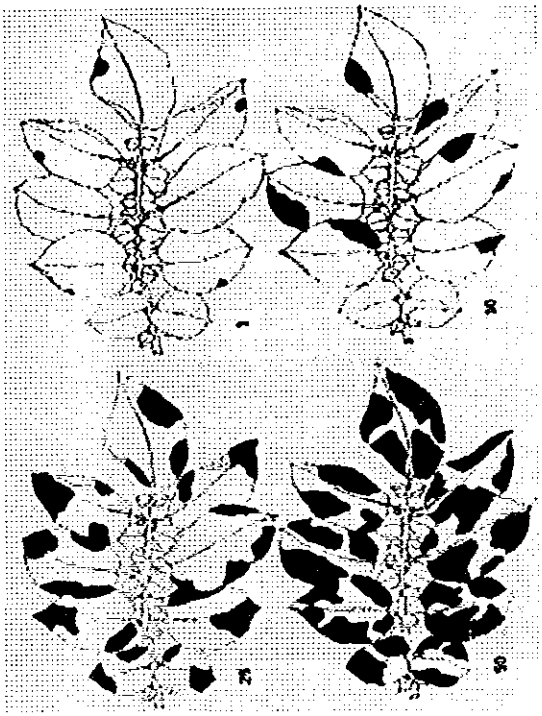


Figure 4. Assessment of percentage of leaf area affected by late blight within the foci. It is scaled as 1, 10, 25 and 50% (James, 1947 cited in Fahim *et al.*, 2003)

Statistical analysis was conducted using the general linear models procedures of the SPSS. Analysis of variance of differences in the treatments and least significance tests were carried out. Significance was evaluated at P=0.05 for the tests.

V. RESULTS

5.1. Antagonistic test between *T. viride* and *P. infestans*

Growth reduction of *P. infestans* was observed with radial growth inhibition of 36.7%. The average radius of *P. infestans* in the dual plates was 1.96 cm and 3.1 cm in the control plates. After the 4th day, the mycelia of *T. viride* started to overgrow the mycelia of *P. infestans*. By the 9th day, *T. viride* had completely overgrown the mycelia of the *P. infestans* as it is shown in Fig.4.

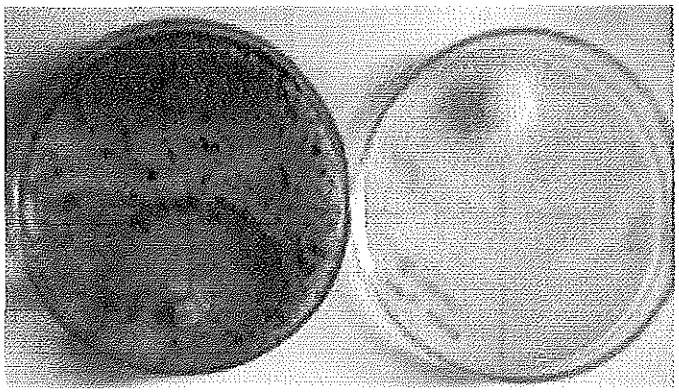
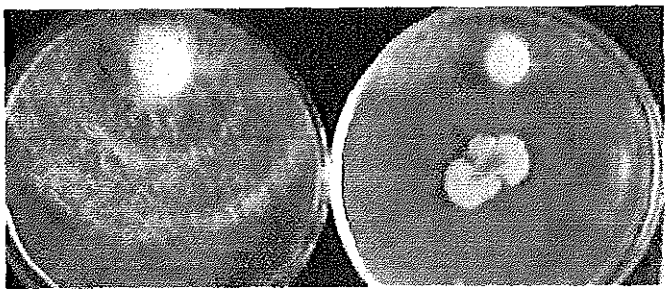


Figure 5. Antagonistic test between *T. viride* and *P. infestans*. The picture shows the complete overgrowth of *T. viride* on *P. infestans* (plate A). Plate A was inoculated with *P. infestans* on the upper portion and a mycelial disc of *T. viride* on the lower part. Plate B is the control plate (*P. infestans* alone).

5.2. Antagonistic test between *P. fluorescens* and *P. infestans*

The average radii of *P. infestans* in the dual and the control plates were 0.3 cm and 2.5 cm respectively, ten days after the inoculation of the pathogen. Therefore, the radial growth inhibition was 88%. A picture is shown in Fig.5 which shows the inhibition of *P. infestans* by *P. fluorescens*. A clear zone of inhibition of *P. infestans* was observed in the dual plate (B).



A B

Figure 6. Antagonistic test between *P. fluorescens* and *P. infestans*. Plate A: the control plate (*P. infestans* alone) and B: dual plate containing *P. fluorescens* at the center and *P. infestans* near the periphery.

5.3. Antagonist compatibility test

The average radius of the fungus on the 4th day in the dual plates was 2.6 cm whereas 3.9 cm in the control plates. By the 9th day, the colonies of the fungus and the bacterium met and neither organism grew any further. Inhibition zone was not seen between the bacterial and the fungal colonies (Fig. 6). The greenish area (A) is the fungus and the yellowish region (B) shows the bacterium.

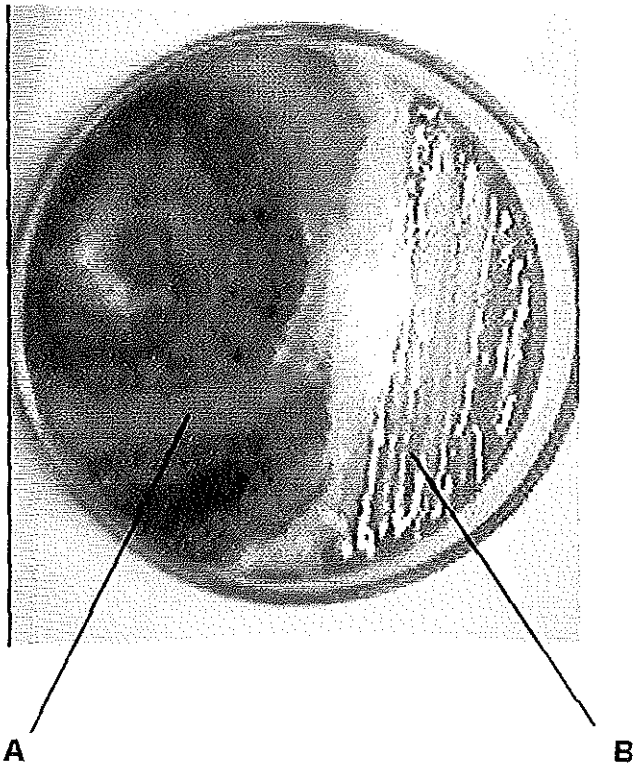


Figure 7. Compatibility test between *T. viride* (A) and *P. fluorescens* (B). The picture was taken on the 9th day after dual inoculation.

5.4. Observation of mycoparasitism by *T. viride*

Microscopic observation of the hyphal interactions between *T. viride* and the *P. infestans* revealed that *T. viride* coiled around or grew along the hyphae of *P. infestans.*

5.5. Foliar spray method

The values presented in Table 1 are the average AUDPC values of nine replicates of each of the six treatments. Each AUDPC value in turn was calculated using the midpoint rule from five disease severity assessments taken every 7 days. Plants sprayed with *T. viride*, *P. fluorescens* and the chemical fungicide mancozeb had significantly (P=0.05) reduced disease severity values compared to the negative controls (inoculated/untreated checks) and the mixed culture treated ones (Table 1). The smallest AUDPC values (less disease severity) was recorded in mancozeb (AUDPC=85.9) treated plants followed by *T. viride* (AUDPC=260) treated plants as shown in Fig.7. Mancozeb and *T. viride* treated ones were not significantly different while they were different from *P. fluorescens* (AUDPC=765.1) which shows the good performance of *T. viride. P. fluorescens* also significantly reduced the disease but to a lesser extent when compared with the performance of *T. viride* and mancozeb. On the contrary, the mixed culture (AUDPC=999) gave the least control of all the treatments which was not significantly different (P >0.05) from the negative control (AUDPC=1045.1) (Table 1). The result is also reflected in Fig.8. The picture shows high disease severity in mixed culture treated plant and the negative control, whereas mancozeb and *T. viride* treated plants are in a very good condition.

Table1. Mean AUDPC (%days) calculated from five consecutive observations of foliage disease severity in the foliar spray method

Treatment	Mean AUDPC
T. viride	260.0 ± 190.0 **c**
P. fluorescens	765.1 ± 218.6 **b**
Mixed culture	999.0 ± 274.5 **a**
Mancozeb	85.9 ± 77.8 **cd**
Negative control (inoculated/untreated)	1045.1 ± 227.2 **a**
Positive control (non-inoculated/untreated)	0.00 ± 0.00 **d**

Means followed by the same letter are not significantly different at P=0.05

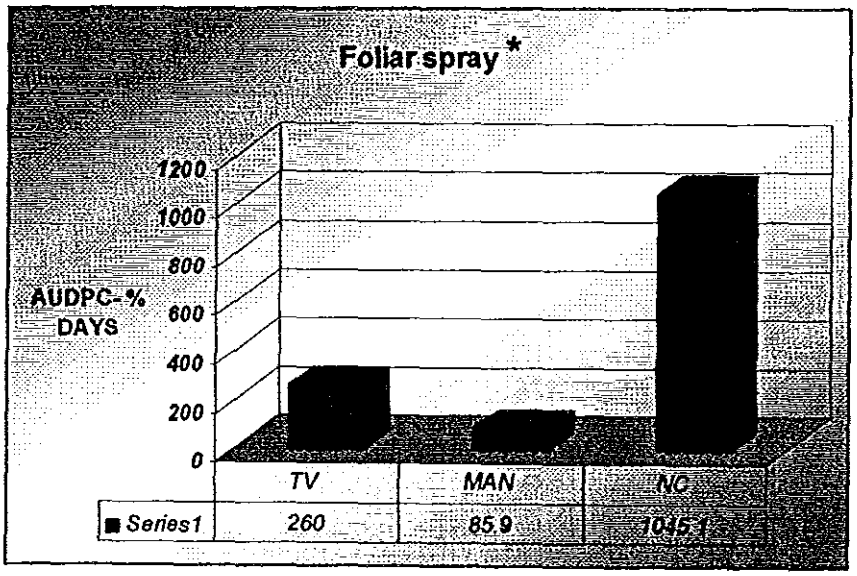


Figure 8. A chart showing the AUDPC values of the *T. viride* (TV), Mancozeb (MAN) and Negative control (NC) in the foliar spray method. It is clear that *T. viride* reduced the disease considerably when compared with the inoculated/ untreated check (Negative control). *The higher the AUDPC value, the more severe the disease over the span of the growing period.

Figure 9. Picture showing the status of the plants in the different treatments at the last date of observation in the foliar spray method. TV (*T. viride*), PF (*P. fluorescens*), MC (mixed culture), MAN (mancozeb), NC (negative control), and PC (positive control). The picture shows that the status of Mancozeb, *T. viride* and the positive control are very good while the mixed culture and the negative control are almost dead. *P. fluorescens* is between the two extremes.

The results of the ratio of infected to non-infected leaves on the main stems at the 14th day after foliar inoculation are presented in Table 2. It can be seen that mancozeb and *T. viride* treated plants had the lowest ratio and were not significantly different from non-inoculated/untreated check (positive control). Fig. 9 shows the result of the ratio of infected to non-infected leaves at each leaf position on the main stem. Highest ratio (0.88) was observed in the 9th leaf of the mixed culture treated plants followed by the 11th leaf of the negative control (0.77). The overall result shows a relatively higher infection between the 5th and the 11th leaves especially in those plants treated with mixed culture, *P. fluorescens* and the negative control. A relatively lower infection was observed in the first three leaves and the last two leaves in most of the treatments.

Table 2. Ratio of infected to non-infected leaves on the main stem in the foliar spray method at the14th day after *P. infestans* inoculation

Treatment	Mean
T. viride	0.066 ± 0.07 **a**
P. fluorescens	0.236 ± 0.16 **b**
Mixed culture	0.333 ± 0.15 **b**
Mancozeb	0.008 ± 0.02 **a**
Negative control	0.307 ± 0.17 **b**
Positive control	0.0 ± 0.0 **a**

Means followed by the same letter are not significantly different at P=0.05

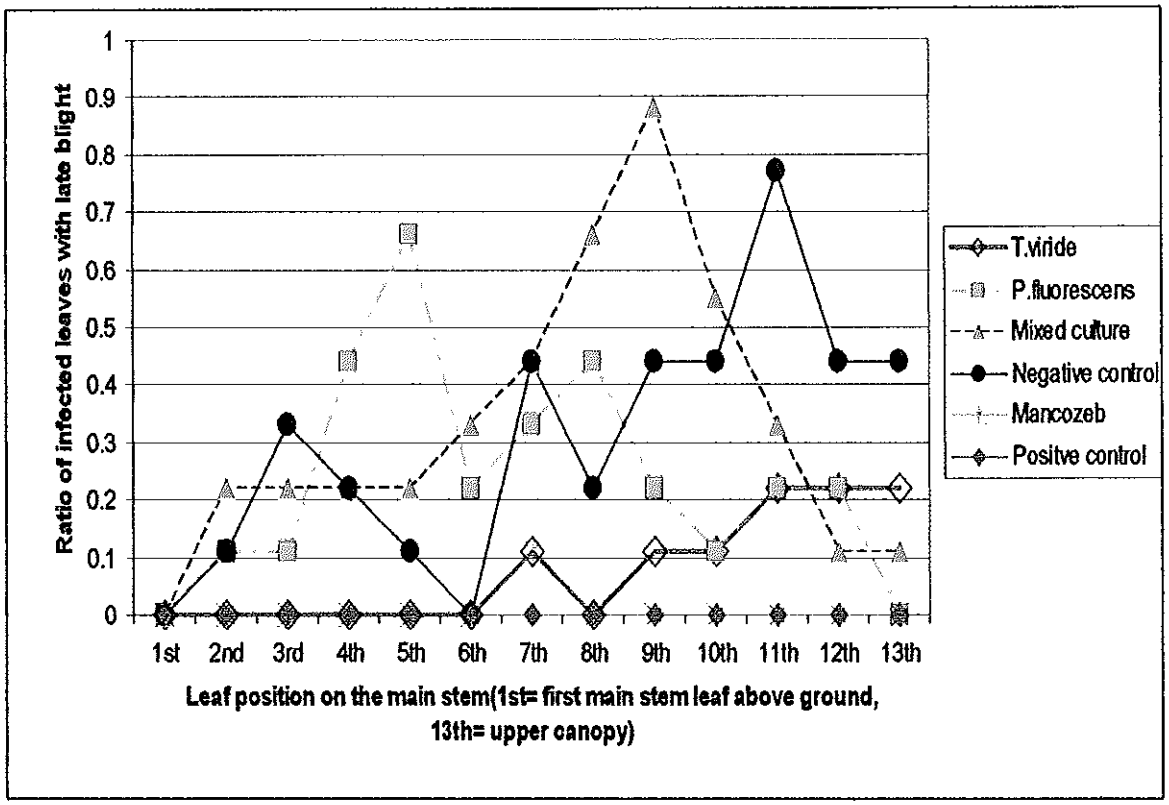


Figure 10. Ratio of infected to non-infected leaves in relation to the position of the leaf on the main stem at the 14th day after foliar inoculation of *P. infestans*.

5.6. Seed treatment method

The AUDPC values presented in Table 3 are the results of six consecutive disease severity assessment values taken every week. Highest AUDPC was observed in the negative control (1677.1) followed by mancozeb treated plants (1513.8) as shown in Fig.10. The AUDPC values presented in Table 3 indicate that no significant (P >0.05) difference was observed among each of mancozeb, mixed culture, *T. viride*, *P. fluorescens* and the negative control. The only significant difference (P<0.05) was observed between the positive control (non-inoculated/untreated check) and the remaining five treatments.

Table 3. Mean AUDPC (%days) calculated from six consecutive observations of foliage disease severity in the seed treatment method

Treatment	Mean AUDPC
T. viride	1279.818 ± 827.24 **a**
P. fluorescens	1109.850 ± 425.74 **a**
Mixed culture	1480.539 ± 788.71 **a**
Mancozeb	1513.789 ± 794.50 **a**
Negative control	1677.083 ± 625.11 **a**
Positive control	0.00 ± 0.00 **b**

Means followed by the same letter are not significantly different at P=0.05

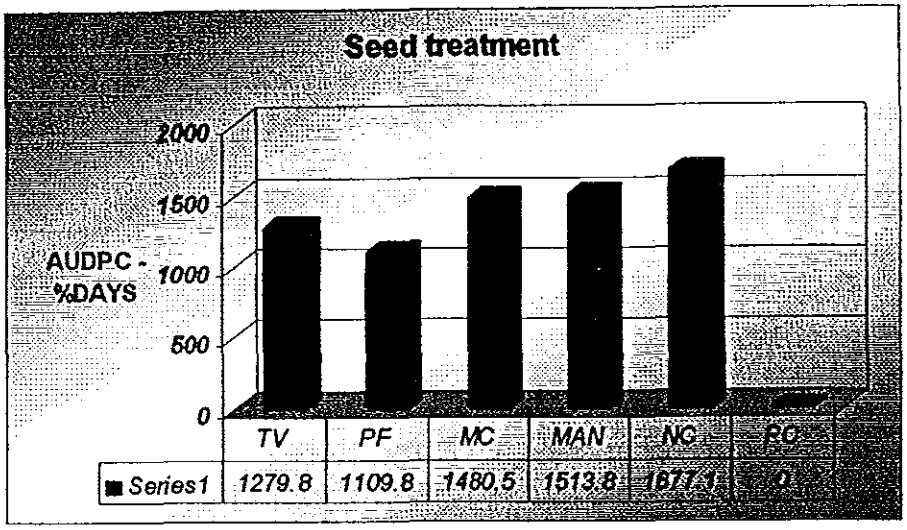


Figure11. A chart showing the AUDPC values of the different treatments in the seed treatment method. No significant difference was observed among each of *T. viride* (TV), *P. fluorescens* (PF), mixed culture (MC), mancozeb (MAN) and negative control (NC). PC refers to positive control.

Data on height of the main stems were taken after all the plants had emerged (18 days after being planted), to see if the different seed treatments could bring differences in the rate of growth (Table 4). Similarly, total number of leaves was also compared among the six treatments (Table 5). In both cases significant difference was observed only between the positive control and the remaining treatments. Significantly higher number of leaves (33.2) and higher growth (25.3 cm) rate was observed in the positive control (non-inoculated/ untreated check). Therefore, no significant differences were observed in height and number of leaves in those treated with *T. viride*, *P. fluorescens*, mixed culture, mancozeb and the negative control as shown in Table 4 and Table 5 respectively.

The ratios of number of stems emerged to total number of sprouts present on the tubers were also compared among the six treatments (Table 6). This was conducted to determine whether the artificially infected tubers have a different ratio compared to the healthy tubers that were used in the positive control. Even though highest ratio (0.934) was observed on the positive control and lowest ratio (0.760) in mancozeb treated plants, the differences were not statistically significant (P >0.05). Thus, as shown in Table 6, no significant difference was observed in any of the six treatments.

Table 4. Average height of the main stems in the six treatments in the seed treatment method. Measurements were taken after all the plants had emerged (18 days after planting)

Treatment	Mean (cm)
T. viride	15.556 ± 6.52 **a**
P. fluorescens	13.444 ± 4.85 **a**
Mixed culture	13.389 ± 7.63 **a**
Mancozeb	12.556 ± 5.66 **a**
Negative control	14.833 ± 6.03 **a**
Positive control	25.333 ± 5.39 **b**

Means followed by the same letter are not significantly different at P=0.05.

Table 5. Total number of leaves in the seed treatment method. Data taken 28 days after being planted.

Treatment	Mean leaf number
T. viride	18.66 ± 9.61 **a**
P. fluorescens	16.56 ± 5.81 **a**
Mixed culture	13.22 ± 7.12 **a**
Mancozeb	16.11 ± 10.03 **a**
Negative control	19.56 ± 12.22 **a**
Positive control	33.22 ± 13.32 **b**

Means followed by the same letter are not significantly different at P=0.05.

Table 6. Ratio of emerged sprouts (stems) to total sprouts among the six treatments in the seed treatment method

Treatment	Mean
T. viride	0.803 ± 0.197 **a**
P. fluorescens	0.819 ± 0.189 **a**
Mixed culture	0.814 ± 0.180 **a**
Mancozeb	0.760 ± 0.255 **a**
Negative control	0.898 ± 0.228 **a**
Positive control	0.934 ± 0.132 **a**

Means followed by the same letter are not significantly different at P=0.05

VI. DISCUSSION

T. viride was shown to retard the radial growth of *P. infestans*. This is most probably related to inhibitory metabolites that are produced by *T. viride*. Moreover, *T. viride* completely overgrew the colonies of *P. infestans*. This could be explained by the mycoparasitic action of *Trichoderma*. This mycoparasitic action was confirmed in the slide culture method in which *T. viride* hyphae coil around or grow along the hyphae of *P. infestans*. Similar inhibition result was seen in a related oomycete pathogen, *Pythium ultimum*, using *Trichoderma* strains (TH1, N47 and T12) (Naseby *et al.*, 2000). The fast growth of *T. viride* seen even at this relatively lower temperature (18°C) gives it advantage in competition for substrates over this pathogen.

In the *in vitro* plate assay, mycelial growth of *P. infestans* was strongly (88%) inhibited by *P. fluorescens*. This is corroborated by the work of Torres-Rubio *et al.* (2000) in which *P. fluorescens* inhibited *P. infestans* to an extent of 74%. The clear inhibition zone that was observed in the dual plates is suggestive of production of antibiotics by the *P. fluorescens*.

In the *in vitro* compatibility test between *T. viride* and *P. fluorescens*, the colonies of the fungus and the bacterium met on the 9th day and no inhibition zone was observed between the two. In addition, no further growth of either organism occurred. The absence of inhibition zone may indicate that antagonistic metabolites (if produced) were not inhibitory to each other. This observation was the basis for testing a combination of the two antagonists as a" Mixed culture " in the greenhouse experiment. A similar *in vitro* compatibility observation was reported between *Pseudomonas aeruginosa* and *Pochonia chlamydosporia* (Siddiqui and Shaukat, 2003).

The foliar spray result indicated that both individual antagonists (*T. viride* and *P. fluorescens*) significantly reduced the severity of foliar phase of late blight infection. The smallest area of leaf infection was observed in mancozeb treated plants and the highest in the untreated checks (negative control). Even though mancozeb treated plants show the least infection, the difference with that of *T. viride* treated

plants was not statistically significant. This shows that the performance of *T. viride* was comparable to that of the chemical fungicide. It is possible that more than one antagonistic mechanism could have been involved in the reduction of the disease. However, with this simple experiment, it is difficult to estimate the relative importance of each of the different mechanisms in the biocontrol of *P. infestans* by the bioagents and needs to be further examined. This is because the relative importance of the mechanisms is dependent on the particular isolate used, the target organism and also the ambient environmental conditions (Tronsmo, 1996).

The rationale behind the use of mixed cultures is that multiple strains allow the deployment of several different biocontrol mechanisms simultaneously. Besides, effective control of the target pathogen over diverse set of environmental conditions could be expected if strains with different ecological requirements are included in the inoculant (Mazzola, 1998). Contrary to what was expected, the performance of the mixed culture was the least and it was not even significantly different from the inoculated/untreated check in the foliar spray method. This requires a thorough study. Perhaps competition among the two antagonists might be one reason. It is desirable that antagonists should be complementary, not competitive (Baker and Cook, 1974). The agar test showed compatibility as no inhibition zone was formed between the *T. viride* and *P. fluore*scens and no overgrowth was seen. Thus it means that the *in vitro* compatibility test did not reflect the *in vivo* compatibility. Therefore, at this point it would be worth mentioning that strain combinations do not ensure improved biological control (Mazzola, 1998). The present result is in part similar to the observation of Hubbard *et al.* (1983). Hubbard *et al.* (1983) added *Trichoderma hamatum* to an iron-deficient soil containing high *Pseudomonas* spp. population density and consequently failed to achieve biological control. They found out that the *T. hamatum* was sensitive to iron deprivation induced by the *Pseudomonas* spp. Thus, competiton for iron between the two antagonists may also be the reason in the present case. However, evidence for the role of siderophores in competition for iron in the phyllosphere is needed if this assumption is to be true.

In the foliar spray method, the control agents were sprayed 3 days ahead of the pathogen. The reason is antagonists should occupy the site eariler than the

pathogen's arrival (Baker and Cook, 1974). In a study by Kexiang *et al.* (2000), *Trichoderma* species were found to give better control of *Botryosphaeria berengeriana* f.sp. *piricola* when inoculated 3 days in advance than when the two are co-inoculated. It is recommended that antagonists should attack or influence the pathogen at a weak point, preferably the most vulnerable to the pathogen (Baker and Cook, 1974). Campbell (1989) stated that germination of fungal spores on the leaf surface is a critical stage in the development of the host-pathogen interface, and one in which the pathogen is often vulnerable. It is known that the motile zoospores of *P. infestans* have no cell wall and are probably extremely vulnerable to adverse conditions. They are also the main infective propagules (Erwin and Ribeiro, 1996). Thus, zoospores can be targeted in biological control of *P. infestans*. The absence of cell wall makes them susceptible to antibiotics and enzymic attack by bioagents. Even the contact fungicide mancozeb makes use of this advantage and acts during the germination and penetration phases (Fernandez-Northcote *et al.*, 2000).

The ratio of infected to non-infected leaves on the main stem was significantly lower in those that were treated with mancozeb and *T. viride* than the rest of treatments. This reflects the strong performance of *T. viride*. The reason for a lower infection that was observed in the lower leaves of most of the treatments is perhaps due to the fact that potato leaves at the base of the stem are less susceptible to late blight infection than leaves closer to the flower (Carnegie and Colhoun, 1982). On the other hand, lower infection ratio of the upper leaves is probably because they are young and had not been there when the pathogen was sprayed 14 days ago.

Survival within the potato tubers provides the only over-wintering mechanism for *P. infestans* in most potato producing regions (Cooke and Little, 2001). Investigation of seed lots showed that up to 20% of tubers can be latently infected with *P. infestans*, even if no signs of the symptoms of tuber blight are visible (Appel *et al.*, 2001). Thus, infected tuber is an important source of initial infection. Consequently, if it is possible to control the transmission of late blight pathogen from infected tubers to emerging sprouts, it will be possible to eliminate or at least reduce initial sources of inoculum in areas where oospores and alternative hosts do not initiate an epidemic. The seed treatment experiment was done from this stand point.

Seed treatment is important because it requires smaller amounts of the bioagents than in-furrow or broadcast application (Papavizas, 1985). It is probably the simplest and least costly in time, material and money of any chemical or biological control measure (Maloy, 1993). Unfortunately, the data on the seed treatment revealed that neither the bioagents nor the chemical fungicide was able to control the development and spread of the pathogen from the infected tubers to the aerial part. This result is similar to a study that involved individual and combinations of four chemical fungicides including mancozeb (Kirk *et al.*, 1999). They found that neither of the chemical treatments effectively controlled late blight spread inside the tuber and the subsequent development of the infection to the leaf. Thus, the present study is in agreement with the above. The reason for such phenomenon is perhaps because the pathogen grows from the tuber to the sprout systemically by way of mycelial growth and finally to the stem (Appel *et al.*, 2001) and hence the control agents will not reach the pathogen.

The result of height measurements and leaf counts among the six treatments in the seed treatment method revealed a significant difference only between the positive controls and the remaining treatments in both cases. Vigorous growth was observed in those plants emerged from healthy tubers (positive controls). On the other hand, in the rest treatments where infected seed tubers had been used, retarded growth was seen regardless of the seed treatments. This indicates that the pathogen inside the tubers influences the growth rate by deteriorating the potential of the tubers. Moreover, the control agents coated on the seed tubers were not able to reverse this condition.

The ratio of emerged stems to total sprouts showed no significant difference in any of the six treatments. No statistically significant difference was observed even between the positive controls (healthy tubers used) on one side and the rest five treatments (infected tubers used) on another side. It may seem that the presence of the pathogen in the tuber does not affect the number of stems that develop from the tuber. This will definitely contradict my expectation and also the observation of Kirk *et al.* (1999) in which low number of plants emerged from infected tubers. The difference between the two results is probably explained by the fact that in the present experiment the tubers used had relatively larger sprouts before being

planted. Consequently, the sprouts must have emerged from the soil before the pathogen can immigrate far enough and kill them. This assumption can be supported by the fact that some stems in the non-positive control treatments were seen to die very soon after emergence. Thus, if potatoes with very little sprouts had been used in the present experiment, it would have probably caused a different result. This is because, as observed, little sprouts will take more time to emerge from the soil and this in turn will provide sufficient time for the pathogen to spread within the tuber and eventually the sprouts will become infected and killed prior to emerging from the soil.

VII. CONCLUSION

The overall result of this study revealed that *T. viride*-TNAU has a good potential in controlling the foliar phase of late blight when used as a foliar spray. *P. fluorescens*-Bak 150 also reduced the disease severity of the foliar blight but to a lesser extent when compared with *T. viride*-TNAU. Amazingly, a combination of the two antagonists performed least of all; the reason requires further study. On the other hand, the bioagents and the chemical fungicide mancozeb, when used as seed tuber treatment, were not effective in controlling or reducing the transmission of the pathogen from the infected tubers to the sprouts and the ensuing progress of the disease in the foliage.

With the current status of late blight control; no alternative control measures can fully substitute the chemical fungicides. Perhaps chemical fungicides will always be needed for control of late blight. However, their use should be integrated with other control methods if a best and environmentally friendly result is to be achieved. For instance, integration of biocontrol agents with reduced doses of chemical agents is one option. This is feasible because it is possible to create fungicide tolerant strains of biocontrol agents.

As any other biocontrol systems, biological control of late blight of potato (if implemented) should be regarded as one facet of the integrated control program rather than a method to be used alone and to be judged on its solo performance.

VIII. RECOMMENDATION

1. The present study was conducted under greenhouse conditions and hence corresponding field trials should be carried out to further confirm the efficacy of *T. viride*-TNAU.

2. In this study, *T. viride*-TNAU spore suspension without any food base and sticker was used. Thus, perhaps a much better result could be achieved by using appropriate formulations.

3. There could also be a chance of enhancing the efficacy of *T. viride*-TNAU by using different spore concentrations other than the one used in this experiment and thus studies should also be done in this direction.

4. In this study only one time spray was tested. Hence, multiple spraying with some time intervals may also result in a better outcome.

5. In addition to *T. viride*-TNAU, there could also be more effective local *Trichoderma* isolates or other antagonists against *P. infestans*. Therefore, more research should also be directed in screening other more efficient bioagents which are well adapted to the highly stressed leaf environment.

6. The possibility of strain improvement should not also be ignored. In parallel, studies on economically-effective large scale production of the bioagents should be conducted.

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