UNIVERSITA' DEGLI STUDI DI NAPOLI

"FEDERICO II"

DOTTORATO DI RICERCA

IN AGROBIOLOGIA E AGROCHIMICA

Indirizzo: PATOLOGIA VEGETALE

XXI CICLO

Novel plant bio-protectants based on *Trichoderma* spp. strains with superior characteristics

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INDEX

1. INTRODUCTION

Climate changes caused by augmented global warming will significantly modify the agricultural environment. Notable increases will be noted in: atmospheric carbon dioxide concentration ([CO_2]), average temperature and tropospheric ozone concentration ([O_3]), the severity and frequency of droughts, the intensity of precipitation events which will lead to increased flooding, the degradation and erosion of soils, and fluctuations in climatic extremes will be more likely to occur (IPCC, 2007). This overall global climatic change will consequently affect geographic distribution, biodiversity and growth of plant species, possibly causing new phenomena, such as the proliferation of pathogen populations, modification of host plant physiology or resistance, or modifications to the presence of natural enemies (GCTE-LUCC, 1998).

In Africa, agriculture is the principle industry and the key to economic development. Agriculture productivity on the Continent is not only the lowest, but it has also remained stagnant whereas all other regions in the world have shown substantial increases in product output (FAO, 1997). Crop production in the northern territories of Africa, where Libya is located, takes place under extremely variable agro-ecological conditions, with very low rainfall, high temperature and occasional sand storms. The current pest management research activities carried out by national and international agricultural research agencies in Africa have been progressively re-oriented to a reduced application of pesticide chemicals while focusing on classical biological control methods to manage crop pests and traditional plant breeding programs to improve host plant resistance (Abate et al., 2000).

In this new scenario, alternative methods for crop protection have focused on the introduction of several beneficial microorganisms as the active ingredients in new formulations of bio-pesticides that represent the basis for many natural products of microbial origin (Montesinos, 2003).

Various strains of the filamentous fungus *Trichoderma* spp. are considered to be among the most useful fungi in industrial enzyme production, agriculture and bioremediation. More recently, these fungi have been utilized extensively as model microorganisms in studies in order to analyze and improve the understanding of the role that these antagonistic fungi have in important biological interactions, for instance with crop plants and phytopathogens (Marra et al. 2006; Woo et al., 2006). New techniques, such as the use of genomic approaches to study the complex and fascinating mechanisms that permit *Trichoderma* to produce large amount of heterologous proteins, control pathogens and effect plant metabolism and physiology are still in their infancy, but they are revealing exciting findings (Marra et al., 2006). Although the need and interest to sustain both structural and functional genomic projects is widely recognized and has led to funding and start up of several new initiatives, very little has been accomplished to date and much further investigation is required.

1.1. What is *Trichoderma*? General description of morphology, life history, and distribution

Trichoderma are filamentous fungi commonly found in the soil community that are facultative saprophytes. They are members of a genus belonging to a group of largely asexually reproducing fungi that includes a wide spectrum of micromycetes that range from very effective soil colonizers with high biodegradation potential to facultative plant symbionts that colonize the rhizosphere. According to MYCONET electronic database (www.umu.se/myconet/myconet6.html), *Trichoderma* combines anamorphic (mitosporic) fungi of genus *Hypocrea* (telomorph) belonging to the *Hypocreaceae* of the *Hypocreales* within the class *Sordariomycetes*.

Trichoderma is usually recognized by the presence of fast-growing colonies producing white, green, or yellow cushions of sporulating filaments, the fertile filaments or conidiophores produce side branches bearing whorls of short phialides that support the spherical to ovoid green colored spores (Fig. 1). *Trichoderma* is

found in nearly all temperate and tropical soils, where samples contained 10^1–10^3 cultivable propagules per gram of soil. These fungi also colonize woody and herbaceous plant materials, in which the sexual teleomorph (genus *Hypocrea*) has most frequently found.

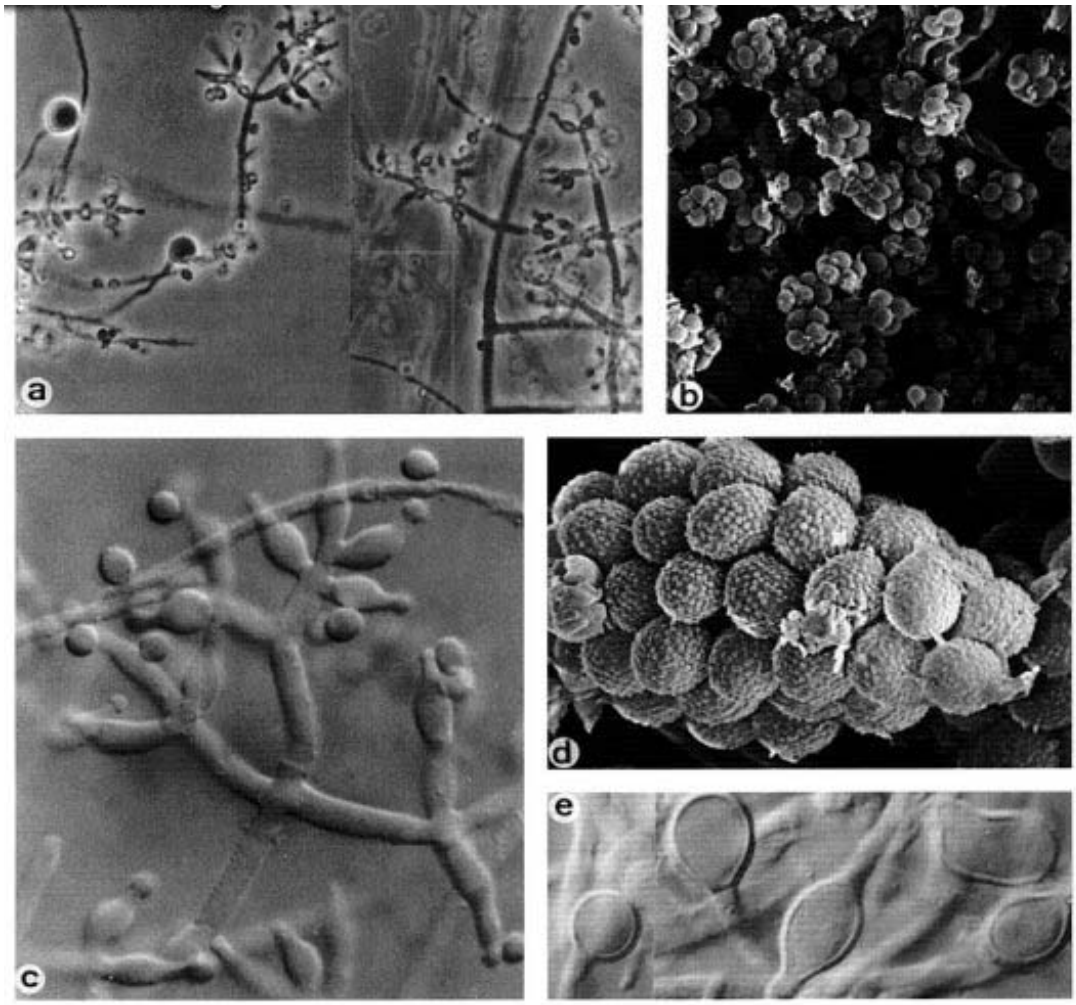


Figure 1. *Trichoderma viride* IFO 30498. a-d Conidiophores and conidia; e chlamydospores. a x512; b x1000; c x1600; d x4400; e x1600. Pictures by de Hoog et al. (2000).

In general, the mycelia of *Trichoderma* spp. on potato dextrose agar (PDA) plate cultures is typically fast growing, with the optimal temperatures between 25-30° C, and growth is usually minimal or absent at temperatures greater than 35° C. The hyphae are initially transparent or whitish, and depending upon the species, the mycelium become greenish, yellowish or less frequently white within one week (Fig. 2). A characteristic sweet or 'coconut' odor is produced by some species such as *T. atroviride*. Conidiophores are highly branched and thus difficult to define or measure. They may be loosely grouped or compactly tufted, and often develop in distinct concentric rings (in correspondence to available light) or are borne along the scant aerial hyphae. Main branches of the conidiophores produce lateral side branches that may be paired or not, the longest branches distant from the tip and often phialides arising directly from the main axis near the tip.

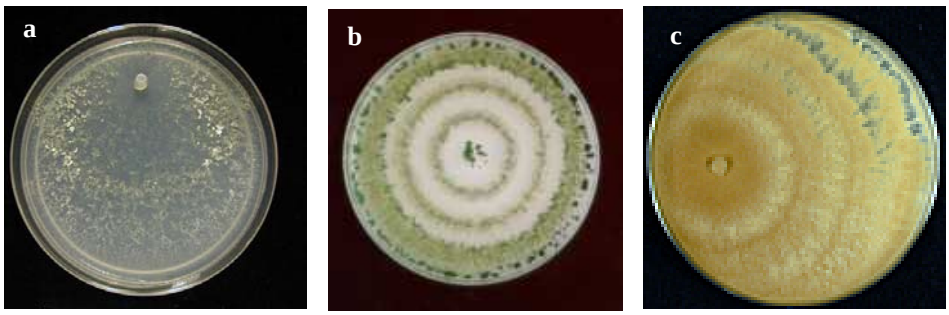


Figure 2. Examples of *Trichoderma* cultures grown in Petri dishes. a *T. atroviride*; b *T. viride*; c *T. harzianum.*

The teleomorphic form, not frequently seen in nature, belongs to the ascomycete genus *Hypocrea* Fr. and is characterized by the formation of fleshy, stromata in shades of light or dark brown, yellow or orange. Typically the stroma is discoidal to pulvinate and not extensive, whereas the stromata of some species are effused, sometimes covering extensive areas. Stromata of some species (*podostroma*) are clavate or turbinate. Perithecia are completely immersed in the

mycelium. Ascospores are bicellular but disarticulate at the septum early in development into 16 partial-ascospores so that the ascus appears to contain 16 ascospores. Ascospores are hyaline or green and typically spinulose. More than 200 species of *Hypocrea* have been described but only few have been grown in pure culture and fewer have been re-described in modern taxonomic terms. However, the majority of the species, including most biocontrol strains, have no known sexual stage and are grouped in the group of deuteromycetes or imperfect fungi.

Many members of the genus *Trichoderma* are prolific producers of extracellular proteins, and best known for their ability to produce enzymes that degrade cellulose and chitin, although they are also capable of producing other useful enzymes for industry and agriculture (Harman and Kubicek, 1998). For example, numerous *Trichoderma* strains produce hundreds of different metabolites that are also known to have antibiotic activity.

Trichoderma species have long been recognized as biological control agents (BCAs) for the control of plant disease and for their ability to increase plant growth and development. They are widely used in agriculture, and some of the most useful strains demonstrate a property known as 'rhizosphere competence', the ability to colonize and grow in association with plant roots (Harman, 2000). Much of the known biology and many of the uses of these fungi have been documented recently (Harman and Kubicek, 1998; Harman et al., 2004a; Kubicek and Harman, 1998). The taxonomy of this fungal genus is continually being revised, and many new species are being described (Komon-Zelazowska et al., 2007; Kubicek et al., 2008; Overton et al., 2006; Samuels, 2006). The mechanisms that *Trichoderma* uses to antagonize phytopathogenic fungi include competition, colonization, antibiosis and direct mycoparasitism (Howell, 2003). This antagonistic potential serves as the basis for effective biological control applications of different *Trichoderma* strains as an alternative method to chemicals for the control of a wide spectrum of plant pathogens (Chet, 1987; Harman and Björkman, 1998).

1.2. Known applications of *Trichoderma* spp.

Trichoderma spp. have been widely studied, and are presently marketed as biopesticides, biofertilizers and soil amendments, due to their ability to protect plants, enhance vegetative growth and contain pathogen populations under numerous agricultural conditions (Harman, 2000; Harman et al., 2004a; Lorito et al., 2006; Vinale et al., 2008a). The commercial success of products containing these fungal antagonists can be attributed to the large volume of viable propagules that can be produced rapidly and readily on numerous substrates at a low cost in diverse fermentation systems (Agosin et al. 1997; 1998). The living microorganisms, conserved as spores, can be incorporated into various formulations, liquid, granules or powder etc., and stored for months without losing their efficacy (Jin et al. 1991; 1992; 1996). To date more than 50 different *Trichoderma*-based preparations are commercialized and used to protect or increase the productivity of numerous horticultural and ornamental crops (Table 1; Lorito et al. 2006).

Table 1. Examples of commercial products containing *Trichoderma* and/or *Gliocladium*.

Commercial Product	Biocontrol Organism(s)	Product Type	Formulation, Application	Uses - Location, Crops	Uses, Pathogens controlled	Manufacturer/Supplier, Country, Internet Reference
Ago Biocontrol Trichoderma 50	*T. harzianum*	Biological fungicide	n/a	Flowers, vegetables, fruits, other crops	*Fusarium*, *Rhizoctonia*, *Alternaria*, *Rosellinia*, *Botrytis*, *Sclerotium*, *Phytophthora* spp	Ago Biocontrol, Colombia (http://www.sipweb.org/directorymcp/fungi.html)
Antagon	*Trichoderma* spp.	Biological fungicide	powder	Horticulture (commercial), parks, recreational areas, sports fields	damping-off diseases	De Ceuster Meststoffen N.V. (DCM), Belgium (http://www.agroBiologicals.com/products/P1609.htm)
Binab T	*T. harzianum*, *T. polysporum*	Biological fungicide	Pellets, wettable powder or granules; spray, drench, mixed in soil	Wood products; ornamental, shade, forest trees; greenhouse, nursery, field; cut flowers, potted plants, vegetables, mushrooms, flower bulbs	Wood rots causing internal decay, or originating from pruning wounds; *Didymella, Chondrostereum, Heterobasidion, Botrytis, Verticillium, Pythium, Fusarium, Phytophthora, Rhizoctonia*	BINAB Bio-Innovation AB, Sweden (http://www.algonet.se/~binab/index2.html); Henry Doubleday Research Association, United Kingdom; Svenska Predator AB, Sweden; E.R. Butts International, Inc., USA
BioFit	*T. viride*	Biological fungicide	Seed treatment, root/tuber dip, drench; Used alone or in combination with chemicals.	Gram, pepper, groundnut, wheat, potato, ginger, turmeric, peas, matki, mung, urid , tomato, bhindi, onion, other vegetables, grapes.	*Pythium, Rhizoctonia, Fusarium, Sclerotium*, other root rots; for *Botrytis* in combination with chemicals	Ajay Bio-tech (India) Ltd., India (http://www.ajaybio.com)
Bio-Fungus (formerly Anti-Fungus), Supresivit	*Trichoderma* spp.	Biological fungicide	granular, wettable powder, sticks, crumbles; soil incorporation; spray or injection	Flowers, strawberries, trees, vegetables	*Sclerotinia, Phytophthora, Rhizoctonia solani, Pythium* spp., *Fusarium, Verticillium*	BioPlant, Denmark (www.bioplant.dk); De Ceuster Meststoffen N.V. (DCM), Belgium

Commercial Product	Biocontrol Organism(s)	Product Type	Formulation, Application	Uses - Location, Crops	Uses, Pathogens controlled	Manufacturer/Supplier, Country, Internet Reference
Combat	*T. harzianum, T. virens (=T. lignorum G. virens), Bacillus subtilis*	Biological fungicide	Talc; seed treatment, broadcast, root dip, drench, foliar spray	Grapes, cotton, pulses, tea, potato, tomato, oil seeds, tobacco, spices, cereals, vegetables, horticultural crops	Downy mildew, powdery mildew, die back, *Verticillium*, *Fusarium*, Panama wilt; pod, seedling, late blight; root, collar, stem, red, soft, clump, dry, bean, fruit, pod rot; black leg, damping off, abnormal leaf fall, black thread, canker	BioAg Corporation USA (http://www.bioag.com/products.html)
Harzian 20 (under development)	*T. harzianum*	Biological fungicide	n/a	orchard crops, vineyards	*Armillaria* spp., *Pythium* spp., *Sclerotinia* spp.	Natural Plant Protection (NPP), France (http://www.agroBiologicals.com/products/P1362.htm)
PlantShield	*T. harzianum*	Biological fungicide	Granules, wettable powder; soil drench, foliar spray	Greenhouse, flowers, ornamentals, herbs, nursery, vegetable crops; hydroponic, orchard trees	*Pythium, Fusarium, Rhizoctonia, Cylindrocladium, Thielaviopsis*; suppresses *Botrytis*	BioWorks, Inc., USA (http://www.bioworksbiocontrol.com)
Primastop	*G. catenulatum*	Biological fungicide	Powder; drench, spray, irrigation	ornamental, vegetable, tree crops	pathogens causing seed, root, stem rot, wilt disease	Kemira Agro Oy, Finland (http://growhow.kemira-agro.com); AgBio Development Inc.USA
Root Pro, RootProtato	*T. harzianum, T. cornedia*	Biological fungicide	Powder; spores mixed with growing media	Seedling, rooting stage in nursery; Horticulture - flowers, vegetables, potatoes	*Rhizoctonia solani, Pythium spp., Fusarium spp., Sclerotium rolfsii*	Mycontrol Ltd., Israel; Efal Agri, Israel (**http://www.efal.com/main.htm**, http://www.agroBiologicals.com/company/C1096.htm)

The benefits of using *Trichoderma* in agriculture are multiple, and depending upon the strain the advantages for the associated plant can include: (i) colonization of the rhizosphere by the BCA (‘‘rhizosphere competence’’), allowing rapid establishment within the rhizosphere of a stable microbial community; (ii) control of phytopathogenic and competitive micro flora or fauna by using a variety of mechanisms; (iii) overall improvement of the plant health; (iv) plant growth promotion, by stimulation of above and below ground parts; (v) enhanced nutrient availability and uptake, and (vi) induced systemic resistance (ISR) similar to that stimulated by beneficial rhizobacteria (Harman et al., 2004a; Howell, 2003; Woo and Lorito, 2006).

Trichoderma biocontrol strains utilize numerous mechanisms for both attacking other soil organisms and enhancing plant and root growth (Benítez et al., 2004; Harman, 2000; Harman et al., 2004a; Vinale et al., 2008a). The colonization of the root system by rhizosphere competent strains of *Trichoderma* results in increased development of root and/or aerial systems and crop yields (Chacón et al., 2007; Harman and Kubicek, 1998; Yedidia et al., 2001). *Trichoderma* has also been described as being involved in other biological activities such as the induction of plant systemic resistance and antagonistic effects on plant pathogenic nematodes (Sharon et al., 2001). Some strains of *Trichoderma* have also been noted to be aggressive biodegraders in their saprophytic phases (Wardle et al., 1993), in addition to acting as competitors to fungal pathogens, particularly when nutrients are a limiting factor in the environment (Simon and Sivasithamparam, 1989). These facts strongly suggest that in the plant root environment *Trichoderma* actively interacts with the components in the soil community, the plant, bacteria, fungi, other organisms, such as nematodes or insects, that share the same ecological niche.

Trichoderma spp. are important participants in the nutrient cycle. They aid in the decomposition of organic matter and make available to the plant many elements normally inaccessible. Yedidia et al. (2001) noted that the presence of the fungus increased the uptake and concentration of a variety of nutrients (copper, phosphorus, iron, manganese and sodium) in the roots of plants grown in hydroponic culture, even under axenic conditions. These increased concentrations indicated an

improvement in plant active-uptake mechanisms. Corn that developed from seeds treated with *T. harzianum* strain T-22 produced higher yields, even when a fertilizer containing 40% less nitrogen was applied, than the plants developed from seed that was not treated with T-22 (Harman 2000; Harman and Donzelli, 2001). This ability to enhance production with less nitrate fertilizer, provides the opportunity to potentially reduce nitrate pollution of ground and surface water, a serious adverse consequence of large-scale maize culture. In addition to effects on the increase of nutrient uptake and the efficiency of nitrogen use, the beneficial fungi can also solubilize various nutrients in the soil, that would be otherwise unavailable for uptake by the plant (Altomare et al., 1999).

The cross-talk that occurs between the fungal BCA and the plant is important both for identification of each component to one another and for obtaining beneficial effects. Somehow, the plant is able to sense, possibly by detection of the released fungal compounds, that *Trichoderma* is not a hostile presence, therefore the plant defense system is not activated as it is when there is pest attack and the BCA is recognized as a plant symbiont rather than a plant pathogen (Woo and Lorito, 2006). Molecules produced by *Trichoderma* and/or its metabolic activity also have potential for promoting plant growth (Chacón et al., 2007; Vinale et al., 2008a,b; Yedidia et al., 1999). Applications of *T. harzianum* to seed or the plant resulted in improved germination, increased plant size, augmented leaf area and weight, greater yields (Altomare et al., 1999; Harman, 2000; 2004b; Inbar et al., 1994; Vinale et al., 2008b).

Numerous studies indicated that metabolic changes occur in the root during colonization by *Trichoderma* spp., such as the activation of pathogenesis-related proteins (PR-proteins), which induce in the plant an increased resistance to subsequent attack by numerous microbial pathogens (Table 2). The induction of systemic resistance (ISR) observed *in planta* determines an improved control of different classes of pathogens (mainly fungi and bacteria), which are spatially and temporally distant from the *Trichoderma* inoculation site. This phenomenon has been observed in many plant species, both dicotyledons (tomato, pepper, tobacco, cotton, bean, cucumber) and monocotyledions (corn, rice). For example, *T.*

harzianum strain T-39, the active ingredient of the commercial product Tricodex™, induces resistance towards *Botrytis cinerea* in tomato, tobacco, lettuce, pepper and bean plants, with a symptom reduction ranging from 25 to 100% (De Meyer et al., 1998). Moreover, *Trichoderma* determined an overall increased production of defense-related plant enzymes, including various peroxidases, chitinases, β-1,3-glucanases, and the lipoxygenase-pathway hydroperoxide lyase (Harman et al., 2004a; Howel et al., 2000; Yedidia and Chet, 1999).

Thus far, *Trichoderma* is able not only to produce toxic compounds with a direct antimicrobial activity against pathogens, but also to generate fungal substances that are able to stimulate the plant to produce its own defense metabolites. In fact, the ability of *T. virens* to induce phytoalexin accumulation and localized resistance in cotton has already been discussed (Hanson and Howell, 2004). In cucumber, root colonization by strain T-203 of *T. asperellum* caused an increase in phenolic glucoside levels in the leaves; the aglycones, which are phenolic glucosides with the carbohydrate moieties removed, are strongly inhibitory to a range of bacteria and fungi (Yedidia et al., 2003).

Table 2. Evidence for, and effectiveness of, induced resistance in plants by *Trichoderma* species (Harman et al., 2004a).

Species and strain	Plant	Pathogens	Evidence or effects	Time after application	Efficacy
T. virens G-6, G-6-5 and G-11	Cotton	*Rhizoctonia solani*	Protection of plants; induction of fungitoxic terpenoid phytoalexins	4 days	78% reduction in disease; ability to induce phytoalexins required for maximum biocontrol activity
T. harzianum T-39	Bean	*Colletotrichum lindemuthianum*; *Botrytis cinerea*	Protection of leaves when T-39 was present only on roots	10 days	42% reduction in lesion area; number of spreading lesions reduced
T. harzianum T-39	Tomato, pepper, tobacco, lettuce, bean	*B. cinerea*	Protection of leaves when T-39 was present only on roots	7 days	25–100% reduction in grey-mould symptoms
T. asperellum T-203	Cucumber	*Pseudomonas syringae* pv. lachrymans	Protection of leaves when T-203 was present only on roots; production of antifungal compounds in leaves	5 days	Up to 80% reduction in disease on leaves; 100-fold reduction in level of pathogenic bacterial cells in leaves
T. harzianum T-22; *T. atroviride* P1	Bean	*B. cinerea* and *Xanthomonas campestris* pv. phaseoli	Protection of leaves when T-22 or P1 was present only on roots; production of antifungal compounds in leaves	7–10 days	69% reduction in grey-mould (*B. cinerea*) symptoms with T22; lower level of control with P1. 54% reduction in bacterial disease symptoms.
T. harzianum T-1 & T22; *T. virens* T3	Cucumber	Green-mottle mosaic virus	Protection of leaves when *Trichoderma* strains were present only on roots	7 days	Disease-induced reduction in growth eliminated
T. harzianum T-22	Tomato	*Alternaria solani*	Protection of leaves when T-22 was present only on roots	3 months	Up to 80% reduction in early blight symptoms from natural field infection
T. harzianum T-22	Maize	*Colletotrichum graminicola*	Protection of leaves when *Trichoderma* strains were present only on roots	14 days	44% reduction of lesion size on wounded leaves; no disease on non-wounded leaves
Trichoderma GT3-2	Cucumber	*C. orbiculare*, *P. syringae* pv. lachrymans	Protection of leaves when *Trichoderma* strains were present only on roots; induction of lignification and superoxide generation	1 day	59% and 52% protection from disease caused by *C. orbiculare* or *P. syringae*, respectively
T. harzianum	Pepper	*Phytophthora capsici*	Protection of stems when *Trichoderma* strains were present only on roots; enhanced production of the phytoalexin capsidiol	9 days	~40% reduction in lesion length
T. harzianum NF-9	Rice	*Magnaporthe grisea*; *Xanthomonas oryzae* pv. oryzae	Protection of leaves when NF-9 was present only on roots	14 days	34–50% reduction in disease

A fundamental part of the *Trichoderma* antifungal capability consists in the production and secretion of a great variety of extracellular cell wall degrading enzymes (CWDEs), including endochitinases, β-N-acetylhexosaminidase (N-acetyl-β-D-glucosaminidase), chitin-1,4-β-chitobiosidases, proteases, endo- and exo-β-1,3-glucanases, endo β-1,6-glucanases, lipases, xylanases, mananases, pectinases, pectin lyases, amylases, phospholipases, RNAses, DNAses, etc. (Benítez et al., 1998; Lorito, 1998). The chitinolytic and glucanolytic enzymes are especially valuable for their CWDE activity on fungal plant pathogens, hydrolyzing polymers not present in

plant tissues (Woo et al., 1999). Each of these classes of enzymes contains diverse sets of proteins with distinct enzymatic activities. Some have been purified, characterized and their encoding genes cloned (Ait-Lahsen et al., 2001; de la Cruz et al., 1992; 1995a; 1995b; García et al., 1994; Limón et al., 1995; Lora et al., 1995; Lorito et al., 1993; 1994a; Montero et al., 2007; Peterbauer et al., 1996; Suárez et al., 2004; Viterbo et al., 2001; 2002). Once purified, many *Trichoderma* enzymes have shown to have strong antifungal activity against a wide variety of phytopathogens, and they are capable of hydrolyzing not only the tender young hyphal tips of the target fungal host, but they are also able to degrade the hard, resistant conservation structures such as sclerozi.

1.3. Novel applications of *Trichoderma* spp.

Trichoderma produces a variety of lytic enzymes that have a high diversity of structural and kinetic properties, thus increasing the probability of this fungus to counteract the inhibitory mechanisms used by neighboring microorganisms (Ham et al., 1997). Further, *Trichoderma* hydrolytic enzymes have been demonstrated to be synergistic, showing an augmented antifungal activity when combined with themselves, other microbial enzymes, PR proteins of plants and some xenobiotic compounds (Lorito et al., 1994a; 1994b; 1996b; 1998; Fogliano et al., 2002; Schirmböck et al., 1994; Woo et al., 2002). In fact, the inhibitory effect of chemical fungicides for the control of the foliar pathogen *Botrytis cinerea* was substantially improved by the addition of minute quantities (10-20 ppm) of *Trichoderma* CWDEs to the treatment mixture (Lorito et al., 1994b).

Extensive testing of *T. harzianum* strain T22 conducted for the registration of this biocontrol agent in the USA by the Environmental Protection Agency (EPA) has found that the CWDEs do not have a toxic effect on humans and animals (ED_{50} and LD_{50}), and that they do not leave residues, but degrade innocuously in the environment. Therefore, these *Trichoderma* hydrolytic enzymes present a novel product for plant disease control based on natural mycoparasitic compounds used by

the antagonistic fungi. Single or mixed combinations of CWDEs with elevated antifungal effects, obtained from fermentation in inducing conditions, over-expression of the encoding genes in strains of *Trichoderma*, or heterologous expression of the encoding genes in other microbes are possible alternatives for pathogen control. These natural substances originating from the BCA are an improvement over the use of the living microorganism in the production of commercial formulations because they are easily characterized, resist desiccation, are stable at temperatures up to 60° C, and are active over a wide range of pH and temperatures in the agricultural environment.

Many purified CWDEs are of interest not only to crop production, but also to the agro-food industry (Harman and Kubicek, 1998). *T. reesei* has a long history of safe use in industrial-scale enzyme production. Applications of cellulases and xylanases produced by this fungus are used widely in the production of human food products, animal feeds, pharmaceuticals, as well as in the textile, pulp and paper industries (Nevalainen et al., 1994). The enormous potential of the β-(1,4)-endoglucanase produced by *T. longibrachiatum* and *T. reesei* has been used to solve filtration problems associated with the presence of β-glucans in beer production. The addition of this enzyme is a frequent practice in this industrial sector. Biotechnological advancements have now transferred the encoding glucanase genes to brewer's yeast (*Saccharomyces* spp.) and these transgenic yeasts are used for making beer (Linko et al., 1998). Furthermore, a β-(1,4)-endoglucanase from *T. longibrachiatum* is also used in the wine industry because the action of this enzyme promotes the liberation of aromatic terpene precursors in grape that leads to the final fruity aroma of wines (Pérez-González et al., 1993). Finally, *Trichoderma* cellulases and hemicellulases have been used for years as an additive to chicken feed formulations to improve digestibility, by partially degrading and reducing the fiber content, thus improving fecal production (Nahm and Carlson, 1985).

In this context, the production of secondary metabolites by *Trichoderma* strains also shows great potential in a variety of applications. *Trichoderma* strains seem to be an inexhaustible source of antibiotics, from the acetaldehydes gliotoxin and viridin (Dennis and Webster, 1971), to alpha-pyrones (Keszler et al., 2000),

terpenes, polyketides, isocyanide derivatives, piperacines, and complex families of peptaibols (Sivasithamparam and Ghisalberti, 1998). All these compounds produce synergistic effects in combination with CWDEs, with strong inhibitory activity to many fungal plant pathogens (Lorito et al., 1996a; Schirmböck et al., 1994). The potential to use many of the genes involved in diverse biosynthetic pathways of antibiotics, i.e. polyketides (Sherman, 2002) and peptaibols (Wiest et al., 2002) production, and apply them to human and veterinary medicine has yet to be explored. In general, the direct use of anti-microbial compounds produced by fungal BCAs, instead of the whole ‘‘live’’ organism, is not only advantageous in industrial and agricultural applications, but it may also be more compliant to public opinion because these biological products do not reproduce and spread. Moreover, the selective production of active compounds may be performed by modifying the growth conditions, i.e. utilizing different culture substrates, temperature of incubation, speed of agitation and pH, etc. (Lorito and Scala, 1999; Woo and Lorito, 2007).

Trichoderma strains may be employed in many different ways in order to obtain beneficial effects to the plant, such as biocontrol and plant growth promotion. Recently, it has been demonstrated that hundreds of genes and gene products are involved in the multiple interaction processes of this BCA: mycoparasitism, antibiosis, competition (for nutrients or space), improvement of plant stress tolerance by enhancing the root and aerial development, solubilization and sequestration of inorganic nutrients, induced resistance, and inactivation of enzymes produced by pathogens (Monte, 2001). Some of these genes have been identified, characterized, patented and used transgenically to improve plant disease resistance against fungal pathogens (Lorito et al., 1998). Bacterial and fungal microorganisms represent huge sources of genes potentially useful to increase disease resistance against different microorganisms, viruses and insects (Lorito and Scala, 1999). A typical example, which involves compounds with direct antimicrobial activity, is the use of antifungal chitinolytic enzymes. The transgenic expression of *Trichoderma* chitinase gene *chit42* in tobacco and potato conferred almost complete resistance to both aerial and soil-borne pathogens, thus overcoming the limits of transgenic expression of plant

chitinases, both in the level and the spectrum of disease resistance to fungal pathogens (Lorito et al., 1998).

Numerous *Trichoderma* strains are resistant to or capable of degrading hydrocarbons, chlorophenolic compounds, polysaccharides and the xenobiotic pesticides used in agriculture (Harman and Kubicek, 1998; Harman et al., 2004b). In fact, the BCA *T. atroviride* P1 was selected for its resistance to benomyl and its cold tolerance – characteristics potentially important for post-harvest, cold storage disease control. Moreover, the compatibility of *T. harzianum* T22 and *T. atroviride* P1 with many organic compounds conventionally acceptable for use in biological farming has also been demonstrated (Vinale et al., 2004). Results indicated a high level of tolerance by the *Trichoderma* strains to concentrations of copper oxychloride varying from 0.1 to 5 mM without negative effects to mycelia growth.

The molecular basis of *Trichoderma* resistance to toxic compounds has been partially elucidated with the recent discovery that different fungal strains produce a set of ATP-binding cassette (ABC) transporters. These ATP-dependent permeases mediate the transport of many different substrates through biological membranes, and overexpression of ABC-transporter genes decreases the accumulation of toxicants in *Trichoderma* cells (Lanzuise et al., 2002). In *Trichoderma* spp., ABC transporters have been shown to be important in many processes. These include resistance to environmental toxicants that are produced by soil microflora or introduced by human activity (for example, fungicides and heavy metal pollutants), and secretion of factors (antibiotics and cell-wall-degrading enzymes) that are necessary for the establishment of a compatible interaction with a host fungus, or for the creation of a favorable microenvironment. ABC transporters are probably necessary for the establishment of mycoparasitic interactions with plant pathogenic fungi. Knock-out mutants of *T. atroviride* P1, lacking specific ABC transporters, were inhibited by the presence of various plant fungal pathogens (*B. cinerea*, *Rhizoctonia solani* and *Pythium ultimum*) in the culture medium, and they exhibited reduced capacity as effective fungal parasites (Ruocco et al., 2008).

Industrialization combined with increased urbanization and changing agricultural practices have caused a rise in the level of contaminants found in the

environment, resulting consequently with a negative impact on human health. Methods used for clean up of polluted sites by the removal of hazardous compounds is a serious problem, which requires a multi-faceted approach for obtaining suitable solutions. Physical and chemical treatments have been the most commonly used methods for remediation of soil pollutants to date, but their high costs, economically and energetically, have increased the search for alternative methods based on biological systems, such as bioremediation (involving microbes) and phytoremediation (involving both microbes and plants) techniques for detoxification of xenobiotic compounds (Eapen et al., 2007).

As mentioned previously, *Trichoderma* is able to establish an intimate association with the plant. The exchange of bioactive molecules between the fungus and the plant establishes a symbiosis, permitting the fungus to colonize, grow and persist on the roots and the plant receives long-term benefits in terms of health, vigor and productivity (Harman et al., 2004a). This molecular communication in the plant-fungus association comprises of various compounds originating from both the plant and fungus such as metabolites, plus substances released (breakdown products of hydrolysis) or factors uniquely synthesized during the interaction. The capacity of these organisms to sequester, metabolize, release and exchange substances may represent a potential application for bioremediation or phytobioremediation in the cleanup of contaminated sites. In this strategy, the BCA fungus could accumulate toxicants or breakdown the compounds, as well as stimulate the growth and development of the plant which in turn augments its capacity to accumulate and metabolize the noxious substances, then these plants could be eventually removed from the site (Harman et al., 2004b, D'Aquino et al. personal communication).

1.4. *Trichoderma* spp. as a pathogen

In the past twenty years, some species of *Trichoderma* have been noted as the causal agent of green mould that has produced severe disease attacks in the edible mushroom industry (Sinden and Hauser, 1953). In 1985, an epidemic of green mould

disease immerged in the production of compost used for the growth of *Agaricus bisporus* (champignon) in Northern Ireland. This was subsequently followed by severe infestations in mushroom farms in the United Kingdom, Spain, Germany, the Netherlands, and across the Altantic in United States and Canada. (Seaby, 1998; Hermosa et al., 1999; Castle et al., 1998). In Italy, a problem with *Trichoderma* infestations appeared in the production of *Pleurotus ostreatus* (oyster mushroom) around 2002 (Woo et al., 2004; 2006).

Among the *Trichoderma* isolates obtained from infested *A. bisporus* compost, three different biological forms of *Trichoderma* were identified, two non-pathogenic and one pathogenic forms. The first biotype was identified as *T. harzianum* (formerly reported as *T. harzianum* biotype Th1), a taxonomic group which includes the ex-neotype of *T. harzianum*, and many biological control strains (Hermosa et al., 2000; Samuels et al., 2002). The second biotype was identified as the causal agent of green mould disease (Muthumeenakshi et al., 1994; 1998; Hermosa et al., 1999; 2000), and was characterized as a new species, *T. aggressivum* forma *europeaum* (formerly reported as *T. harzianum* biotype Th2) (Samuels et al., 2002). The third, non-pathogenic biotype, was identified as *T. atroviride* (formerly reported as *T. harzianum* biotype Th3). The *Trichoderma* pathogens to mushroom production in North America were identified as different from the European pathogen, and this latter fourth biotype was taxonomically classified as *T. aggressivum* f. *aggressivum* (formerly reported as *T. harzianum* biotype Th4) (Chen et al., 1999a; 1999b; Samuels et al., 2002).

The infestations caused by the two different green mould pathogens of *A. bisporus* are apparently geographically separate. In practise, they are morphologically indistinguishable, although minute differences could be noted in some characters at the beginning of development in vitro (Muthumeenakshi et al. 1998; Samuels et al., 2002). However, various molecular markers to distinguish the aggressive forms from the non-pathogenic forms associated with mushroom production (Muthumeenakshi et al. 1998; Castle et al., 1998). Furthermore, analysis of ITS1 and ITS2 sequences of different *Trichoderma* species, including *T. aggressivum* f. *europeaum* and *T. a.* f. *aggressivum* determined that these two

biotypes were taxonomically diverse from one another, as well as the other biotypes found associated with *A. bisporus* (Hermosa et al., 2000; Ospina-Giraldo et al. 1998; Samuels et al., 2002).

The problems associated with *Trichoderma* spp. in the production of *P. ostreatus* is relatively new in comparison to that found with *A. bisporus* production (Woo et al., 2005). Although *Trichoderma* has been found occasionally with oyster mushrooms (Samuels et al., 2002; Largeteau-Mamoun et al., 2002), little is known or indicated in the literature, i.e. the origins of the inoculum, the stages of infection, if the *Trichoderma* is a mycoparasite etc. Recently, Komoń-Zelazowska et al. (2007) identified two different but genetically closely related *Trichoderma* species that originated from the compost of *Pleurotus* originating from various European countries, including Italy, and they described these new species as *T. pleurotum* and *T. pleuroticola*. These two species belong to the Harzianum clade of *Hypocrea/Trichoderma* which also includes the *T. aggressivum* complex, the causative agent of green mold disease of *Agaricus*.

During recent years, attention has been drawn towards the possible health risks of handling, producing and using biocontrol fungi (Doekes et al., 2004, Jensen et al., 2002). Human exposure to these fungi in occupational settings, homes and outdoor environments, where they naturally occur or are applied as biocontrol agents, are important factors to consider for risk assessment on the use of fungal BCAs. It is now recognized that the exposure of respiratory airways to various microorganisms in occupational environments is associated with a wide range of adverse health effects (Douwes et al., 2003). Respiratory symptoms and lung function impairment are probably the most widely studied among organic dust-associated health effects. Fungi are well-known sources of allergens and are also sources of β-glucan, which causes non-allergic respiratory symptoms (Douwes et al., 2003). Several species of the saprophytic genus *Trichoderma* have been identified as the cause of infections in immuno-suppressed humans (Gautheret et al., 1995; Jacobs et al., 1992; Munoz et al., 1997; Richter et al., 1999; Tanis et al., 1995). In one instance, *T. harzianum* has been identified as the causal agent of peritonitis in a dialysis patient (Guiserix et al., 1996). On the other hand, clinical effects caused by

short-term human exposure to *T. harzianum* were not greater than effects observed in the placebo (Meyer et al., 2005). Recent molecular studies have determined that the majority of all human infections are caused by a single taxonomic 'section' composed of *T. longibrachiatum* (Kuhls et al., 1999).

1.5. Scope of the thesis

Global warming caused by the greenhouse effect represents one of the main threats to the environment and subsequently humanity. Climatic changes towards increased temperatures changes the biological biodiversity, and regions that are presently subjected to intense conditions will become even more severe. Further, this situation will consequently alter the geographical distribution of host and pathogen populations, thus affecting the natural physiology of their interaction and reducing the efficacy of both chemical and biological control strategies presently in use. These climatic changes will alter the agro-ecosystems continually and new management practices need to be used. In perspective to this scenario, the main task of this thesis work is to isolate and characterize new biocontrol agents of the genus *Trichoderma* from Libya, where these fungi are among the most applied antagonists used in the country's agriculture. The intention is to obtain microbes having a natural adaptability to function in adverse climate conditions (low rainfall, drought, extreme temperatures, poor soil quality, etc.), test their efficacy as biological control agents against different plant pathogens and determine their potential as active ingredients in novel biological formulations for use in agriculture and industry.

Although numerous commercial products containing *Trichoderma* are available for use in greenhouse and field, the effectiveness and reliability of these products under diverse environmental conditions, i.e. temperature, can limit growth and development. Recently, in Libya, interest has been oriented to the potential use of biocontrol in agriculture. However, there is a general lack of information on the efficacy of these commercial products in the Libyan environment. Further, little is known about the natural populations of local antagonists present – their identity,

efficacy, ability to interact with commercial products and possible applications. The isolation and characterization of new *Trichoderma* isolates may be useful for the development of a plethora of biotechnological applications, among which the use of selected strains for the biological control of various phytopathogenic fungi is the most notable.

The main objectives of this thesis are:

1. Isolation, identification and characterization of several *Trichoderma* strains, obtained from different Libyan soils. An integrated approach to species characterization comprising morphological, physiological, and molecular analyses will be used. Moreover, biochemical analysis and *in vitro* antagonistic activity of the selected strains will be determined.
2. The biotechnological use of the isolated strains in bioremediation projects will also be evaluated.
3. Evaluation of new possible applications of the selected *Trichoderma* strains as plant growth promoters and inducers of systemic resistance.
4. Development and analysis of new formulations based on the selected *Trichoderma* isolates able to effectively control fungal disease.

2. MATERIALS AND METHODS

2.1. Isolation of fungi and evaluation of growth at different temperatures

Triplicate soil samples were randomly collected from soil depths ranging from 0 to 30 cm, at nine agricultural areas in the northwestern part of Libya, including Al-Khums, Al-Garabulli, Tajoura "sites 1 & 2", Al-Nofleen, Tareek Al-Matar, Ghasser Ben-Ghasheer, El-Azizia and Yefren, in order to determine the fungal population density and obtain a representative set of isolates. Soil samples were placed in polyethylene bags, and stored at 5° C until plated. The fungal isolations were performed by using a serial dilution technique (Tuite, 1969).

Potato dextrose agar (PDA; SIGMA, St. Louis, MO, USA) medium was prepared according to the manufacturer's instructions, and augmented with Lactic acid and Rose Bengal to suppress bacterial growth, then poured into 90 mm Petri plates. One hundred grams of soil samples were added to 100 ml distilled water and homogenized for 1 min.; then a dilution series was prepared (0, 10, 10^2, 10^3, 10^4) in sterile water. One hundred microliters of each dilution was inoculated to the surface of plates containing PDA, spread evenly with a sterile spreader and incubated in the dark for 5-7 days at 25° C. Emerging fungal colonies were isolated, stained with methylene blue, identified by observations under a microscope. Colonies of *Trichoderma* were selected, transferred to new PDA plates, then pure cultures were obtained, and maintained on PDA slants at 25° C. Conidia from 4 day old cultures were collected in water and any mycelial debris was separated by filtration through filter paper (Whatman No. 4; Brentford, UK). Conidial concentration was determined using with a haemocytometer and adjusted when necessary. Spore suspensions were stored at -20° C in 20% v/v glycerol solution until used. *T. atroviride* strain P1 (ATCC 74058) and *T. harzianum* strain T22 (ATCC 20847), commonly used as biocontrol agents (Harman, 2000; Tronsmo, 1989), were included as controls.

Agar plugs of the *Trichoderma* cultures were inoculated to the center of plates containing PDA or agarized (1.5%) Salt Medium (SM) and incubated at 25° and 30° C in the dark. The growth of the fungal colony was measured daily throughout the incubation period. The composition of SM in one liter of water was as follows: KH_2PO_4 680 mg L^{-1}, K_2HPO_4 870 mg L^{-1}, KCl 200 mg L^{-1}, NH_4NO_3 1 g L^{-1}, $CaCl_2$ 200 mg L^{-1}, $MgSO_4$. $7H_2O$ 200 mg L^{-1}, $FeSO_4$ 2 mg L^{-1}, $MnSO_4$ 2 mg L^{-1}, $ZnSO_4$ 2 mg L^{-1}, Sucrose 10 g L^{-1}, agar 10 g L^{-1} (all purchased from SIGMA).

2.2. *In vitro* confrontation bioassays of *Trichoderma* isolates by microscopy observations

Cultures of the three local (Lib1, Lib2, Lib3) and two non-local *Trichoderma* biocontrol isolates (*T. harzianum* T22, *T. atroviride* P1) were screened for their ability to interact with the plant pathogens *Rhizoctonia* sp., *Alternaria* sp. and *Fusarium* sp., that are important plant pathogens worldwide causing significant yield loss to a range of crops. The phytopathogens were obtained from the collection of the Department of Arboricolture, Botany and Plant Pathology, Università degli Studi di Napoli "Federico II" (Naples, Italy), maintained on PDA slants at room temperature and sub-cultured bimonthly.

2.2.1. Confrontation dual plate cultures

This experiment was conducted at two temperatures (25° and 30° C) to test the efficacy of the isolates to different climatic conditions. The treatments consisted of factorial combinations of the five *Trichoderma* isolates, three pathogens and the two temperatures. Agar plugs from actively growing plate cultures of the antagonist and host are inoculated at separate distinct points, near the periphery of 90 mm Petri plates containing PDA then incubated at 25° C and 30° C in the dark. Evaluations were made of the growing mycelia, involving the measurement of fungal growth

rate, and noting the development of a "clearing" zone between the two fungi which indicates hyphal interference at 24 h intervals for seven days.

2.2.2. Slide culture interactions

Petri plates containing sterilized wet filter paper and glass rods were prepared. A thin layer of PDA (1x1 cm) was cut and placed on a sterile microscope slide. From actively growing plate cultures of the antagonist and the host, an agar plug of each fungus was inoculated at the edge of the PDA; a cover slip was then placed on the slide-cultures, and incubated for 5 days at 30° C. Once the fungi showed clear and proper growth, microscopic observations were performed by transferring the cover slip to another microscope slide, and adding lactophenol-cotton blue to stain the fungi.

2.3. Detoxification and compatibility with toxic pollutants

Liquid cultures of the three Libyan *Trichoderma* isolates (Lib1, Lib2, Lib3) were screened for their ability to growth in presence of Methyl *tert*-butyl ether (MTBE), a common contaminant of ground water when gasoline with MTBE is spilled or leaked at gas stations. Fungal inoculum (prepared from plate cultures as described above) was inoculated in flasks containing sterile medium (SM) amended with different concentrations of MTBE (SIGMA). The cultures were incubated at 25° C, in orbital agitation of 150 rpm for 6 d. The mycelial biomass was collected by filtration, dried at 120° C for 2 h (or until dry) and then weighed.

Moreover, the ability of the isolates to degrade the toxic compound was quantified by determining the residue of MTBE present in the culture filtrate after removing the fungal mycelium. Separation and quantification of MTBE in the liquid culture was performed by using Gas Chromatography - Flame Ionization Detector (GC-FID) on an Agilent 7890A gas chromatographer (Agilent Technologies) with an HP-5 column. The sample injection port was maintained at 300° C, and all samples

were eluted through the column with a flow rate of 1.2 ml/min. The concentration of the MTBE was determined by comparison to a standard curve with concentrations ranging from 0.1 to 10% (v/v). All samples were analyzed at least in duplicates.

2.4. ITS sequence analysis

Genetic analysis of Ribosomal DNA internal transcribed spacer (ITS) sequences were determined following the method of Gruber et al. (1990). Spores of three *Trichoderma* strains (Lib1, Lib2 and Lib3) were inoculated in potato dextrose broth (PDB, SIGMA) and incubated at 25° C on a orbital shaker (250 rpm) for 5 days. Mycelia were harvested and genomic DNA isolated, in order to analyse ribosomal DNA. We used a PCR based approach to amplify, by the use of primers SR6R (5'-AAGTAGAAGTCGTAACAAGG-3') and LR1 (5'-GGTTGGTTTCTTTTCCT-3'), fragments containing the internal transcribed spacer 1 (ITS-1), the 5,8 S rDNA and the ITS-2 regions. The following parameters were used: 1 min initial denaturation at 94° C, followed by 30 cycles of 1 min denaturation at 94°C, 1 min primer annealing at 50° C, 90 sec extension at 72° C, and a final extension period of 7 min at 72° C. The PCR products were gel electrophoresed, for quantification and assessment of PCR specificity, and sequenced. Sequence alignment and phylogenetic studies were carried out by the use of the MEGA version 3.1 software (Kumar et al., 2004).

2.5. Isolation and characterization of secondary metabolites

Secondary metabolites were isolated from the *Trichoderma* culture filtrates as described in Vinale et al. (2006). Briefly, two 7-mm diameter plugs of each Libyan *Trichoderma* isolate, obtained from actively growing margins of PDA cultures, were inoculated into 5 L conical flasks containing 1 L of sterile one-fifth (1/5 X) strength PDB. The stationary cultures were incubated for 31 days at 25° C. The cultures were filtered under vacuum through filter paper (Whatman No. 4), and the filtrates stored at 2° C for 24 h. The filtered culture broth (2 L) of each isolate was extracted

exhaustively with ethyl acetate (EtOAc). The combined organic fraction was dried (Na_2SO_4) and evaporated under reduced pressure at 35° C. The recovered red-brown residue was subjected to flash column chromatography (Si gel; 50 g), eluting with a gradient of EtOAc:petroleum ether (8:2 to 10:0). Column chromatography was carried out using silica gel 60 GF_{254} and GF_{60} 35-70 mesh (Merck, Darmstadt, Germany).

Analytical and preparative thin-layer chromatographies (TLC) were performed on silica gel (Kieselgel 60, GF_{254}, 0.25 and 0.5 mm, respectively, Merck); compounds were detected with UV radiation (254 or 366 nm) and/or by spraying the plates with $CeSO_4$ (10% w/v in water) or H_2SO_4 (5% v/v in ethanol) and heating at 110° C for 10 min. Fractions showing similar TLC profiles were combined and further purified by using RP-18 column (H_2O: Methanol gradient form 100 to 0 of H_2O).

All purified compounds were analyzed by ^{1}H, ^{13}C NMR and LC/MS. ^{1}H and ^{13}C NMR spectra were recorded with a Bruker AM 500 spectrometer operating at 500 (^{1}H) and 125 (^{13}C) MHz using residual and deuterated solvent peaks as reference standard. Low and high resolution mass spectra were obtained by using a VG Autospec mass spectrometer (EI mode).

2.6. Effect of *Trichoderma* strains on plant productivity

Ten grams of tomato seeds from three different cultivars (*Solanum lycopersici* cv. San Marzano, Principe Borghese and Corbarino) were coated with a conidial suspension of each Libyan *Trichoderma* isolate containing 1 x 10^7 conidia/ml. The same concentration of conidial suspensions of *T. atroviride* strain P1 and *T. harzianum* strain T22 was used as seed treatments for reference controls; finally, water was used for seed coating in the untreated control (C). Seeds were planted in 14-cm vases containing sterile soil (sterilized for 1 h at 122° C) at a depth of 4 cm, incubated in a growth chamber at 25° C with 16h light, and kept under humid conditions. Seed germination and plant growth parameters were monitored for 3

weeks. At the end of the experiment, the effect of the *Trichoderma* treatments on the root system was evaluated by the determination of the fresh and dried weights of the recovered. The experiments were repeated at two different times.

2.7. Effect of *Trichoderma* strains on plants inoculated with the fungal pathogen *Botrytis cinerea*

The conidial suspension of the foliar pathogen *B. cinerea* was obtained from 10 day-old sporulating cultures on PDA in 0.1% Tween-20 solution, filtered through glass wool and diluted to a final concentration of 5 $x10^5$ conidia ml^{-1}. Ten grams of tomato seeds were coated with a conidial suspension of each *Trichoderma* isolate containing 1 x 10^7 conidia/ml, or water in the case of the control. When tomato plants had developed to the stage where four true leaves had emerged, the leaf surface of treated and control plants was inoculated at two different inoculation points with a 15 µl *B. cinerea* spore suspension in germination buffer (20 mM glucose and 20 mM KH_2PO_4). Inoculated plants were incubated at 25° C with 16h light in a humid chamber. After 48 h the leaves were evaluated for disease symptoms, and the diameter of each necrotic zone was measured. Two inoculations were made per leaf on four leaves per plant for three plants per treatment and two replicates for each experiment. The experiments were repeated at two different times. The statistical analyses included an analysis of variance of treatment means with a significance level of $P < 0.05$.

2.8. Conditions applied for fermentation processes

In order to study new formulations based on *Trichoderma* spores and metabolites (enzymes), the major parameters which optimized the fungal growth and metabolite production (temperature, pH, aeration, etc) were first monitored in small-scale production. A *Trichoderma* spore suspension (1 $x10^6$ conidia ml^{-1}) was

inoculated in flasks containing PDB and allowed to growth on an orbital shaker (150 rpm) for 72h at 25° C. The cultures were filtered through filter paper (Whatman), and transferred in to a 50L fermenter, where different operating conditions were applied. The temperature was set at 25° C and the base cultivation substrate was either Salt Medium (SM) or Shiping Medium (SpM). The composition of The Sp.M. was as follows for one liter: 0.05M NO_3, 0.095M KH_2PO_4, 0.0065M $MgSO_4.7H_2O$, 1.2 $x10^{-4}$M $FeCl_3$, $9x10^{-6}$M $ZnSO_4.7H_20$, $8x10^{-7}$M $CuSO_4.5H_2O$, $6x10^{-6}$M $MnSO_4.H_2O$, $4x10^{-7}$M $(NH_4)_6Mo7O_{24}.4H_2O$, $2x10^{-5}$M $(NH_4)_2SO_4$, 0.002M $CaCl_2$, $9x10^{-6}$M $FeSO_4.7H_2O$, $4x10^{-6}$M $CoCl_2.6H_2O$. The SM or SpM substrates were then amended with lyophilized champignon mushrooms (*Agaricus bisporus*), wheat (*Triticum durum*) fiber or chitin extracted from crab shells, as the main carbon and energy sources, as follows:

1) 0.5% (w/v) lyophilized mushrooms + 0.2% (w/v) wheat fiber;
2) 0.5% (w/v) lyophilized mushrooms + 0.3% (w/v) wheat fiber;
3) 0.5% (w/v) chitin + 0.3% (w/v) wheat fiber.

The different experimental conditions applied in each fermentation are summarized in Table 3. All cultures were conducted in a small scale fermenter of 50L; only the last fermentation (VI) was performed in a 200L fermenter to determine the effect on the fungal development in an industrial-scale production.

Table 3. Conditions used for the fermentation of *Trichoderma* in liquid culture.

Fermentation N.	Substrate	Orbital shaking (ppm)	Aeration (vvm)
I	SM + 0.5% lyophilized mushrooms + 0.2% wheat fiber	200	0.7
II and III	SM+ 0.5% lyophilized mushrooms + 0.3% wheat fiber	100	0.5
IV	SM + 0.5% chitin extract from crab shells + 0.3% wheat fiber	100	0.5
"Shiping"	SpM + 0.5% lyophilized mushrooms + 0.3% wheat fiber	200	0.3
V	SM+ 0.5% lyophilized mushrooms + 0.3% wheat fiber	100; after 48h →200	0.5; after 48h →0.3
VI	SM+ 0.5% lyophilized mushrooms + 0.3% wheat fiber	100; after 72h →200	0.5; after 72h →0.3

2.9. Analysis of the novel formulation

Samples were collected throughout the fermentation process, from each treatment, twice per day, for a total of 7 days of fermentation. The samples were examined under microscope and the fungal concentration was determined. Cultures collected from the fermenter were centrifuged at 5000 rpm for 25 min. and filter sterilized through a 0.22 μm filter, and then stored at 4° C until used. Total protein concentration was determined according to the method described by Bradford (1976) and all samples were standardized before conducting enzyme assays.

Enzyme activities in the culture filtrates were assayed as previously described (Harman et al., 1993; Di Pietro et al. 1993; Lorito et al. 1993). In general, the substrates for the different hydrolytic enzymes were prepared in potassium phosphate buffer (50 mM, pH 6.7) at a concentration of 0.3 mg/ml. The enzyme activity was determined in colorimetric assays by quantifying the amount of p-nitrophenyl, conjugated with various enzyme substrates, that was released by the enzyme as

measured in a spectrophotometer at an absorbance of 405 nm (Harman et al., 1993). Overall chitinase activity was determined by using a 4-nitrophenyl-ß-D-N',N'',N'''-triacetylchitotriose as well as a reducing sugars assay. The different enzyme activities were determined on the substrates as follows: exochitinase (N-acetyl-ß-D-glucosaminidase (NAGase) on *p*-nitrophenyl N-acetyl-ß-D-glucosaminide (Sigma) (colorimetric assay), chitin 1,4-ß-chitobiosidase (chitobiosidase) on *p*-nitrophenyl ß-D-N,N'-diacetylchitobiose (Sigma), exo-glucanase on *p*-nitrophenyl ß-D-glucopyranoside (Sigma), and glucan 1,3-ß-glucosidase (glucanase, β-1,3 glucanase) (EC 3.2.1.58) on laminarin (a polymer of glucose with β-1,3 bonds, used in reducing sugar assay). For xylanase and cellulase activities, commercial kits were used (Xylazyme AX Test Tablets and Cellazyme AX Test Tablets, respectively; Megazyme, UK). Each enzymatic assay was repeated three times with three replicates per sample.

The direct count of the concentration of mycelia fragments and spores in the sample suspension was determined by using a haemocytometer. To determine the number of colony forming units (CFUs), 1 ml of the samples was vortexed and prepared in a dilution series (10^4, 10^5, 10^6), 0.1 ml for each dilution and plated onto PDA, incubated at 28° C in the dark. After 16 h, the CFUs were calculated.

In vivo biocontrol assays against the fungal pathogen *B. cinerea* were performed on tomato and lettuce plants. Briefly, a 3 ml of the culture obtained from the fermentation process was sprayed to the plants by using an atomizer (Pelikan) and left to dry. Then the leaf surface was inoculated at two different inoculation points with a 15 µl *B. cinerea* spore suspension (5 x 10^5 conidia ml^{-1}) in germination buffer. Inoculated plants were incubated at 25° C with 16h light in a humid chamber. After 48 h the leaves were evaluated for disease symptoms, and the diameter of each necrotic zone was measured. Two inoculations were made per leaf on four leaves per plant for three plants per treatment and two replicates for each experiment. The experiments were repeated at two different times.

The growth promotion activity of the formulation was analyzed *in vitro*. Tomato seeds (*Solanum lycopersici* cv. San Marzano) were surface sterilized with a 1% hypochlorite solution for 1 min, rinsed twice with sterile water, then placed in

Petri dishes containing the *Trichoderma* formulation amended with 1.5% agar, in order to obtain a solid medium. Controls were performed by using 1.5% water agar as substrate. Plates were incubated at 25° C with 16h light in a humid chamber. Root length was measured after 7 d. Experiments were performed in triplicates and repeated twice.

The effect of different processing treatments (spray drying and lyophilization techniques) on the stability of the novel formulation was evaluated by determining the chitinolytic and N-acetylglucosaminidase activities and the spore concentration (as previously reported), before and after treatments. Moreover, the addition of glycerol to the samples to a final concentration of 20% (v/v) was evaluated to determine if it protected spore vitality.

Formulation stability was also monitored at 45 and 110 d after fermentation by evaluating spore viability and chitinolytic activities. The addition of different substances were tested for their stabilizing effect on the liquid formulation: mineral oil (30% v/v); glycerol (20% v/v); ampicillin (100 ppm); ampicillin (100 ppm) + 3mM phenylmethylsulfonyl fluoride (PMSF).

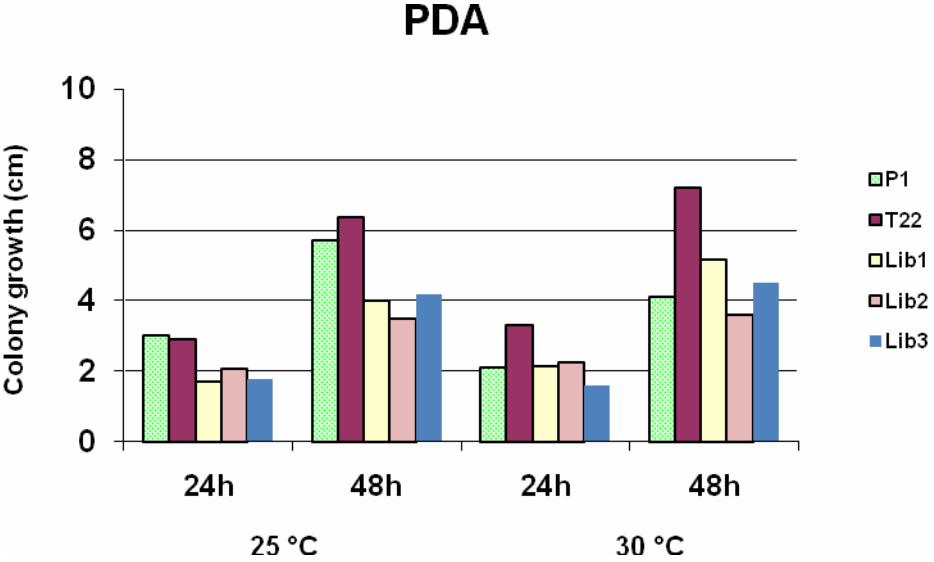
3. RESULTS

3.1. Isolation and morphological characterization of *Trichoderma* isolates

Three pure cultures of Libyan isolates, hereby named Lib1, Lib2 and Lib3, were obtained and maintained on PDA plates at 25° C. The morphological characterization of the fungal isolates was performed by measuring the mycelium growth, the time necessary to sporulate and the amount of spores produced on different solid media (PDA and SM) at two different temperatures (25° and 30° C). There were substantial differences between the isolates of *Trichoderma* originating from Libya and the biocontrol strains (*T. harzianum* strain T22 and *T. atroviride* strain P1, used as reference controls). In particular, on PDA the isolates from Libya showed much less growth than the two biocontrol isolates at 25° C. Better growth was noted for the Libyan *Trichoderma* at the higher temperature of 30° C in comparison to the P1 control, whereas T22 grew more rapidly than all isolates at both temperatures after 48 h (Fig. 3A). On salt medium (SM), where only 1% glucose was present as a carbon source, the Libyan isolates showed similar results as compared to the non-local isolates at 25° and 30° C after 24 h, and improved growth over P1 at 30° C after 48 h(Fig. 3B).

On PDA plates the controls and particularly the commercial strain T22 showed the highest growth rate and sporulation at 30° C after 3 days (Fig. 4). This was probably due to the high concentration of nutrients present in the medium, which this strain is able to degrade and utilize more quickly than the other isolates examined.

A)



B)

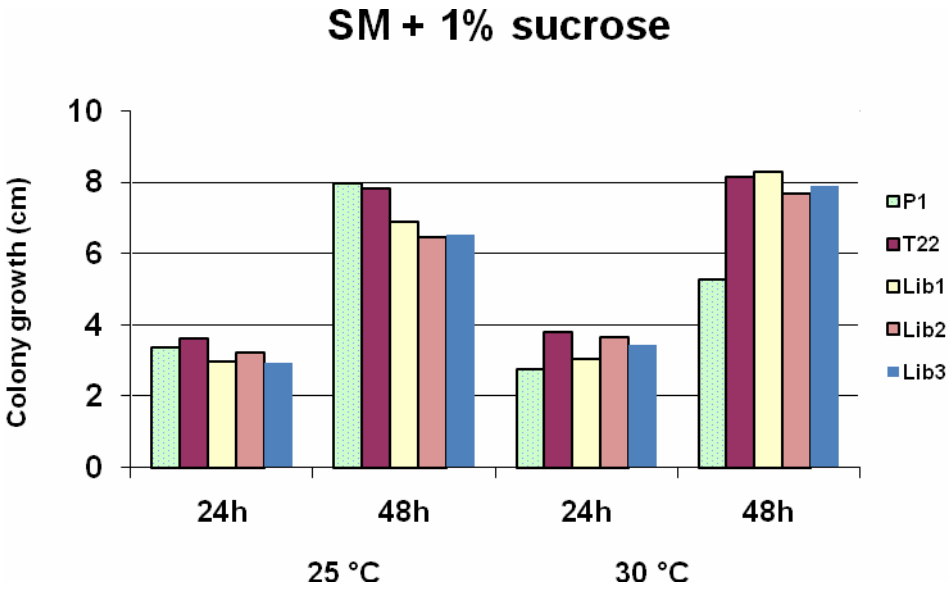


Figure 3. Mycelia growth (diameter of colony growth in cm) of different *Trichoderma* isolates from Libya (Lib 1, Lib2 and Lib3) and biocontrol strains *T. atroviride* P1 (P1) and *T. harzianum* T22 (T22) at 25° C and 30° C evaluated 24 and 48 h after inoculation. The experiments were performed on Petri dishes containing (A) PDA or (B) SM + 1% (w/v) sucrose.

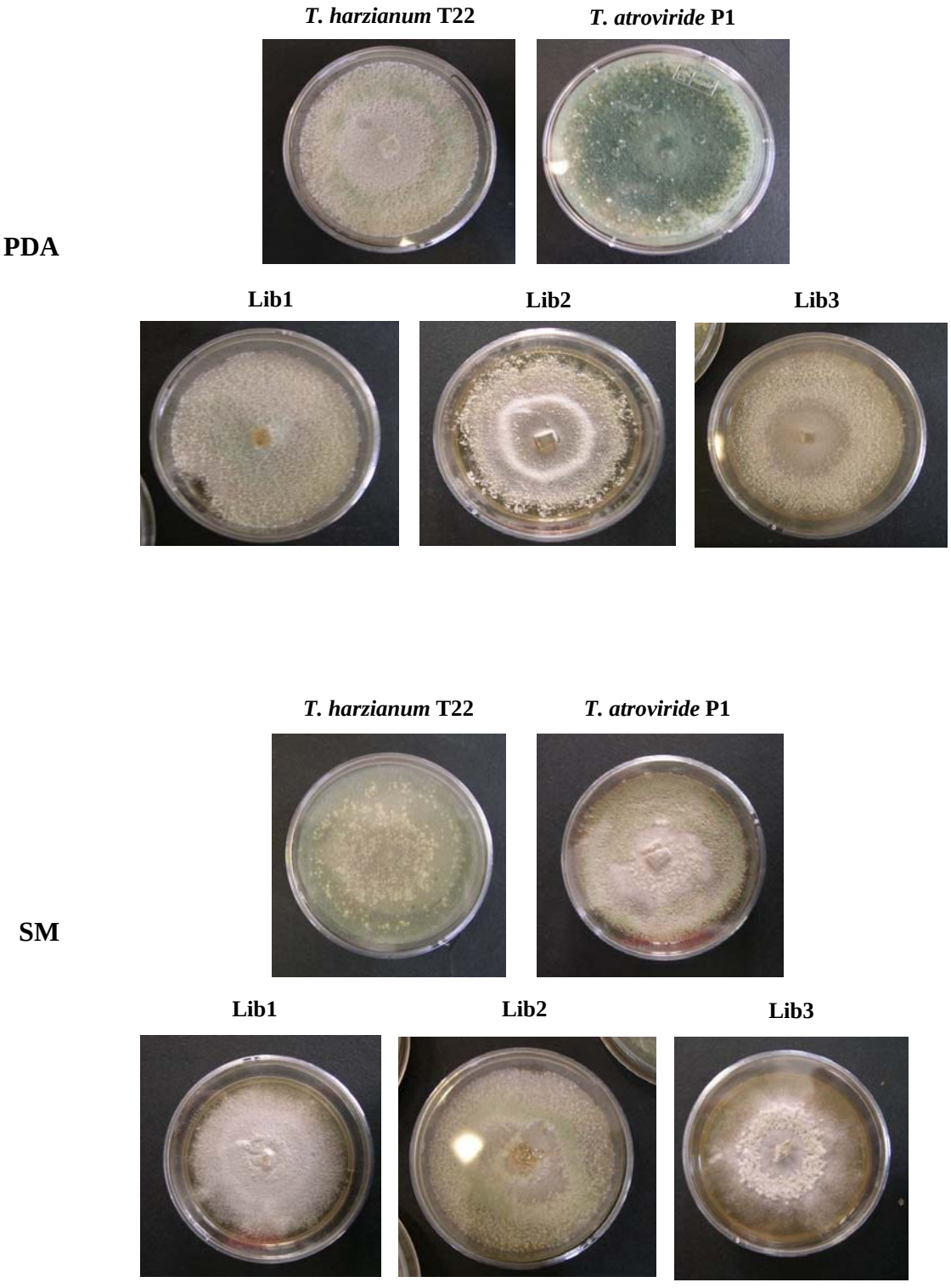


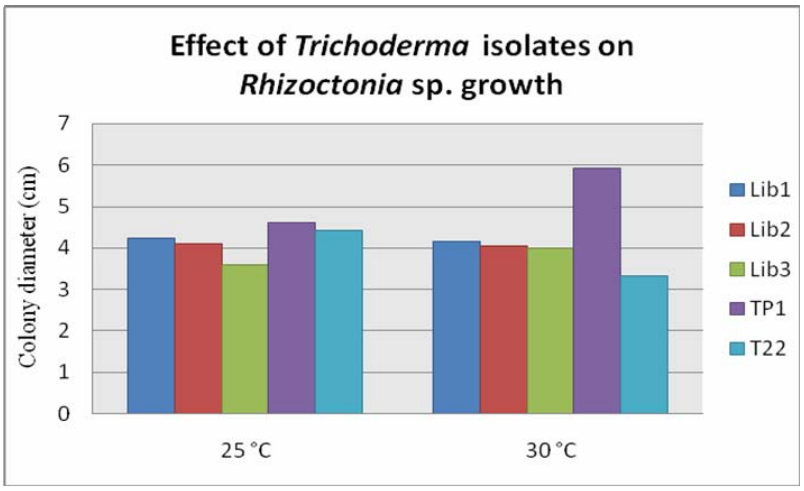
Figure 4. Growth and sporulation of *Trichoderma* isolates after 3 days on plates containing PDA (top) or SM (bottom) at 30° C.

3.2. *In vitro* plate confrontation assays

The greatest proliferation of the fungal cultures was observed 7 days after inoculation. The Libyan isolates were able to parasitize the host fungi with different levels of growth inhibition depending upon the pathogen tested and the temperature used (Fig. 5). At both 25° and 30° C, Libyan isolates were able to reduce the growth of *Rhizoctonia,* like the biocontrol strain T22 (Fig. 5A), whereas the higher temperature greatly reduced the antagonistic ability of *T. atroviride* strain P1.

The temperature had little effect on the antagonistic abilities of the Libyan isolates against *Alternaria*, but the inhibition of pathogen growth was always lower than that observed with the two biocontrol strains at the same temperatures (Fig. 5B). A slightly greater inhibition of *Fusarium* mycelia was noted at 30° C than at 25° C, and the limitation of pathogen growth was similar to P1 and T22. However, among the local isolates examined, Lib2 and Lib3 showed the better performance of antagonistic activities at both 25° and 30° C (Fig. 5C). Mycoparasitism of *Rhizoctonia* sp. and *Fusarium* sp. by *Trichoderma* local isolates and loss of turgidity in host hyphae were also observed by microscopy slide observations (Fig. 6).

A)



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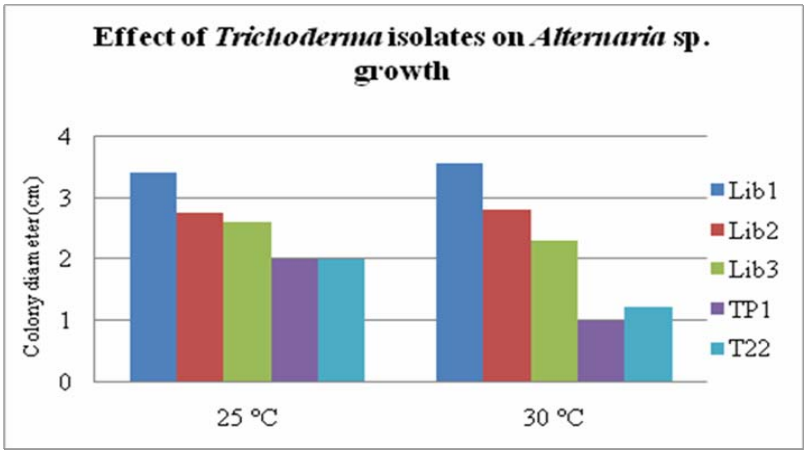


C)

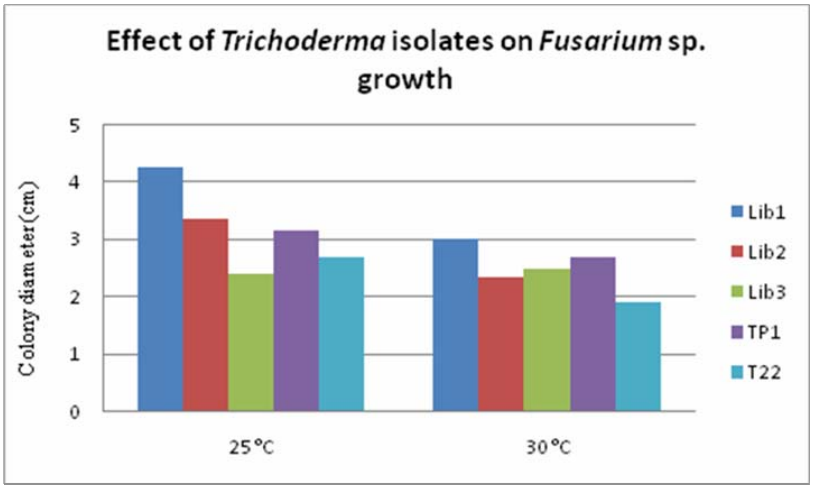


Figure 5. Effect of different *Trichoderma* strains on the mycelial growth (diameter of fungal colony in cm) of plant pathogens *Rhizoctonia* sp. (A), *Alternaria* sp. (B) and *Fusarium* sp. (C) in plate confrontation assays on PDA, 7 days after incubation at 25° C and 30° C. Lib1, Lib2, Lib3 = *Trichoderma* isolates obtained from Libya; TP1 = *T. atroviride* strain P1; T22 = *T. harzianum* strain T22.

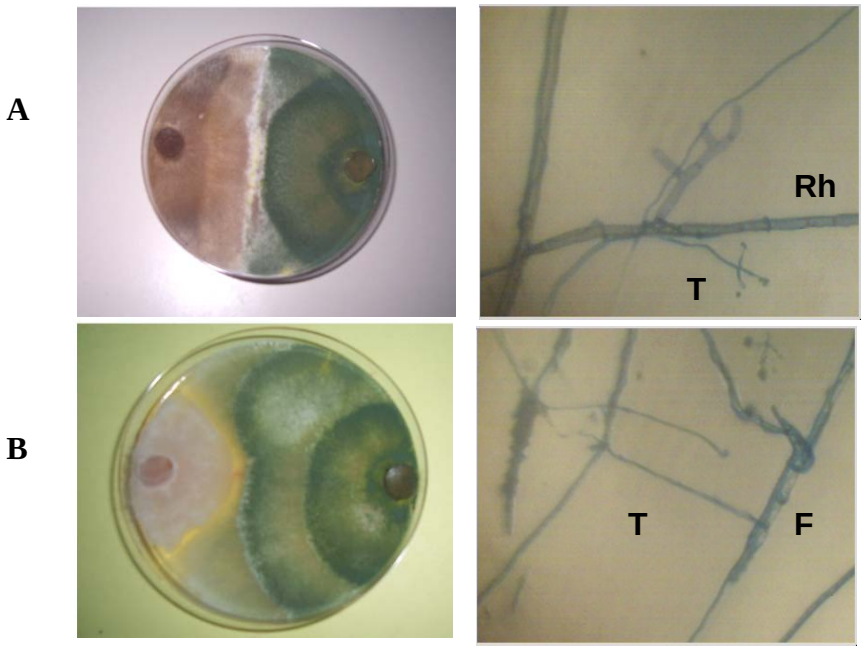


Figure 6. *In vitro* interaction between Libyan isolate Lib1 and fungal pathogens (**A**) *Rhizoctonia* sp.; and (**B**) *Fusarium* sp., after 7 days at 30° C. Left: PDA plate confrontation assay. Right: Micrographs observed by light microscopy of the fungal interaction from slide cultures (x400). T: *Trichoderma*; Rh: *Rhizoctonia* sp.; F: *Fusarium*.

3.3. Detoxification abilities of Libyan isolates

The *Trichoderma* strains isolated in Libya were also tested for their ability to growth in contaminated substrates, in order to evaluate their possible biotechnological application as "bioremediating microbes". *In vitro* assays were performed to analyze their growth in liquid medium amended with different concentrations of the toxic pollutant Methyl *tert*-butyl ether (MTBE) ranging from 0.1 to 1.5 % (v/v). Both Lib1 and Lib2 isolates showed good tolerance to the pollutant up to a concentration of 0.4%, compared to the untreated control. Conversely, the biomass of isolate Lib3 was negatively affected by the presence of MTBE even at lower concentrations (Fig. 7).

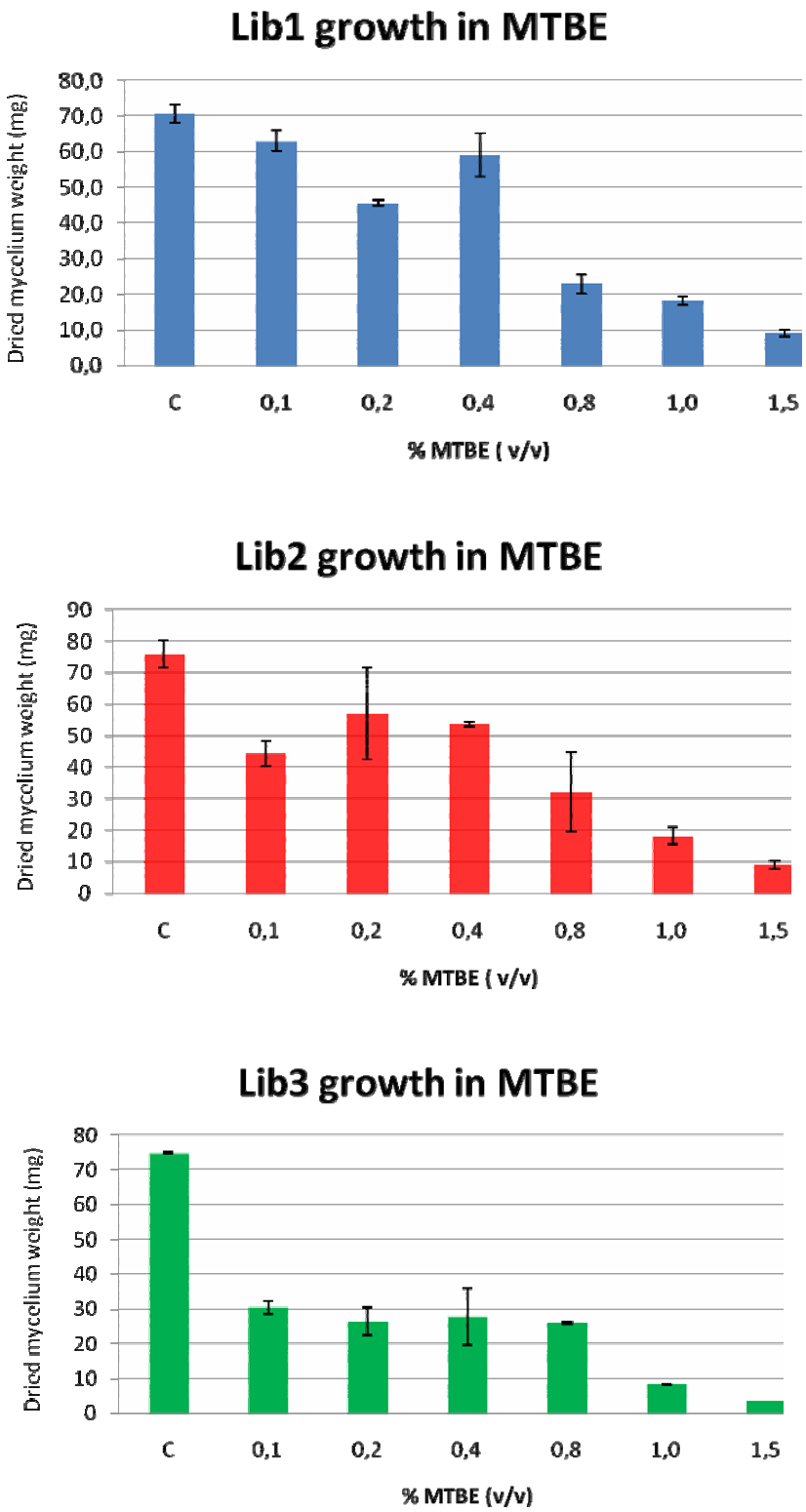


Figure 7. *In vitro* growth of three Libyan isolates (top Lib1, middle Lib2, bottom Lib3) in the presence of different concentrations of MTBE (0.1 to 1.5 % v/v). C = control without MTBE. Fungal mycelium was harvested by filtration, dried and weighed.

Analysis by gas chromatography of the fungal culture filtrates grown in the presence of MTBE showed a decrease in quantity of MTBE with all three of *Trichoderma* isolates from Libya. Although all isolates demonstrated similar trends in their chromatographic profiles, the Lib2 isolate showed the highest degradation of the contaminant particularly at 4 days after inoculation, as compared to the other two isolates, and only results from this representative are shown (Fig. 8).

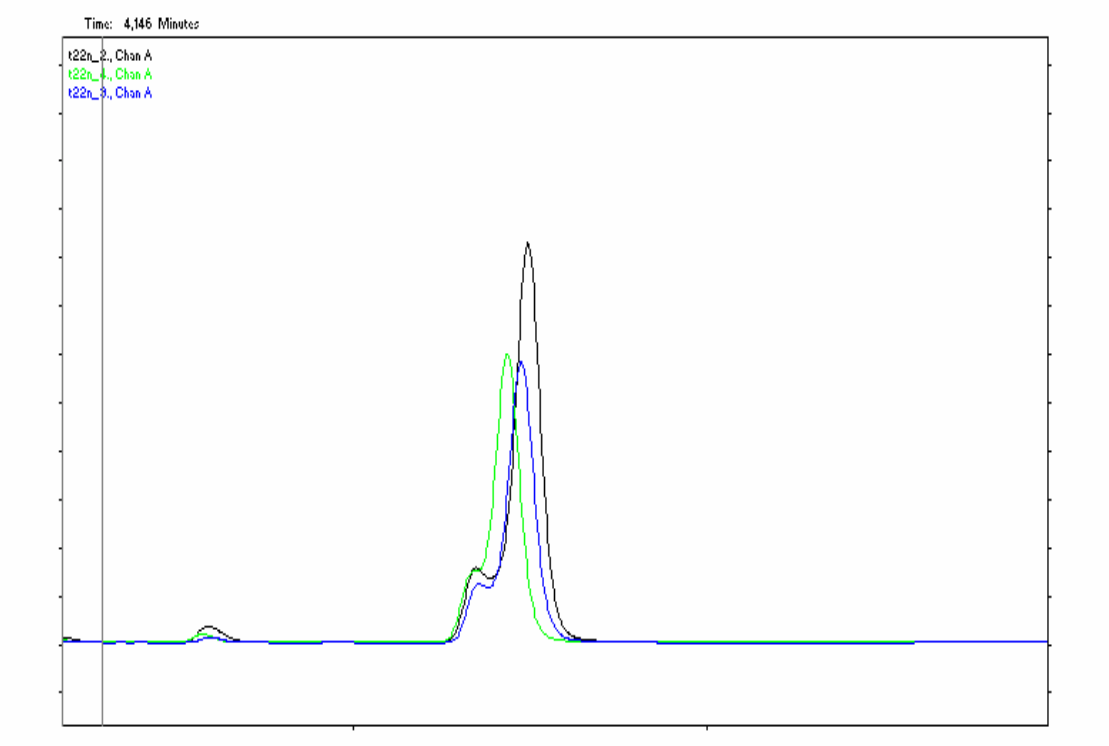


Figure 8. GC-FID analysis of Lib2 isolate culture filtrate grown in presence of 0.2% MTBE after removal of fungal mycelium. Black line: 2 d after inoculum; Green Line: 4 d after inoculum; Blue line: 6 d after inoculum.

3.4. *Trichoderma* species identification

Primers SR6R and LR1 were used to amplify internal transcribed spacer 1 (ITS-1), the 5.8 S rDNA and the internal transcribed spacer 2 (ITS-2) from the fungal rDNA. Sequence analysis of the ITS-1, 5.8S and ITS-2 regions from the three *Trichoderma* isolates from Libya revealed no variation within the 5,8 S gene, while

low but informative variation in both ITS-1 and ITS-2. Slight length variation was observed among the three characterized *Trichoderma* strains. The length of ITS-1 was 222 bp for Lib1, 199 bp for Lib2 and 221 bp for Lib3. Differences in ITS-2 length variation (Lib1 169 bp, Lib2 172 bp, Lib3 169 bp) were less than those noted in ITS-1. Homology searches between the ITS-1-5.8S-ITS-2 nucleotide sequences of the Lib1, Lib2 and Lib3 strains using BLAST with the sequences deposited in NIH GenBank identified both strain Lib1 (99% homology with *T. longibrachiatum* strain UAMH 7955) and Lib3 (100% homology with *T. longibrachiatum* strain UAMH 7956) as *T. longibrachiatum* species, while Lib2 (100% homology with *T. harzianum*) was identified as a *T. harzianum* strain (Fig. 9).

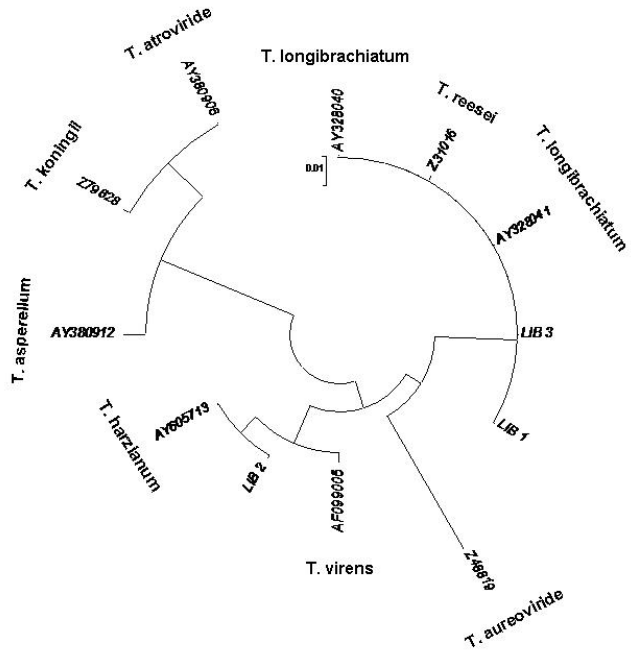


Figure 9. Radial dendrogram showing *Trichoderma* phylogeny based on ITS-1, 5.8S and ITS-2 regions of Libyan isolates and known *Trichoderma* species. Sequence alignment and phylogenetic studies were carried out by the use of the MEGA version 3.1 software (Kumar et al., 2004).

3.5. Metabolic profile of Libyan isolates

Although our data confirmed that the Libyan *Trichoderma* strains do not produce 6-*n*-pentyl-*6H*-pyran-2-one (TLC analysis), the most characterised and important of the *Trichoderma* antibiotics (Ghisalberti et al., 1990), other compounds with antibiotics activity were detected. Unfortunately the organic fractions obtained from culture filtrates of Lib2 and Lib3 isolates didn't allow to properly identify the secondary metabolites produced.

On the other hand, when the methanolic fraction extracted from Lib1 culture filtrate was analyzed, the mixture showed two major components, corresponding to lipo-carbohydrate and lipeptaibol. This fraction was further separated by preparative RP flash chromatography. Fraction n. 4 gave a major component that was further analysed by using NMR spectroscopy. The isolated compound showed ^{1}H (Fig. 10-A) and ^{13}C (Fig. 11) spectra similar to those reported in literature (Fig. 10–B) (Auvin-Guette et al., 1992). Moreover, the COSY bidimensional NMR spectrum of fraction n. 4 (Fig. 12-A) suggested that the isolated compound could be assigned to the lipopeptaibols class of natural compounds, and in particular resulted closely related to the Trichogin A IV, previously isolated from *T. longibrachiatum* (Peggion et al., 2003; Fig. 12-B).

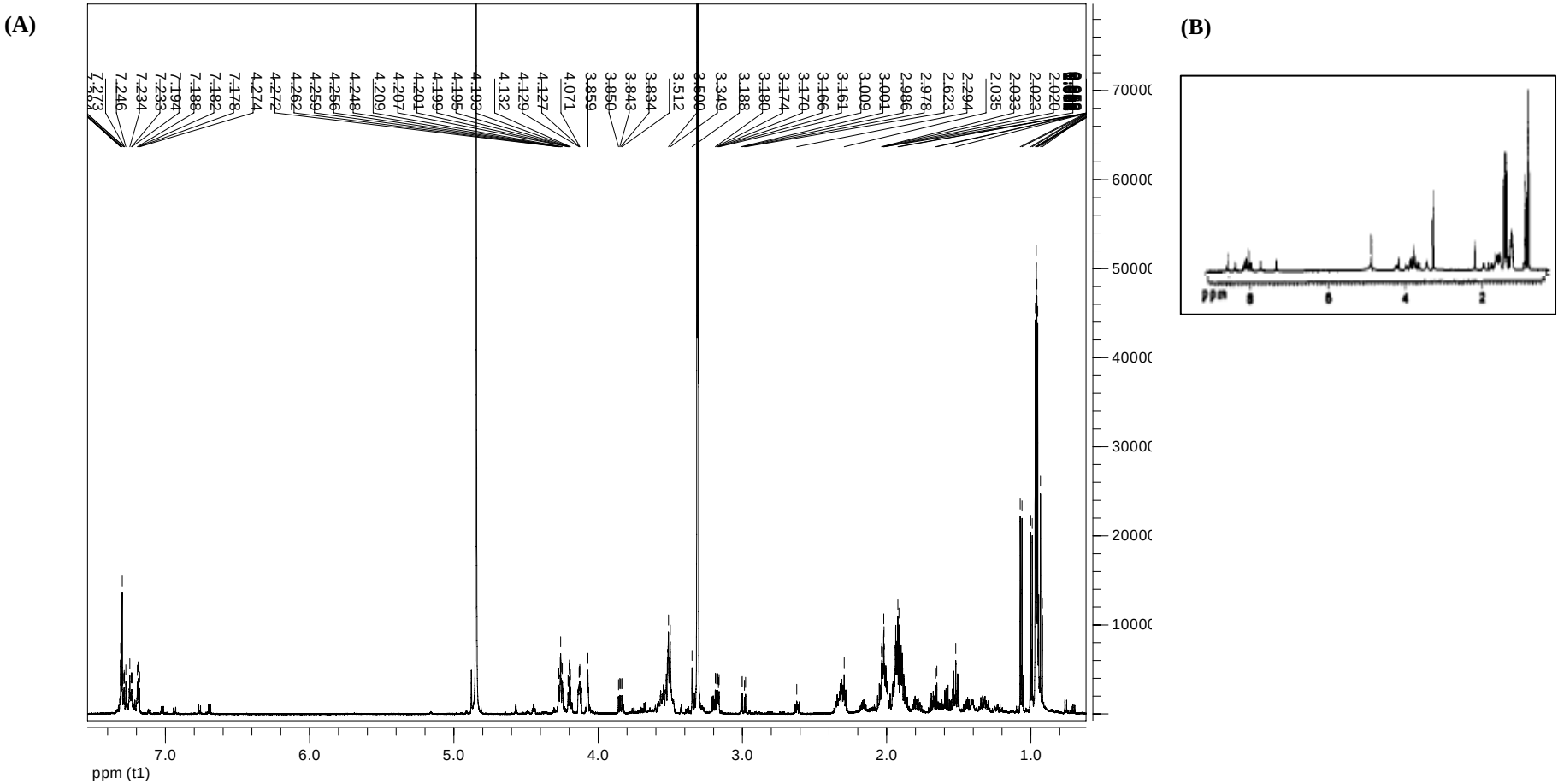


Figure 10. ^{1}H NMR spectra of fraction n° 4 isolated from Lib1 culture filtrate and recorded in CD_3OD (A) and Trichogin A IV recorded in *d6*-DMSO (B) (Auvin-Guette et al., 1992). Instrument: Burker 600 MHz.

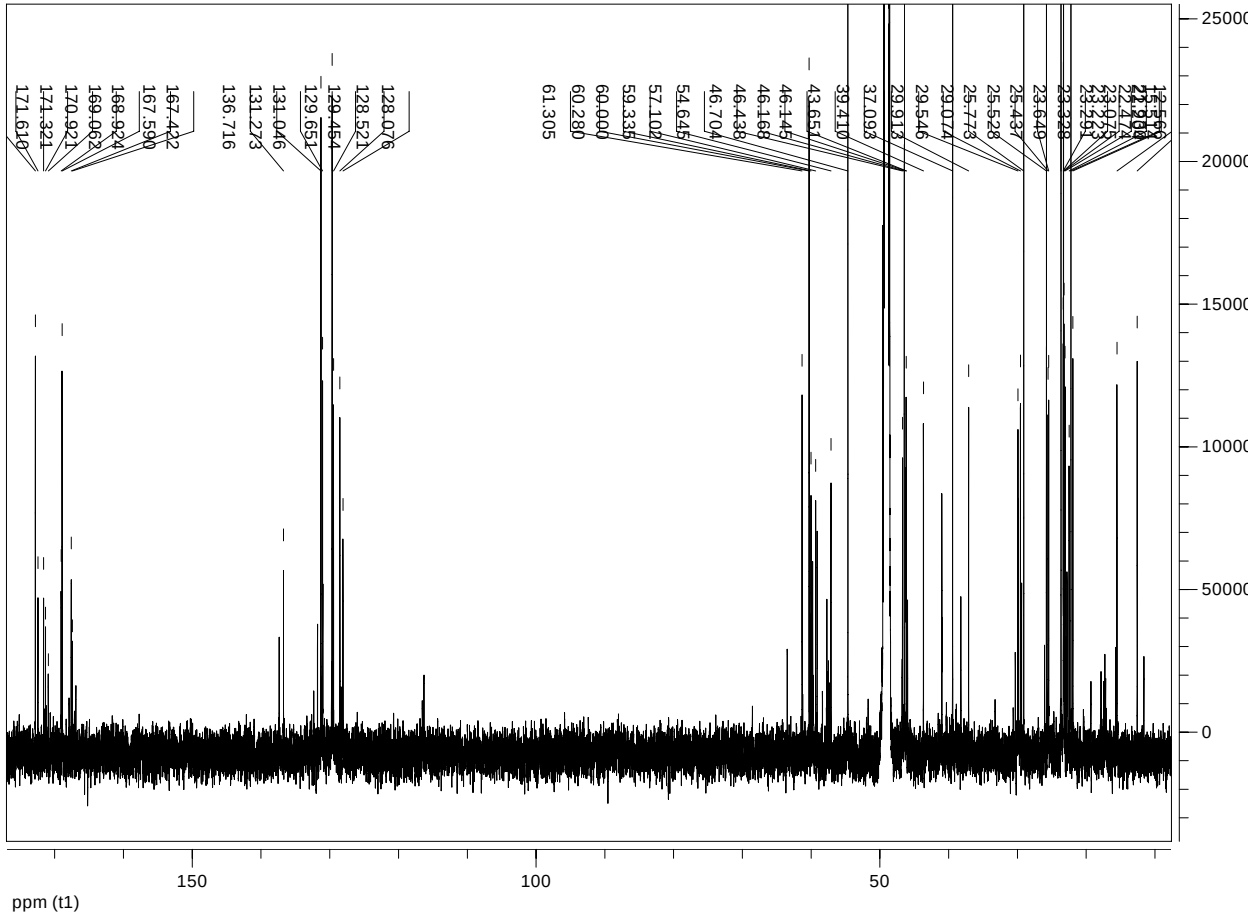


Figure 11. ^{13}C NMR spectrum of fraction n° 4 isolated from Lib1 culture filtrate and recorded in CD_3OD. Instrument: Burker 600 MHz.

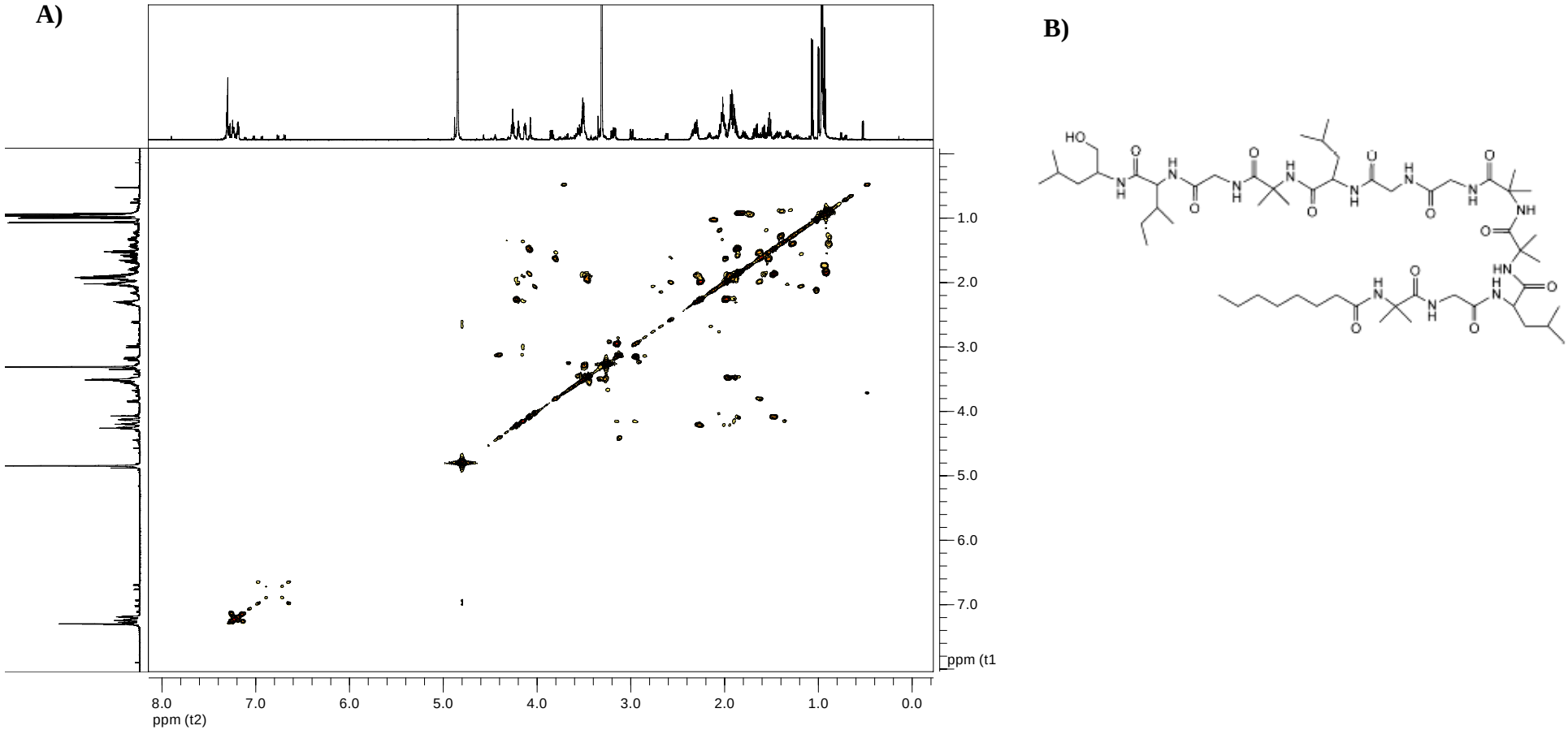


Figure 12. COSY bidimensional NMR spectrum of fraction n° 4 isolated from Lib1 culture filtrate (A) and structure of Trichogin A IV isolated from *T. longibrachiatum* by Peggion et al., 2003 (B).

3.6. Induction of plant growth and systemic resistance

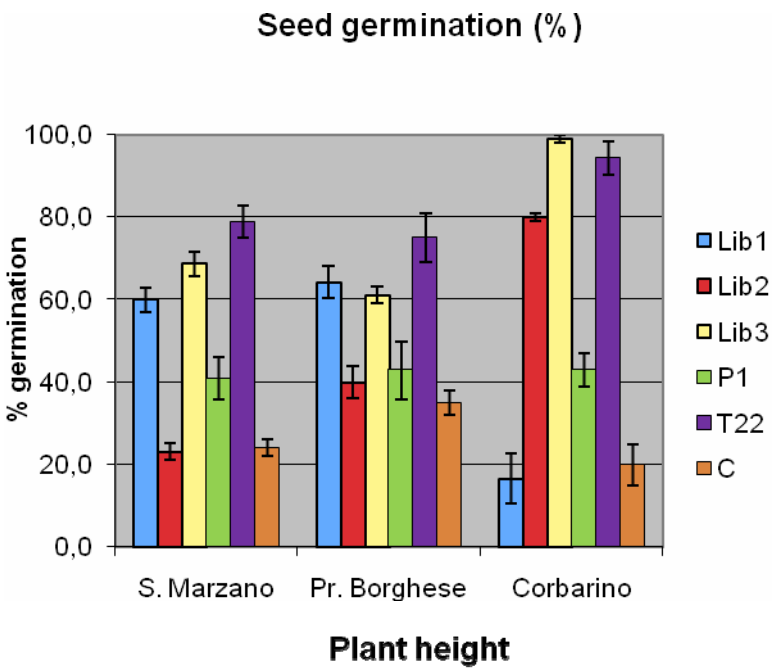
To test if Libyan isolate applications affect plant growth, tomato seeds from 3 different lines were coated with a *Trichoderma* conidial suspension and planted in sterile soil. Seed germination and plant growth were monitored for 3 weeks. Interestingly, tomato seeds of cv. S. Marzano and Principe Borghese showed at least 60% seed germination when treated with isolates Lib1 and Lib3, both identified as *T. longibrachiatum*, while 100% germination was observed in cv. Corbarino, whose seeds were coated with isolate Lib3 (Fig. 13-A).

The height of treated plants varied among the lines and according to the fungal isolate challenged. The plants treated with Lib1 showed a significant increase of growth compared with untreated samples (Fig. 13-B), whatever tomato line considered. On the other hand, Lib2 increased only the height of tomato plants cv. S. Marzano, but no differences were observed in the other lines or by applying the isolate Lib3, as compared with controls.

The mean number of leaves/plant was also calculated, but no great differences were found among the isolates and the controls (Fig. 13-C). The only exceptions were represented for tomato cv. S. Marzano by the isolate Lib2, and for tomato cv. Corbarino by the isolates Lib1 and Lib2, which determined a significant increase of the mean number of leaves *per planta*.

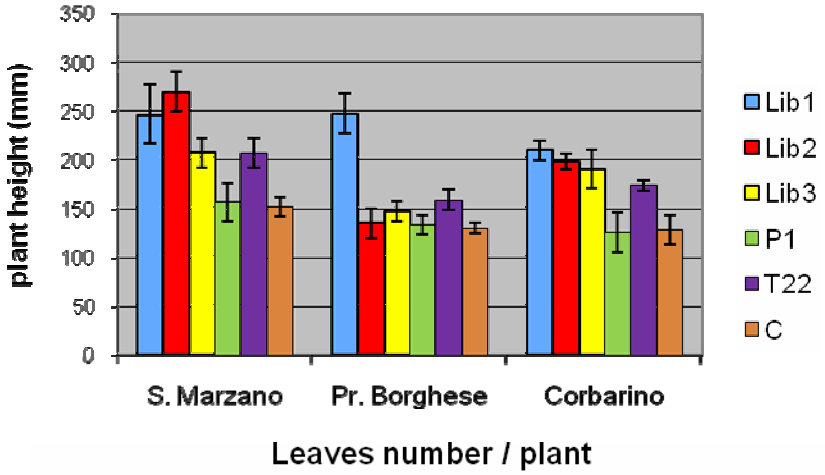
Figure 13 (next page). Effect of the Libyan *Trichoderma* isolates Lib1, Lib2 and Lib3 on the growht of different tomato cvs. (*S. lycopersici* cv. San Marzano, Principe Borghese and Corbarino). Plant seeds were coated with a *Trichoderma* spore suspension and planted in sterile soil. After 3 weeks, seed germination (**A**), plant lenght (**B**) and the number of leaves *per planta* (**C**) were evaluated. The antagonistic strains *T. atroviride* P1 (P1) *T. harzianum* T22 (T22) were used as controls. Water-treated plants were used as untreated controls (C).

A)



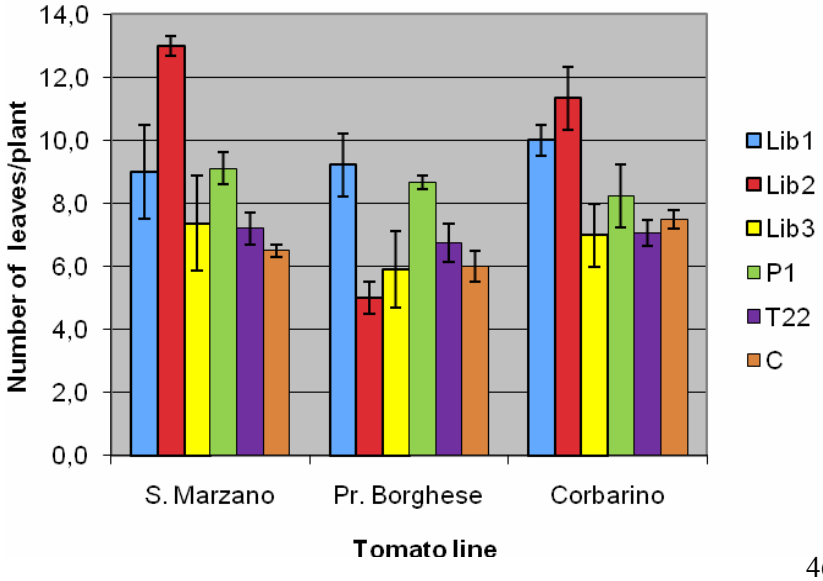
Plant height

B)



Leaves number / plant

C)



Fresh and dried weights of plant roots were also examined (Fig. 14). Inoculations of Libyan isolates caused mainly similar or lower effects compared to the antagonistic strains P1 or T22; only the tomato plant cv. Corbarino showed significant increases of both fresh and dried root weights when treated with the isolates Lib1 and Lib3, as well as with the antagonist *T. harzianum* strain T22.

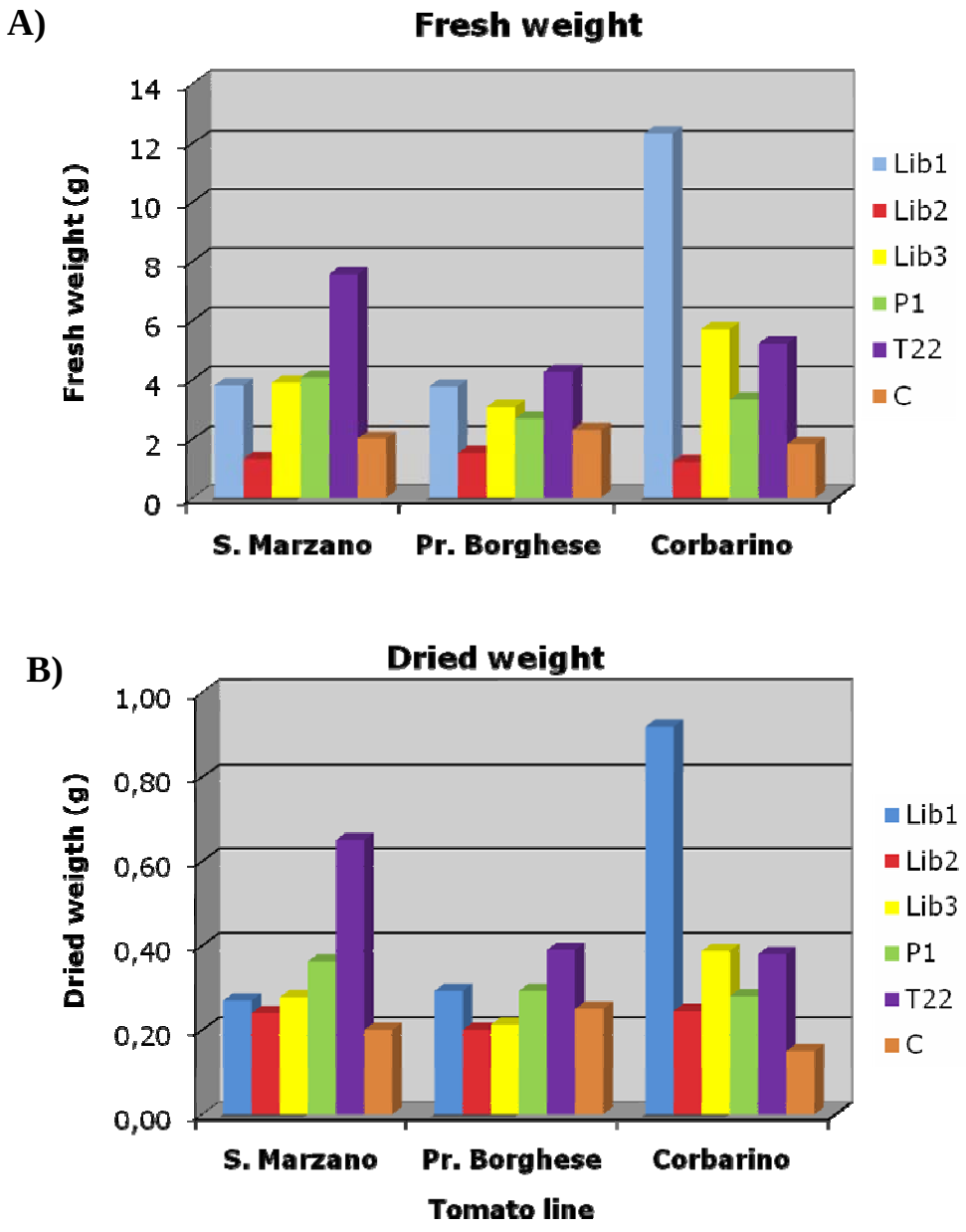


Figure 14. Effect of the Libyan *Trichoderma* isolates Lib1, Lib2 and Lib3 on plant productivity of different tomato cv. (*S. lycopersici* cv. San Marzano, Principe Borghese and Corbarino). Plant seeds were coated with a *Trichoderma* spore suspension and planted in sterile soil. After 3 weeks, the roots were cut and the fresh (**A**) and dried (**B**) weights were determined. The antagonistic strains *T. atroviride* P1 (P1) and *T. harzianum* T22 (T22) were used as controls. Water-treated plants were used as untreated controls (C).

In vivo tests were also performed to evaluate the ability of Libyan isolates to induce systemic resistance (ISR) against the foliar pathogen *Botrytis cinerea*. Seed coating with *Trichoderma* spore suspension was performed as above. Leaf surface was inoculated with the pathogen spore suspension and the diameter of necrotic area was measured after 48 h. As expected, the ISR effect varied according to the plant genotype. However, the fungi isolated in Libya significantly reduced the pathogen infection, showing a decrease of disease symptoms similar or sometimes higher to that observed with the biocontrol agents P1 or T22 (Fig. 15).

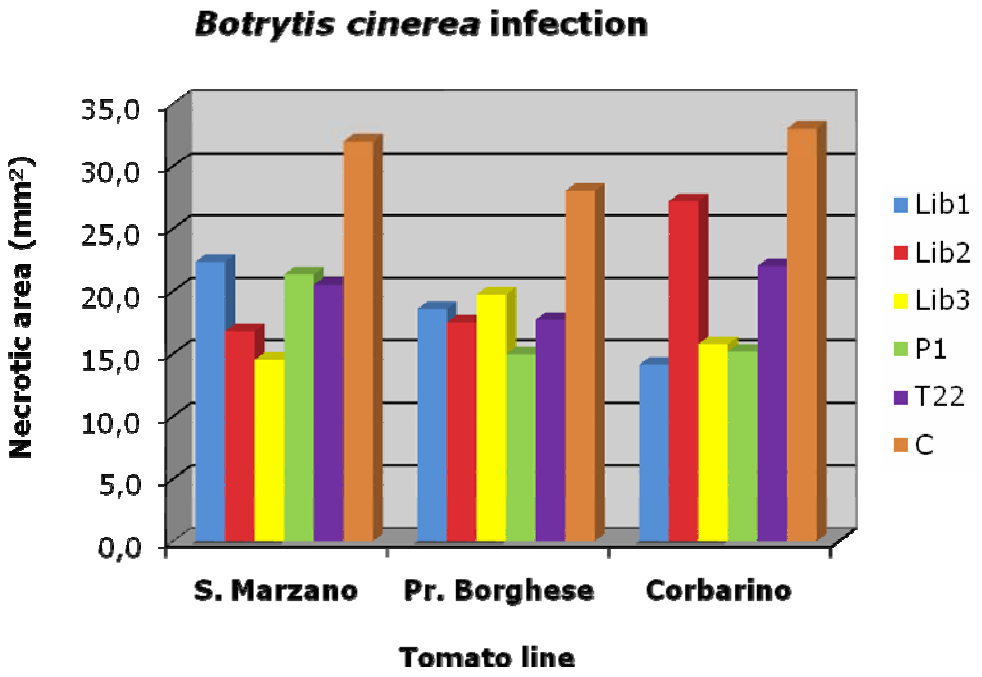


Figure 15. Effect of Libyan *Trichoderma* isolates Lib1, Lib2 and Lib3 on plant resistance of different tomato cv. (*S. lycopersici* cv. San Marzano, Principe Borghese and Corbarino) against the foliar pathogen *B. cinerea*. The development of disease symptoms (necrotic area on infected leaves) was evaluated 48 h after inoculation. *T. atroviride* strain P1 (P1) and *T. harzianum* strain T22 (T22) were used as controls. Water-treated plants were used as untreated controls (C).

3.7. Production of novel liquid formulations of bio-pesticides based on *Trichoderma* spores and metabolites

The development of a new formulation based on a *Trichoderma* isolate and/or its metabolites could represent a useful biotechnological application. The isolated fungi have demonstrated their potential ability to both control *in vitro* and *in vivo* plant pathogens, and simultaneously promote plant growth. Because of its performances in terms of fungal antagonism, plant growth promotion and induction of disease resistance, the isolate Lib1 was chosen for the development of a new liquid bio-fungicide. Therefore, the major parameters which allowed to optimize the fungal growth and metabolite production (temperature, pH, aeration, etc) were monitored in a small-scale process. The antagonistic fungus was cultivated in liquid medium and then transferred in to a 50L fermentor, where different operating conditions were applied to gain highest fungal growth and enzymatic activity. Samples were collected from each treatment twice per day for a total of 7 days of fermentation.

The first fermentation (I) was performed by transferring the *Trichoderma* starter culture into the fermentor containing 40L of Salt Medium (SM). In order to stimulate enzymatic activities, lyophilized *Agaricus bisporus* (0.5% w/v) + wheat fiber (0.2% w/v) were added as the only carbon sources. The temperature was set at 25° C, while 200 rpm and 0.7 vvm were used as orbital shaking and aeration parameters, respectively.

As a consequence of microbial growth, oxygen pressure (pO2) rapidly decreased during the first 36h of fermentation and then reached a plateau around 93%. In parallel, the pH increased from 6.2 to 6.9 (Fig. 16).

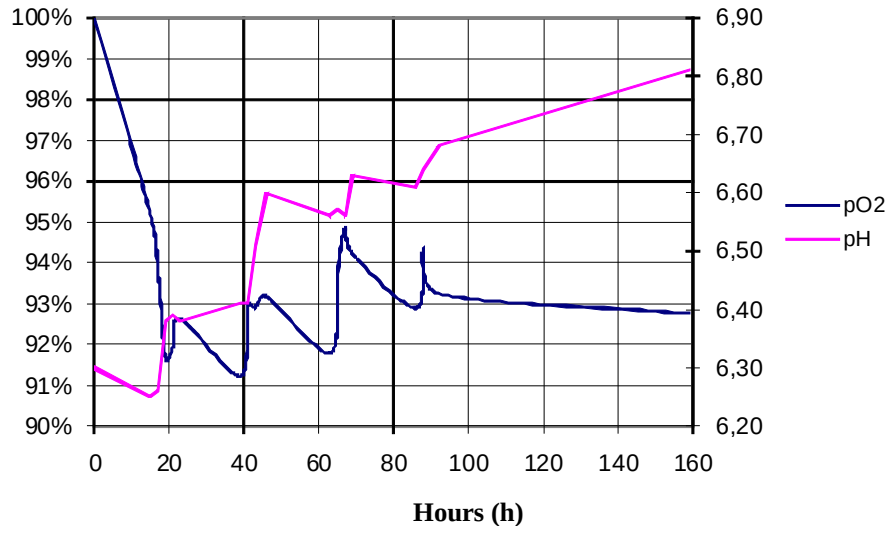


Figure 16. Monitoring of fermentation parameters (pO_2 and pH) during the first 150h after inoculum (fermentation I).

Total protein concentration was also determined as well as enzymatic activities. Maximum protein content was 4 µg/ml after 48h of fermentation and then slowly decreased till 2,36 µg/ml were reached after 144h (Fig. 17).

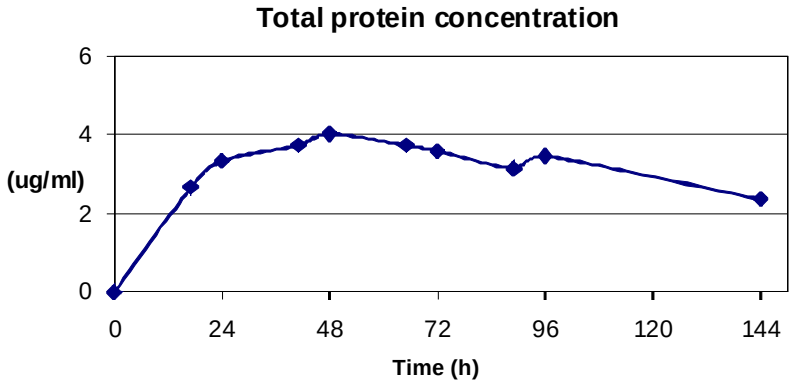


Figure 17. Total protein concentration (µg/ml) during the I fermentation.

The levels of enzymatic activities were not as higher as expected. In particular, for chitinolytic activity, both endo- and esochitinases increased their accumulation till reaching a maximum after 72h, corresponding to absorbance (Abs) values of 0.276 for N-acetylglucosaminidase, 0.450 for chitobiosidase and 0.242 for endochitinase, respectively (Fig. 18). After 72h, the Abs values remained constant, then started to decrease at the end of the fermenting process; this was probably due to the exhaustion of the inducing substrate or to the production of proteases by the fungus itself.

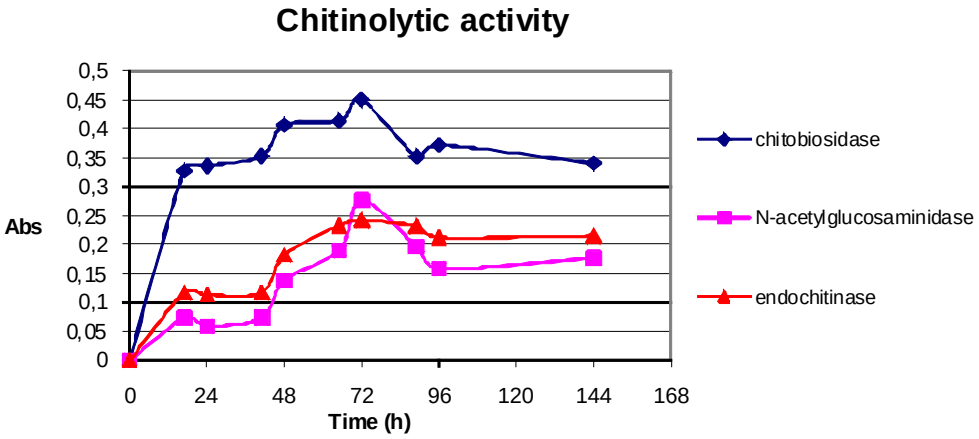


Figure 18. Chitinolytic activity of culture broths obtained in the I fermentation.

Similarly, β-1,3glucanase increate after 72h up to 0.245 Abs value, while glucanolytic activity increased after 96h till the end of the fermentation (Fig. 19).

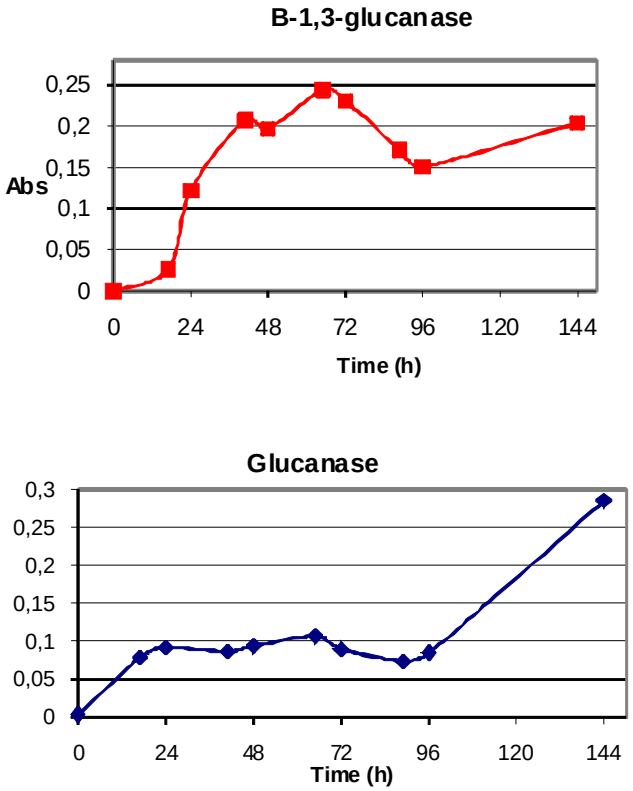


Figure 19. β-1,3glucanase (left) and glucanolytic (right) activities of culture broths obtained in the I fermentation.

The xilanase activity increased progressively during the fermentation, reaching the maximum value after 144h (Fig. 20).

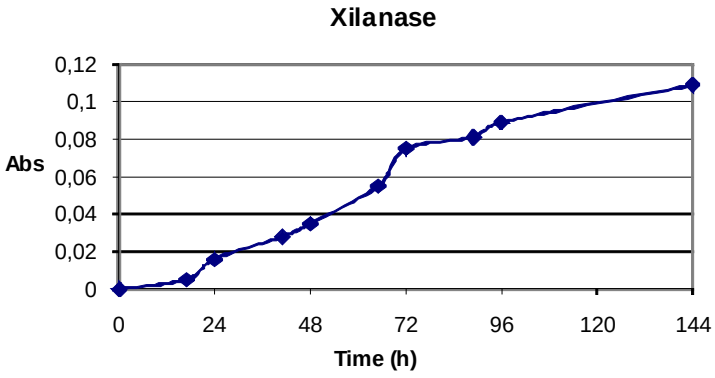


Figure 20. Xilanase activity of culture broths obtained in the I fermentation.

Finally, the spore concentration was measured by microscope direct counting. The results showed an increasing sporulation from the beginning till the end of the fermentation, with a maximum concentration at 1.3 x 10^6 spore/ml (Fig. 21).

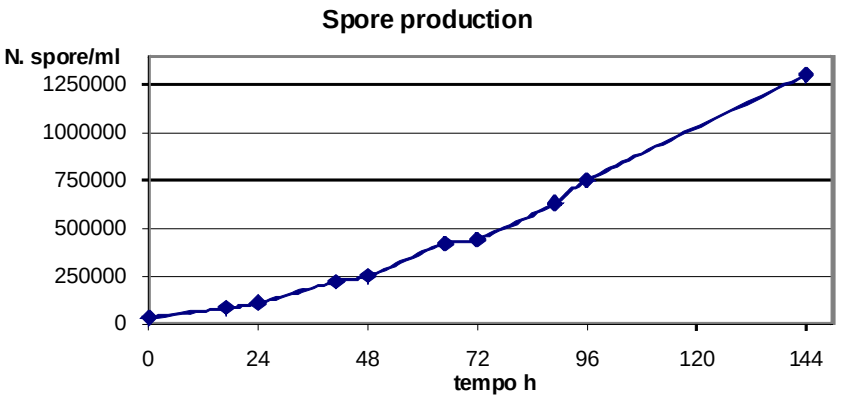


Figure 21. Spore production (Number of spore /ml) obtained in the I fermentation.

At the microscope observation, the fungal mycelium appeared extremely fragmented with filamentous hyphae of reduced dimensions, probably because of the turbulence generated by the high orbital shaking and aeration conditions applied. The mycelium development and the enzymatic activities resulted negatively affected by the operation conditions used in the first fermentation. Thus, in order to improve the fungal growth, a second experiment was performed where aeration and shaking values were reduced respectively at 0.5 vvm and 100 rpm; moreover, to maximize the enzymatic production, the wheat fiber was added at 0.3% (w/v). The experiment was repeated twice (II and III fermentation) and the results obtained in terms of total protein concentration, enzymatic activities and spore production are summarized in Figures 22 and 23.

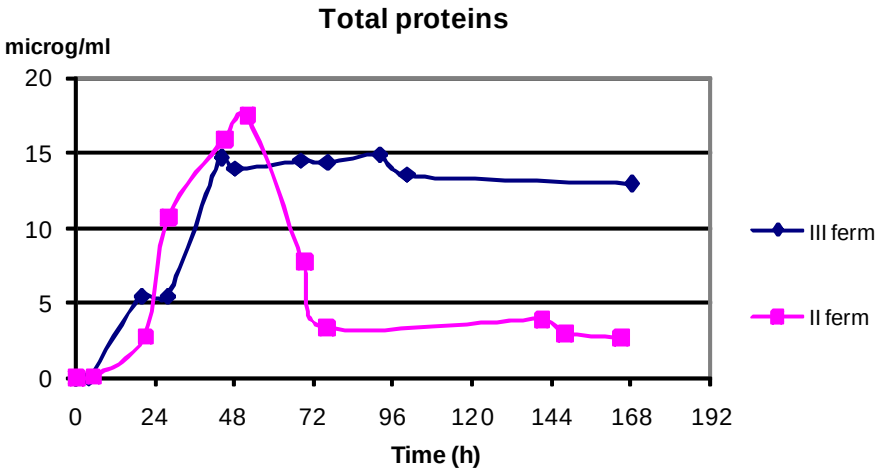
The results obtained in the II and III fermentations, both in terms of protein concentration and enzymatic activities, were more promising; in fact, by changing

the process parameters, after only 48 h total protein content increased up to 4 times, reaching 17.51 μg/ml and 14 μg/ml in the II and III fermentation, respectively (Fig. 22). Moreover, chitinolytic activities (both endo- and eso-) were similar to the ones observed in the first experiment, but higher values were obtained.

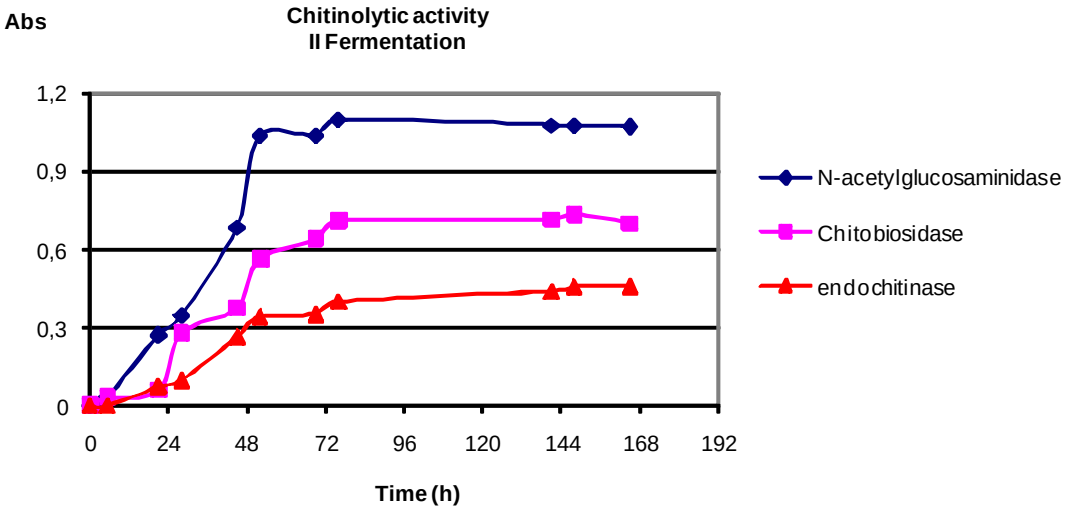
The enzymatic activity of β-1,3 glucanase was similar to that observed in the previous fermentation, while the glucanolytic activity was extremely higher after 72h, reaching 0.253 and 0.319 Abs values in the II and III fermentation, respectively (Fig. 23). Therefore, xilanase activity after 72h was 6 times higher than that observed before.

During the II and the III fermentations the spore concentration reached the final values of $2{,}12 \times 10^6$ and $7{,}0 \times 10^6$ spore/ml (data not shown). This difference could be due to the fact that at the end of the III fermentation the shaking was increased up to 4000 rpm for 10 min to recover the fungal biomass.

A



B



C

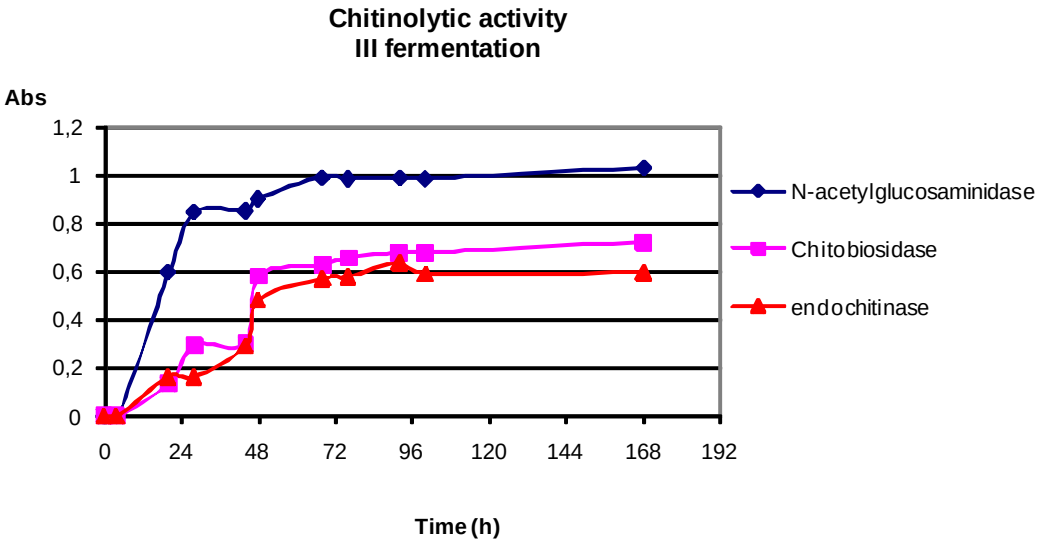
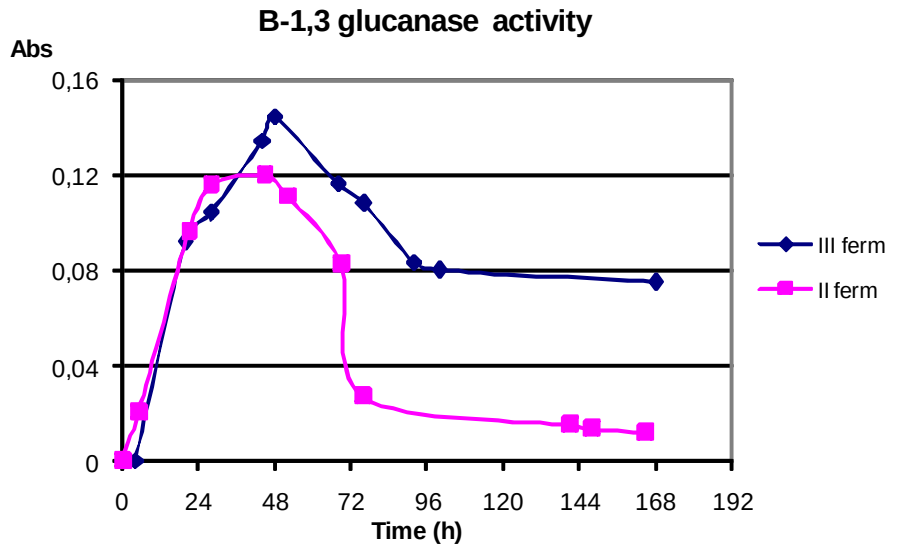
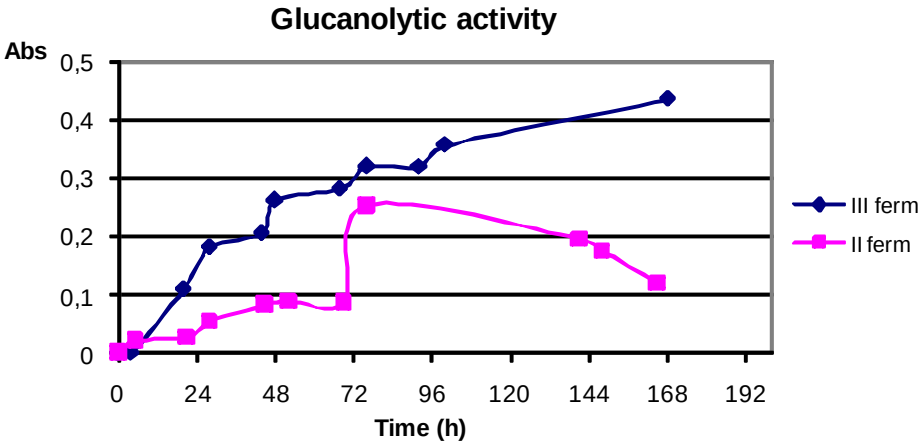


Figure 22. Total protein concentration (µg/ml) (**A**) and chitinolytic activities (**B** and **C**) of culture broths obtained in II and III fermentations.

A



B



C

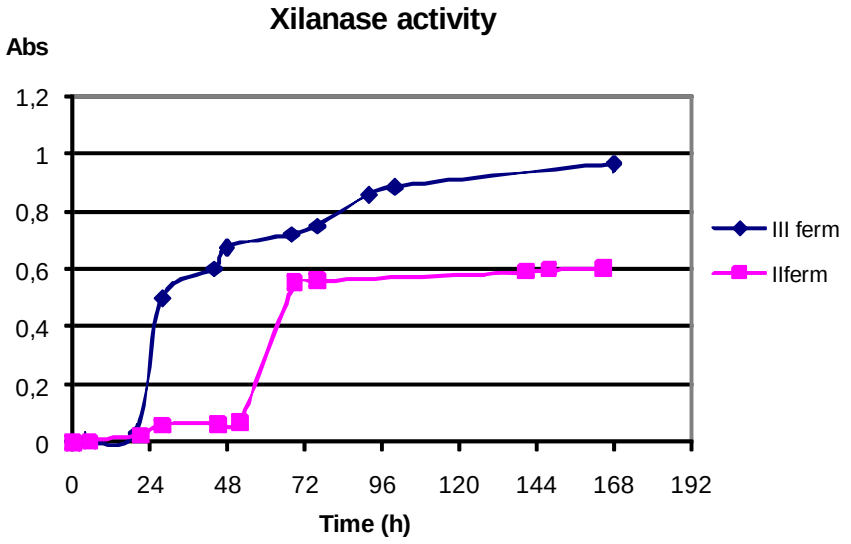
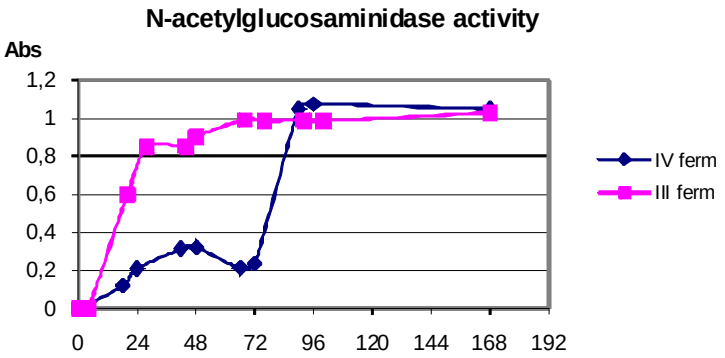


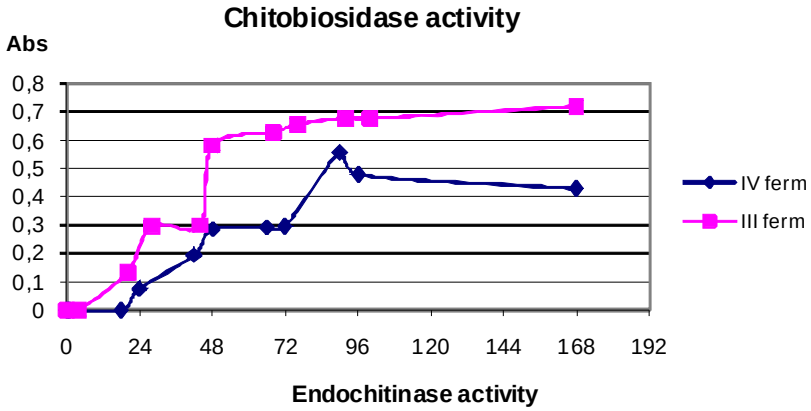
Figure 23. β-1,3glucanase (**A**), glucanolytic (**B**) and xilanase (**C**) activities of culture broths obtained in II and III fermentations.

The effect of medium composition was also evaluated by using nutrients less expensive and/or more efficient in stimulating fungal growth and enzymatic activities. Thus, the substrate used for fermentation number IV was SM containing as carbon sources 0.5% (w/v) chitin extract from crab shells + 0.3% (w/v) wheat (*T. durum*) fiber for enzymatic induction. The results, showed in Figure 24 (where enzymatic activities obtained during III and IV fermentations were compared), clearly indicated that chitin extract from crab shells is a lower inducer of enzymatic activity compared to lyophilized mushrooms. Chitinolytic enzymes showed lower levels of activity and their production was delayed (72h after inoculum). Similar results were observed for glucanase activity, while xilanase was not induced. Final spore concentration was 1.01 $x10^6$ spore/ml (data not shown).

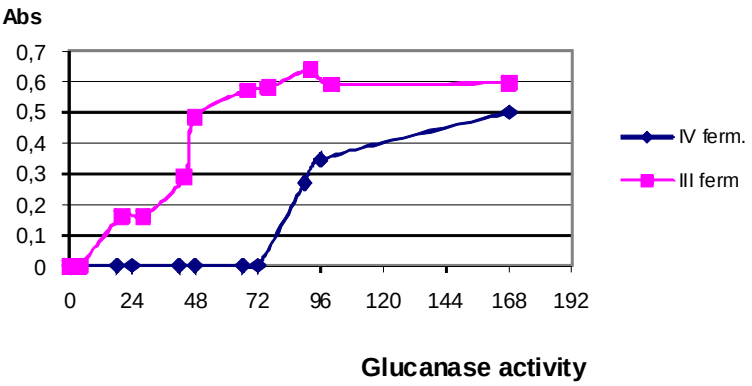
A



B



C



D

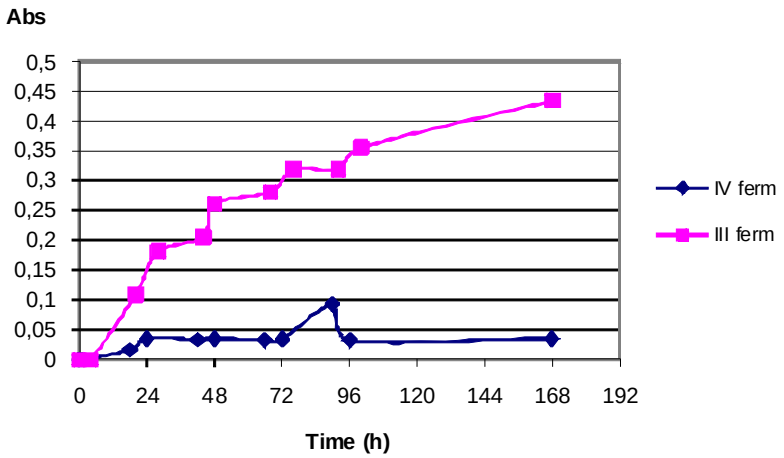
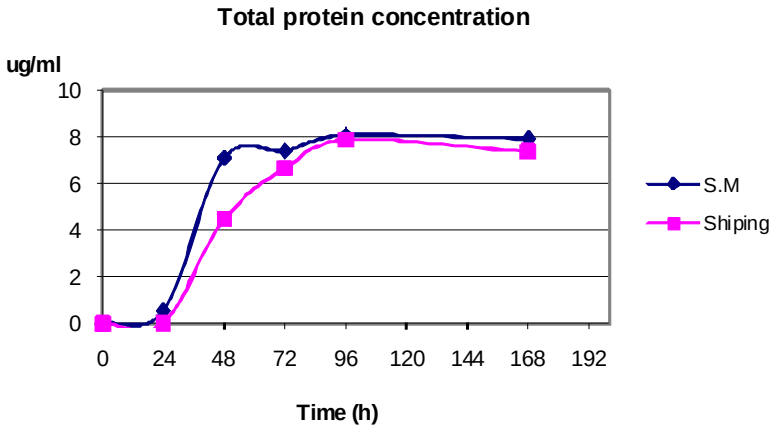


Figure 24. Chitinolytic (N-acetylglucosaminidase =**A**, chitobiosidase =**B** and endochitinase =**C**) and glucanase (**D**) activities of culture broths obtained in III and IV fermentations.

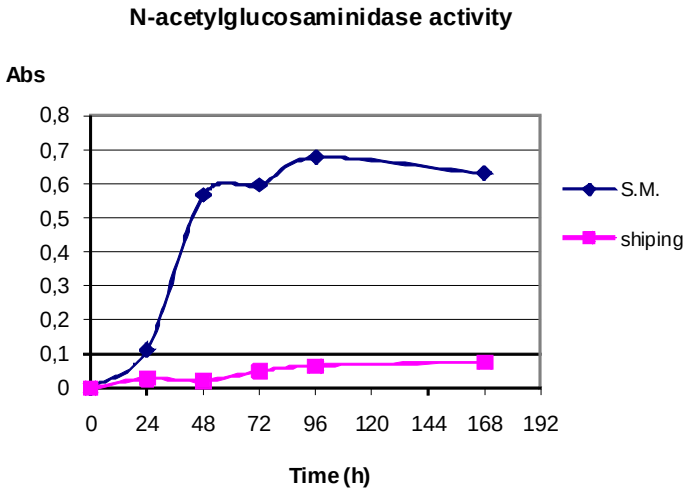
Once established that the presence of 0.5% (w/v) lyophilized mushrooms (*A. bisporus*) + 0.3% (w/v) wheat (*T. durum*) fiber represented the most inducing condition, the influence of mineral salts present in the substrate was also evaluated. Two different fermentations were performed in 1L flasks containing 250ml of SM or Shiping Medium, both minimal medium whose composition was regulated according to the stechiometric proportions of nutrients (particularly N, P, Mg, K, Fe, Zn, Co, Mn, and other microelements) necessary for microbial growth. Fermentations were performed for 7d at 25° C with a orbital shaking of 200 rpm and 0.3 vvm as aeration. Samples from fermenting cultures were evaluated in terms of total protein content and enzymatic activities. The results showed in Fig. 25 and 26 demonstrated that, even if both conditions determined similar protein concentration values and trends, the enzymatic activities were more induced when cultivation in SM was performed.

Thus, the most efficient inducing condition was represented by use of salt medium (SM) amended with lyophilized *A. bisporus* (0.5% w/v) + wheat fiber (0.3% w/v). Moreover, by using 100 rpm and 0.5 vvm as orbital shaking and aeration parameters, respectively, the maximum enzyme production was reached only 72h after inoculum.

A



B



C

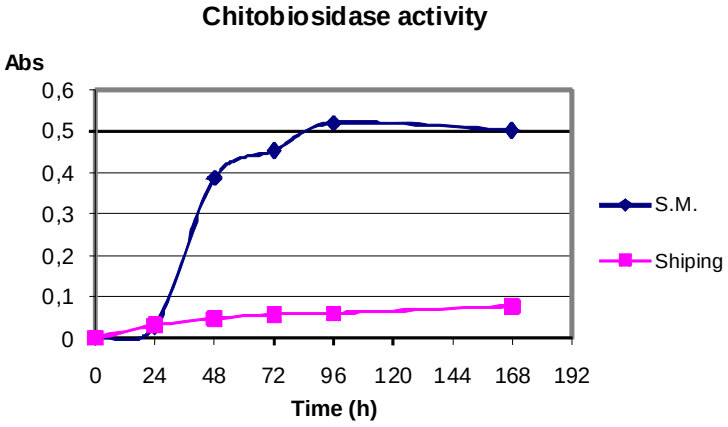


Figure 25. Total protein concentration (µg/ml) (**A**) and chitinolytic (N-acetylglucosaminidase =**B** and chitobiosidase =**C**) activities of *Trichoderma* culture broths obtained by cultivating the fungus in different substrates (salt medium = SM or Shiping Medium).

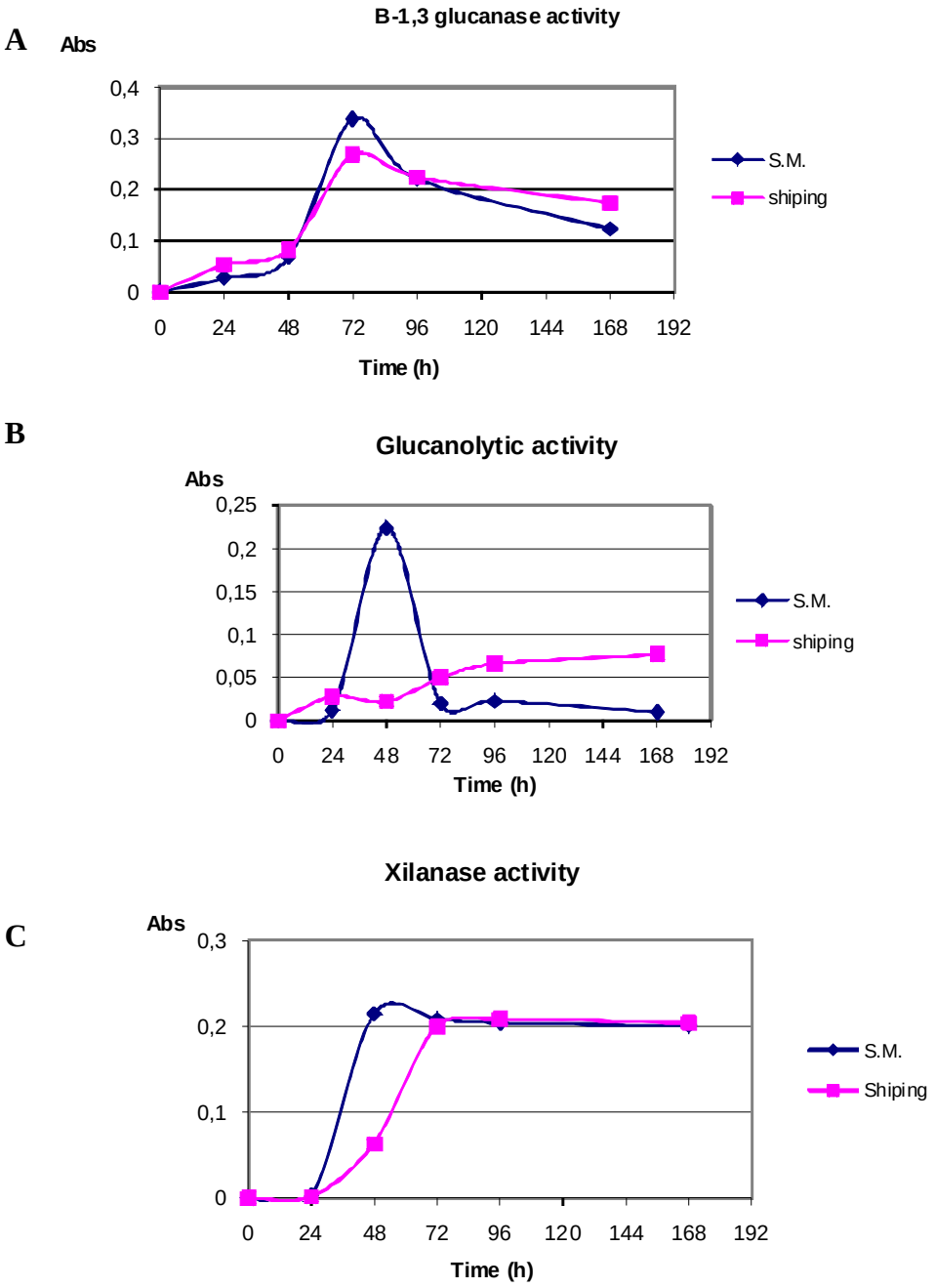
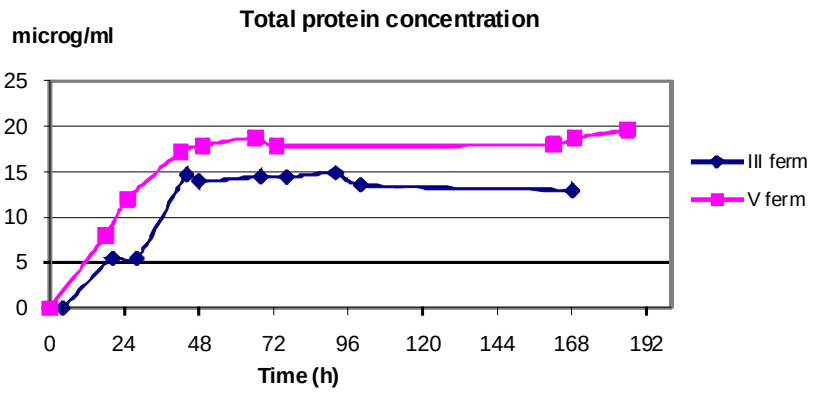


Figure 26. Chitinolytic (endochitinase =**A**), glucanolytic (**B**) and xilanase (**C**) activities of *Trichoderma* culture broths obtained by cultivating the fungus in different substrates (salt medium = SM or Shiping Medium).

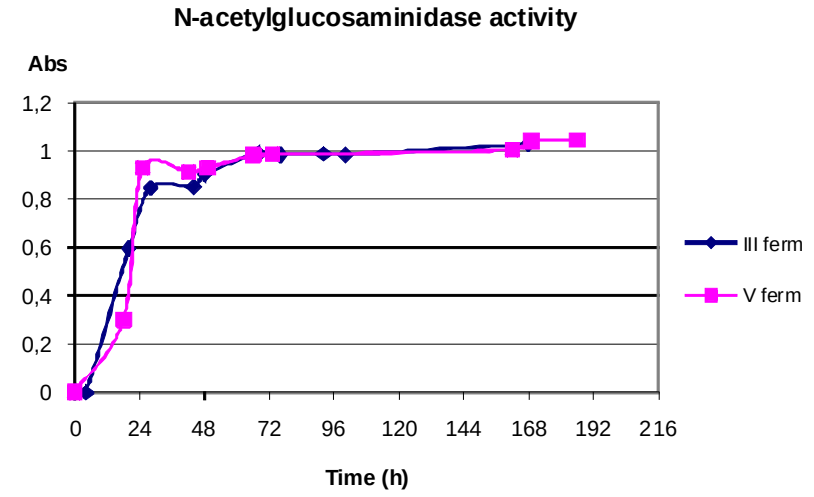
Finally, in order to induce a higher fungal sporulation without negatively affect the enzyme production, another fermentation (V) was performed: in this case at 48h after inoculum the shaking was increased up to 200 rpm and the aeration decreased to 0.3 vvm. The reduced oxygen availability and the turbulence obtained with high shakes represent more stressful conditions for the fungus. This promotes the fungal sporulation and reduces the mycelium growth (Felse and Panda, 1999, Jsten et al., 1996). The results reported in Fig. 27 and 28 showed a comparison between enzymatic activities obtained in the V and III fermentation and confirmed this hypothesis. In particular, from 48h after inoculum chetobiosidase, endochitinase and glucanase activities were slightly lower in the V fermentation, while the spore concentration determined at the end of this experiment was $1.03x10^7$ spore/ml (data not shown).

Therefore, changing the parameters during the fermenting process could improve the spore production, without interfering significantly with enzymatic activities.

A



B



C

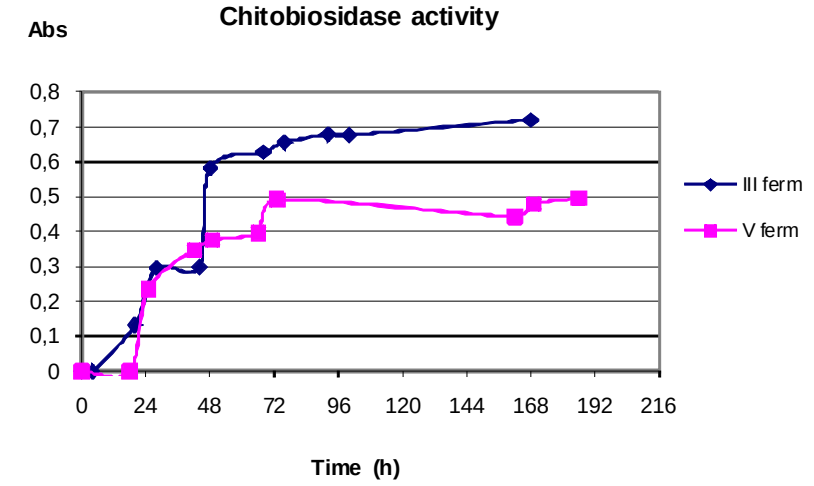


Figure 27. Total protein concentration (µg/ml) (**A**) and chitinolytic activities (N-acetylglucosaminidase =**B** and chitobiosidase = **C**) of culture broths obtained in III and V fermentations.

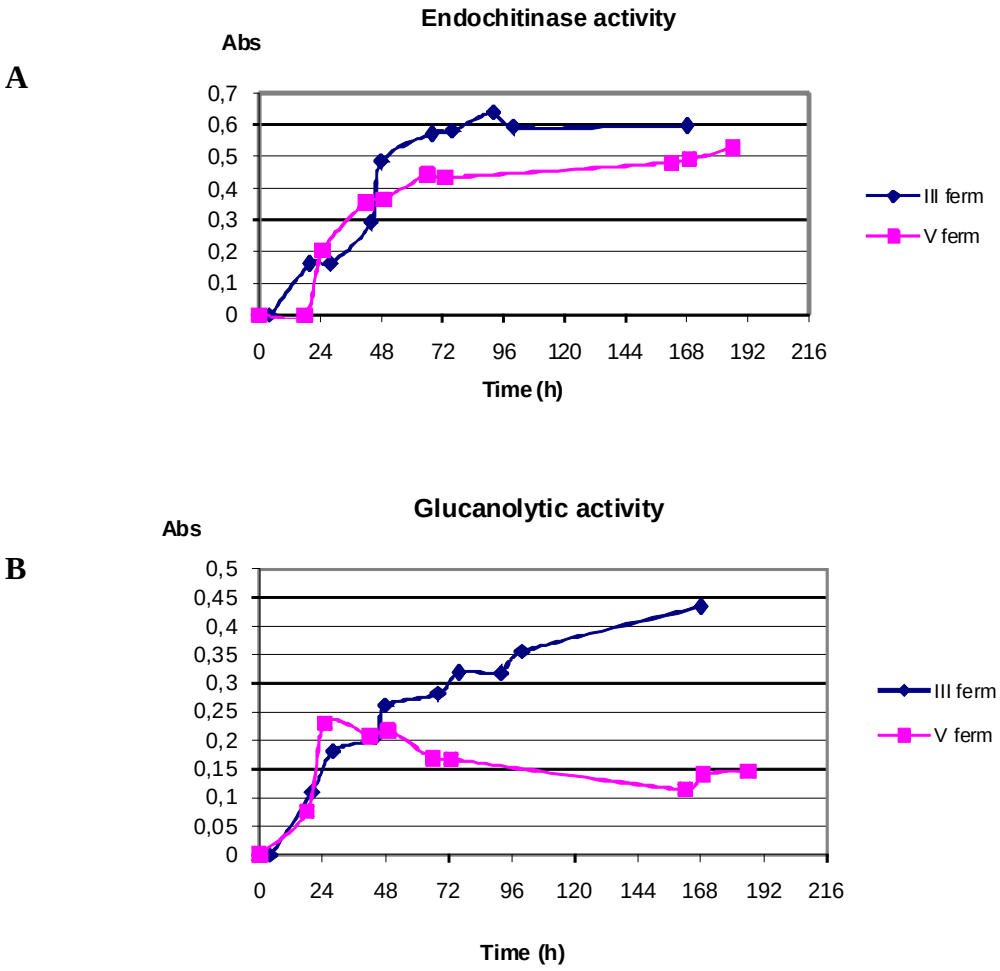
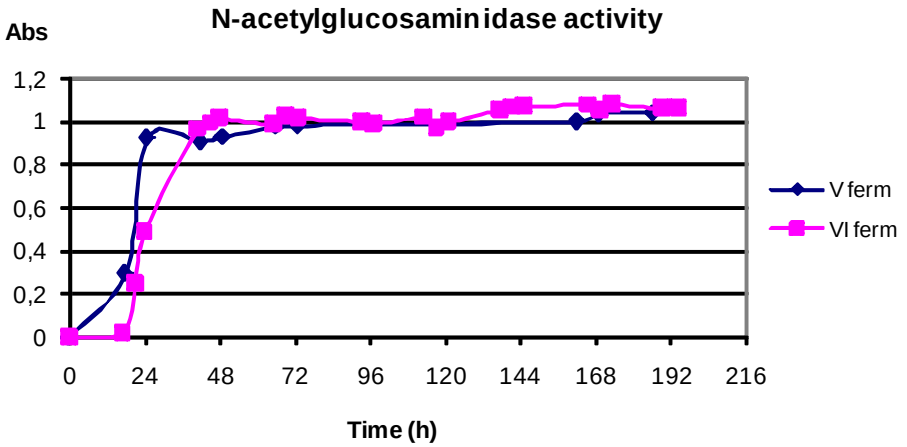


Figure 28. Chitinolytic (endochitinase =**A**) and glucanolytic (**B**) activities of culture broths obtained in III and V fermentations.

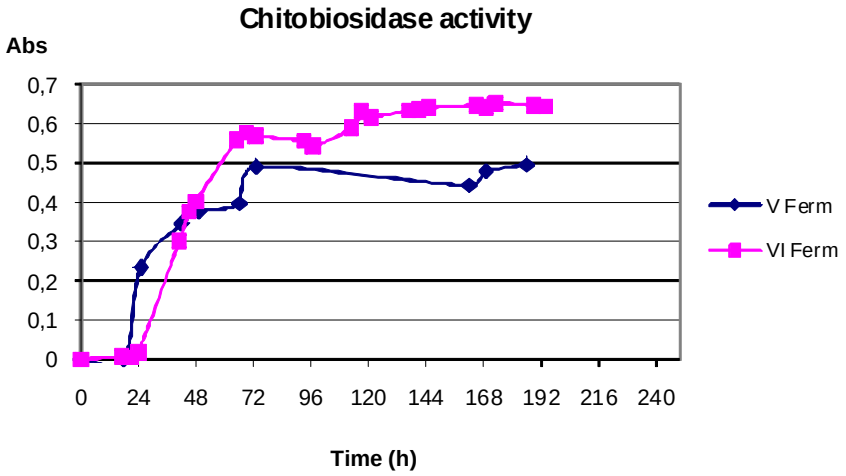
The operative conditions applied on a little-scale process (50L) were used on a 200L fermentor, in order to develop an industrial-scale process. To minimize the effects on enzymatic production, the process parameters (aeration, shaking) were modified after 72h after inoculum. The data obtained in the VI fermentation were similar with those referred to the previous one; chitinolytic and glucanolytic enzymes showed similar levels of activity, while β,1-3 glucanase e xilanase activities were slightly reduced compared to the values of the V fermentation (Fig. 29 and 30).

In conclusion, the modification of shaking and aeration parameters after 72h represents a good strategy to get a fermentative broth with high yields of spores and lytic enzymes. Moreover, aiming in reducing the costs, it should be desirable to stop the fermentation 120h and not 194h after inoculum, as no significant changes occurred.

A



B



C

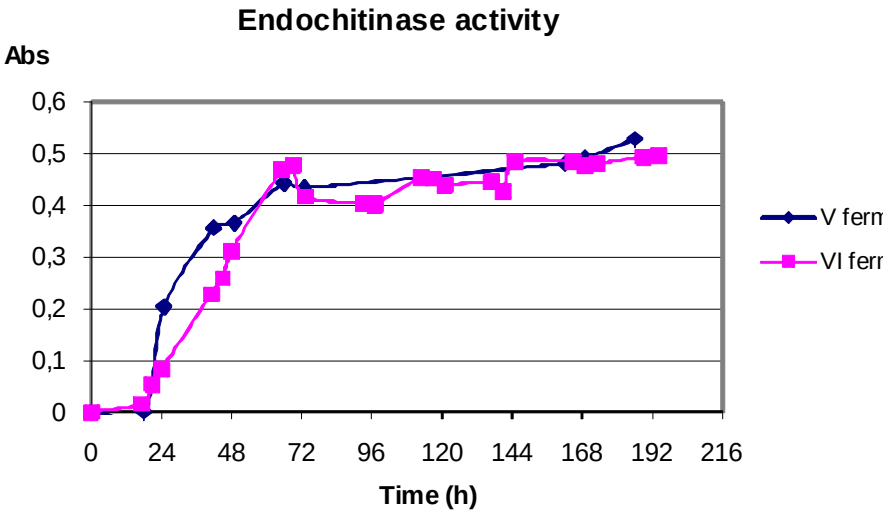
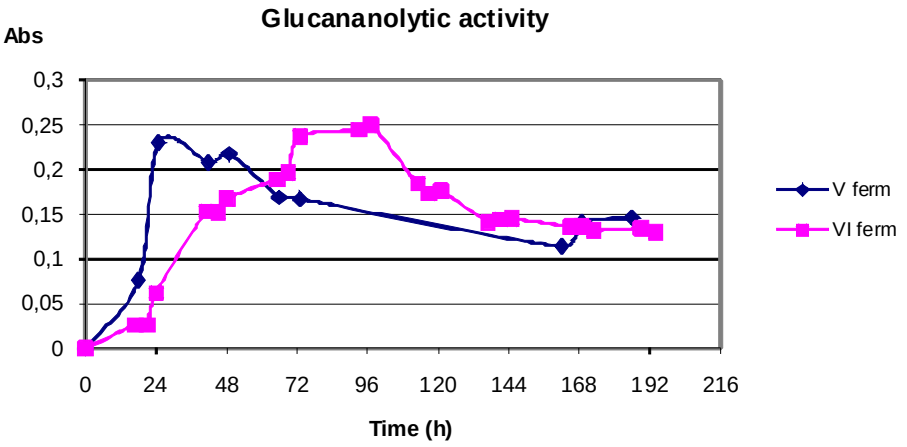
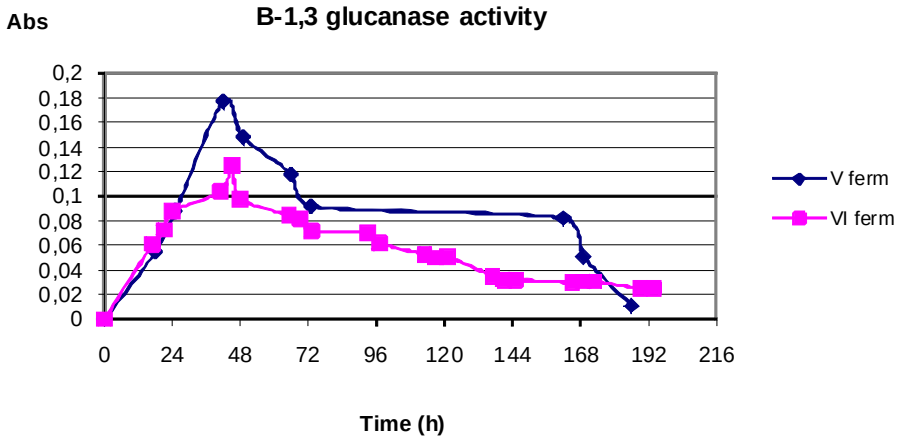


Figure 29. Chitinolytic (N-acetylglucosaminidase =**A,** chitobiosidase = **B** and endochitinase =**C**) activities of culture broths obtained in V and VI fermentations.

A



B



C

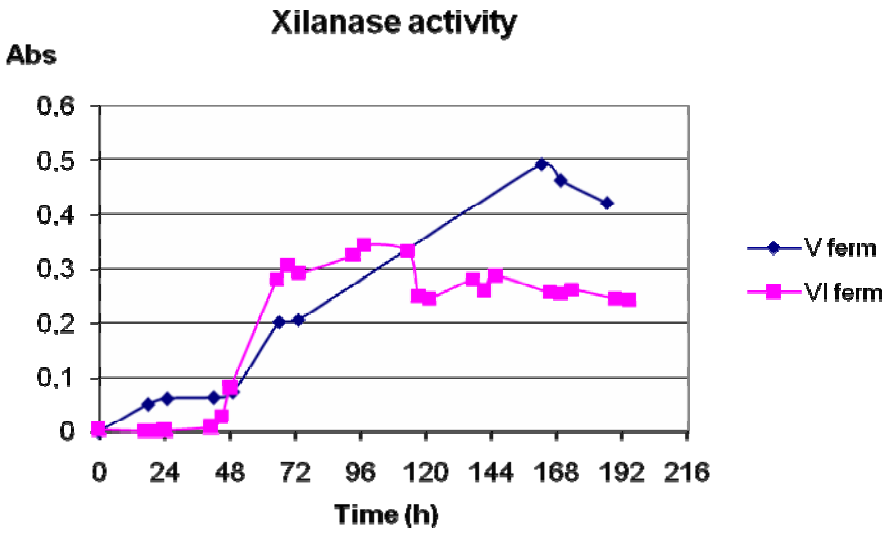


Figure 30. Glucanolytic (**A**), β-1,3 glucanase (**B**) and xilanase (**C**) activities of culture broths obtained in V and VI fermentations.

3.8. Plant growth promotion and disease control by a novel bio-fungicide based on the Lib1 isolate

In vivo bioassays were performed by using samples obtained from culture broths produced in III, V and VI fermentations in order to evaluate their biocontrol activity in plant-pathogen interactions. Tomato and lettuce seeds germinated in sterile soil were sprayed with 3ml of culture broth and then inoculated on leaf surface with *B. cinerea* spore suspension. The biocontrol activity was determined according to the chlorotic and necrotic area developed as a consequence of pathogen development on plant tissues.

On lettuce plants the use of fermentation broths totally reduced the disease symptoms, that was particularly evident 96h after inoculum (Fig. 31).

Figure 31. Disease symptoms developed on lettuce leaves inoculated with *B. cinerea* after treatment with *Trichoderma* culture broths from fermentations V and VI. **A**: infected control (no *Trichoderma* culture broth); **B**: V fermentation broth + *B. cinerea*; **C**: VI fermentation broth + *B. cinerea*.

On tomato plants, the use of the novel formulates greatly reduced the number of leaves showing disease symptoms, compared with control. Moreover, the dimension of pathogenic lesions decreased as well; the lesions produced 120h after inoculum were smaller, with a reduction of damage around 50% (Fig. 32 and 33).

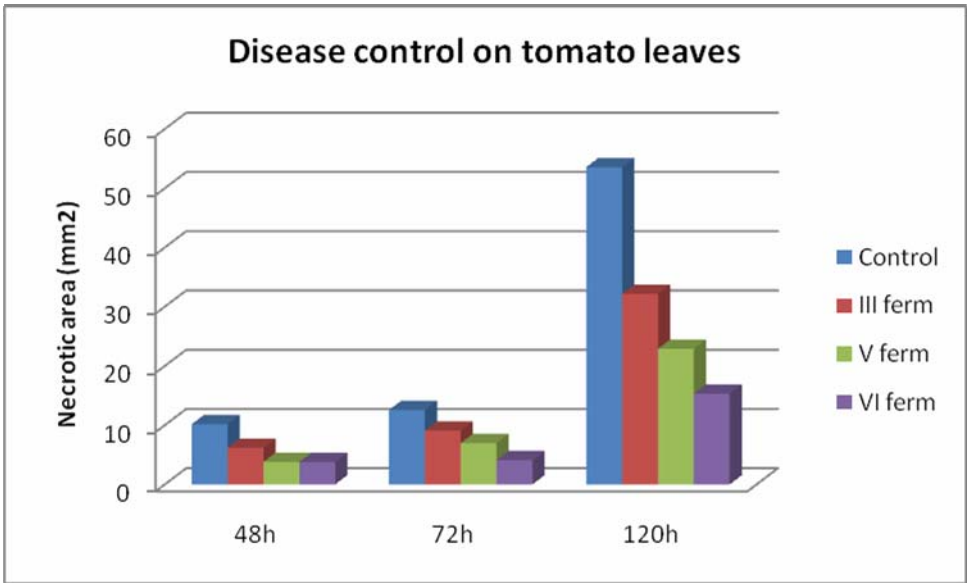


Figure 32. Disease symptoms development on tomato leaves inoculated with *B. cinerea* after treatment with *Trichoderma* culture broths from fermentations III, V and VI, compared with control (tomato leaves treated with *B. cinerea* only).

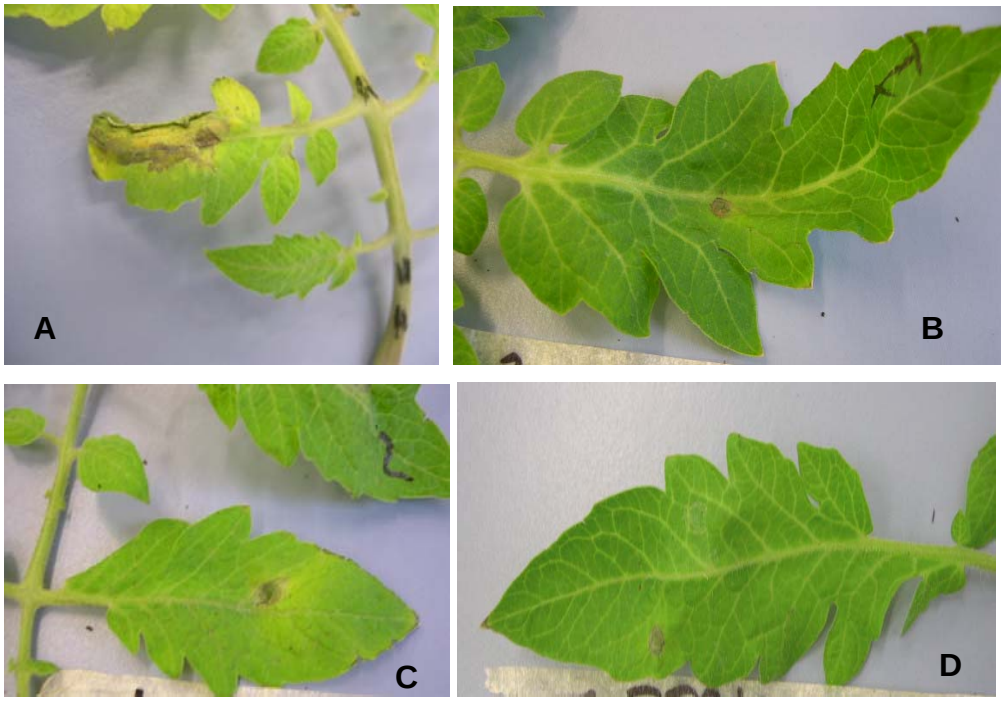


Figure 33. Disease symptoms development on tomato leaves inoculated with *B. cinerea* after treatment with *Trichoderma* culture broths from different fermentations. **A**: infected control (no *Trichoderma* culture broth); **B**:III fermentation broth + *B. cinerea*; **C:** V fermentation broth + *B. cinerea*; **D**: VI fermentation broth + *B. cinerea*.

The effect of the novel formulate on plant growth was also analyzed. Tomato seedlings were allowed to germinate in Petri dishes containing the novel formulate + 1.5% agar. The growth promotion effect was clearly visible by comparing the root length of treated with untreated plants (Fig. 34)

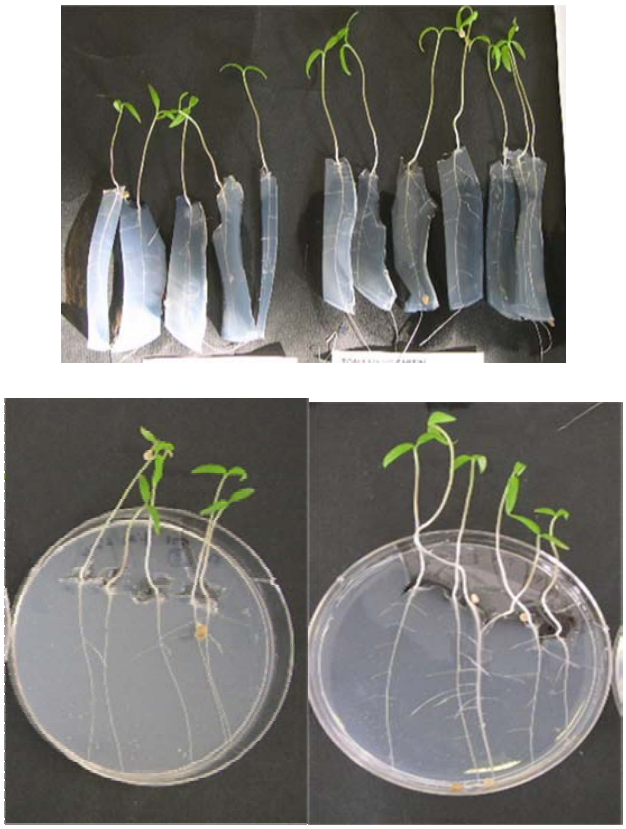


Figure 34. Plant growth promotion effect of *Trichoderma* novel formulate on tomato seedlings. Left = control (water agar as substrate); right = treated tomato plants.

3.9. Development of a new formulate: concentration and stability assessments

In order to develop a marketable formulate, part of the culture broth obtained in the III fermentation was concentrated by using spray drying and lyophilization

techniques. Glycerol was added to the samples (20% v/v) to better preserve the spore vitality. Results showed in Figure 35 showed no significant differences in terms of chitinolytic activity before after treatments. Moreover, spore vitality was not significantly affected by the lyophilization when glycerol was added; without glycerol, the spore concentration reduced from 7.0×10^6 to 1.8×10^6 spore/ml after treatment.

Conversely, the sample treated by spray drying lost completely its activity and no enzymatic activity were registered at all.

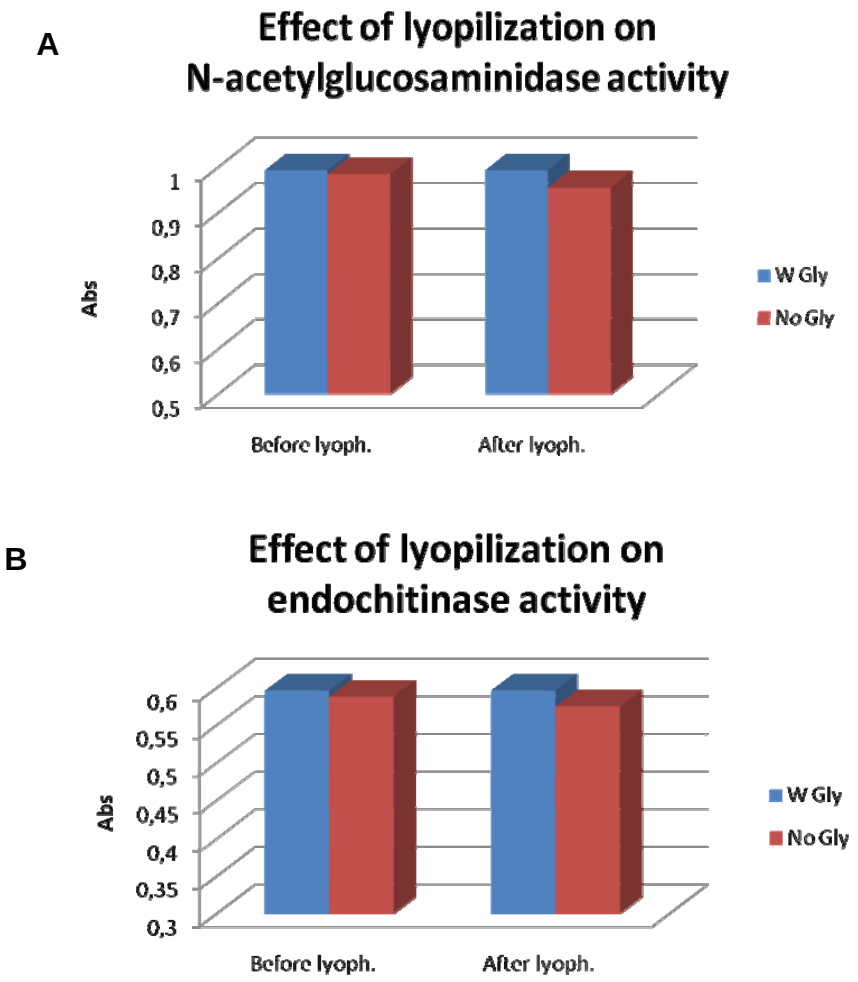
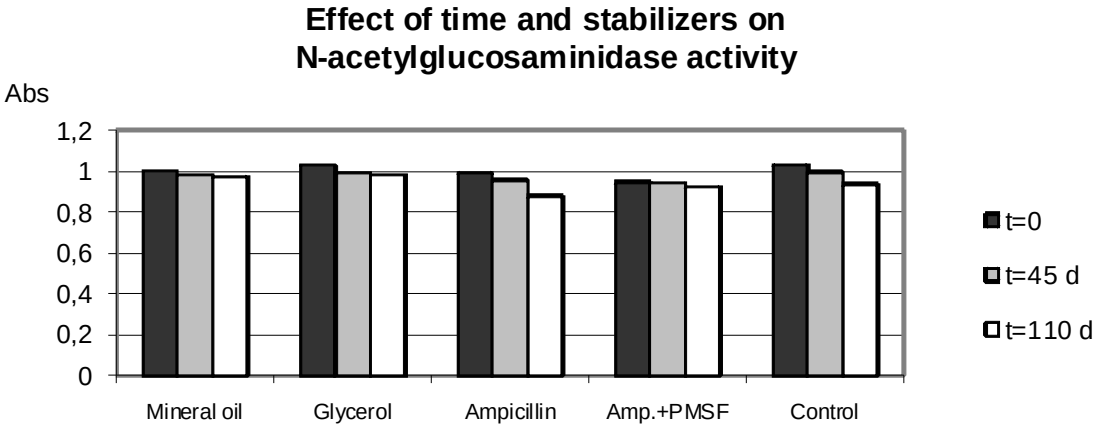


Figure 35. Effect of lyophilization on chitinolytic (N-acetylglucosaminidase =**A** and endochitinase =**B**) activities of culture broths obtained in III fermentations, with (W) or without (No) glycerol added into the sample (final concentration: 20% v/v).

To assess the stability of the novel formulate, the decreases of spore vitality and enzymatic activities were monitored, as well as the effect of different stabilizing compounds (ampicillin, mineral oil, glycerol, PMSF). The results showed no considerable reduction of both spore vitality and chitinolytic activities at 45 and 110 d after fermentation (Fig. 36). Moreover, the different stabilizing treatments did not differ each other significantly.

A



B

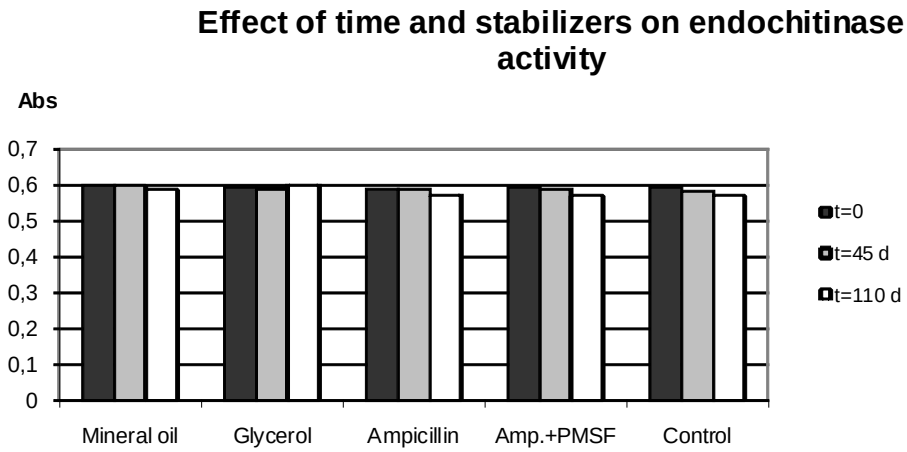


Figure 36. Effect of time and different stabilizers on chitinolytic (N-acetylglucosaminidase =**A** and endochitinase =**B**) activities of culture broths obtained in III fermentation at 0, 45 or 110 days after inoculum.

4. DISCUSSION

The concerns about the worldwide impact of global warming are continuously growing. The 1997 Kyoto Protocol to the UN Framework Convention on Climate Change established an international policy context for the reduction of carbon emissions and increases in carbon sinks in order to address the effect of human activity on the climate system. It is clear that it will be necessary to find means to reduce emission production and prevent increases in (or better, cut back) the present levels to obtain positive effects to improve the current trend in climate change (FAO, 2000; IPCC, 2001; The Royal Society 2001; Watson et al., 2000). The current measures to diminish the warming trend are largely focused on the reduction in the consumption of fossil fuels in industry and transportation, i.e. motorized vehicles. The consequences of climate variation will dramatically alter the natural ecosystem as we presently know it. Other than the obvious changes in climate and weather conditions caused by higher temperatures, notable modifications will also occur in: the geography of the planet, caused by rising sea levels, melting of the glaciers, desertification, etc.; the diversity and distribution of flora and fauna will shift with the migration of tropical species towards more temperate zones; timing and duration of the seasons. Many of the scenarios initially predicted by the U.N. Intergovernmental Panel on Climate Change (IPCC) have been modified in the Fourth Assessment Report because there are indications that these changes are occurring much more rapidly than originally anticipated (http://www.ipcc.ch/).

An analysis of the detrimental effects caused by and caused to agriculture need to be fully investigated in respect to other industrial sectors, since the consequences are of utmost importance on the human and domestic animal food crop production. Agriculture is the major contributor to increasing levels of greenhouse gases such as methane and nitrous oxide into the atmosphere (Fig. 37). The methods of land use management in farming contribute negatively to deforestation, desertification and erosion, as well as the high production of carbon dioxide emissions due to the consumption of fossil fuels in the cultivation practices. Global warming and changes

in precipitation will cause shifts in the crop species presently cultivated. In order for people, particularly those living in developing countries of Africa that live on subsistent farming, to survive this climatic impact and sustain future agriculture production, they will need to adapt and change their current cropping systems to less impacted crops (Llobell et al., 2008.). We tend to forget that even though we are able to improve agricultural productivity utilizing various technologies such as plant genetic improvement, gene transfer biotechnology, development of new agronomic methods and products etc., we are always dependent upon the weather as a determining factor in all aspects of farm production, as well as its influence on soil properties and effects on the native biota. On the other hand, the ability to overcome or diminish the effects of adverse climatic conditions on farm productivity will be determined or aided by the acceptance and application of new technological advancements by the producers (Brown and Funk, 2008)

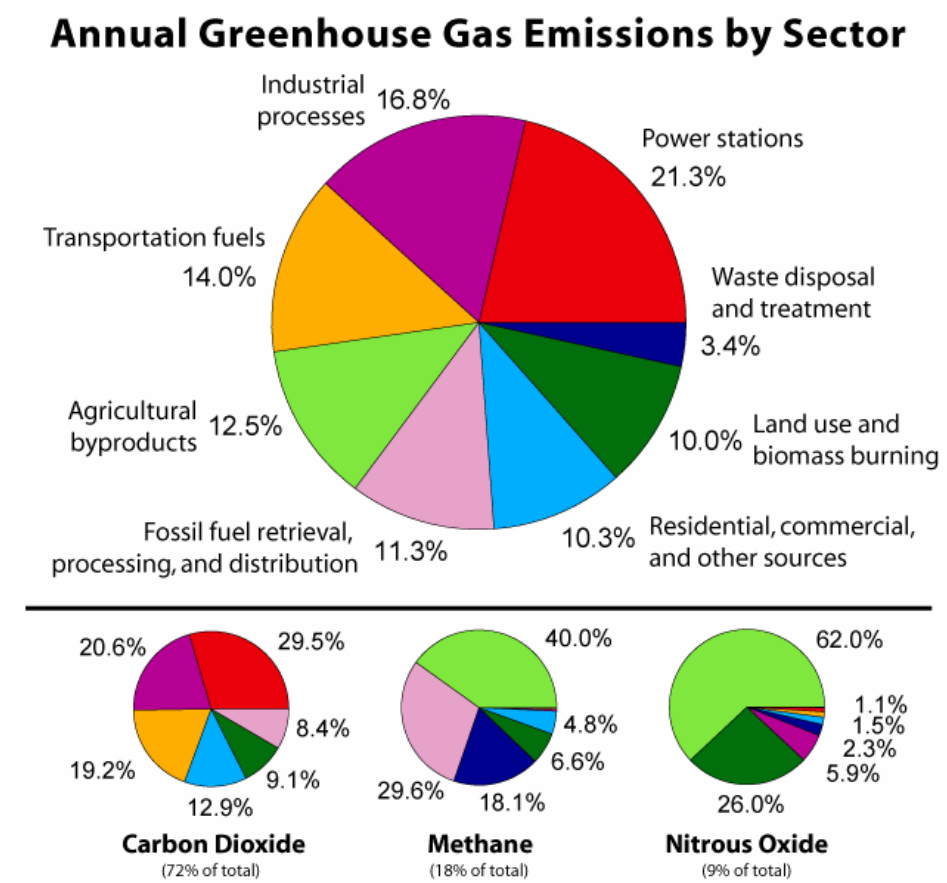


Figure 37. Relative fraction of manmade greenhouse gases coming from different sources, as estimated by the Emission Database for Global Atmospheric Research version 3.2, fast track 2000 project. The upper graph shows the sum over all man-made greenhouse gases, weighted by their global warming potential over the next 100 years. The lower graphs indicate the distribution of the three primary greenhouse gases, sectors are the same color as above. (Source http://en.wikipedia.org/wiki/Image:Greenhouse_Gas_by_Sector.png**).**

The geography of Libya is comprised of a sea coast along the Mediterranean Sea to the north, and to the south it covered by the Libyan and Sahara Deserts. In fact, 90% of the country is desert. The climate is mostly dry, and some regions are known have only erratic rainfalls once in 5-10 years. Temperatures exhibit large fluctuations, are mild similar to the Mediterranean climate of Italy in the northern region, but they can reach maximums around 55° C particularly in desert regions. In

perspective to discussions about the future changes to climate caused by global warming, Libya represents the potential extremes here in present day.

The possible consequences of climatic changes to agriculture can be numerous and vary in their impact. Global warming could produce: an effect on crop productivity in terms of the quantity of the yield and the quality of the harvested products; modifications to current agricultural practices, different agronomic methods of cultivation, water use (irrigation) or selection of plant varieties, and diverse techniques for plant pest control including alternatives to the traditional use of chemical products such as fungicides, herbicides, insecticides and fertilizers; effects to the environment, in particular, resulting from changes in soil properties including aspects of drainage, erosion, availability of cultivatable land; a transformation in the rural economy due to losses and gains of farmland ownership and applications; changes in biodiversity and roles that organisms have in the agro-ecosystem which consequently influence the characteristics that a farmer desires or needs to select for to optimize production, i.e. cultivars that are drought resistance. Advances in the understanding of crop-environment interactions at the molecular, biochemical, physiological, and agronomic level, as well as their relevance to biotechnological crop improvement, have been extensively reviewed. These include discussions of the response mechanisms and potential targets for improving crop response to different abiotic stresses (Lorito et al., 2002), including drought (Barnabás et al., 2008; Chaves and Oliveira, 2004; Parry et al., 2005; Wang et al., 2003), flooding (Agarwal and Grover, 2006), low temperature (Nakashima and Yamaguchi-Shinozaki, 2006; Wang et al., 2003), high temperature (Barnabás et al., 2008; Iba, 2002; Wahid et al., 2007), and low nutrient availability (Hirel et al., 2007). It follows that a number of companies, including Monsanto, Syngenta, and Pioneer-DuPont, have drought-tolerant, heat-tolerant, cold-tolerant, or nitrogen-use efficient germplasm in their research and development programs.

There will be a need to alter the methods of plant disease management as result of the global climate change, because new phytopathogens will arise and spread, “new” crops will become susceptible due to modifications in pest composition, environmental factors, as well as the pathogen distribution patterns, i.e. from tropical

or sub-tropical areas to more temperate regions. This will have an enormous effect on the precedent native populations and affect the capacity of beneficial microorganisms to control disease causing agents, i.e. changes to the composition of natural antagonists in the soil community will reduce soil suppressivity. Moreover, if environmental conditions change, the efficacy of pesticides presently used in agriculture could be reduced due to shifts from the optimal temperatures, humidity etc. required for effective action, particularly important in microbial-based biological formulations. Therefore, it will become increasingly important to select not only resistant crop varieties able to withstand extremes in temperature or in water, but also to single out specific strains of a potential biological control agent that will be effective in the diverse conditions.

In this context *Trichoderma* spp., many which are natural antagonists of numerous plant pathogens, represent a great resource for the development of efficient biological products, since it is ecologically adaptable to a wide range of climatic conditions, able to resist or degrade natural and man-made chemicals and toxins (Harman and Kubicek, 1998; Harman et al., 2004b). The different mechanisms used by *Trichoderma* spp. in the biocontrol process depend not only upon the strain, the fungal host and the crop plant used, but also on the environmental conditions, including temperature, nutrient availability, pH, and iron concentration (Benítez et al., 2004; Harman, 2000; Harman et al., 2004a; Vinale et al., 2008a). Indigenous *Trichoderma* strains already adapted to high temperatures or low rainfall, such as those naturally found in the northern sub-Sahara regions of Africa, could be better adapted to contrast pathogens in the climate conditions that will prevail with the onslaught of global warming. Further, these fungal strains could already have the ability to interact with existing plant varieties that are resistant to growing in extreme environmental conditions and presently cultivated, such as the cultivars utilized in the semi-desert regions of Libya.

This thesis describes the isolation and characterization of novel *Trichoderma* isolates obtained from 9 different areas in Libya. The fungal population of soil samples was analyzed and 3 local *Trichoderma* pure cultures were obtained. These strains, named Lib1, Lib2 and Lib3, were further characterized for their adaptability

to grow at different temperatures (25° C and 30° C) and on different culture media (with high- or low-nutrient content). The *in vitro* tests showed an improved ability of the Libyan isolates to grow on rich medium (PDA) at high temperature, compared to controls, while no differences on minimal medium were observed.

The adaptability of Libyan isolates to high temperatures was also assessed by performing *in vitro* plate confrontation assays. The temperature didn't affect the antagonistic abilities of Libyan isolates; in fact, both at 25° C and 30° C the growth of *Rhizoctonia* was reduced by the Libyan isolates as well as by the biocontrol strain T22, but a lower antagonistic effect was noted against *Alternaria* and *Fusarium* spp. Lib1 was the best antagonist of the three isolates tested against the pathogens, in particularly in the inhibition of *Alternaria* and *Fusarium* growth at both temperatures.

Microscopy slide observations demonstrated that mycoparasitism of *Rhizoctonia* sp. and *Fusarium* sp. by *Trichoderma* local isolates was characterized by the loss of turgidity in the host hyphae. This process is the consequence of *Trichoderma* ability to attach to the host, coil around it and form appressoria-like structures on the fungal host surface (Benítez et al., 2004; Harman et al., 2004a), confirming confocal microscopy observations by Lu et al. (2004). Attachment is probably mediated by the binding of carbohydrates in the *Trichoderma* cell wall to lectins on the target fungus (Inbar et al., 1996). Once in contact, the *Trichoderma* produces several fungitoxic cell-wall-degrading enzymes (Chet et al., 1998), and probably also secretes peptaibol antibiotics (Schirmböck et al., 1994). The combined activities of these compounds result in parasitism of the target fungus and dissolution of the cell walls. At the sites of the appressoria-like structures, holes are produced in the target fungus by lytic digestion, and direct entry of *Trichoderma* hyphae into the hypha of the target fungus occurs. There are at least 20–30 known genes, proteins and other metabolites that are directly involved in this interaction, which is typical of the complex systems that are used by these fungi in their interactions with other organisms (Marra et al., 2006).

A large portion of the Libyan economy is supported by the petroleum industry. During the refining process many pollutants may be released in the environment, air

and groundwater sources. Methyl *tert*-butyl ether (MTBE) is a compound frequently added to gasoline in order to increase octane number. Unfortunately, it frequently contaminates groundwater when gasoline containing MTBE is spilled or leaked in storage and is difficult to clean up due to its high solubility in water. In order to test if the fungal isolates could tolerate, not only high temperatures, but also toxic compounds (i.e. hydrocarbons, benzene, toluene, styrene, and pyrene, etc.), and to determine their ability to degrade and survive such substance, investigations were conducted in presence of MTBE. Contaminated liquid media were inoculated with *Trichoderma* strains and the toxic content, and fungal growth was monitored. Preliminary results demonstrated that the growth of the local isolates Lib1 and Lib2 didn't differ from controls until a concentration of 0.4% MTBE, however, Lib3 showed a reduced biomass weight also at lower doses. Lib1 grew the best in the presence of this toxic compound.

The ability of *Trichoderma* isolates to degrade MTBE in liquid culture was also confirmed by GC-FID demonstrating a significant reduction in the level of this pollutant even 4 days after inoculation. In particular, Lib2 performed the best among the Libyan isolates. These results open another scenario of possible biotechnological applications for the isolated microbes in decontamination of polluted areas, as used alone or in combination with plants (phytoremediation). Various microorganisms are being studied to see if they can remediate various chemicals often present at contaminated industrial sites. Also, scientists are currently looking into genetically engineering certain microorganisms to increase their ability to metabolize specific chemicals, such as hydrocarbons, in contaminated sites. More research needs to be done in order to completely understand the complex microbial processes which make bioremediation possible, especially the bioremediation of metals. Also, researchers are trying to understand why some microorganisms are better at degrading one kind of chemical than another.

Another consideration in this context is the use of *Trichoderma* in the recovery of contaminated/polluted sites (Harman et al., 2004b). Some *Trichoderma* strains are strongly rhizosphere competent which permits them to colonize roots, grow and persist on roots thus providing long-term benefits (Harman, 2000; Harman et al.,

2004a). This capability also permits the fungi to form durable and robust plant associations in a wide variety of soil conditions. The symbiotic nature of the interaction permits the fungus to gain nutrients from the plants, produce molecules that stimulate plant growth and activate plant resistance to biotic and abiotic stresses, plus produce metabolites that are useful to the plants (Lorito et al., 2002). Some of these compounds, including enzymes, may be highly useful in degradation of toxic soil pollutants; this capability is enhanced by the fact that *Trichoderma* spp. possess high intrinsic resistance to some toxic compounds, possibly due to its ability to detoxify certain substances (Ruocco et al., 2008, in press). Further, the rhizosphere competent *Trichoderma* spp. may be able to enhance root growth, thus enhance the capability of hyper-accumulating plants to remove toxic metals (Harman et al., 2004b). Research indicates that the presence of the fungi increases removal of arsenic from soils by hyperaccumulating ferns in the genus *Pteris* (Harman et al. 2004b). Other data demonstrates that root colonization by *T. harzianum* T22 increases the efficiency of nitrogen uptake by corn from fertilizer applications, but that there is a strong specificity to cultivars tested (Harman, 2000). The use of positive responding cultivars of corn could aid in reducing the use of nitrogen fertilizers and consequently reduce nitrate pollution of waterways and decrease spending by producers. Further, some *Trichoderma* spp. such as T22 produce enzymes that degrade cyanide, and they able to accumulate and degrade metallocyanides such as Prussian blue (Harman et al., 2004b). The idea of phyto-bioremediation is to combine rhizosphere competent *Trichoderma* strains with plants that can take up and degrade toxic compounds to provide novel and effective solutions to environmental problems and contamination of surface and groundwater sources.

The analysis of variation of the ribosomal DNA ITS sequences, being one of the most reliable methods for genetic analysis of fungal species, enabled the identification of the fungal isolates to the species level. Homology searches in GenBank database indicated that Lib1 and Lib3 are species of *T. longibrachiatum*, while Lib2 is a *T. harzianum* strain. Even if both species are cosmopolitan, their characteristics are quite different, because *T. harzianum* is frequently utilized in

biological control applications (Harman et al., 2004a; Vinale et al., 2008a), while *T. longibrachiatum* is a common species in the environment and it has been noted as a human pathogen in particular conditions (Chouaki et al., 2002).

In further analysis, the metabolic profile of Libyan isolate Lib1 resulted similar to that observed in a *T. longibrachiatum* strain able to produce Trichogin A IV as major compound (Peggion et al., 2003). This secondary metabolite belongs to the class of peptaibols, which are linear peptides synthesized by fungi, and were isolated initially in 1967 from cultures of *T. viride* (Reusser, 1967). They are produced mainly in soilborne and plant-pathogenic fungi of the genera *Acremonium* (Sharman et al., 1996), *Paecilomyces* (Rossi et al., 1987), *Emericellopsis* (Berg et al., 1996), as well as several species of *Trichoderma*. Those compounds exhibits antimicrobial activity and are characterized by the occurrence of several non-proteinogenic amino acids such as a-aminoisobutyrate (Aib) and isovaline (Iva) (Wada et al., 1995). The N-terminal group of the peptide is usually acetylated, and the C-terminus is an amino alcohol such as phenylalaninol, or in some cases valinol, leucinol, isoleucinol or tryptophanol. The name "peptaibol" is formed from the names of the components: **pept**ide, **Aib** and amino alcoh**ol**. The biological activity of peptaibols is attributed to channel formation in lipid membranes. They present an amphipathic nature, and this property allows many of them to form voltage-dependent ion channels in lipid bilayermembranes (Béven et al., 1998).

Many biocontrol agents, such as fungi, bacteria and viruses, are not only able to control the pathogens that cause plant disease, but are also able to promote plant growth and development (Harman et al., 2004a). In greenhouse and field trials, the ability of *T. harzianum* T22 and *T. atroviride* P1 to improve the growth of lettuce, tomato and pepper plants under field conditions was investigated (Vinale et al., 2004). Crop productivity was increased up to 300%, as determined by comparing the treated plots with the untreated controls and measuring fresh/dry root and above ground biomass weights, height of plants, number of leaves and fruits.

In this thesis, *Trichoderma* Libyan isolates were applied to tomato seeds in order to evaluate their effect on emergence and plant growth. In general, the effects on the plants obtained by the Libyan isolates seed coatings were comparable to those

obtained in the seed treatments with the biocontrol strain T22, whose growth promoting activity has been well documented (Harman, 2000 ; Harman et al., 2004a). The results varied according to the combination of the plant cultivar and the *Trichoderma* strain used. Isolate Lib1 improved the seed germination in cultivars San Marzano and Principe Borghese, but not Corbarino; Lib2 performed best on cv. Corbarino and poorly on the other two cultivars; and Lib3 functioned well on all three cultivars, but best on cv. Corbarino even outperforming the biocontrol isolate T22. When considering the effect of the *Trichoderma* seed treatments on aerial plant growth and development results were different than those noted in germination with the plant cultivar and fungal isolate combinations. Plant height and foliar development on all three tomato cultivars were greatly improved over that of the untreated control by seed treatments with Lib1, whereas Lib2 increased highly the growth in cv. San Marzano and moderately in cv. Corbarino. All three Libyan isolates had none to little effect on the root systems of tomato plants cv. S. Marzano and Pr. Borghese. Only Lib1 produced an extremely positive stimulation of root development in cv. Corbarino, with about a 67% greater increase in growth than the T22 treatment.

The beneficial effect of the isolates from Libya on the plant was also confirmed in assays whereby tomato was treated with *Trichoderma* and then later, the plant leaves were inoculated with the foliar pathogen *B. cinerea*. Interestingly, all of the *Trichoderma* treated tomato cultivars showed inhibition in disease development, suggesting an effect of induced systemic resistance. In general the Libyan isolates performed as well as the two biocontrol strains of P1 and T22, and on the cv. Corbarino, Lib1 and Lib3 actually outperformed T22 in the reduction of disease symptoms.

Present findings that these three *Trichoderma* isolates from Libya (in particular Lib1) are able to improve plant growth and contemporarily withstand adverse environmental conditions, such as high temperature and pollutants, plus reduce the development of disease may represent novel applications for biocontrol in Libya. Moreover, the Libyan isolated fungi, especially Lib1 and Lib3, significantly reduced *B. cinerea* infection on tomato plants, as well as and in some cases better than known

biocontrol agents. The induction of plant resistance mechanisms mediated by the presence of *Trichoderma* antagonistic fungi has been a well documented aspect (De Meyer et al., 1998; Hanson and Howell, 2004; Harman et al., 2004a; Yedidia et al., 2003), and appears to be similar to the effect that is elicited by rhizobacteria, which are able to enhance the plant defence system without stimulating the production of pathogenesis-related (PR) proteins that are normally activated during pathogen attack (Harman et al., 2004a; Stacey and Keen, 1999; Van Loon et al., 1998). During the interaction of *Trichoderma* with the plant, different classes of metabolites may act as elicitors or resistance inducers (Harman et al., 2004a; Woo and Lorito, 2007; Woo et al., 2006). These molecules could include: serine proteinases, xylanases, endopolygalacturonidase, chitin deacetylase, chitinases and other enzymes; peptides and proteins that induce terpenoid phytoalexin biosynthesis and peroxidases; various *Trichoderma*-specific effector proteins such as Sm1 or swollenin; and/or homologues of effector proteins found in pathogens, i.e. AvrE, Nip1, and AVR-PTA (Djonovic et al., 2007, Marra et al., 2006, Shoresh and Harman, 2008). Aequorin-expressing soyabean cell suspension cultures treated with a mix of *Trichoderma* metabolites found in the culture filtrates, produced by the antagonist alone or grown in the presence of the *B. cinerea,* indicated a differential perception by the cells to the fungal compounds and a consequent activation of both Ca^{2+}-mediated signalling and cell responses typical to those launched in plant defence to pathogen attack such as: the accumulation reactive oxygen species (ROS), reduced cell viability, programmed cell death (PCD) in contrast to necrosis (observed by the induction of caspase 3-like activity, chromatin condensation and other morphological cell alterations; Navazio et al., 2007). Moreover, the production of endochitinase (*ech*42 has been found to play a key role in mycoparasitism of *T. atroviride* P1; Woo et al., 1999) was found to determine a reaction by the plant and affect the plant response to *Trichoderma*, as found in comparative testing with wild type or the disrupted mutant strains culture filtrates on the soyabean tissues. In absence of the endochitinase production, the Ca^{2+}-signal produced in the cell cultures almost completely disappeared within 10 minutes after treatment with the metabolite mixture of the mutant strain. The increasing importance of the ability of some *Trichoderma* strains

to cause ISR is becoming more and more apparent as a mechanism used by these fungi in the biocontrol of plant pathogens (particularly fungi) instead of the exclusive action of direct mycoparasitism as previously assumed (Howell, 2003; Harman, 2000; Harman et al., 2004a; Woo and Lorito, 2006; Woo et al. 2006). The concept of "vaccinating" the plant to future pathogen attack, in order to stimulate its defence system, by using extracts from antagonistic fungi is a new potential strategy for biological control.

The compounds produced by the BCA in the fungal culture filtrates contained various secondary metabolites, like peptaibols, which may also act as elicitors of plant defence mechanisms against pathogens. In fact, the application of peptaibols were found to activate a defence response in tobacco plants (Benítez et al., 2004; Viterbo et al., 2007). Similarly, the peptaibol isolated and identified from the Lib1 culture could represent a molecular factor possibly involved in the induction of defence mechanisms in *Trichoderma*-treated plants.

Many secondary metabolites produced by *Trichoderma* have antibiotic activity and have been demonstrated to play a role in biological control against various phytopathogens, however, their effect on the plant in the BCA-plant interaction are not known. Recently, Vinale et al. (2008b) have found that some *Trichoderma* compounds, such as 6-pentyl-α-pyrone (6PP) acted as effectors on plant growth, possibly by acting in an auxin-like manner or by stimulating the hormone production in the plant, thus enhancing growth of the root system and plant size. Further, when some fungal BCA secondary metabolites were applied to tomato or canola plants, they stimulated ISR to subsequent treatments with the foliar pathogens *B. cinerea* or *Leptosphaeria maculans,* respectively, and activated the production of several PR-proteins associated with plant defense. Other studies have also indicated that *Trichoderma* effectors may be used effectively for disease control, as foliar spray applications or in post-harvest treatments for the conservation of fruits and vegetables in long term storage (Vinale et al., 2008- unpublished).

The important discovery of factors secreted by *Trichoderma* that are involved in the biological control of phytopathogens and responsible for producing other beneficial effects to the plant, a new liquid formulation can be proposed for

applications in agriculture that is comprised both of the live BCA organism and its naturally produced substances. The development of a microbial pesticide requires several steps: selection of a potential BCA strain, identification of ideal characteristics, screening of efficacy by means of *in vitro* or *in vivo* bioassays in controlled conditions, then applications to actual field conditions with determinate crop varieties in diverse geographical regions. Moreover, once a putative BCA has passed these selective tests for efficacy, the aspects of commercial production and delivery need to be confronted. It is important to produce the biocontrol agent at an industrial scale (in solid state or liquid fermentation), determine the culture conditions necessary to produce the desired biocontrol characteristics, determine the conditions essential for preservation and conservation, find a formulation for commercialization and application of the final product (Agosin and Aguilera, 1998; Jin et al., 1992; Montesinos, 2003). Independent of the method used for fermentation, the overall aim is to achieve the highest yield possible with the lowest economic cost of production (Agosin and Aguilera, 1998; Jin et al., 1992).

The objective of this thesis is to develop a novel bio-formulation containing a synergistic combination of the living fungus, both in forms of mycelia and conidia, and of a powerful mixture of *Trichoderma* "effectors" capable of stimulating plant defense response and growth, as well as directly controlling pathogenic microbes. The proposed product will be prepared by liquid fermentation in selected low-cost medium, demonstrate sufficient shelf-life, retain the multiple beneficial effects of the fungus (antimicrobial, plant growth promotion, ISR inducing activity) and be applied as a foliar spray or soil drench in most agricultural applications.

On the basis of the consistently good results obtained with the *in vitro*, *in vivo* and *in planta* testing with the *Trichoderma* isolate Lib1 from Libya, this isolate was selected for investigations on the growth parameters in liquid fermentation necessary to obtain massive production of biomass and high enzymatic activity. Testing in small scale cultures determined that the most efficient condition to induce overall enzyme production was found by the use of a minimal salt medium SM that was amended with lyophilized biomass of the commonly available edible champignon mushroom *A. bisporus* (0.5% w/v) + wheat fiber (0.3% w/v). In order to obtain high

levels of fungal biomass, particularly the production of spores which are stable dormancy structures that are more stable in retaining the viability of the fungus, different conditions of agitation and aeration were tested, as well as the time of application of these conditions during the fermentation. Optimal spore production was found when the cultures were initially grown with orbital shaking at 100 rpm and aeration at 0.5 vvm, then after 72 h of fermentation, these parameters were changed with an increase in orbital shaking up to 200 rpm and aeration reduced to 0.3 vvm. This beginning stage permitted the fungal biomass to develop well by promoting vegetative growth, then the second phase created an environment that "stressed" the fungus thus inducing the production of spores for protection of the fungus, structures important for the conservation of the fungus in adverse conditions. Thus, the selected growth parameters permitted the production of a culture with a good combination of characteristics for producing a formulation with a high concentration of spores and significant levels of enzyme activities. Moreover, with considerations to reducing the production costs, energy and time that fermenter needs to be occupied, the fermentation process could be stopped at 120 h and not the 194 h after inoculation, without causing any significant changes in the final product.

More importantly, the novel liquid formulation based on the Lib1 *Trichoderma* isolate maintained their good biological activity in the large-scale fermentation. The obtained culture was able to significantly reduce the disease symptoms caused by *B. cinerea* on lettuce and tomato leaves in *in vivo* testing. The conservation of the antagonistic activity against the target pest or pathogen is a critical step to overcome in the commercialization process because not all selected putative biocontrol microorganisms are able to pass all of the requirements imposed by industrial production. In order to confirm the efficacy of isolate Lib1, the present bioassays which were performed in a small-scale controlled-environment on a single target pathogen, will need to be further expanded with testing on different pathogens and plant species/varieties, as well as in the real field conditions to assess the potential of the bio-formulation to be applied in the agricultural environment.

The final important factors to consider in a commercial bio-formulation are product stability, the capacity to produce consistent results by preserving the

characteristics producing the biological effects; the storability of the material, the ability to be conserved in unspecialized conditions similar to those of chemical pesticides; and a reasonable shelf-life or time that the product can be stored and used without compromising the efficacy (Agosin and Aguilera, 1998; Agosin et al., 1997; Jin et al., 1991; 1992; 1996; Jones, 1993; Powell and Jutsum, 1993). When a formulation contains the living microorganism component, the treatment must consist of stabilizing the viability of the BCA. For liquid formulations this can be achieved by maintaining the product in refrigeration (<10° C) or by freezing in the presence of cryoprotectant substances. However, conservation of a commercial product in these conditions is not economic for maintaining low temperatures or efficient because the liquid is both bulky and heavy, plus it is difficult to sustain these conditions in storage and transportation. In comparison, it is preferable to obtain formulations that contain a dehydrated product, stored as a powder, granule, talc, etc. This work demonstrated that lyophilization did not reduce chitinolytic activity and spore vitality when the fermented cultures were treated with compounds that protect the osmotic integrity of the living material such as glycerol. Generally, lyophilization is the method that best maintains viability, but its cost is very high. At the industrial level and in order to obtain a low-cost product, the methods preferred are spray- or fluidized bed- drying. Many products are obtained by spray-drying, but this method produces a high loss of viability in some microorganisms (observed also in this formulation), due to the thermal treatment. Moreover, different compounds (ampicillin, mineral oil, glycerol, PMSF) were added to determine if they aided in to maintaining the stability of the formulation. The enzyme activity in samples assayed over time were not effected neither positively nor negatively by the addition of the compounds in comparison to the untreated control. Obviously, it is very important to maintain good sanitary conditions throughout the fermentation process and during packaging in order to avoid possible contamination that will compromise the product during storage.

In spite of the relatively abundant number of patents filed for microbial pesticides, the number of commercial applications has not been as dramatic as expected (Montesinos, 2003). In Europe, the limiting factor for registration, apart

from the cost, is undoubtedly the slow process of decision-taking. As an example, the first application for patenting a biopesticide, *Paecilomyces fumosoroseus*, was submitted to the European Union in 1994 and approved only in 2001. In most cases, excessive specificity is a problem difficult to solve because it is intrinsic to the biological control system. In fact, success depends on three living systems: the pathogen or pest, the BCA and the host plant. Biosafety and environmental concerns are also major limiting factors for microbial pesticide prospects. Furthermore, the registration procedure to approve a biopesticide formulation on the market has not been altered to consider the biological aspects of the product, criteria which are different than those considered for the testing of chemical based products.

Consumer concerns related to the use of living microbes in association to agricultural products and the limited information available these microbes cannot be overlooked. Certainly, emerging food-borne diseases and the threat of bioterrorism does not help to create a socially receptive environment for microbial pesticides. The future of microbial pesticides is not only in developing new active ingredients derived from microorganisms beneficial to plants, but in producing self-defending plants (so-called plant-incorporated pesticides) to potential pathogen attack by transforming agronomically high-value crop plants with genes from BCAs (Lorito and Scala, 1999; Lorito et al., 1998), or by using microbial metabolites derived from the living organisms or other natural substances to "vaccinate" the plants by improving disease resistance or to promote plant growth and vigor (Vinale et al., 2008b; Woo and Lorito 2006). The identification and the application of various derivatives from a fungal antagonist such as *Trichoderma* provides novel alternatives to the classical applications of the live microorganism in the biological control field. The main advantages are also practical – no more concerns for the production, viability or conservation of a commercial product containing a live organism, but also more safe, thus addressing public concerns for pesticide safety. Further, it is easier to industrially produce the fungal substances in greater quantities, to maintain higher and consistent control of quality and to practically apply such products in the greenhouse, field or storage facilities.

Global warming trends predicted in the 2007 Intergovernmental Panel on Climatic Change (IPCC) report are likely to change the temporal and geographical distribution of infectious diseases, and thus impact on the evolution of pathogens and hence diseases associated with crop production. It will be necessary, or better unavoidable, to update the set of biotechnological products based on microbial agents which are available today on the agricultural market. New biopesticides and biofertilizers exhibiting higher adaptability to the climate changes most likely to occur need to be formulated. This thesis study on the production of a novel liquid formulation based on *Trichoderma* isolates, selected for their beneficial effects to the plant, originating from the extreme meteorological environments of Libya, which may represent the climatic conditions caused global warming, serves as a useful initiative for confronting the difficulties and resolving the problems in light of the agricultural perspectives of the future.

5. REFERENCES

Abate T, van Huis A, Ampofo JKO (2000). Pest management strategies in traditional agriculture: An African perspective. Annu. Rev. Entomol., 45:631–659.

Agarwal S, Grover A (2006). Molecular biology, biotechnology and genomics of flooding-associated low O_2 stress response in plants. Crit. Rev. Plant Sci., 25: 1–21.

Agosin E, Volpe D, Munoz G, San Martin R, Crawford A (1997). Effect of culture conditions on spore shelf life of the biocontrol agent *Trichoderma harzianum*. World J. Microbiol. Biotechnol., 13: 225-232.

Agosin E, Aguilera JM (1998). Industrial production of active propagules of *Trichoderma* for agricultural use. In: *Trichoderma* and *Gliocladium*. Volume 2, Enzymes, Biological control and commercial applications, G.E. Harman and C.P. Kubicek eds., Taylor & Francis Ltd., London, UK, pp. 205-227.

Ait-Lahsen H, Soler A, Rey M, de la Cruz J, Monte E. and Llobell A (2001). An antifungal exo-α-1,3-glucanase (AGN 13.1) from the biocontrol fungus *Trichoderma harzianum*. Appl. Environ Microbiol., 67: 5833-5839.

Altomare C, Norvell WA, Björkman T, Harman GE (1999). Solubilization of phosphates and micronutrients by the plant-growth-promoting and biocontrol fungus *Trichoderma harzianum* Rifai 1295-22. Appl. Environ. Microbiol., 65, 2926–2933.

Auvin-Guette C, Rebuffat S, Prigent Y, Bodo B (1992). Trichogin A IV, an 1 1-Residue Lipopeptaibol from *Trichoderma longibrachiatum*. J. Am. Chem. Soc., 114, 2170-2174.

Barnabás B, Jäger K, Fehér A (2008). The effect of drought and heat stress on reproductive processes in cereals. Plant Cell Environ., 31: 11–38.

Benítez T, Delgado-Jarana J, Rincón AM, Rey M, Limón MC (1998). Biofungicides: *Trichoderma* as a biocontrol agent against phytopathogenic fungi. In: Pandalai SG (eds) Recent research developments in microbiology, vol. 2. Research Signpost, Trivandrum, pp. 129-150.

Benítez T, Rincón AM, Limón MC, Codón AC (2004). Biocontrol mechanisms of *Trichoderma* strains. Int. Microbiol., 7: 249-260.

Berg A, Ritzau M, Ihn W, Fleck W., Heinze S, Gräfe U (1996). Isolation and structure of bergofungin, a new antifungal peptaibol from *Emericellopsis donezkii* HKI 0059. J. Antibiot., 49 (8), 817-820.

Béven L, Duval D, Rebuffat S, Riddell FG, Bodo B, Wróblewski H (1998). Membrane permeabilisation and antimycoplasmic activity of the 18-residue peptaibols, trichorzins PA. Biochem. Biophys. Acta, 1372 (1), 78-90.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254.

Brown ME, Funk CC (2008). Food Security Under Climate Change. Science, 319 (5863), 580.

Castle A, Speranzini D, Rghei N, Alm G, Rinker D, Bissett J (1998). Morphological and molecular identification of *Trichoderma* isolates on North American mushroom farms. Appl Environ Microbiol., 64(1): 133-137.

Chacón MR, Rodríguez-Galán O, Benítez T, Sousa S, Rey M, Llobell A, Delgado-Jarana J (2007). Microscopic and transcriptome analyses of early colonization of tomato roots by *Trichoderma harzianum*. Intern. Microbiol., 10: 19–27.

Chaves MM, Oliveira MM (2004). Mechanisms underlying plant resilience to water deficits: prospects for water-saving agriculture. J. Exp. Bot., 55: 2365–2384.

Chen X, Romaine CP, Tan Q, Schlagnhaufer B, Ospina-Giraldo MD, Royse DJ, Huff DR (1999). PCR-based genotyping of epidemic and preepidemic *Trichoderma* isolates associated with green mold of *Agaricus bisporus*. Appl Environ Microbiol., 65(6): 2674-2678.

Chet I (1987). *Trichoderma*-Application, mode of action, and potential as a biocontrol agent of soil-born pathogenetic fungi. In: Innovative approaches to plant disease control (Chet I. Ed.) John Wiley and Sons, pp. 137-160.

Chet I, Benhamou N, Haran S (1998). In: *Trichoderma* and *Gliocladium* Vol. 2 (eds Kubicek CP and Harman GE) pp. 153–172, Taylor and Francis, London, UK.

Chouaki T, Lavarde V, Lachaud L, Raccurt CP, Hennequin C (2002). Invasive infections due to *Trichoderma* species: report of 2 cases, findings of in vitro susceptibility testing, and review of the literature. Clin. Infect. Dis., 35(11): 1360-1367.

de Hoog GS, Guarro J, Gené J, Figueras MJ (2000). Atlas of clinical fungi, 2nd ed. Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

de la Cruz J, Hidalgo-Gallego A, Lora JM, Benítez T, Pintor-Toro JA and Llobell A (1992). Isolation and characterization of three chitinases from *Trichoderma harzianum*. Eur J. Biochem, 206: 859-867.

de la Cruz J, Pintor-Toro JA, Benítez T and Llobell A (1995a). Purification and characterization of an endo-β-1,6-Glucanase from *Trichoderma harzianum* that is related to its mycoparasitism. J. Bacteriol, 177: 1864-1871.

de la Cruz J, Pintor-Toro JA, Benítez T, Llobell A and Romero L (1995b). A novel endo-β-1,3-glucanase, BGN 13.1, involved in the mycoparasitism of *Trichoderma harzianum*. J. Bacteriol, 177:6937-6945.

De Meyer G, Bigirimana J, Elad Y, Hofte M**.** (1998). Induced systemic resistance in *Trichoderma harzianum* T39 biocontrol of *Botrytis cinerea*. Eur. J. Plant Pathol., 104: 279–286.

Dennis C, Webster J (1971) Antagonistic properties of species-groups of *Trichoderma*. III. Hyphal interaction. Trans. Brit. Mycol. Soc. 57, 363-369.

Di Pietro A, Lorito M, Hayes CK, Broadway RM, Harman GE (1993). Endochitinase from *Gliocladium virens*: isolation, characterization and synergistic antifungal activity in combination with gliotoxin. Phytopathol., 83: 308-313.

Djonovic S, Vargas WA, Kolomiets MV, Horndeski M, Wiest A, Kenerley CM (2007). A proteinaceous elicitor Sm1 from the beneficial fungus *Trichoderma virens* is required for induced systemic resistance in maize. Plant Physiol., 145: 875-889.

Doekes G, Larsen P, Sigsgaard T, Baelum J (2004). IgE sensitization to bacterial and fungal biopesticides in a cohort of Danish greenhouse workers: the BIOGART study. Am. J. Ind. Med., 46, 404-407.

Douwes J, Thorne P, Pearce N, Heederik D (2003). Bioaerosols health effects and exposure assessment: progress and prospects. Ann. Occup. Hyg., 47, 187-200.

Eapen S, Singh S, D'Souza SF (2007). Advances in development of transgenic plants for remediation of xenobiotic pollutants. Biotech. Adv., 25: 442–451.

Felse AP, Panda T (1999). Submerged culture production by *Trichoderma harzianum* in stirred tank bioreactors-the influence of agitator speed. Biochem. Eng. J., 4: 115-120.

FAO (1997). Production Yearbook 1997, Vol. 51. Rome. pp 239.

FAO (2000). Carbon sequestration options under the clean development mechanism to address land degradation. World Soil Resources Report no. 92. UN Food and Agricultural Organization and International Fund for Agricultural Development, Rome.

Fogliano V, Ballio A, Gallo M, Woo S, Scala F, Lorito M (2002). *Pseudomonas* lipodepsipeptides and fungal cell wall-degrading enzymes act synergistically in biological control. Mol Plant Microbe Interact., 15(4): 323-333.

García I, Lora JM, de la Cruz J, Benítez T, Llobell A, Pintor-Toro JA (1994). Cloning and characterization of a chitinase (chit42) cDNA from the mycoparasitic fungus *Trichoderma harzianum*. Curr Genet., 27(1): 83-89.

Gautheret A, Dromer F, Bourhis JH, Andremont A (1995). *Trichoderma pseudokoningii* as a cause of fatal infection in a bone marrow transplant recipient. Clin. Infect. Dis., 20: 1063–1064.

GCTE-LUCC (1998). The Earth's Changing Land. GCTE-LUCC Open Science Conference on Global Change. Barcelona: Inst. Cartogr. Catalunya.

Ghisalberti EL, Narbey MJ, Dewan MM, Sivasithamparam K (1990). Variability among strains of *Trichoderma harzianum* in their ability to reduce take-all and to produce pyrones. Plant Soil 121, 287–291.

Gruber F, Visser J, Kubicek CP, de Graaff LH (1990). The development of a heterologous transformation system for the cellulolytic fungus *Trichoderma reesei* based on a pyrG-negative mutant strain. Curr. Genet., 18(1): 71-76.

Guiserix J, Ramdane M, Finielz P, Michault A, Rajaonarivelo P (1996). *Trichoderma harzianum* peritonitis in peritoneal dialysis. Nephron 74: 473–474.

Ham KS, Wu SC, Darvill AG, and Albersheim P (1997). Fungal pathogens secrete an inhibitor protein that distinguishes isoforms of plant pathogenesis-related endo-β-1,3-glucanases. Plant J., 11:169-179.

Hanson LE, Howell CR (2004). Elicitors of Plant Defense Responses from Biocontrol Strains of *Trichoderma viren*. Phytopathol., 94(2): 171-176.

Harman GE (2000). Myths and dogmas of biocontrol: changes in perceptions derived from research on *Trichoderma harzianum* T-22. Plant Dis., 84: 377–393.

Harman GE, Bjorkman T (1998). Potential and existing uses of *Trichoderma* and *Gliocladium* for plant disease control and plant growth enhancement. In: Kubicek CP and Harman GE (eds.), *Trichoderma* and *Gliocladium* Vol. II. London: Taylor and Francis, London, pp. 1-393.

Harman GE, Donzelli BGG (2001). In: Enhancing Biocontrol Agents and Handling Risks (eds Vurro, M. et al.) pp. 114–125, IOS, Amsterdam, The Netherlands.

Harman GE, Kubicek CP (1998). *Trichoderma* and *Gliocladium*. Vol. II. Taylor and Francis, London.

Harman GE, Hayes CK, Lorito M, Broadway RM, Di Pietro A, Peterbauer CK, Tronsmo A (1993). Chitinolytic enzymes of *Trichoderma harzianum*: purification of chitobiosidase and endochitinase. Phytopathol., 83: 313–318.

Harman GE, Howell CR, Viterbo A, Chet I, Lorito M (2004a). *Trichoderma* species - Opportunistic, avirulent plant symbionts. Nat. Rev. Microbiol., 2: 43-56.

Harman GE, Lorito M, Lynch JM (2004b). Uses of *Trichoderma* spp. to alleviate or remediate soil and water pollution. Adv. Appl. Microbiol., 56: 313-330.

Hermosa MR, Grondona I, Monte E (1999). Isolation of *Trichoderma harzianum* Th2 from commercial mushroom compost in Spain. Plant Dis. 83: 591.

Hermosa MR, Grondona I, Iturriaga EA, Diaz-Minguez JM, Castro C, Monte E, Garcia-Acha I (2000). Molecular characterization and identification of biocontrol isolates of *Trichoderma* spp. Appl Environ Microbiol., 66(5): 1890-1898.

Hirel B, Le Gouis J, Ney B, Gallais A (2007). The challenge of improving nitrogen use efficiency in crop plants: towards a more central role for genetic variability and quantitative genetics within integrated approaches. J. Exp. Bot., 58: 2369–2387.

Howell CR (2003). Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts. Plant Dis., 87, 4–10.

Howell CR, Hanson LE, Stipanovic RD, Puckhaber LS (2000). Induction of terpenoid synthesis in cotton roots and control of *Rhizoctonia solani* by seed treatment with *Trichoderma virens*. Phytopathol., 90: 248–252.

Iba K (2002). Acclimative response to temperature stress in higher plants: approaches of gene engineering for temperature tolerance. Annu. Rev. Plant Biol., 53: 225–45.

Inbar J, Abramsky M, Coen D, and Chet I (1994). Plant growth enhancement and disease control by *Trichoderma harzianum* in vegetable seedlings grown under commercial conditions. Eur. J. Plant Pathol., 100: 337-346.

Inbar J, Menendez A, Chet I (1996). Hyphal interaction between *Trichoderma harzianum* and *Sclerotinia sclerotiorum* and its role in biological control. Soil Biol. Biochem., 28: 757–763.

IPCC (2001). Climate change 2001: impacts, adaptation and vulnerability. Third Assessment Report. Intergovernmental Panel on Climate Change, IPCC Secretariat, Geneva, Switzerland. (Available from http://www.ipcc.ch/).

IPCC (2007). Climate change 2007: the physical science basis. In: S Solomon, D Qin,MManning, Z Chen,MMarquis, KB Averyt,MTignor, HL Miller, eds, Contribution of Working Group I to the Fourth Annual Assessment Report of the

Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, UK, pp 996.

Jacobs F, Byl B, Bourgeois N, Coremans-Pelseneer J, Florquin S, Depre G, Van de Stadt J, Adler M, Gelin M, Thys JP (1992). *Trichoderma viride* infection in a liver transplant recipient. Mycoses, 35: 301–303.

Jensen GB, Larsen P, Jacobsen BL, Madsen B, Wilcks A, Smidt L, Andrup L (2002). Isolation and characterization of *Bacillus cereus*-like bacteria from faecal samples from greenhouse workers who are using *Bacillus thuringiensis*-based insecticides. Int. Arch. Occup. Environ. Health, 75(3), 191-196.

Jin X, Harman GE, Taylor AG (1991). Conidial biomass and desiccation tolerance of *Trichoderma harzianum* produced at different medium water potentials. Biol. Control, 7: 267-243.

Jin X, Hayes CK, Harman GE (1992). Principles in the development of biological control systems employing *Trichoderma* species against soil-borne plant pathogenic fungi. In: Frontiers in Industrial Mycology, G.F. Leatham (ed.), Chapman and Hall, New York, pp. 174-195.

Jin X, Harman GE, Taylor AG (1996). Development of media and automated liquid fermentation methods to produce desiccation-tolerant propagules of *Trichoderma harzianum*. Biol. Control, 1: 267-274.

Jones DG (1993). Exploitation of microorganisms. Chapman Hall, London, UK.

Jsten P, Paul GC, Thomas CR (1996). Biotechnol. Bioeng. 52:972.

Keszler A, Forgács E, Kótai L, Vizcaíno JA, Monte E, García-Acha I (2000). Separation and identification of volatile components in the fermentation broth of *Trichoderma atroviride* by solid-phase extraction and gas chromatography-mass spectrometry. J Chromatogr. Sci., 38(10): 421-424.

Komon-Zelazowska M, Bissett J, Zafari D, Hatvani L, Manczinger L, Woo S, Lorito M, Kredics L, Kubicek CP, Druzhinina IS (2007). Genetically closely related but phenotypically divergent *Trichoderma* species cause green mold disease in oyster mushroom farms worldwide. Appl. Environ. Microbiol., 73(22):7415-26.

Kubicek CP, Harman GE (1998). *Trichoderma* and *Gliocladium*. Vol. II. Taylor and Francis, London.

Kubicek CP, Komon-Zelazowska M, Druzhinina IS (2008). Fungal genus *Hypocrea*/*Trichoderma*: from barcodes to biodiversity. J. Zhejiang Univ. Sci. B. 9(10): 753-763.

Kuhls K, Lieckfeldt E, Samuels GJ, Meyer W, Kubicek CP, Borner T (1997). Revision of *Trichoderma* sect. Longibrachiatum including related teleomorphs based on analysis of ribosomal DNA internal transcribed spacer sequences. Mycologia, 89: 442–460.

Kumar S, Tamura K, Nei M (2004). MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinform., 5: 150-163.

Lanzuise S, Ruocco M, Scala V, Woo SL, Scala F, Vinale F, Del Sorbo G and Lorito M (2002). Cloning of ABC transporter-encoding genes in *Trichoderma* spp. to determine their involvement in biocontrol. J. Plant Pathol., 84: 184.

Largentau-Mamoun ML, Mata G, Savoie JM (2002). Green mold disease: adaptation of *Trichoderma harzianum* Th2 to mushroom compost. In "Mushroom Biology and Mushroom Products" (Sanchez et al. eds), pp. 179-187, UAEM, Cuernavaca, Mexico.

Limón MC, Lora JM, García I, de la Cruz J, Llobell A, Benítez T, Pintor-Toro JA (1995). Primary structure and expression pattern of the 33-kDa chitinase gene from the mycoparasitic fungus *Trichoderma harzianum*. Curr Genet., 28(5): 478-483.

Linko M, Haikara A, Ritala A, and Penttilla M (1998). Recent advances in the malting and brewing industry. J. Biotech., 65:85-98.

Lobell DB, Burke MB, Tebaldi C, Mastrandrea MD, Falcon WP, Naylor RL. 2008. Prioritizing climate change adaptation needs for food security in 2030. Science, 319 (5863): 607–10.

Lora JM, De la Cruz J, Llobell A, Benítez T, Pintor-Toro JA (1995). Molecular characterization and heterologous expression of an endo-beta-1,6-glucanase gene

from the mycoparasitic fungus *Trichoderma harzianum*. Mol Gen Genet., 247(5): 639-645.

Lorito M (1998). Chitinolitic enzymes and their genes. In *Trichoderma* and *Gliocladium.* Harman GE and Kubicek CP,Vol.II: 73-99, Taylor and Francis, London.

Lorito M, Scala F (1999). Microbial genes expressed in transgenic plants to improve disease resistance. J. Plant Pathol., 81: 73-88.

Lorito M, Harman GE, Hayes CK, Broadway RM, Tronsmo A, Woo SL, Di Pietro A (1993). Chitinolytic enzymes produced by *Trichoderma harzianum*: antifungal activity of purified endochitinase and chitobiosidase. Phytopathol. 83, 302–307.

Lorito M, Hayes CK, Di Pietro A, Woo SL, Harman GE (1994a). Purification, characterization and synergistic activity of a glucan 1,3-bglucosidase and an N-acetyl-b-glucosaminidase from *Trichoderma harzianum*. Phytopathol. 84, 398–405.

Lorito M, Peterbauer C, Hayes CK, Harman GE (1994b). Synergistic interaction between fungal cell wall degrading enzymes and different antifungal compounds enhances inhibition of spore germination. Microbiol. 140, 623–629.

Lorito M, Farkas V, Rebuffat S, Bodo B, Kubicek CP (1996a). Cell wall synthesis is a major target of mycoparasitic antagonism by *Trichoderma harzianum*. J. Bacteriol. 178: 6382–6385.

Lorito M, Woo SL, D'Ambrosio M, Harman GE, Hayes CK, Kubicek CP, Scala F (1996b). Synergistic interaction between cell wall degrading enzymes and membrane affecting compounds. Mol. Plant Microbe Int. 9: 206–213.

Lorito M, Woo SL, Garcia Fernandez I, Colucci G, Harman GE, Pintor-Toro JA, Filippone E, Muccifora S, Lawrence CB, Zoina A, Tuzun, S and Scala F (1998). Genes from mycoparasitic fungi as a novel source for improving plant resistance to fungal pathogens. PNAS USA, 95: 7860-7865.

Lorito M, Del Sorbo G, Scala F (2002). Molecular Approaches for increasing plant resistance to biotic and abiotic stresses. In: Breeding for Ornamentals: Classical

and Molecular Approaches, A. Vainstein ed., Kluwer Academic Publishers, the Netherlands, pp. 197-218.

Lorito M, Woo S, Iaccarino M, Scala F (2006). Microrganismi antagonisti. In: Microrganismi benefici per le piante, M. Iaccarino (ed). Idelson-Gnocchi s.r.l., Napoli, Italia, pp. 146-175.

Lu Z, Tombolini R, Woo SL, Zeilinger S, Lorito M, Jansson JK (2004). *In vivo* study of *Trichoderma*-pathogen-plant interactions, using constitutive and inducible green fluorescent protein reporter systems. Appl. Envir. Microbiol.,: 3073-3081.

Marra R., Ambrosino P, Carbone V, Vinale F, Woo SL, Ruocco M, Ciliento R, Lanzuise S, Ferraioli S, Soriente I, Gigante S, Turrà D, Fogliano V, Scala F, Lorito M (2006). Study of the three-way interaction between *Trichoderma atroviride*, plant and fungal pathogens by using a proteomic approach. Curr. Genet., 50: 307-321.

Meyer HW, Jensen KA, Nielsen KF, Kildesø J, Norn S, Permin H, Poulsen LK, Malling HJ, Gravesen S, Gyntelberg F (2005). Double blind placebo controlled exposure to molds: exposure system and clinical results. Indoor Air, 15(Suppl 10), 73-80.

Monte E (2001). Understanding *Trichoderma*: Between biotechnology and microbial ecology. Int. Microbiol., 4: 1-4.

Montero M, Sanz L, Rey M, Llobell A, Monte E (2007). Cloning and characterization of bgn16.3, coding for a beta-1,6-glucanase expressed during *Trichoderma harzianum* mycoparasitism. J. Appl. Microbiol., 103(4): 1291-1300.

Montesinos E (2003). Development, registration and commercialization of microbial pesticides for plant protection. Int. Microbiol., 6: 245–252.

Munoz FM, Demmler GJ, Travis WR, Ogden AK, Rossmann SN, Rinaldi MG (1997). *Trichoderma longibrachiatum* infection in a pediatric patient with aplastic anemia. J. Clin. Microbiol., 35: 499–503.

Muthumeenakshi S, Mills PR, Brown AE, Seaby DA (1994). Intraspecific molecular variation among *Trichoderma harzianum* isolates colonizing mushroom compost in the British Isles. Microbiol., 140 (Pt 4): 769-777.

Muthumeenakshi S, Brown AE, Mills PR (1998). Genetic comparison of the aggressive weed mould strains of *Trichoderma harzianum* from mushroom compost in North America and the British isles. Mycol. Res. 102 (4): 385-390.

Nakashima K, Yamaguchi-Shinozaki K (2006). Regulons involved in osmotic stress-responsive and cold stress-responsive gene expression in plants. Physiol. Plant 126: 62–71.

Nahm KH, Carlson CW (1985). Effects of cellulase from *Trichoderma viride* on nutrient utilization by broilers. Poult Sci., 64(8): 1536-1540.

Navazio L, Baldan B, Moscatiello R, Zuppini A, Woo SL, Mariani P, Lorito M (2007). Calcium-mediated perception and defense responses activated in plant cells by metabolite mixtures secreted by the biocontrol fungus *Trichoderma atroviride*. BMC Plant Biol., 7: 41-49.

Nevalainen H, Suominen P, Taimisto K (1994). On the safety of *Trichoderma reesei*. J. Biotechnol., 37(3): 193-200.

Ospina-Giraldo MD, Royse DJ, Chen X, Romaine CP (1998). Molecular phylogenetic analyses of biological control strains of *Trichoderma harzianum* and other biotypes of *Trichoderma* spp. associated with mushroom green mold. Phytopathology, Vol. 89, No. 4, pp. 308-313.

Overton BE, Stewart EL, Geiser DM (2006). Taxonomy and phylogenetic relationships of nine species of *Hypocrea* with anamorphs assignable to *Trichoderma* section *Hypocreanum*. Stud. Mycol., 56: 39-65.

Parry MAJ, Flexas J, Medrano H (2005). Prospects for crop production under drought: research priorities and future directions. Ann. Appl. Biol., 147: 211–226.

Peggion C, Formaggio F, Crisma M, Epand RF, Epand RM, Toniolo C (2003). Trichogin: a paradigm for lipopeptaibols. J. Peptide Sci., 9: 679–689.

Pérez-González JA, González R, Querol A, Sendra J, Ramón D (1993). Construction of a recombinant wine yeast strain expressing β–(1,4)-endoglucanase and its use in microvinification processes. Appl. Environ. Microbiol., 59: 2801-2806.

Peterbauer CK, Lorito M, Hayes CK, Harman GE and Kubicek CP (1996). Molecular cloning and expression of the *nag1* gene (N-acetyl-D-glucosaminidase-encoding gene) from *Trichoderma harzianum* P1. Curr. Genet., 30:325–331.

Powell KA, Jutsum AR (1993) Technical and commercial aspects of bicontrol products. J. Pestic. Sci., 37: 315–321.

Reusser F (1967). Biosynthesis of Antibiotic U-22,324, a Cyclic Polypeptide. J. Biol. Chem., 242: 243-247.

Richter S, Cormican MG, Pfaller MA, Lee CK, Gingrich R, Rinaldi MG, Sutton DA (1999). Fatal disseminated *Trichoderma longibrachiatum* infection in an adult bone marrow transplant patient: species identification and review of the literature. J. Clin. Microbiol., 1154–1160.

Rossi C, Tuttobello L, Ricci M, Casinovi CG, Radics L. (1987). Leucinostatin D, a novel peptide antibiotic from *Paecilomyces marquandii*. J. Antibiot., 40 (1), 130-133.

Ruocco M, Lanzuise S, Vinale F, Marra R, Turrà D, Woo SL, Lorito M (2008). Identification of a new biocontrol gene in *Trichoderma atroviride*: the role of an ABC transporter membrane pump in the interaction with different plant pathogenic fungi. Mol. Plant Microbe Inter. *(in press)*

Samuels GJ (2006). *Trichoderma*: systematics, the sexual state, and ecology. Phytopathol., 96(2): 195-206.

Samuels GJ, Dodd SL, Gams W, Castlebury LA, Petrini O (2002). *Trichoderma* species associated with the green mold epidemic of commercially grown *Agaricus bisporus*. Mycologia, 94: 146–170.

Schirmböck M, Lorito M, Wang YL, Hayes CK, Arisan-Atac I, Scala F, Harman GE and Kubicek CP (1994). Parallel formation and synergism of hydrolytic enzymes and peptaibol antibiotics, molecular mechanisms involved in the antagonistic action of *Trichoderma harzianum* against phytopathogenic fungi. Appl. Environ. Microbiol., 60: 4364-4370.

Seaby DA (1998). *Trichoderma* as a weed mould or pathogen in mushroom cultivation. In "*Trichoderma* and *Gliocladium*" Volume 2, Enzymes, biological

control and commercial applications (G.E. Harman & C.P. Kubicek eds.), Taylor & Francis Ltd., London, UK, pp.267-287.

Sharman GJ, Try AC, Williams DH, Ainsworth AM, Beneyto R, Gibson TM, Mcnicholas C, Renno DV, Robinson N, Wood KA, Wrigley SK (1996). Structural elucidation of XR586, a peptaibol-like antibiotic from *Acremonium persicinum*. Biochem. J., 320 (Pt 3), 723-728.

Sharon E, Bar-Eyal M, Chet I, Herrera-Estrella A, Kleifeld O, Spiegel Y (2001). Biological Control of the Root-Knot Nematode *Meloidogyne javanica* by *Trichoderma harzianum*. Phytopathol., 91(7): 687-693.

Sherman DH (2002). New enzymes for "warheads". Nature Biotechnol., 20: 984-985.

Shoresh M, Harman GE (2008). The molecular basis of shoot responses of maize seedlings to *Trichoderma harzianum* T22 inoculation of the root: a proteomic approach. Plant Physiol., 147: 2147-2163.

Simon A, Sivasithamparam K (1989). Pathogen suppression: a case study in biological suppression of *Gaeumannomyces graminis* var. tritici in soil. Soil Biol. Biochem., 21: 331-337.

Sinden J, Hauser E (1953). Nature and control of three mildew diseases of mushrooms in America. Mushroom Sci., 2: 177–180.

Sivasithamparam K, Ghisalberti EL (1998). In: *Trichoderma* and *Gliocladium.* Kubiecek CP and Harman GE (eds), Taylor and Francis, London, Vol 1, pp. 139-191.

Stacey G, Keen NT (1999). Plant–Microbe Interactions, vol. 4. APS Press, St. Paul.

Suárez B, Rey M, Castillo P, Monte E, Llobell A (2004). Isolation and characterization of PRA1, a trypsin-like protease from the biocontrol agent *Trichoderma harzianum* CECT 2413 displaying nematicidal activity. Appl. Microbiol. Biotechnol., 65(1): 46-55.

Tanis BC, van der Pijl H, van Ogtrop ML, Kibbelaar RE, Chang PC (1995). Fatal fungal peritonitis by *Trichoderma longibrachiatum* complicating peritoneal dialysis. Nephrol. Dial. Transplant., 10: 114–116.

The Royal Society (2001). The role of land carbon sinks in mitigating global carbon change. Policy Document 10/01.

Tronsmo A (1989). *Trichoderma harzianum* used for biological control of storage rot on carrots. Norw. J. Agric. Sci., 3, 157–162.

Tuite J (1969). Plant Pathological Methods: Fungi and Bacteria. Burgess Publishing Co, Minneapolis, MN, USA.

van Loon LC, Bakker PAHM, Pieterse CMJ (1998). Systemic resistance induced by rhizosphere bacteria. Annu. Rev. Phytopathol., 36, 453–483.

Vinale F, D'Ambrosio G, Abadi K, Scala F, Marra R., Turrà D, Woo SL, Lorito M (2004). Application of *Trichoderma harzianum* (T22) and *Trichoderma atroviride* (P1) as plant growth promoters, and their compatibility with copper oxychloride. J. Zhejiang Univ., 30(4): 2-8.

Vinale F, Marra R, Scala F, Ghisalberti EL, Lorito M, Sivasithamparam K (2006). Major secondary metabolites produced by two commercial *Trichoderma* strains active against different phytopathogens. Lett. Appl. Microbiol., 43: 143148.

Vinale F, Sivasithamparam K, Ghisalberti EL, Marra R, Woo SL, Lorito M (2008a). *Trichoderma*–plant–pathogen interactions. Soil Biol. Biochem., 40: 1–10.

Vinale F, Sivasithamparam K, Ghisalberti EL, Marra R, Barbetti MJ, Li H, Woo SL, Lorito M (2008b). A novel role for *Trichoderma* secondary metabolites in the interactions with plants. Physiol. Mol. Plant Pathol., 72: 80–86.

Viterbo A, Haran S, Friesem D, Ramot O, and Chet I (2001). Antifungal activity of a novel endochitinase gene (chit36) from *Trichoderma harzianum* Rifai TM. FEMS Microbiol. Lett., 200: 169-174.

Viterbo A, Montero M, Ramot O, Friesem D, Monte E, Llobell A, and Chet I (2002). Expression regulation of the endochitinase chit36 from *Trichoderma asperellum* (*T. harzianum* T-203). Curr. Genet., 42: 114-122.

Viterbo A, Wiest A, Brotman Y, Chet I, Kenerley C (2007). The 18mer peptaibols from *Trichoderma virens* elicit plant defence responses. Mol. Plant Pathol., 8(6), 737–746.

Wada S, Iida A, Akimoto N, Kanai M, Toyama N, Fujita T (1995). Fungal metabolites. XIX. Structural elucidation of channel-forming peptides, trichorovins-I-XIV, from the fungus *Trichoderma viride*. Chem. Pharm. Bull., 43 (6), 910-915.

Wahid A, Gelani S, Ashraf M, Foolad MR (2007) .Heat tolerance in plants: an overview. Environ. Exp. Bot. 61: 199–223.

Wang W, Vinocur B, Altman A (2003). Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta, 218: 1–14.

Wardle DA, Parkinson D, Waller JE (1993). Interspecific competitive interactions between pairs of fungal species in natural substrates. Oecologia, 94: 165-172.

Watson RT, Noble IR, Bolin B, Ravindranath NH, Verardo DJ, Dokken DJ (2000). In: IPCC Special Report on Land Use, Land-Use Change and Forestry. A special report of the Intergovernmental Panel on Climate Change. IPCC Secretariat, World Meteorological Organisation, Geneva, Switzerland.

Wiest A, Grzegorski D, Xu BW, Goulard C, Rebouffat S, Ebbole DJ, Bodo B, Kenerley CM (2002). Identification of peptaibols from *Trichoderma virens* and cloning of a peptaibol synthetase. J. Biol. Chem., 277: 20862-20868.

Woo SL, Lorito M (2006). Exploiting the interactions between fungal antagonists, pathogens and the plant for biocontrol. In: Novel Biotechnologies for Biocontrol Agent Enhancement and Management, M. Vurro and J. Gressel (eds.) Springer, the Netherlands, pp. 107-130.

Woo SL, Lorito M (2007). Exploiting the interactions between fungal antagonists, pathogens and the plant for biocontrol. In: Vurro, M., Gressel, J. (Eds.), Novel Biotechnologies for Biocontrol Agent Enhancement and Management. pp. 107–130, IOS, Springer Press, Amsterdam, the Netherlands.

Woo SL, Donzelli B, Scala F, Mach R, Harman GE, Kubicek CP, Del Sorbo G, Lorito M (1999). Disruption of the ech42 (endochitinase-encoding) gene affects biocontrol activity in *Trichoderma harzianum* P1. Mol. Plant Microbe Int., 12 (5):419-429.

Woo S, Fogliano V, Scala F, Lorito M (2002). Synergism between fungal enzymes and bacterial antibiotics may enhance biocontrol. Ant. van Leeuw., 81: 353-356.

Woo SL, Formisano E, Di Benedetto P, Senatore M, Abadi K, Gigante S, Soriente I, Ferraioli S, Scala F, Lorito M (2004). Identification and characterization of *Trichoderma* species aggressive to *Pleurotus* in Italy. J. Plant Pathol., 86 (4, Special issue): 337.

Woo SL, Ruocco M, Vinale F, Turrà D, Marra R, Ambrosino P, Lanzuise S, Ferraioli S, Soriente I, De Martino M, Lorito M (2005). Lotta a ceppi di *Trichoderma* deleteri per la produzione del fungo edule *Pleurotus*. In "Annali della Facoltà di Agraria della Università degli Studi di Napoli Federico II – Portici. Serie Quinta- Vol. II- Anno 2005". pp. 15-35.

Woo SL, Scala F, Ruocco M, Lorito M (2006). The molecular biology of the interactions between *Trichoderma* spp., phytopathogenic fungi, and plants. Phytopathol., 96, 181–185.

Yedidia I, Chet I (1999). Induction of defense responses in cucumber plants (*Cucumis sativus*) by the biocontrol agent *Trichoderma harzianum*. Appl. Environ. Microbiol., 65: 1061-1070.

Yedidia I, Srivastva AK, Kapulnik Y, Chet I (2001). Effect of *Trichoderma harzianum* on microelement concentrations and increased growth of cucumber plants. Plant Soil, 235:235-242.

Yedidia I, Shoresh M, Kerem Z, Benhamou N, Kapulnik Y, Chet I (2003). Concomitant induction of systemic resistance to *Pseudomonas syringae* pv. *lachrymans* in cucumber by *Trichoderma asperellum* (T-203) and accumulation of phytoalexins. Appl. Environ. Microbiol., 69: 7343-7353.