Università degli Studi della Tuscia
Facoltà di Agraria
Dipartimento Geologia e Ingegneria Meccanica, Naturalistica ed Idraulica per il Territorio
(GEMINI)

PhD in Horticulture – 22nd cycle
Scientific-disciplinary sector: Agr/04

Thesis

CONTROL OF PHYTOPATHOGENIC FUNGI IN HORTICULTURAL CROPS BY NATURAL PLANT EXTRACTS

PhD student
Eva Švecová

Coordinator
Prof. Alberto GRAIFENBERG

Tutors
Dr. Paola CRINO'
ENEA C.R. Casaccia, Roma

Dr. Giuseppe Colla
Università degli Studi della Tuscia, Dipartimento GEMINI

Academic year 2009/2010

Mamince

To my mum

INDEX

1

GENERAL PART

1.1 INTRODUCTION

Plants and their pathogens have evolved together for millions of years. Losses of crop yield from diseases have had severe effects on the human race, for example the Irish potato famine in the 1800's, caused by late blight, a fungal disease of potato; in the mid 19th century, downy mildew grape disease was accidentally introduced in Europe and almost destroyed the vineyards in many countries; massive European epidemics caused by ergot of rye grain frequently occurred during medieval times; and in the early 20th century, the American Chestnut tree was wiped out by an Asian blight disease.

For disease to occur, three critical factors or conditions must exist: (i) a pathogen, (ii) a susceptible host plant, and (iii) the right mix of environmental conditions. The relationship of these factors is represented by the DISEASE TRIANGLE (*Fig. 1.1*), which represents a central concept of plant pathology. It is based on the principle that infectious diseases develop when a susceptible host and a disease-causing pathogen meet in a favorable environment. If any one of these three conditions were not met, there would be no disease.

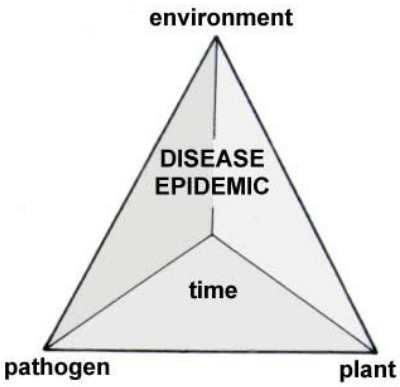


Fig. 1.1: Disease triangle

PATHOGENS (fungi, bacteria, viruses, mycoplasma) are microorganisms causing disease and, because they are living, they are called biotic agents. Each of them has a different life cycle, which includes an infectious stage. Most pathogens are host-specific to a particular plant species, genus or family, while some diseases, such as powdery mildews, produce similar symptomes on different plants. Fungi are usually host-specific.

A SUSCEPTIBLE HOST has a genetic background that permits the development of a particular disease. The genetic defence against a disease is called disease resistance. This resistance can be represented by physical, chemical, and growth patterns of the plant. For disease to occur, the host plant must be at a specific stage of development.

Specific ENVIRONMENTAL CONDITIONS must exist for pathogens to cause infection and they vary for different pathogens. High moisture and specific temperature ranges, for example, are necessary for many fungal diseases. These conditions must continue for a critical period of time, while the pathogen is in contact with the host, for infection to occur. Each pathogen has a specific temperature range for growth and activity. Soil temperature can also be critical for pathogen infection. Cool, wet soils promote fungal root diseases. Moisture, temperature, wind, sunlight, nutrition and soil quality affect plant growth. If one of these factors is out of balance for the culture of a specific plant, that plant may have a greater tendency to become diseased.

The DISEASE CYCLE is characterized by five stages: (i) inoculation, (ii) incubation, (iii) penetration, (iv) infection, and (v) symptoms. Pathogen is carried on the plant (inoculation) by rain, wind, insects, birds, people or it is transmitted by seeds or plant debris. During the second stage of disease development (incubation), pathogen changes or grows into a form that can enter the new host plant; in many fungal diseases, it arrives on the plant as a spore, which must germinate before it can grow into the plant, where it sends out the hyphae penetrating the plant through wounds or natural pores. In the fourth stage of the disease cycle (infection), the pathogen grows within the plant and begins damaging the plant tissue. Then, as the pathogen consumes nutrients, the plant reacts by showing symptoms. Symptoms are the evidence of the damages caused by the pathogen to the plant. In response to disease, the plant defence response often involves a hypersensitive response (HR), visible as flecks of dead cells at sites of attempted entry.

Successful DISEASE CONTROL requires knowledge of the causal agent and the disease cycle, the host-pathogen interactions in relation to environmental factors, and cost. Disease control starts with the best variety, seed, or planting stock available and continues throughout the life of the plant. For harvested crops, disease control must be extended through transport, storage, and marketing. Relatively few diseases are controlled by a single method; the majority require several approaches. These often need to be integrated into a broad program of biological, cultural, and chemical methods to control as many different diseases on a given crop as possible.

There are at least 50,000 diseases of crop plants and new diseases are discovered every year. Pathogens multiply and mutate rapidly. The use of appropriate agronomical practices and an understanding of plant pathology are the first line of defence against pathogens. Almost all control methods are aimed at protecting plants from becoming diseased but only few plant diseases can be controlled satisfactorily by therapeutic means in the field. The

various control methods can be classified as regulatory, cultural, biological, physical, and chemical, depending on the nature of the agent employed (Agrios, 2005). Regulatory control measures aim at excluding a pathogen from a host or from a certain geographic area. Most cultural control methods help plants to avoid contact with a pathogen, so creating environmental conditions unfavorable to the pathogen, as well as eradicating or reducing the amount of a pathogen in a plant (host eradication), a field (crop rotation), an area (sanitation) and improving plant growing conditions. Most biological methods use living organisms or natural plant-derived products to reduce the pathogen inoculum. Other type of biological control consist in the use of transgenic plants that exhibit resistance to a certain disease. Finally, physical (i.e. sterilization, heat treatment of plant organs, refrigeration, and radiations) and chemical methods (i.e. soil treatment, soil fumigation, and seed treatment with chemicals) aim at protecting plants from pathogen inoculum or curing an infection that is already in progress. Some chemicals operate by activating the defences of the plant (systemic resistance) against pathogens (Agrios, 2005). Synthetic fungicides are still the most important components in the management of fungal diseases. More than 100 fungicides have been developed, and several hundreds of fungicide formulations are available (Vidhyasekaran, 2004).

Resistance of numerous fungi to some systemic fungicides, all of which containing a benzene ring, began to appear in the 1960s. To date, fungicide resistance has been reported in *Phytophthora infestans, Peronospora parasitica,* and *Bremia lactucae* against phenylamides (metalaxyl compounds); *Ustilago nuda* and *Ustilago maydis* against carboxamides (carboxin compounds); *Erysiphe graminis* f. sp. *hordei* against hydroxypyrimidine; *E. graminis* f. sp. *tritici, E. graminis* f. sp. *hordei,* and *Pyrenophora teres* against triazoles (propiconazole); and *Botrytis cinerea, Botrytis fabae, Septoria tritici, Pyrenopeziza brassicae, Cercospora beticola, Fusarium nivale, Fusarium culmorum,* and *Pseudocercosporella herpotrichoides* against benzimidazoles (carbendazim, benomyl, thiophanate-methyl compounds). Some other important pathogens such as *Alternaria*, *Colletotrichum*, *Verticillium*, *Sphaerotheca*, *Mycosphaerella*, *Aspergillus*, *Penicillium*, *Pythium*, and *Venturia inaequalis* are known to have produced strains resistant to one or more of the systemic fungicides (Agrios, 2005; Vidhyasekaran, 2004).

In addition, some efficient fungicides may be phased out globally because they are seriously harmful for the environment, e.g. methyl bromide that, since 2005, was banned in European Union countries (Batchelor, 2002) because considered as ozone-depleting compound (Anonymous, 1998). Therefore, integrated control methods has been widely

involved in plant disease management and are usually aimed against all diseases affecting a crop (Agrios, 2005). As there is a great interest to decrease the impact of chemicals applied to the crops, novel natural products are evaluated for possible use in plant disease control. Such products can be either of microbial or plant origin.

Thousands of plant species are known to have medicinal properties due to their chemical compounds. Plant-derived products such as plant extracts are traditionally used in local medicine all over the world. Therefore, these extracts are supposed to have antimicrobial properties in plant disease management as well. Such preparations may have many advantages for both grower and consumer, e.g. in the crop culture the environment is less stressed by natural products and fungi and resistance against such products has not been acquired. In this way, natural products are often prefered by consumers as they are considered helthier due to minor content of chemical residues.

1.2 FUNGAL PLANT PATHOGENS

Fungi are one of the most important groups of plant pathogens. To date, there are about 10,000 species of fungi, that can cause diseases in plants. All plants are attacked by some kinds of fungi, and each of the parasitic fungi can attack one or many kinds of plants (Vidhyasekaran, 2004; Agrios, 2005). Some species of fungi, the mycorrhizae, live symbiotically on or in the roots of many plants. This relationship is basically parasitic but in many situations is probably beneficial to both the plant and the fungus. The growth of the plant is promoted by the improved uptake of some mineral nutrients while the fungus gains access to organic nutrients and shelter.

The majority of phytopathogenic fungi belong to the Ascomycetes and the Basidiomycetes. Fungi are reproduced both sexually and asexually by production of spores that may be spread long distances by air or water or may be soil borne. Fungi are common in soil, in air (mainly as spores) and on plant surfaces throughout the world.

1.2.1 AIR-BORNE FUNGI

Many Ascomycetes and imperfect fungal pathogens are airborne and attack plant shoots. Air-borne fungi are transmitted mainly by air currents or water droplets and they attack plant penetrating through aerial organs.

1.2.1.1 *Botrytis cinerea*

Botrytis cinerea Pers. is a necrothrophic fungus, probably the most common and widely distributed among vegetable, ornamental, and fruit greenhouse and field-grown crops throughout the world. *Botrytis cinerea* is pathogenic on over 200 species of plants.

Botrytis diseases appear primarily as blossom blights and fruit rots, but also as damping-off, stem cankers or rots, leaf spots, and tuber, corm, and bulb rots. Some of the most serious diseases caused by *Botrytis* include gray mold of strawberry, grapes and of many vegetables, calyx end rot of apples, onion blast and neck rot, blight or gray mold of many ornamentals, bulb rot of amaryllis, corm rot of gladiolus, and others. *Botrytis* also causes secondary soft rots of fruits and vegetables in storage, transit, and market (Agrios, 2005; Webster and Weber, 2007; Beever and Weeds, 2007). In greenhouse culture, *Botrytis cinerea* is well-known cause of considereable damages in tomato.

The Pathogen

The pathogen *Botrytis cinerea*, a *Deuteromycete*, produces abundant gray mycelium and long branched conidiophores with rounded apical cells bearing clusters of colorless or gray, one-celled, ovoid conidia. The conidiophores (*Fig. 1.2*) and clusters of conidia resemble a grape-like cluster. Hyaline conidia (asexual spores) are released readily in humid weather and are carried by air currents. The fungus frequently produces black, hard, flat, irregular sclerotia as survival structures in older cultures. It overwinters as sclerotia or intact mycelia, both of which germinate in spring to produce conidiophores. The conidia are dispersed by wind and rain-water and cause new infections.

Fig. 1.2: Conidiophore of *B. cinera*

It is usually referred to the fungus with its anamorph (asexual form) name, because the sexual phase is rarely observed. The teleomorph (sexual form) is an ascomycete, ***Botryotinia fuckeliana*** (Jarvis, 1980). Some species of *Botrytis* occasionally produce a *Botryotinia* perfect stage in which ascospores are produced in an apothecium (Agrios, 2005).

Symptoms

In the field, blossom blights often precede and lead to fruit rots and stem rots. The fungus becomes established in flower petals and there it produces abundant mycelium with

conidia forming whitish-gray or light brown mold. The fungus later moves from the petals into the fruit and causes a blossom end rot of the fruit, which may destroy part or all of the fruit (*Fig. 1.3*). Rot development is rapid and quite common in the field. Infected fruit and succulent stems become soft, watery, and light brown. As the tissue rots, the epidermis cracks and the fungus fruits abundantly. Flat black sclerotia may appear on the surface. Damping-off of seedlings due to *Botrytis* occurs primarily in cold frames, where humidity is high. Some species of *Botrytis* cause leaf spots on their hosts e.g. on gladiolus, onion, and tulip. Infection of below ground parts, such as bulbs, corms, tubers, and roots, may begin while these organs are still in the ground or at harvest (Agrios, 2005; Maas, 2004).

Fig. 1.3: B. cinerea infection on strawberry

Disease development

Botrytis overwinters in the soil as mycelium in decaying plant debris and as conidia and sclerotia (*Fig. 1.4*). Each part of the fungus thallus can serve as a survival structure. Mycelia, sclerotia and conidia have different abilities for survival and dispersal, and the relative roles of these structures will vary greatly depending on ecosystem and season. Sclerotial types are over winter favoured on perennial hosts while conidial types on annual hosts with abundant susceptible flowers (Beever and Weeds, 2007; Holz *et al.*, 2007). The fungus requires cool (18–25°C) and damp weather (humidity >90%) for best growth, sporulation, spore release and germination, and establishment of infection. The pathogen, active at low temperatures, may cause considerable losses on crops kept for long periods in storage, even at the temperatures between 0 and 10°C. *Botrytis* sclerotia usually germinate by producing mycelial threads that can infect directly but, in a few cases, sclerotia germinate by producing apothecia and ascospores (Agrios, 2005; Ponti and Laffi, 1985).

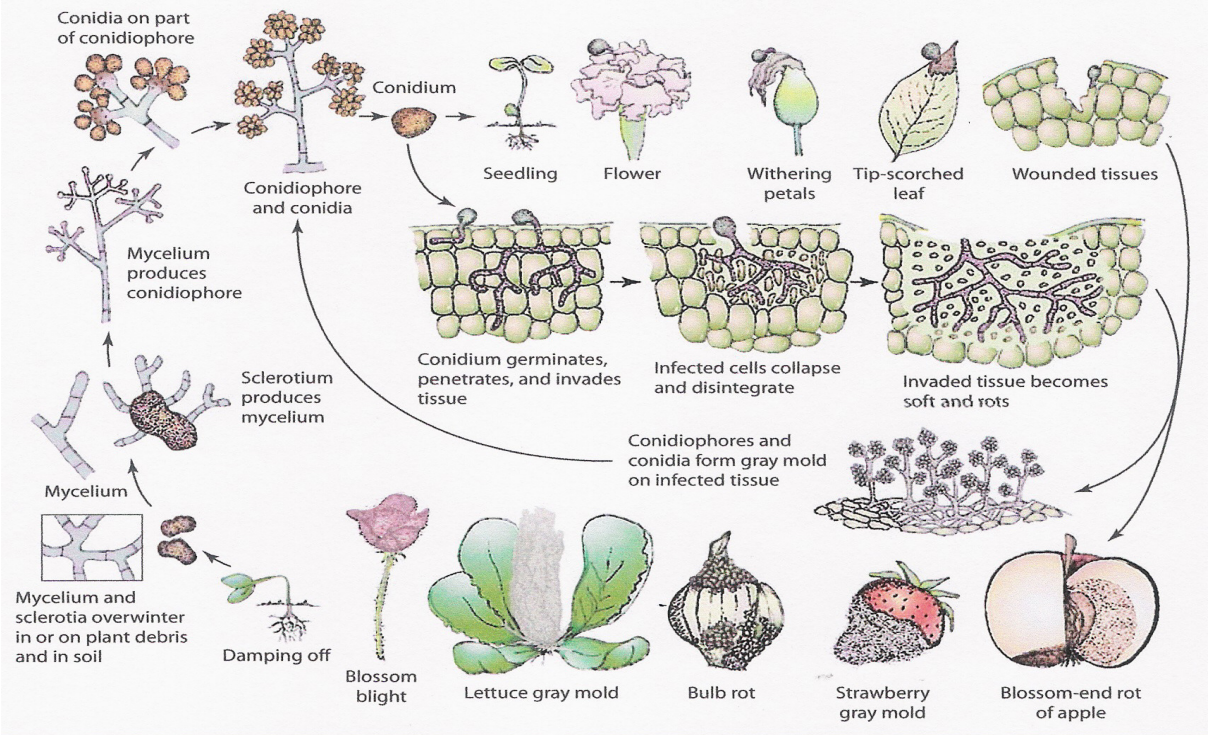


Fig. 1.4: Life cycle of *Botrytis cinerea* (from Agrios, 2005)

Control

The control of *Botrytis* diseases is difficult to obtain. It is aided by the removal of infected debris from the field and by providing storage conditions with proper aeration. Other measures that can be taken only to minimize losses due to *Botrytis* fruit rot include prevention of excessive vegetative growth by regulating plant density, timely nitrogen applications, harvesting fruit before it is fully ripe to avoid injuries and prompt transfer of harvested fruit to refrigerated storage. Storage organs such as onion bulbs can be protected by keeping them at 32 to 50°C for 2 to 4 days to remove excess moisture and then keeping them at 3°C in dry environment. In greenhouses, humidity should be reduced by ventilation and heating.

Biological control of *Botrytis* gray mold may be obtained with spray application of spore suspensions of certain antagonistic fungi and with mixtures of several biocontrol fungi and bacteria. A number of microbial products available commercially for control of *Botrytis* diseases indicates that biocontrol can succeed. Especially *Trichoderma* and *Ulocladium* have given the greatest success in *Botrytis* control. Sprays with a number of broad-spectrum or systemic fungicides control *Botrytis* on a wide variety of crops. The most efficient fungicides are Vinclozolin, Procimidone, and Iprodione. Other fungicides in use against *Botrytis* are Captano, Folpet, Captafol, Clorotalonil, and Tiram. However, control of *Botrytis* in the field through chemical sprays has been only partially successful. Application of botryticides just

before or after harvest is desirable but this practice is restricted by the toxicological risks of their residues. *Botrytis* strains resistant to several systemics and even to some broad-spectrum fungicides have been found in various crops, in particular against benzimidazoles, phenylcarbamates, and dicarboximides (Agrios, 2005; Vidhyasekaran, 2004; Leroux, 2007; Maas, 2004; Ponti and Laffi, 1985).

1.2.1.2 *Ascochyta rabiei*

Ascochyta blight is a serious disease of cool-season grain legumes (chickpea, faba bean, lentil, and pea) caused by fungal species of the anamorphic genus *Ascochyta* and related genera (White and Chen, 2007). *Ascochyta rabiei* (Pass.) Labr. causes the most destructive disease (*Fig. 1.5*) in many chickpea growing countries (Basandrai *et al.*, 2007).

Fig. 1.5: Chickpea field damaged by *A. rabiei*

The pathogen

This pathogen undergoes heterothallic sexual reproduction on infested residues, resulting in air-borne ascospores, which are capable of spread over long distances. Rapid polycyclic spread within crops occurs through splash-borne asexual conidia (pycnidiospores) (Davidson and Kimber, 2007).

Symptoms

The disease affects all above ground parts of the plant. Symptoms are characterised by necrotic lesions (*Fig. 1.6*), which under favourable conditions, can girdle stems leading to breakage and severe yield reduction on susceptible cultivars. Wilting causes the death of the plant. Seed quality may also be reduced through seed discolouration or retardation of seed development (Davidson and Kimber, 2007).

Fig. 1.6: Disease symptoms of *A. rabiei*

Disease development

Under favourable conditions, conidia germinate forming new hyphae which penetrates through epidermal cells into the plant tissue. In consequence, this necrotrophic pathogen in advance kills the plant cells of further mycelial development. Therefore, toxins and cell-wall degrading enzymes are often presumed to be important biochemical determinants of pathogenesis. The necrotic lesions develop after 4-5 days in susceptible varieties (Alam *et al.*, 1989; Pandey *et al*., 1987). The lesions on the stems and branches enlarge, sometimes covering entire plant organ. Such infected tissues cause death of the plant as the tissues break easily and are not any more able to give the mechanical support to the plant. Sometimes, numerous picnidia with abbundant conidia are formed on the necrotic lesions. In resistant varieties, fungus penetration is limited and it is followed by much rapid necrosis of the cells with consequent suppression of disease development (Porta-Puglia, Crinò, 1993). The rapid development of the disease is favoured by temperatures between 10 and 20^{o}C with relative humidity over 60%. The temperatures higher than 25 ^{o}C reduce the pathogen attack (Reddy and Singh, 1990; Weltzien and Kaack, 1984).

Control

The control methods include destroying inoculum sources, manipulating sowing times, using crop rotations, disease-free seed, application of seed and foliar fungicides, and adopting cultivars with improved resistance. Implemented fungicide strategies differ according to cultivar resistance and the control efficacy of fungicides. The effectiveness of genetic resistance varies according to seasonal conditions. The combination of strategies is determined by economics, availability of cultivar resistance and disease epidemiology (Davidson and Kimber, 2007).

1.2.1.3 *Phytophthora infestans*

An oomycete, *Phytophthora infestans* (Mont.) De Bary is the causal agent of late blight both of potatoes and tomatoes. The crop cultures are attacked by the fungus both under field and greenhouse conditions. The losses in yield caused by *P. infestans* are of a world-wide importance as potato and tomato make, an essential part of human alimentation throughout the world (Crinò, 1993; Ponti and Laffi, 1985).

The Pathogen

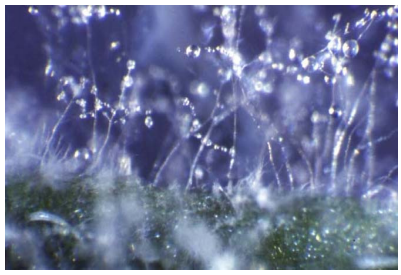


Fig. 1.7: Mycelium of *P. infestans*

The mycelium produces branched sporangiophores with lemon-shaped sporangia at their tips (*Fig. 1.7*). At the places where sporangia are produced, sporangiophores form swellings that are characteristic for this oomycete (Agrios, 2005). This organism is heterothallic and requires two mating types for sexual reproduction by gametangia contact. Sexual reproduction is not necessary for the survival of the organism as the mycelium may survive in infected tubers. Several biflagellated zoospores are formed and are liberated from each sporangium. Zoospores on leaves encyst and germinate via germ tubes, which form appressoria (Ament and Trigiano, 2004). Although there are potato and tomato strains of *P. infestans*, each is capable of infecting the other host (Horst, 2008).

Symptoms

Symptoms (*Figs. 1.8, 1.9*) appear at first as water-soaked spots, usually at the edges of the lower leaves. In moist weather, the spots enlarge rapidly and form brown, blighted areas with indefinite borders. A white zone appears at the border of the lesions on the undersides of the leaves. Under continuously wet conditions, blighted plant organs give off a characteristic odor due to secondary bacterial and fungal invaders. In dry weather, existing lesions stop enlarging, turn black, curl, and wither. Infected potato tubers are small with sunken lesions, stained purple or brown. On tomato seedlings, small and dark spots on stems or leaves are followed by death within 2 or 3 days. On mature tomato plants, blight starts with dark,

Fig. 8: Disease symptoms of *P. infestans* in potato

Fig. 1.9: Disease symptoms of *P. infestans* in tomato

watersoaked leaf spots and large dark brown spots on fruit, with most of the leaves soon hanging lifeless and fruit rotting on the ground. The rot, however, continues to develop after the tomato fruit or potato tuber is harvested (Agrios, 2005; Ament and Trigiano, 2004; Horst, 2008).

Disease development

Entire plants and entire fields may become blighted and die in a few days or a few weeks. The development of epidemics depends on the prevailing humidity and temperature during the different stages of the life cycle of the oomycete (*Fig. 1.10*).

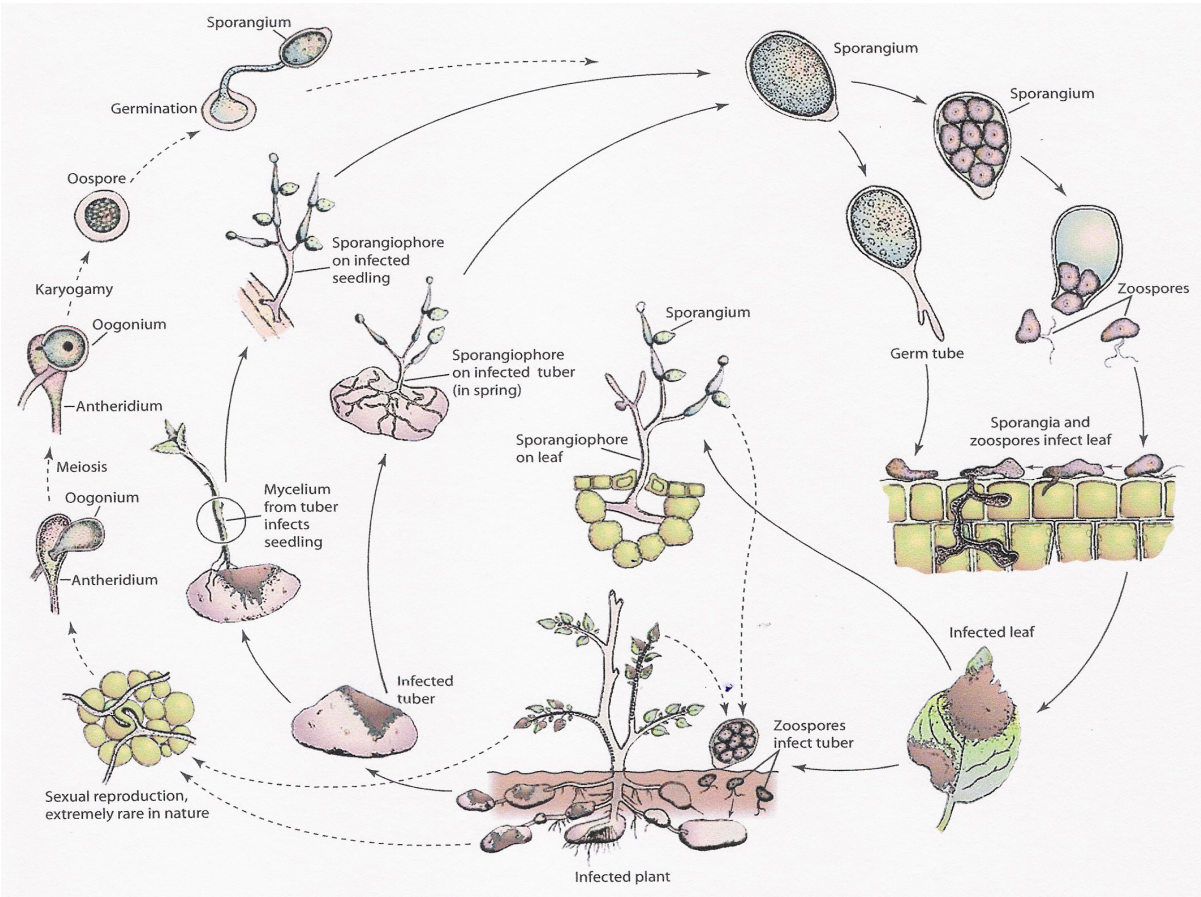


Fig. 1.10: Life cycle of *P. infestans* (from Agrios, 2005)

P. infestans grows and sporulates abundantly at a relative humidity near 100% and at temperatures between 15 and 25°C (optimal temperature is 20-23°C). Temperatures above 30°C slow or stop the growth of the oomycete in the field, and the oomycete can start to sporulate again when the temperature and humidity become favorable. Sporangia germinate by the zoospores at temperatures up to 12 or 15°C, whereas above 15°C sporangia may germinate directly by producing a germ tube. Oospores may survive in the soil for 3–4 years.

Mycelium from infected potato tubers or from germinating oospores and zoospores spreads into shoots produced from infected or healthy tubers and cause discoloration and collapse of the cells. When the mycelium reaches the aerial parts of plants, it produces sporangiophores, which emerge through the stomata of the stems and leaves, and produce sporangia. The sporangia, when ripe, become detached and are carried off by the wind or are dispersed by rain and infect new plants. A large number of asexual generations and new infections may be produced in one growing season (Agrios, 2005; Ponti and Laffi, 1985).

Control

Late blight can be controlled successfully by a combination of sanitary measures, partially resistant varieties, and chemical sprays. Only disease-free plant material should be used for seed. All volunteer potato plants in the area should be destroyed, as they can be a source of late blight infection (Agrios, 2005). Only the most resistant varieties should be planted. However, no cultivars of both tomato and potato are completely resistant to this fungus (Crinò, 1993). Several potato varieties resist to one or more races of oomycete. Many varieties possess so-called field resistance, which is a partial resistance of varying degrees effective against all races of the fungus. Even resistant varieties should be sprayed regularly with fungicides to eliminate the possibility of becoming suddenly attacked by races of *P. infestans* to which they are not resistant. Several broad-spectrum and systemic fungicides are used for late blight control. The most effective are the copper fungicides. Protective spraying of foliage usually affects a considerable reduction in tuber infection. However, the new strains of *P. infestans* are resistant to some of the systemic fungicides (metalaxyl) (Agrios, 2005; Ponti and Laffi, 1985).

1.2.2 SOIL-BORNE FUNGI

Soil-borne fungi survive in the soil and and in residues on the soil surface; they attack plant penetrating through lesions into the roots or collets. Thus the soil is a reservoir of inoculum of these pathogens, the majority of which are widely distributed in agricultural soils.

These diseases are difficult to control because they are caused by pathogens which can survive for long periods in the absence of the normal crop host and often have a wide host range including weeds; chemical control often does not work well or is not practical or too

expensive and it is difficult to develop resistant varieties of plants. These diseases are often very difficult to diagnose accurately. Damages to root and crown tissues is hidden in the soil. Thus, these diseases may not be noticed until the above-ground (foliar) parts of the plant are affected severely showing symptoms such as stunting, wilting, chlorosis and death.

1.2.2.1 *Fusarium oxysporum*

Fusarium vascular wilts are *F. oxysporum* is a widespread and destructive fungus that causes *Fusarium wilt* disease in more than a hundred species of plants, many ornamental and horticultural crops included. It does so by colonizing the water-conducting vessels (xylem) of the plant. As a result of this blockage and breakdown of xylem, symptoms appear in plants such as leaf wilting, yellowing and eventually plant death.

In solid media culture, such as potato dextrose agar (PDA), the different special forms of *F. oxysporum* can have varying appearances. In general, the aerial mycelium first appears white (*Fig. 1.11*), and then may change to a variety of colors - ranging from violet to dark purple - according to its special form.

Fig. 1.11: Mycelium of *Fusarium* sp.

The fungus can persist in the soil also for prolonged periods. It is characterized by production of three types of asexual spores (microconidia, macroconidia and chlamydospores). Microconidia (*Fig. 1.12*), which have one or two cells, are the most frequently and abundantly produced spores under all conditions within the wessels of infected plants. Macroconidia are the typical "*Fusarium*" spores; they are three to five celled, have gradually pointed and curved ends, and appear commonly on the surface of plants killed by the pathogen. Chlamydospores are one-or two celled, thick-walled, round spores produced either within or terminally on older mycelium or in macroconidia. Only chlamydospores can survive in the soil for a long time (Agrios, 2005). The life cycle of this fungus is divided in two phases: (1) saprofitical and (2) parasitical.

Morphological characterization of *F. oxysporum* is based on the shape of macroconidia, the structure of microconidiophores, and on the formation and disposition of the chlamidydospores. Asexual reproduction of *Fusarium* is accomplished by macroconidia and microconidia.

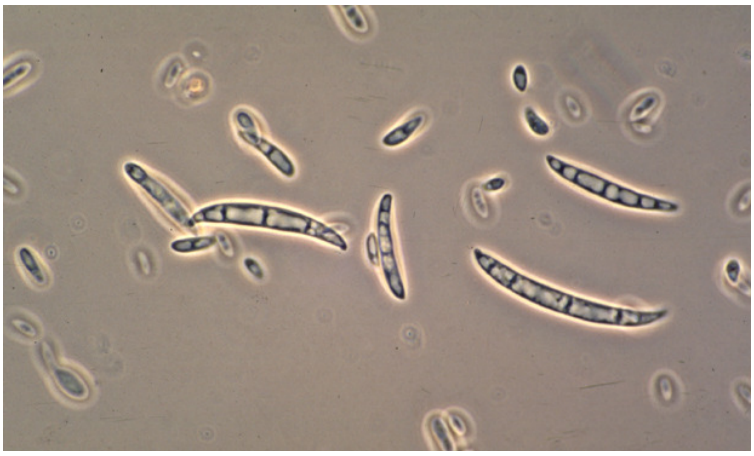


Fig. 1.12: Micro and macroconidia produced by *F. oxysporum*

1.2.2.1.1 *Fusarium oxysporum* f.sp. *basilici*

Fusarium wilt, caused by *F. oxysporum* f. sp. *basilici* (Dzidzariya) Arms. *et* Arms. (FOB), is one of the main problems for sweet basil because of its very destructive nature (*Fig. 1.13*) under field, hydroponic, and greenhouse conditions. The pathogen has a narrow host range and is considered specific for basil (Keinath, 1994; Garibaldi *et al.*, 1997; Reuveni *et al.*, 1997). FOB has been disseminated to new growing areas throughout the world via imported seeds (Elmer, 2001) and is now present in most countries where sweet basil is cultivated.

Fig. 1.13: Basil plant damaged by Fusarium wilt

Unfortunately, there is no treatment for infected basil plants. Fungicides are ineffective and although a few biological control organisms have been tested, none have reliably provided control. Infected plants should be removed immediately because spores from infected stems can move on air currents or splashing water to infect nearby basil plants. Prevention is the only way to control Fusarium wilt of basil.

Typically, basil Fusarium wilt management relies on the integration of different control measures, such as soil and substrate disinfestation, raised bench cultivation, seed dressing, and use of antagonistic *Fusarium* spp. (Garibaldi *et al*, 1997; Minuto *et al.*, 1994, 1995, 1997). However, considerable potential for soil contamination and reinfestation through infected seed and airborne propagules makes soil disinfestation only partially effective against *F. oxysporum* f. sp. *basilici* (Chiocchetti *et al.*, 1999). The low efficacy of chemical control measures, the limited availability of resistant cultivars (Reuveni *et al.*, 1997), and the

unsatisfactory level of control sometimes offered by the commercially available formulations of biocontrol agents (Keinath, 1994; Minuto *et al.*, 1997) boost the urgency for seed and transplant certification procedures on sweet basil.

Probably the best option is to use a variety that is tolerant or resistant to the disease. 'Nufar' is a variety resistant to fusarium wilt originated from a few healthy plants found in the middle of a diseased field in Israel. The variety is now commercially available in the United States through several seed companies.

The pathogen

The mycelium is colorless at first, but later it becomes cream-colored, pale yellow or pink. Microconidia are the most frequent primary inoculum.

Symptoms

This pathogen causes wilt of plants at all growth stages, brown and black discoloration of roots and lower stems, black lesions on stems, root and crown rot, blackening and drying of vegetative apices, growth retardation, and xylem discoloration (*Figs. 1.14, 1.15*). The stems are covered withpink-orange layer consisting mainly of macroconidia (Gamliel *et al.*, 1996).

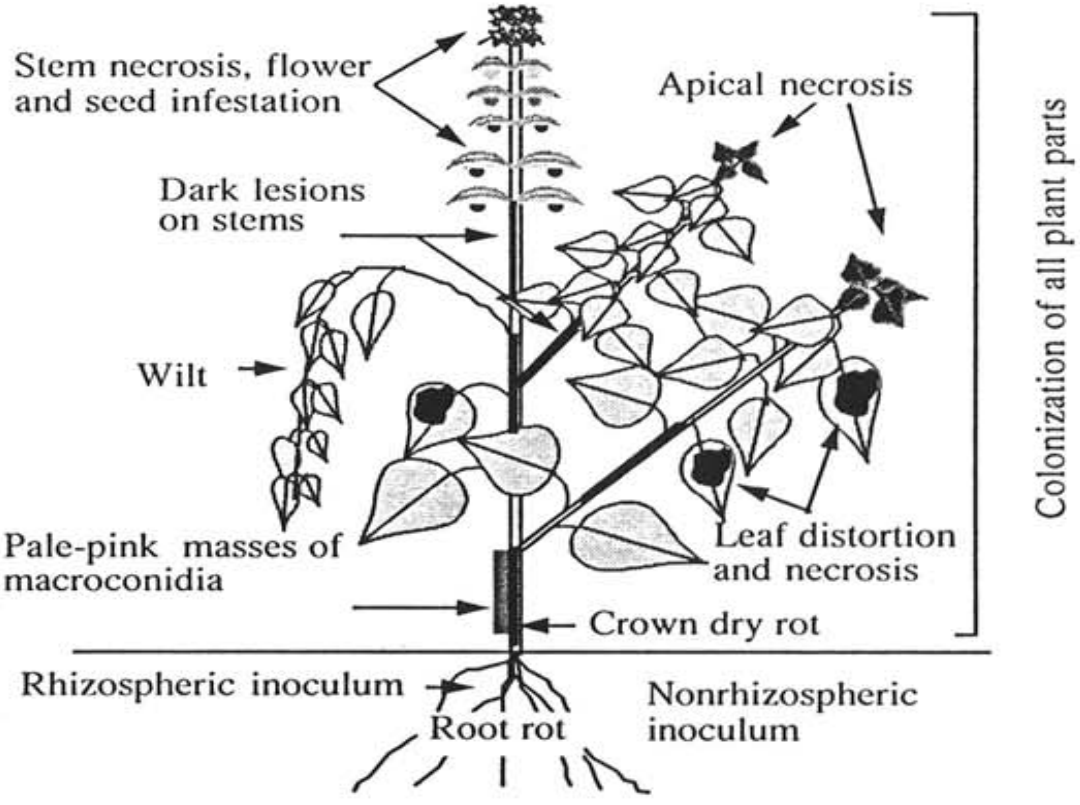


Fig. 1.14: The main symptoms of Fusarium disease in basil (from Gamliel *et al.*, 1996)

Fig. 1.15: Symptoms caused by *F. oxysporum* f. sp. *basilici* on basil.
(a) internal discolouration of basil stems infected with fusarium wilt. (b) young basil plant with Fusarium wilt

Disease development

The fungus penetrates into the roots from the soil. First, the pathogen causes leaf chlorosis. It is followed by wilting of the leaves, epinasty and stunting which lead to death of the plant. The temperatures favourable to the disease development are rather higher, about 27^{o}C. Although, *Fusarium* is a soil-borne pathogen, Rekah (2000) and Gamliel *et al.* (1996) reported the importance of air-borne inoculum as well.

Control

Currently, no efficient fungicide is available to control the disease. Also the availability of commercially acceptable resistant cultivars is limited. Therefore, there is still a need for alternative ways to control the disease. Moreover, the current trend to near-zero market tolerance for pesticide residues in fresh herbs also leads to search for non-chemical means to control the disease. For instance, in the plants grown in soilless culture, the choice of the medium can affect the disease proliferation rate. Also the use of compost can induce protection against *F. oxysporum* f. sp. *basilici* and reduce the severity of the visual symptoms of Fusarium wilt (Reuveni *et al.*, 2002; Minuto *et al.*, 1997). Moreover, Reuveni *et al.* (2002) suggest that under practical conditions, compost, combined with the resistant cultivar 'Nufar' could provide full protection for basil plants against Fusarium wilt.

1.2.2.1.2 *Fusarium oxysporum* f. sp. *lycopersici*

Fusarium oxysporum f. sp. *lycopersici* (Sacc.) W.C. Snyder and H.N. Hans causes Fusarium wilt specifically in tomato for which it is one of the most prevalent and damaging diseases. This disease, first described in England in 1895, it is of worldwide importance because at least 32 countries had reported the disease.

Fusarium wilt is most destructive in warm climates and sandy soils of temperate regions. The disease causes great losses when soil and air temperatures are rather high during

much of the season. Infected plants become stunted and soon wilt and finally die. Occasionally, entire fields of tomatoes are killed or damaged severely before a crop can be harvested (Agrios, 2005). The development and use of resistant cultivars have nearly eliminate the concern over this disease.

The Pathogen

The mycelium of *F. oxysporum* f. sp. *lycopersici* is colorless at first, but with age it becomes cream-colored, pale yellow, pale pink, or purplish (Agrios, 2005). Three physiological races of the fungus have been reported. Race 1 is the most widely distributed and has been reported from most geographical areas. Although race 2 was first reported in Ohio in 1940, it did not become widespread or of economic concern until its discovery in Florida in 1961. Since then, it was rapidly reported in several other countries, including Australia, Brazil, Great Britain, Israel, Mexico, Morocco, the Netherlands, and Iraq. Race 3 was reported in 1966 in Brazil. Thereafter, it has been found in Australia and in Florida and California.

The mycelium is delicate white to pink, often with purple tinge, and is sparse to abundant. The fungus produces the three types of spores, microconidia, macroconidia, and chlamydospores (*Fig. 1.16*). Microconidia are borne on simple phialides arising laterally and are abundant, oval-ellipsoid, straight to curved, 5-12 x 2.2-3.5 µm, and nonseptate. Macroconidia, sparse to abundant, are borne on branched conidiophores or on the surface of sporodochia and are thin walled, three- to five-septate, fusoid-subulate and pointed at both ends, have pedicellate base. Three-septate spores are more common. Chlamydospores, both smooth and rough walled, are abundant and form terminally or on an intercalary basis. They are generally solitary, but occasionally form in pairs or chains. No perfect stage is known.

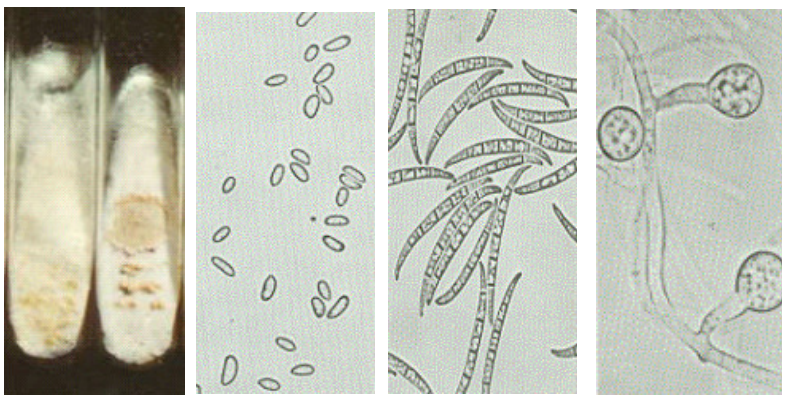


Fig. 1.16: Main traits of *F. oxysporum* f. sp. *lycopersici.*
(a) mycelium; (b) microconidia; (c) macroconidia; (d) chlamydospores

Symptoms


Fig. 1.17: Symptoms of Fusarium wilt in tomato

Fusarium wilt can affect tomato plants in every developmental stage (Ponti and Laffi, 1985). The first symptoms appear as slight vein clearing on the outer and younger leaflets (*Fig. 1.17*). Subsequently, the oldest and lowest leaves show yellowing and epinasty caused by drooping of the petioles. Plants infected at the seedling stage usually wilt and die soon. As the disease progresses, growth is typically stunted, and little or no fruit develops. Older plants in the field may wilt and die suddenly, if the infection is severe and if the weather is favorable for the pathogen. However, in older plants, vein clearing and leaf epinasty are more commonly followed by stunting of the plants, yellowing of the lowest leaves, occasional formation of adventitious roots, wilting of leaves and young stems, defoliation, marginal necrosis of the remaining leaves, and finally death of the plant. After an initial period of stunting, the smallest side roots rot. Also fruit may occasionally become infected and then it rots and drops off (Agrios, 2005). If the main stem is cut, dark brown streaks may be seen running lengthwise through the stem. This discoloration often extends far up the stem and is especially noticeable in a petiole scar. The browning of the vascular system is characteristic of the disease and generally can be used for its identification.

Disease development

The disease development depends on the plant growth stage. In young plants, the disease development is fast and leads to early death. The pathogen is soilborne and remains for up to ten years in infested soils where it survives as mycelium and in all its spore forms in infected plant debris in the soil; as chlamydospores, it can survive for many years in the soil. In contaminated soil, the germ tube of spores or the mycelium penetrates root tips directly or enters the roots through wounds or at the point of formation of lateral roots. The mycelium travels through the vessels toward the stem and crown of the plant; it spread throughout the plant by the vascular system. In the vessels, the mycelium branches and produces microconidia, which are detached, carried upward in the sap stream and then germinate. When the leaves transpire more water than the roots and stem can transport to them, the stomata close, the leaves wilt and finally die, followed by death of the rest of the plant. The fungus then invades all tissues of the plant extensively, reaches the surface of the dead plant, and there sporulates profusely.

The spores may be disseminated to new plants or air by wind or water (Agrios, 2005). Dissemination of the pathogen is via seed, tomato stakes, soil, and infected transplants or infested soil adhering to transplants. Local dissemination is by transplants, tomato stakes, windborne and waterborne infested soil, and farm machinery.

The optimal temperatures for development of Fusarium wilt in tomato are between 27 and 30^{o}C. For this reason the disease developes rather in the summer (Ponti and Laffi, 1985). Too warm (34°C) or too cool (17-20°C) soils retard wilt development. If soil temperatures are optimum but air temperatures below optimum, the pathogen will extend into the lowest parts of the stem, but without exhibiting external symptoms. In general, factors favoring wilt development are: soil and air temperatures of 28°C, soil moisture optimum for plant growth, plants pre-conditioned with low nitrogen and phosphorus and high potassium, low soil pH, short day length, and low light intensity. Virulence of the pathogen is enhanced by micronutrients, phosphorus, and ammonium nitrogen and decreased by nitrate nitrogen.

Control

Use of tomato varieties resistant to the fungus and solarization of field soil by covering with transparent plastic film are the most practical measures for controlling the disease in the field. Soil sterilization should be always practiced also for greenhouse-grown tomato plants. Use of healthy seed and transplants is mandatory, and hot-water treatment of seed should precede planting (Agrios, 2005). The seed can be treated also with benzimidazole fungicides. Crop rotation of 4-5 years is recommended (Ponti and Laffi, 1985). Recently, biological control of Fusarium wilt seems to be successful. Such control methods include prior inoculation of plants with nonpathogenic strains of *F. oxysporum* or the use of antagonistic fungi, such as *Trichoderma* and *Gliocladium*, *Pseudomonas fluorescens* and *Burkholderia cepacia* bacteria. It was shown that spraying tomato plants with a suspension of zoospores of the oomycete *Phytophthora cryptogea* induces systemic acquired resistance. Although promising, none of these methods have been used for control of Fusarium wilt in practice so far (Agrios, 2005).

1.2.2.1.3 *Fusarium oxysporum* f.sp. *melonis*

Fusarium wilt [*Fusarium oxysporum* Schlecht f.sp. *melonis* Snyder and Hansen. (*Fom)*] is one of the most devasting and widely distributed diseases of melon both under greenhouse and field conditions (*Fig. 1.18*). It can attack the plant at any developmental stage.

Fig. 1.18: Melon plants damaged by Fusarium wilt

The pathogen

The fungus produces pink or purplish mycelium and three types of asexual spores: macroconidia, microconidia, and chlamydospores (*Fig. 1.19*). Conidia are produced on monophialiades and in sporodochia, and are scattered loosely over the surface of a mycelium. Microconidia are predominantly uninucleate and germinate poorly and variably. The macroconidia are produced abundantly, are multinucleate and germinate rapidly, thereby reproducing the fungus efficiently. Chlamydospores are viables, asexually produced accessory spores resulting from the structural modification of a vegetative hyphal segment or conidia cell possessing a thick wall. Its function is primarily survival in soil.

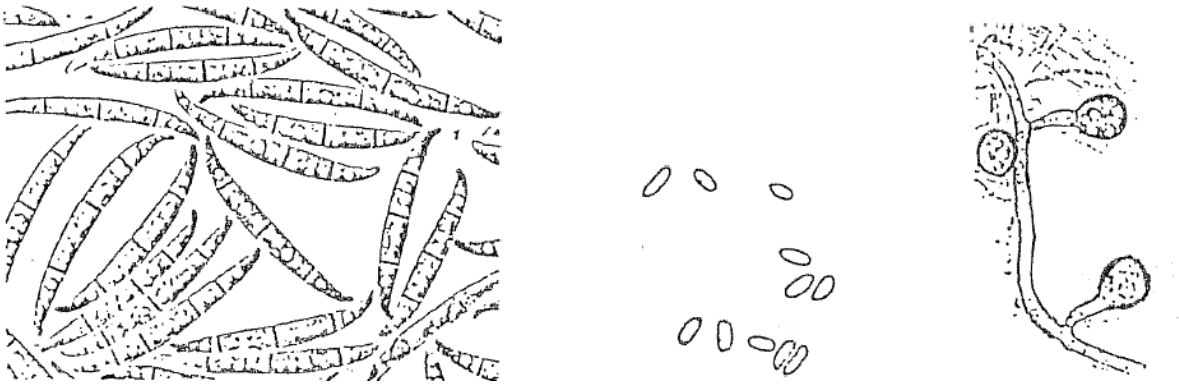


Fig. 1.19: Three kinds of spores of *F. oxysporum* f. sp. *melonis*

Four fysiological races of this fungus are known as 0, 1, 2, and 1.2 (Risser *et al.*, 1976) based on the interaction between two major genes of resistance of the host and variants of the pathogen. The dominant resistance genes *Fom-1* and *Fom-2* provide resistance to *Fom* races 0 and 2, and to races 0 and 1, respectively. Race 1.2 of *Fom* is able to overcome these two resistance genes, and was classified into pathotype Y, which causes symptoms of yellowing, and pathotype W, which causes wilt symptoms.

Symptoms

The first symptoms, leaf-yellowing and wilting, appear before flowering. In some cases, wilting proceeds so fast that the leaves conserve a part of the chlorophyl that gives them a grey-greenish colour. When the stem of diseased plant is cut horizontaly, the browning of the vascular system is visible (*Fig. 1.20*). Usually, yellowing proceeds slowly starting from

the nerves. The brown-orange layer of exudates covering the stems is the most characteristic symptom. Fruit rot usually begins at leaf-stalk. On the dead plants or gravely damaged plants, the white-pink mould can be observed (Ponti and Laffi, 1985).

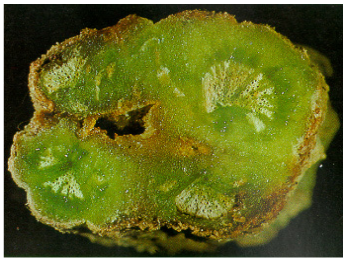


Fig. 1.20: Browning of melon vascular system caused by Fusarium wilt

Disease development

The fungus survives in the soil mainly as chlamydospores, which can remain vital up to ten years. It can be transmitted also by infected seeds. Propagules can be moved from field to field in soil on farm machinery, infected crop debris, and irrigation water.

In optimal conditions (temperatures about 20°C), the fungus penetrates into the roots through microlesions. In the xylem vascular tissues, the fungus grows fast and the first symptoms are visible in 10-15 days (Ponti and Laffi, 1985; Ferrari, 1998).

Control

Use of resistant varieties, when available, must be preferred. Until now many sources of resistance to *Fom* races 0, 1, and 2 have been reported (Alvarez *et al.*, 2005), but the same does not occur for race 1.2. Only partial resistance to race 1.2 of *Fom* have been found in several accessions (Risser and Rode 1973; Perchepied and Pitrat 2004). These genotypes allowed breeding for partially resistant lines to *Fom* race 1.2, such as 'Isabelle' and, recently, two double-haploid lines 'Nad-1' and 'Nad-2', which show higher resistance than other genotypes (Ficcadenti *et al.,* 2002). However, Herman and Perl-Treves (2007) found recently that, in genotype BIZ, two complementary recessive genes are required to obtain full resistance to race 1.2 of *Fom.* Herman and Perl-Treves (2007) demonstrated that alleles encoding for *Fom* race 1.2 resistance may behave as dominant, co-dominant, or recessive depending on the environment conditions.

Apart use of resistant varieties, also grafting results as an efficient measure to control this fungus. In any case, seed treatment with benzimidazole is recommended, as well as soil disinfestation. The lesions on the roots should be avoided during transplanting (Ponti and Laffi, 1985). Dong and Cohen (2001) showed that dry mycelium of *Penicillium chrysogenum* is an inducer of resistance against *F. oxysporum* f.sp. *melonis.* Also movement field to field by washing equipment has to be minimized.

1.2.2.2 *Phytophthora* spp.

Phytophthora spp. belong to Oomycetes, Peronosporales. This important genus contains many species causing destructive diseases on different types of plants ranging from seedlings of annual vegetables or ornamentals to fruit and forest trees. Most species cause root rots, damping-off of seedlings, and rots of lower stems, tubers, and corms similar to those caused by *Pythium* spp. Others cause rots of buds or fruits, and some cause blights of the foliage, young twigs, and fruit. Some species attack only one or two species of host plants, but others may cause similar or different symptoms on many different kinds of host plants. The best known species is *Phytophthora infestans*, the causal agent of late blight of potatoes and tomatoes, but several other species also cause extremely destructive diseases on their hosts. *Phytophthora cactorum*, *P. cambivora*, *P. cinnamoni*, *P. citrophthora*, *P. fragariae*, *P. palmivora*, and *P. syringae* cause primarily root and lower stem rots, but also some cankers, twig blights, and fruit rots of woody ornamentals and of fruit and forest trees as well as of vegetables and other herbaceous plants. Several other species, such as *P. capsici*, *P. cryptogea*, *P. megasperma*, and *P. parasitica*, cause root, stem, and fruit rots of many vegetables, ornamentals, and field crops, but also of some woody plants (Agrios, 2005).

The life history of *Phytophthora* species differs in detail from that of most *Pythium* species. The most notable difference between the two genera is that zoospores of *Phytophthora* species are delimited and functional within the sporangium, whereas in *Pythium* species, zoospores are formed from the cytoplasm of sporangia that have migrated into a vesicle. *Phytophthora* species also have indeterminate sporangia, but many species have very differentiated sporangiophores or sporangia. For example, *P. infestans* produces sympodially branched sporangiophores, which have swollen nodes, and produce lemon-shaped, papillate sporangia. Some *Phytophthora* species produce haustoria unlike closely related *Pythium* species (Ament, Trigiano, 2004). Sporangia, formed successively on sporangiophores, slender, sparsely branched hyphae emerging from stomata, germinate either by a germ tube or by zoospores. The sexual spore is an oospore (Horst, 2008).

The oomycete requires two mating types for sexual reproduction. When the two mating types grow adjacently, the female hypha grows through the young antheridium (male reproductive cell) and develops into a globose oogonium (female reproductive cell) above the antheridium. The antheridium then fertilizes the oogonium, which develops into a thick-walled and hardy oospore. Oospores germinate by means of a germ tube that produces a sporangium, although at times the germ tube grows directly into the mycelium (Agrios, 2005).

1.2.2.2.1 *Phytophthora capsici*

P. capsici Leon. is an oomycete plant pathogen that infects a wide host range that including cucumber, squash, melon, pumpkin, pepper, tomato, eggplant (Roberts *et al.*, 2004).

On pepper, the disease was first reported in New Mexico, USA in 1922 (Leonian, 1922). The disease is now distributed worldwide throughout North and South America, Europe, and Asia. Extensive losses of pepper occur during an epidemic (Roberts *et al.*, 2004).

The pathogen

P. capsici is reproduced asexually by producing ellipsoid to pyriform sporangia with papilla (*Fig. 1.21*). The sporangium is usually oblong, average 30-60 µm. It is nearly spherical to pyriform (pear shape), hyaline (colorless), papillate (pointed at the tip), deciduous (spores fall from the colony) and have a long pedicel (stalk) attached to the base of the spore., zoospores. In water, sporangia form and release several (15-25) spores called zoospores that are flagellated and are chemotactically attracted to plant and root exudates. Sporangia also germinate directly by producing several germ tubes that begin new fungal colonies. Hyphae are fairly coarse and irregularly branched.

Fig. 1.21: Sporangia and spores of *P. capsici*

P. capsici is heterothallic and has two mating types (A1 and A2). Both mating types are needed for the abundant production of sexual spores called oospores. These are thick walled and results from the pairing of an oogonium with an antheridium. Some species of *Phytophthora* are induced to form oospores when the mating type of another species is present.

The pathogen does not produce chlamydospores (Roberts *et al.*, 2004). It grows well between 25 and 30 C^{o}C.

Symptoms

The pathogen is capable of infecting all parts of the pepper plant (*Fig. 1.22*). It causes seedling death, crown and stem lesions, root rot, leaf blight and fruit rot. Stem lesions,

Fig. 1.22: Pepper plant damaged by Phytophthora blight

whether at the soil line or higher, initially appear first dark green and water-soaked, but then turn dark brown to black and girdle the stem. Infected roots are dark brown and rotten.

Leaf blights begin as small, irregular to round shaped and water-soaked areas on the underside of leaves, which rapidly enlarge. Later, the lesion becomes dry, papery, and turns a light tan to grayish brown. During warm, wet weather, infected areas may be bordered by white fungal growth. Entire branches may wilt and die from infection at the forked part of stems. Pepper fruit are frequently infected through wounds. The fruit tissue becomes dark, shrunken, and white fungal growth of mycelium and sporangia is apparent on its surface. Infected fruit remain attached to the stem (Roberts *et al.*, 2004). Symptoms on squash are similar; green leaf lesions spreading over the blade, a basal stem rot, and wilting (Horst, 2008).

The pathogen moves into stems from infected leaves and sections of the plant that are killed. Rots which develop at the soil line or affect major branches cause the plant to wilt or die. Root rots can be severe and stunt the plant or cause plant decline. Fruit rots are irregular in shape and olive green or light green with water soaked borders. Rots expand rapidly and fruits can be completely diseased and desiccated, causing the formation of "mummified" fruits. Infected seeds are brown and shriveled.

Disease development

Phytophthora capsici survives on plant debris and in the soil as oospores, mycelium, sporangia or zoospores. Phytophthora blight is a polycyclic disease in which the pathogen reproduces and infects new plants several times within a single season. Epidemics of pepper fields are caused by *P. capsici* during wet weather. Environmental conditions such as water temperature, nutrition, pH, and other factors determine the length of time in which zoospores continue to swim. Encysted zoospores germinate by producing a thin fungal hypha or thread. The germ tube commonly penetrates the leaf through stomates which are natural opening in the leaf epidermis. Larger germ tubes produced by sporangia also penetrate the leaves.

In pepper fields, the fungus is soil borne and initial infections of roots, collars, and lower leaves occur. The fungus grows within the host and produces sporangia on the surface of diseased tissue, especially leaves. Sporangia are spread by splashing water from irrigation or rain. With moisture present, zoospores are formed and released. Released zoospores swim in a film of water for a few minutes to more than an hour to reach host tissue before encysting. Periodic flooding and saturated soil conditions from either rainfall or irrigation stimulates the release of zoospores from sporangia (Bowers and Mitchell, 1990). The

pathogen and its sporangia are dispersed by splashing rain and wind or overhead irrigation (Bowers *et al.,* 1990, Ristaino, 1991). Sporangia are also moved within the field by contact with field equipment, clothing, gloves, tools, etc. Movement of soil from one field to another on equipment or boots will move this pathogen. Dissemination is also through infected transplants and contaminated soil and equipment. Root to root contact spreads the disease. Wet soil and high temperatures encourage blight. The application of water made frequently through drip irrigation causes earlier onset of the disease compared to applications of drip irrigation made less frequently (Ristaino, 1991; Ristaino *et al.,* 1993; Horst, 2008). Optimum temperature for growth of *P. capsici* is about 27^{o}C (Cristinzio, 1993).

Control

The control of this oomycete is difficult due to its great adaptability on many different hosts. In addition, it has developed a resistance against some systemic fungicides such as Metalaxyl. Therefore, it is necessary to applicate an integral disease management such as crop rotation with non-susceptible crops rather than exclusive use of chemical fungicides (Cristinzio, 1993) to reduce the amount of *P. capsici* surviving in soil. Disinfested equipment and fungicide-treated seed as well as sterile potting media should be used to establish healthy transplants. Fields should be well drained without volunteer cucurbit and solanaceous plants. Soil should be fumigated before planting. The use of black plastic mulch can be used as a barrier to dispersal of inoculum (Ristaino *et al.,* 1997). Fungicides, both contact and systemic, should be applied to the foliage and used preventively. However, some isolates are insensitive to mefenoxam. Soil solarization may reduce populations of the pathogen (Yucel, 1995). Soil amendments, such as compost and manure, are a possibility for disease management, as they can develop suppressive soils. Resistant varieties should be planted, where available (Roberts *et al.*, 2004). Some cultivars are more tolerant, and better resistance to *P. capsici* is being developed in pepper.

1.2.2.2.2 *Phytophthora cactorum*

Crown rot, caused by *Phytophthora cactorum* (Leber *et* Cohn) Schroeter, is an important strawberry disease in Europe and other temperate to subtropical regions. First identified on cacti, this oomycete is capable of infecting an extremely large number of hosts and is problematic in low-lying or wet field conditions. It can limit production for many economically important crops such as strawberry (*Fig. 1.23*), apple, pear, rhododendron, and azalea. The disease is apparently caused by a distinct pathotype of *P. cactorum* (Maas, 2004).

P. cactorum can cause root, collar, and crown rots, as well as foliar and fruit infections. The fungus causes also increased culling of seedlings in forest nurseries (Lilja *et al.*, 2006).

Fig. 1.23: Strawberry affected by *P. cactorum* crown rot

The pathogen

P. cactorum forms a white mycelium. Sporangia are distinctively papillate, and are usually borne terminally. Sporangial shapes vary widely, and can be broadly ellipsoidal, obpyriform, ovoid or spherical (Ellis, 1998; Erwin and Ribeiro, 1996). A distinctive characteristic of this pathogen, compared to other *Phytophthora*, is represented by the caducous sporangia with pedicels less than 40 µm. Each sporangium may contain more than 50 zoospores. Chlamydospores are generally terminal but may occasionally be intercalary. *P. cactorum* is homothallic, and oospores can be found on the plant debris. All antheridia are paragynous and are generally spherical or club-shaped. Oogonia are usually hyaline and smooth-walled, and oospores are plerotic (*Fig. 1.24*; Ellis, 1998).

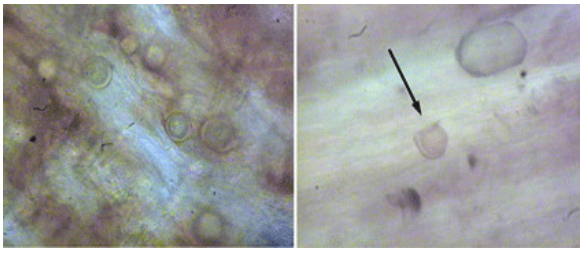


Fig. 1.24: Oospores of *P. cactorum* in infected strawberry root tissue

Symptoms

Although *P. cactorum* can parasitize a wide range of plant species, pathogenicity may vary widely across hosts. In general, this pathogen can cause a number of symptoms and diseases depending on the host. In strawberry, *P. cactorum* causes crown and root rot, and fruit leather rot (*Fig. 1.25*).

Fig. 1.25: Fruit leather rot caused by *P. cactorum*

Symptoms typically develop during early-mid summer, when the disese often appears in the field as a sudden wilting or collapse of the plants. The youngest leaves turn bluish-green. When lifted, diseased plants may easily break at the crown. Wilting will quickly spread throughout the plant, leading to plant death. Extensive internal dark-brown crown necrosis and vascular disintegration are characteristics of this disease. *P. cactorum* crown rot symptoms are most evident at the basal stolon attachment area or at the crown. Roots typically are not infected but die when the upper

part of the plant dies. Plants that do not succumb and appear to recover remain stunted and unproductive (Pettitt and Pegg, 1994; Maas, 2004).

Disease development

The most important propagule for this pathogen is represented by the zoospores, which originate from hyphae or germinating oospores and sporangia (*Fig. 1.26*). Oospores in soil or in strawberry plant debris provide initial sources of inoculum. The oospores germinate to produce zoosporangia and zoospores which infect plants, usually at wound sites. In many cases, this pathogen may enter a field through infected transplants.

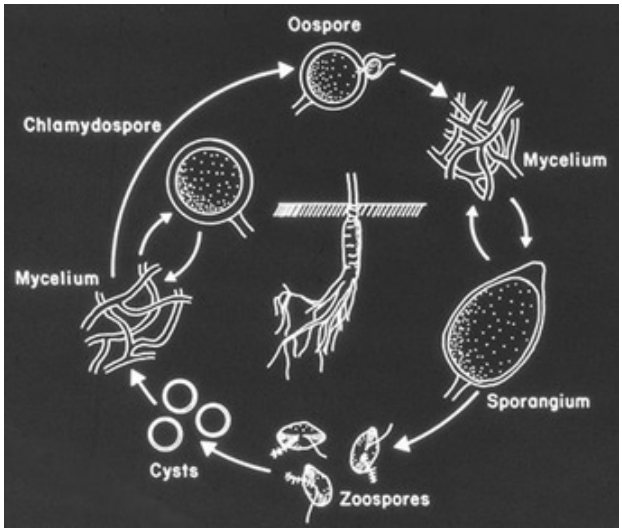


Fig. 1.26: Life cycle of *P. cactorum*

The fungus requires warm temperatures and prolonged wetness periods for inoculum production and for infection development. Motile zoospores are released from sporangia during saturated soil conditions and enter through wounds (Seemuller, 1998). Once the zoospore reaches a host, it infects and developing hyphae of the fungus colonize the host.

Crowns damaged by frost injury prior to plant harvesting or by low temperatures during cold storage are predisposed to infection (Bell *et al.,* 1997; Pettitt and Pegg, 1994). Planting time also affects symptom development; plantings established in spring to early summer are more quickly affected than those established in mid-summer. Disease development is accelerated by high temperatures and water stress (Molot and Nourrisseau 1974; Lederer and Seemüller, 1992). Low temperatures delay disease progression, but does not halt it. Infections may become latent as temperatures become lower, especially during cold storage (Maas, 2004).

Control

An integrated approach is the most effective way to reduce damages caused by *P. cactorum*. Prevention and sanitation are extremely important as this pathogen is often introduced through infected propagative material (*Fig. 1.27*).

Fig. 1.27: Phytophthora blight in production of strawberry plugs: (a) plug production (b) plugs infected with *P. cactorum,* (c) in-line chemigation.

Cultural control may be beneficial as adequate soil drainage and low soil pH may help to reduce disease. In general, site and soil conditions should be managed to ensure rapid water run-off and prevention of soil water-logging. Planting in low, wet areas should be avoided as fields that have a history of crown rot disease. Healthy runner plants should be used for transplantation (Maas, 2004). Cultivars vary in their resistance to crown rot, but the degree of resistance is slight and depend on predisposing factors and resistance screening methods (Bell *et al.,* 1997; Hancock *et al.,* 1990). Systemic fungicides such as metalaxyl can be used as a preventive measure. Also mefenoxam is recommended for strawberry (Louws, 2004). However, there is the risk of development of the resistant strains of *P. cactorum* to fungicides. Biological control with either *Enterobacter aerogenes* or *Trichoderma* has been successful as well (Erwin and Ribeiro, 1996).

1.2.2.3 *Pythium* spp.

Pythium sp. is a genus of parasitic oomycete and one of the most common and most important causes of seed rot, seedling damping-off, and root rot of all types of plants, and also of soft rots of fleshy fruits in contact with the soil. *Pythium* damping off is a very common problem in fields and greenhouses, where the organism kills newly emerged seedlings (*Fig. 1.28*). The greatest damage, is done during germination on seed and seedling roots either before or after emergence. This disease complex usually involves other pathogens such as *Phytophthora* and *Rhizoctonia*. Pythium wilt is caused by zoospore infection of older plants leading to biotrophic infections that become necrotrophic in response to colonization/reinfection pressures or environmental stress. Crop rotation alone is often not capable of eradicating the pathogen.

Fig. 1.28: Seedlings affected by *Pythium* sp.

In field crops, damages by *Pythium* spp. are often limited to the area affected, as the motile zoospores require ample surface water to travel long distances. Additionally, the capillaries formed by soil particles act as a natural filter and effectively trap many zoospores. However, in hydroponic systems inside greenhouses, where extensive monocultures of plants are maintained in plant nutrient solution that is continuously recirculated to the crop, *Pythium* spp. cause extensive and devastating root rot and is often difficult to prevent or control. The root rot affects entire operations within two to four days due to the inherent nature of hydroponic systems where roots are exposed to the water medium, in which the zoospores can move freely.

Losses vary considerably with soil moisture, temperature, and other factors. Older plants develop root and stem lesions and root rots; their growth may be retarded considerably (Agrios, 2005). The genus *Pythium* is best known for its saprotrophic soilinhabiting members, many of which are opportunistic pathogens especially in young plants. There are also obligately pathogenic *Pythium* spp. Many *Pythium* species, along with their close relatives, *Phytophthora* species are plant pathogens of economic importance in agriculture. *Pythium* spp. tend to be very generalistic and unspecific in their host range. Generally, *Pythium* spp. parasitizes a wider diversity of hosts than *Phytophthora* (Webster and Weber, 2007).

The pathogen

Pythium produces a white and rapidly growing mycelium, usually characterized by production of coenocytic hyphae with sporangia. Sporangia germinate directly or by a short hypha with a balloon-like secondary sporangium called a vesicle containing 100 or more zoospores. When released, they form a cyst, and then germinate by producing a germ tube. The germ tube penetrates the host tissue and starts a new infection.

Mycelium also gives rise to spherical oogonia (*Fig. 1.29*) and club-shaped antheridia. Oospores serve as survival stage of the fungus. The type of germination of both sporangia and oospores is determined primarily by the temperature; those above 18°C favor germination by germ tubes, whereas temperatures between 10 and 18°C induce germination by zoospores.

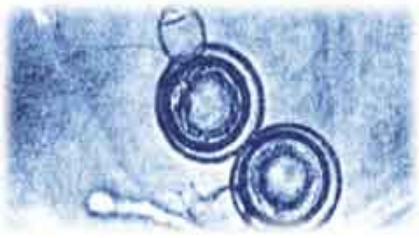


Fig. 1.29: Oogonia produced by *Pythium* spp.

Pythium species occur in surface waters and soils throughout the world. They live on dead plant and animal materials as saprophytes or as parasites of fibrous roots of plants. The pathogen needs free water for its zoospores to swim and infect (Agrios, 2005).

Symptoms

When seeds are attacked by *Pythium*, they fail to germinate, become soft and mushy, and then turn brown, shrivel, and finally disintegrate. Young seedlings can be attacked before emergence, the invaded cells collapse, and the seedling dies (pre-emergence damping-off). Seedlings that have already emerged are usually attacked at the roots and sometimes in the stems or below the soil line. The invaded areas become water soaked and discolored and they soon collapse. The basal part of the seedling stem becomes softer and thinner and at the end the seedling falls over on the soil. The fungus continues to invade the fallen seedling, which quickly withers and dies (postemergence damping-off) (Agrios, 2005).

Symptoms include black discolorations on roots (*Fig. 1.30*). The entire root often darken, look unhealthy, and lack new growth. Foliage is reduced in size, has poor color, and wilts in warm weather. Although many roots rot away completely, they lack the red core discoloration typical of red stele.

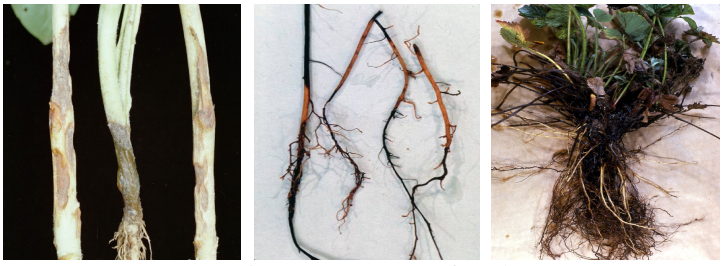


Fig. 1.30: Disease symptoms of *Pythium* sp.

Development of Disease

The fungus enters the seeds or seedling tissues by direct penetration. Enzymes secreted by the oomycete macerate the tissue and then the pathogen grows among and through the cells. As a result, infected seeds and young seedlings are killed and turn into a rotten mass. When the invasion of *Pythium* is limited to the cortex of the belowground stem of the seedling, it can still live for a short time before it falls over and dies. If the infection occurs in well developed seedlings, the advance of the oomycete is stopped and only small lesions develop. Rootlets can be attacked at any stage of plant growth. Invasion of older roots is usually limited to the cortex. *Pythium* can also infect fleshy vegetable fruits and other organs in the field or in storage. Infections of the fruit begin at the point of contact with wet infested soil. As the infection progresses, sporangia begin to appear, followed by the production of oospores, inside and outside the host tissues (*Fig. 1.31*; Agrios, 2005).

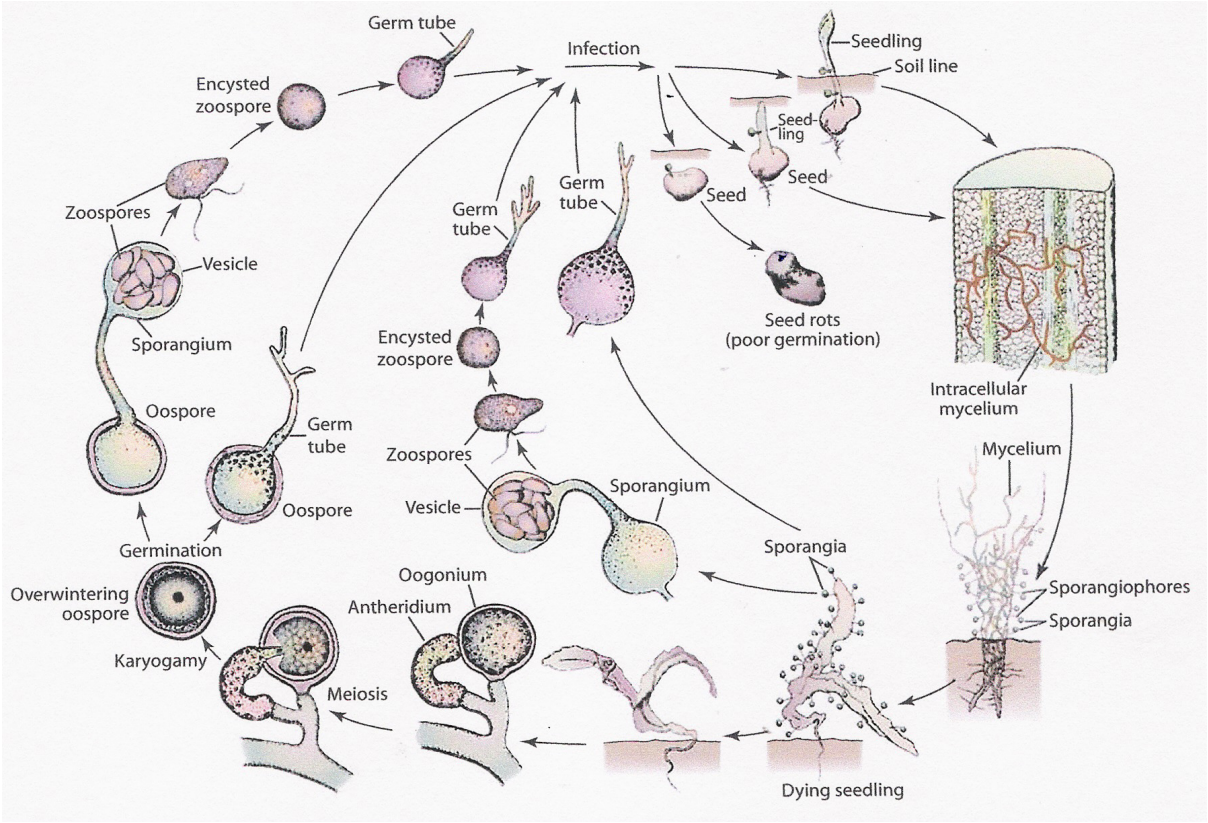


Fig. 1.31 : Disease cycle of damping-off and seed decay caused by *Pythium* spp. (from Agrios, 2005)

Control

So far, no commercial varieties of plants resistant to *Pythium* are available (Agrios, 2005). Management of *Pythium* diseases is primarily carried out through cultural practices and fungicides, although some biocontrol tactics are used to control some oomycetes species. Good soil drainage and management of soil moisture are important cultural practices used to control diseases caused by *Pythium* species in both field and greenhouse situations. Some other practices such as crop rotation and avoiding excess of nitrogen fertilization can be effective. Seeds may be treated with captan or other contact fungicides to prevent root rots. Plants may be drenched or sprayed with one of several systemic acylalanine fungicides (metalaxyl or mefenoxam) either to prevent or control infection (Trigiano *et al.*, 2004).

1.2.2.4 *Monosporascus cannonballus*

Monosporascus cannonballus Pollack & Uecker is a soilborne, root infecting Ascomycete causing root rot and vine decline in melons and watermelons and less commonly in other members of the *Cucurbitaceae* family.

The disease occurs in areas with semiarid climate, high summer temperatures, and saline and alkaline soils. Such areas include for example the southwestern United States, north Africa, Spain, Israel, Iran, India and Japan. The disease affects primarily muskmelon and watermelon. It appears as a root rot and a sudden collapse causing successively death of the plants in the field (*Fig. 1.32*). Losses in melon producing areas infested with *M. cannonballus* fluctuate from year to year from 10 to 25% of the crop, but the crop may be also destroyed completely (Agrios, 2005).

Fig. 1.32: Melon field: healthy (left), destroyed by M. cannonballus (right)

M. cannonballus has only recently emerged as important problem in melon production, but is thought to have been present for many years. Proper identification of the pathogen and shifts in cultural production of melons, such as changing from overhead to drip irrigation and planting of hybrid cultivars, has resulted in a high importance of this disease (Martyn, 2002).

The Pathogen

Root rot and vine decline of melons is caused by *M. cannonballus* and by another related species, *M. eutypoides*, that seems to be responsible for the disease in southeast Asia.

The fungus produces dark spherical perithecia (*Fig. 1.33*) that contain 200 or more asci each, but each ascus contains only one, spherical, cannonball-like ascospore (*Fig. 1.34*). This represents the unique feature of the fungus. The fungus does not have an imperfect stage, i.e. it does not produce conidia (Agrios, 2005).

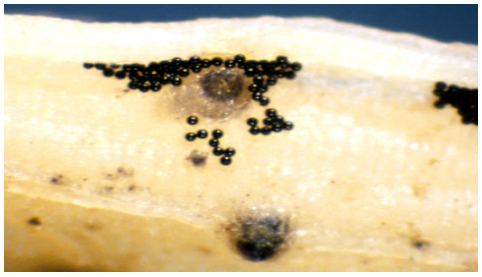


Fig. 1.33: Dark spherical perithecia of the fungus

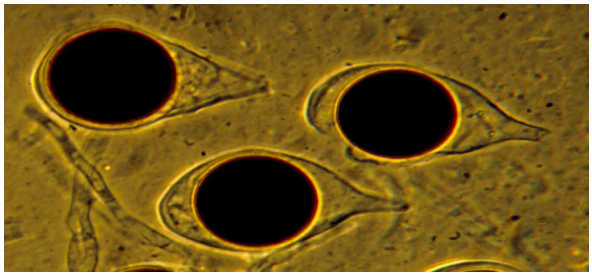


Fig. 1. 34: Immature ascospores inside the asci of *M. cannonballus*

Symptoms

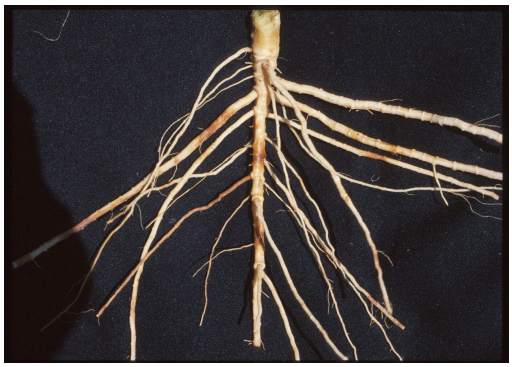


Fig. 1.35: Lesions on melon root caused *by M. cannonballus*

The aboveground symptoms of the disease appear as stunting, yellowing, and necrosis of the leaves in the innest parts of the crown. This is followed by progressive necrosis of the leaves until about 10 to 14 days before harvesting, when the entire canopy of the crop collapses. The fruit, exposed to intense solar radiation due to callapsed leaves, fails to ripe properly. The roots of affected plants show lesions (*Fig. 1.35*), especially at root junctions, the feeder and secondary roots decay and slough off. Some roots may have numerous perithecia embedded in the root cortex (Agrios, 2005; Martyn and Miller, 1996).

Disease development

The fungus survives in the soil as ascospores within or without perithecia. In the vicinity of melon roots, ascospores germinate and penetrate the roots; infection may occur through direct action of the mycelium. At this point, the plant begin to show signs of water stress, yellowing and wilting of leaves, and may collapse and die suddenly. Infections of larger roots result in the formation of perithecia in the root cortex (*Fig. 1.36*) and the appearance of swellings on the root surface, which turn black and finally the ascospores are released in the soil.

Fig.1.36: Perithecia of *M. cannonballus*

Initial infection is believed to occur early in the season. Both tissue colonization and perithecia formation in the roots are encouraged as the soil temperature rises during the production season. When disturbed, the perithecia will release ascospores.

Dissemination of *M. cannonballus* is unknown. It is likely that it is spread by movement of infested soil or infected plant material. Ascospores may also be moved via furrow water or heavy rains. Airborne spread is unlikely due to the large ascospores. Vegetative mycelium is effective at inhabiting decaying tissue but it will not survive even moderate desiccation.

The fungus grows better at high temperatures (30–35°C) (Agrios, 2005). The progression of symptoms is affected by a variety's fruit load and stage of maturity (Martyn and Miller, 1996a). It is presumed that *Monosporascus* root rot and vine decline is a

monocyclic disease (Martyn and Miller, 1996b), since no known asexual (anamorph) stage has never been identified.

Control

Management of *M. cannonballus* has proven to be difficult due to its heat tolerance, the thick-walled resting structures (ascospores), the growing list of host plants and the lack both of genetic resistance in melons and common cultural practices such as drip irrigation and black plastic mulch that favor the pathogen and disease development (Martyn, 2002).

There are no effective controls against the melon root rot and vine decline. A combination of resistant varieties with grafting, soil fumigation, application of fungicides may reduce the disease. Biological control is possible in the laboratory but not so far in the field (Agrios, 2005). Soil fumigation with methyl bromide has been the standard for controlling *M. cannonballus*. However with the phase-out of methyl bromide, alternatives have been examined (Cohen *et al.*, 2000;.Koike *et al.*, 2007). Compared to fumigation, fungicide treatments are often effective and less expensive. Post-plant chemigation with fluidoxonil or thiophanate-methyl, applied through buried drip irrigation beginning at plant emergence has shown to be effective for the control of Monosporascus root rot and vine decline. Crop rotation with non-susceptible hosts should be a standard for control of the disease.

Integrated Pathogen Management represents a great option for Monosporascus root rot and vine decline. It includes manipulation of the structure of the root system to promote a larger, more prolific system that can help the plant overcome wilting. This can be achieved through forms of irrigation encouraging wide root systems, such as overhead or furrow irrigation (however this may favour spread of ascospores), as well as with direct seeding. Traditional soil solarization is not effective for this pathogen because of its extreme heat tolerance. However, if it is combined with reduced rates of fumigation there is potentiality for the control. Also, reduction of build up of inoculum (ascospores) in the soil by pulling roots out of the ground directly after final harvest or destroying the roots with a fumigant can be effective for the control of the fungus (*Fig. 1.37*).

Fig. 1.37: Removal of vines

1.3 BIOLOGICAL CONTROL OF FUNGAL PLANT PATHOGENS

Beyond good agronomic and horticultural practices, growers often rely heavily on chemical fertilizers and pesticides. Such inputs to agriculture have contributed significantly to the improvement in crop productivity and quality over the past 100 years. However, environmental pollution caused by excessive use of agrochemicals has led to considerable changes in people's attitudes towards the use of pesticides in agriculture. Efforts have been focused on developing alternative inputs to synthetic chemicals for controlling diseases and pests. One of these alternatives is represented by the biological control.

In plant pathology, the term "biological control" or "biocontrol applies to the use of microbial antagonists to suppress diseases but, more broadly, the same term is also applied to the use of the natural products extracted or fermented from various sources. These formulations may be represented by very simple mixtures of natural ingredients with specific activities or complex mixtures with multiple effects on the host as well as on the target pathogen. Most broadly, if growers' activities are considered relevant, cultural practices such as the use of rotations and planting of disease resistant cultivars could be also included in the definition.

Plants produce a remarkably diverse array of over 100,000 low-molecular-mass natural products (secondary metabolites), many of which have evolved to confer selective advantage against microbial attack. Secondary metabolites are distinct from the components of intermediary (primary) metabolism in that they are generally nonessential for the basic metabolic processes. This rich diversity (*Fig. 1.38*) results in part from an evolutionary process driven by selection for acquisition of improved defence against microbial attack or insect/animal predation.

Natural products such as the plant-derived ones, e.g. plant extracts and essential oils, and antagonistic microorganisms as a method of biological control have recently acquired a great scientific interest. This is due to toxicity problems such as water contamination, persistence of residues both in the environment and in the food or accumulation in animal and human fat tissues, and to the resistance of parasites caused by chemical fungicides (Banerji *et al.*, 1985; Butt *et al.*, 2001). In some cases, other effective methods of control are not available and chemical pesticides are banned (e.g. organochlorines) or being phased out (e.g. methyl bromide). Consumer perceptions worldwide are that chemical usage in agricultural

production needs to be significantly reduced. In order to satisfy this demand, biological control strategies, especially for the growing organic market, are urgently required (Butt *et al.*, 2001).

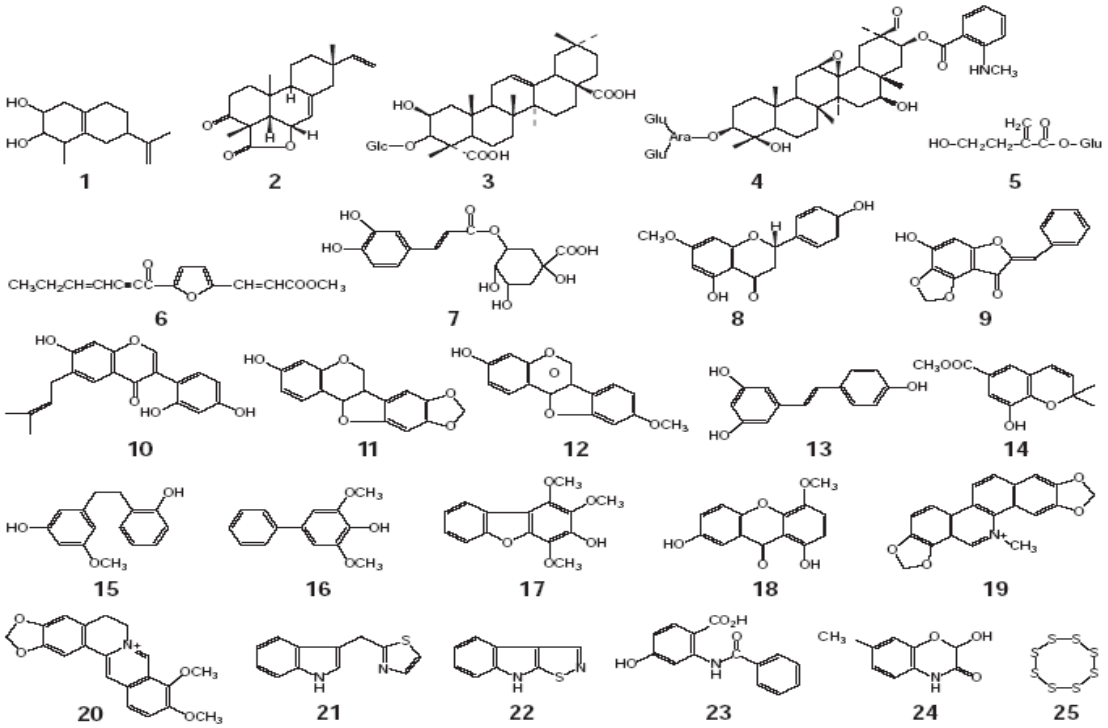


Fig. 1.38: Chemical diversity of constitutive and inducible antimicrobial plant natural products. The chemical class of the compound is followed in parentheses by the trivial name (if available), a selected species of origin, and an indication as to whether the compound is produced constitutively (c) or is inducible (I). TERPENOIDS: **1.** sesquiterpene (rishtin, *Nicotiana tabacum*, I); **2.** diterpene (momilactone A, *Oryza sativa*, I); **3.** saponin (medicagenic acid 3-0-glucoside, *Dolichos kilimandscharicus,* C); **4.** saponin (avenacin A, *Avena sativa*, C). ALIPHATIC ACID DERIVATIVES: **5.** butyrolactone precursor (tuliposide A, *Tulipa* spp., C.); **6.** furanoacetylene (wyerone, *Vicia faba*, I). PHENOLICS AND PHENYLPROPANOIDS: **7.** hydroxycinnamic acid ester (chlorogenic acid, *Nicotiana tabacum*, C); **8.** flavanone (sakuranetin, *Ribes nigrum*, C; *Oryza sativa*, I); **9.** aurone (*Cephalocereus seniis*, I); **10.** isoflavone (luteone, *Lupinus albus*, C); **11.** pterocarpan (maackiain, *Cicer arietinum*, I); **12.** pterocarpan (medicarpin, *Medicago sativa*, I); **13.** stilbene (resveratrol, *Vitis viniferis*, I); **14.** chromene (*Piper aduncum*, C); **15.** bibenzyl (batatasin IV, *Dioscorea batatas*, C); **16.** biphenyl (aucuparin, *Malus pumila*, I); **17.** benzofuran (*Cotoneaster* spp., I); **18.** xanthone (*Polygala nyikensis*, C). NITROGEN-AND/OR SULPHUR-CONTAINING COMPOUNDS: **19.** benzophenanthridine alkaloid (sanguinarine, *Papaver bracteatum*, I); **20.** benzylisoquinoline alkaloid (berberine, *Berberis* spp., I); **21.** indole (camalexin, *Arabidopsis thaliana*, I); **22.** indole (brassilexin, *Brassica* spp., I); **23.** anthranilamide (*Dianthus caryophyllus*, I); **24.** benzoxazinone (DIMBOA, *Zea mays*, C); **25.** elementhal sulphur (*Theobroma cacao*, I). (from Dixon, 2001)

Most of the naturally active substances show fungistatic as well as fungitoxic characteristics. Due to their rich chemical composition, it is difficult to identify exactly which component has the antifungal effect. In practice, numerous components have similar fungicidal characteristics, so the spectrum of their activity against different fungal species can be judged to be a synergistic effect (Pepeljnjak *et al*., 2003).

Recent advances in molecular technology, aided by the enormous power of large-scale genomics initiatives, are leading to a more complete understanding of the enzymatic machinery that underlies the often complex pathways of plant natural product biosynthesis. Meanwhile, genetic and reverse genetic approaches are providing evidence for the importance of natural products in host defence. Metabolic engineering of natural product pathways is now a feasible strategy for enhancement of plant disease resistance.

1.3.1 PLANT-DERIVED PRODUCTS

Although many plant-derived products are used in the traditional medicine, they are supposed to be efficient also in plant disease control. In fact, historically, plant products have a determinant role in the fields of agriculture and food preservation. References from Egypt, Greece, and China, more than 4,000 years ago, noted the protective effects of inorganic materials such as iodine, arsenic, and ashes, along with that of organic products, such as tannins and vinegar. Also reports from the fourth century B.C. to the sixteenth century in Asia and Europe enumerate many plant species with properties useful for combating pests (Banerji *et al.*, 1985; Hostettman and Lea, 1987; Mendes, 1989). It is reported that about 60 percent of the essential oils obtained from plants possess antifungal activity (Suresh *et al.*, 1997).

Recently many studies have appeared describing antifungal compounds isolated from various plants and tested *in vitro*. However, only few of these compounds have been tested for crop-disease management under field conditions.

Wild marjoram (*Origanum syriacum* Sieb. Exs. Et. L.) extracts completely inhibited the mycelial growth and gave nearly complete inhibition of spore germination of *Botrytis cinerea*, *Alternaria solani*, *Penicillium* sp., *Cladosporium* sp., *Fusarium oxysporum* f.sp. *melonis* and *Verticillium dahliae*. Moreover, the petroleum ether extracts showed higher efficacy for the control of fungi than the methanolic extracts and inhibition of spore germination was higher than inhibition of mycelial growth. Other plants were also found to have antifungal activities such as mint (*Mentha longifolia* (L.) Huds.), sage (*Salvia fruticosa* L.), wild chicory (*Cichorium intybus* L.), inula (*Inula viscosa* (L.) Ait.), centaury (*Centaurea pallescens* Del.), eryngo (*Eryngium creticum* L.), fennel (*Foeniculum vulgare* Mill.), Chinaberry tree (*Melia azedarach* L.) (*Abou-Jawdah et al.*, 2002).

The fungistatic and fungicidal effect of powders, and aqueous and ethanolic extracts of seeds and leaves of huamuchil (*Pithecellobium dulce*) was shown against *Botrytis cinerea*, *Penicillium digitatum*, and *Rhizopus stolonifer* on strawberry fruit. Powder application on strawberry fruit compared with aqueous extracts had a better fungicidal effect, since powders were not subjected to an extraction process; powders may contain also various and different active compounds that might be acting all together. Moreover, kaempferol may be responsible for the fungicidal effect (Bautista-Baños *et al.*, 2003). Also the essential oil extracted from *Thymus vulgaris* L. showed efficiency against *Botrytis cinerea* and *Rhizopus stolonifer* on strawberry fruit (Bhaskara Reddy *et al.*, 1997).

The methanol extract of stems of *Catalpa ovata* G. Don., containing an active compound dehydro-α-lapachone, exhibited potent *in vivo* antifungal activity against *Magnaporthe grisea* on rice plants, *Botrytis cinerea* and *Phytophthora infestans* on tomato plants, *Puccinia recondita* on wheat plants and *Blumeria graminis* f.sp. *hordei* on barley plants (Cho *et al.*, 2006).

An antifungal activity against *Botrytis cinerea* was demonstrated *in vitro* by plant extracts of garlic (*Allium* spp.) and pepper (*Capsicum* spp.), essential oils of palmarosa (*Cymbopogon martini*) and red thyme (*Thymus zygis*), cinnamon leaf (*Cinnamomum zeylanicum*), and clove buds (*Eugenia caryophyllata*). The most frequently costituents of essential oils showing antifungal activity were: D-limonene, cineole, β-myrcene, α-pinene, β-pinene and camphor (Wilson *et al.*, 1997). The antifungal effect of garlic extract was observed also against *Alternaria brassisicola*, *Botrytis cinerea*, *Plectosphaerella cucumerina*, *Magnaporthe grisea*, and *Phytophthora infestans* (Curtis *et al.*, 2004). *Cymbopogon martini* oil showed antifungal activity *in vitro* also against *Pyricularia oryzae, Drechslera oryzae, Rhizoctonia solani, Colleotrichum lindemuthianum, Colleotrichum capsici, Macrophomina phaseolina, Alternaria alternata, Phylostica* sp*., Pestalotia theae, Curvularia lunata, Fusarium oxysporum, Granaria uvicola, Sclerotinia sclerotiorum, Mysosphaerella* sp*., Aspergillus niger, Aspergillus flavus, Botryodiplodia theobromae, Cercospora nicotiana* and *Phoma* sp. (Sridhar *et al.*, 2003).

Rosemary and lavender leaf extracts were found to be effective in reducing germination of *Phytophthora capsici*, *P. megakarya*, and *P. palmivora* zoospores causing black pod disease of cacao (Widmer and Laurent, 2006).

The essential oils from leaves and stems of *Orthosiphon stamineus* Benth., their methanol extract and derived fractions of methanol extract displayed a great potential of antifungal activity as a mycelial growth inhibition against phytopathogenic fungi such as *Botrytis cinerea*, *Rizoctonia solani*, *Fusarium solani*, *Colleotrichum capsici*, and *Phytophthora capsici* (Hossain *et al.*, 2008).


Argemone mexicana

Aqueous extract of *Argemone mexicana* L., when applied in soil, greatly suppressed root-infecting fungi in tomato such as *Fusarium solani*, *Rhizoctonia solani*, and *Macrophomina phaseolina* (Siddiqui *et al.*, 2002).

The crude extract of seeds of *Lupinus mexicanus* containing alcaloid lipanine showed inhibitory activity of the mycelial growth of *Rizoctonia solani* and *Sclerotium rolfsii* (Zamora-Natera *et al.*, 2008).

Hexane and ethyl acetate phases of the methanol extract of *Macaranga monandra* showed fungal growth inhibition of *Colleotrichum aculatum, C. fragariae, C. gleosporoides, Fusarium oxysporum, Botrytis cinerea, Phomopsis obscurans* and *P. viticola* (Salah *et al*., 2003).

The essential oils from *Arnica longifolia*, *Aster hisperius* and *Chrysothamnus nauseosus* and their pure compounds, carvacrol and β-bisabolol, as well as the essential oils from fruit, leaves, stems and roots of *Scaligeria tripartita* (Kalen.) Tamamsch showed antifungal activity against the strawbery anthracnose-causing fungal plant pathogens *Colleotrichum acutatum, C. fragariae and C. gleosporoides* (Tabanca *et al.*, 2006, 2007).

A dichlormethane and a methanol extract of the liverwort *Bazzania trilobata* (L.) S.F. Gray showed antifungal activity against *Botrytis cinerea, Cladosporium cucumerinum, Phytophthora infestans, Pyricularia oryzae* and *Septoria tritici* (Scher *et al.*, 2004).

A novel quinolone alcaloid isolated from *Ruta graveolens* L. leaves was highly active against *Botrytis cinerea* and *Phomopsis* spp. (Oliva *et al.*, 2003).

Essential oil from *Ocimum gratissimum* L., containing eugenol as main compound, inhibited growth of *Botryosphaeria rhodina, Rhizoctonia* sp. and *Alternaria* sp. (Faria *et al*., 2006).

A novel antifungal compound, fistulosin (octadecyl 3-hydroxyindole), isolated from roots of Welsh onion *(Allium fistulosum)* showed high activity against *Fusarium oxysporum,* primarily inhibiting protein synthesis (*Phay et al.*, 1999).

Leaf extracts of *Annona cherimola, Bromelia hemisphaerica,* and *Carica papaya* inhibited sporulation and reduced fruit rot infection and development of *Rhizopus stolonifer,* the pathogen of "circula" fruit *(Spondias purpurea)* rot (Bautista-Baños *et al.*, 2000).

Garlic juice and grapefruit juice (BioSept) effectively controlled rose powdery mildew (*Sphaerotheca pannosa* var. *rosae*), and their efficacy was equal to that of the standard fungicide triforine (Wojdyla, 2000).

Commercial formulations of various plant extracts, such as pepper/mustard (chilli pepper extract and the essential oil of mustard), cassia (extract of cassia tree), clover (70 percent clove oil), and neem (90 percent neem oil) showed their efficacy to control muskmelon wilt caused by *Fusarium oxysporum* f. sp. *melonis* (Bowers and Locke, 2000).

Other studies showed the efficacy of *Decalepis hamiltonii* (Wight&Arn) extracts against important phytopathogenic fungi causing diseases in sorghum, maize and paddy such as *Fusarium* spp., *Drechslera* spp., *Aspergilus* spp., *Penicillium* spp. and *Alternaria alternata*. Among different solvent extracts tested, petroleum ether extracts showed the highest antifungal activity. *In vitro* antifungal activity assay revealed that up to the minimal inhibitory concentration for the respective fungi, the active principle is fungistatic, whereas at higher concentrations it is fungicidal. It was also observed that the mycelium treated with active compound (2-hydroxy-4-methoxybenzaldehyde) showed curved nature and was generally fragmented. In addition, the treated seeds have shown marked improvement in germination and seed vigour at the concentration of 1.000 $\mu g \cdot ml^{-1}$, when the compound is fungicidal and not phytotoxic to the plants. Moreover, the active compound resulted more efficient than synthetic fungicides in controlling the growth of *Penicilium* spp. (Mohana *et al.*, 2008).

The extracts of *Deterium microcarpum* containing saponins, tannins, alkaloids, flavonoids and balsams were effective against some fungal pathogens such as *Fusarium oxysporum*, *Aspergillus niger* and *Penicillium digitatum* (Doughari e Nuya, 2008).

Sisti *et al.* (2007) successfully tested a crude methanolic extract from micropropagated shoots of *Rubus ulmifolius* Schott against 37 potentially pathogenic fungi such as *Alternaria* sp., *Aspergillus* spp., *Botrytis* sp., *Drechslera* sp., *Fusarium* sp., *Penicillium* sp., *Rhizopus* sp., *Trichoderma* sp., *Ulocladium* sp. and *Verticillium* sp.. The phenolic fractions of the extract rich in tannins resulted active against fungal strains tested, whereas those containing chlorogenic, caftaric acid, and caffeoyl derivates resulted partially effective.

Three antifungal lignans isolated from methanol extract of nuttmeg tree [*Myristica fragrans* (Houttyn)] showed activity against *Alternaria alternata, Colleotrichum coccodes, C. gleosporoides* and *Magnaporthe grisea*. The antifungal activity varied according to compound and target species. Under *in vivo* conditions, all the lignans effectively suppressed the development of rice blast and leaf rust. In addition, some of the lignans were highly active

also against the development of barley powdery mildew and tomato late blight. They also moderately inhibited the development of rice sheath blight (Cho *et al.*, 2007).

Park *et al.* (2008) tested methanol extracts from 27 medicinal plant species for their *in vivo* fungicidal activities against six pathogenic fungi. Very strong fungicidal activity was produced by extracts of *Boswellia carterii, Saussurea lappa, Glycyrhizza uralensis, Piper nigrum, Rheum coreanum, Lysimachia foenum-graecum, Evodia officinalis, Santalum album* and *Curcuma longa* at the concentration of 2 mg/ml. At 1 mg/ml, *S. Album, P. nigrum* and *L. foenum-graecum* showed potent activity against *Blumeria graminis* f.sp. *hordei, Puccinia recondita* and *Magnaporthe grisea*, respectively. *L. foenum-graecum* exhibited strong antifungal activity against *M. grisea* at 0.5 mg/l.

The methanol extract of *Annemarrhena aphodeloides* Bunge rhizomes exhibited strong antifungal activity against *Magnaporthe grisea, Rhizoctonia solani* and *Phytophthora capsici*. Its active compound, nyasol, effectively inhibited mycelial growth of *Colleotrichum orbiculare, P. capsici, Pythium ultimum, R. solani* and *Cladosporium cucumerinum* in a range of 1-50 µg/ml. In addition, treatment with nyasol was significantly effective in suppressing *Phytophthora* blight on pepper plants in greenhouse test (Park *et al.*, 2003).

Hwang *et al.* (2005) have evaluated the antifungal effect of wood vinegar of *Cryptomeria japonica* sapwood and its constituents against *Phytophthora capsici, Fusarium oxysporum* and *Pythium splendens*. The wood vinegar constituents such as phenols and guaiacols had a strong antimicrobial effect, but methanol and acetic acid exhibited little or no microbial activity.

The aqueous extracts of the leaves of paw-paw (*Carica papaya*) and bitter leaf (*Vernonia amygdalina*) revealed that, in the field conditions, they can be used as a bio-fungicides against foliar fungal pathogens of groundnut (*Arachis hypogaea* L.) such as cercospora leaf spots and rust, web blight caused by *Phoma arachidicola*, botrytis blight (Botrytis cinerea) and pepper spot (*Leptosphearrulina crassiaca*). However, some combinations of the extracts were toxic to the seeds of groundnut (Ogwulumba *et al.*, 2008). Linear growth, mycelium dry weight and sporulation of *Fusarium moniliforme*, isolated from groundnut seeds, was significantly reduced by chloroform extracts of *Azadirachta indica* leaves. A seed treatments with *A. indica* extracts to evaluate its effect on *in vitro* germination of seeds revealed that it was comparable with NaOCl treatments. Therefore, the *A. indica* leaf extracts may be acceptable substitutes for treating groundnut seeds before planting (Wokoma and Nwaejike, 2008).

Taiga *et al.* (2008) observed by *in vitro* tests the fungicidal properties of the extracts of five plants (*Aloe barbadensis, Azadirachta indica, Nicotiana tabacum, Tridax precubens* and *Carica papaya*) against growth of *Fusarium oxysporum* mycelium.

Aqueous extracts of leaves of *Moringa oleifera* Lam, *Vernonia amygdalina* and *Annona muricata* had significant inhibitory growth effect in controlling *Colleotrichum destructivum* on cowpea (*Vigna uniguculata*) seeds; extracts of *Nicotiana tabacum* leaves significantly controlled the growth of the pathogen *in vitro*. In addition, *M. oleifera* was compared favorably with a systemic fungicide benomyl in the control of the pathogen. However, the extracts of *Ricinus communis* at higher concentration acted as growth promoter to the pathogen; its mycelium had a better sporulation and fluffiness than that of the controlled plate (Akinbode and Ikotun, 2008).

The percent mycelial inhibition with essential oils of three *Cymbopogon* spp. on *Fusarium solani*, *F. moniliforme*, *Curvularia lunata, Exserohilum rostratum, Phoma sorgina, Colleotrichum graminicola, Bipolaris sorokiniana* and *Acremonium strictum* were similar or better to those obtained with the chemical control. Moreover, these essential oils reduced significantly sorghum and pearl millet seed contamination by selected funghi. The lowest rates of infected seeds were generally recorded on seeds treated with 10 µl and/or 15 µl of essential oil per g of seeds (Zida *et al.*, 2008).

Crude ethanol extracts from *Vincetoxicum rossicum* (Kleopow) Barbar. roots and fruits significantly inhibited the growth of *Fusarium* spp., *Botrytis cinerea*, *Alternaria alternata*, *Sclerotinia sclerotiorum* and *Verticillium dahliae*. In addition, an inhibitor compound (-)-antofine was identified through bioassay-guided fractionation of crude extract (Mogg *et al.*, 2008).

Ross *et al.* (2008) investigated an antifungal activity of some seagrasses. Whole plant tissues from *Thalassia testudinum, Halodule wrightii* and *Syringodium filiforme* prevented *in vitro* overgrowth of *Fusarium s*pp.. Moreover, the crude extract of *Ruppia maritima* exhibited the highest antifungal activity against *Fusarium* spp. These seagrases appear to use a combined strategy to combat fungal infection, including microbial chemical defences and signalling pathways observed in terrestrial plants. A plant-derived chemical, coumarin (1,2-Benzopyrone), with antifungal properties was found in various plants such as clover, sweet woodruff and grasses. The coumarins are highly active group of molecules with a wide range of antimicrobial activity against both fungi and bacteria. Halogenated coumarin derivates work very effectively to inhibit *in vitro* fungal growth of *Macrophomina phaseolina* (charcoal rot), *Phytophthora* spp. (damping off and seedling rot), *Rhizoctonia* spp. (damping off and

root rot) and *Pythium* spp. (seedling blight). In addition, these compounds might be used also for seed treatments to prevent fungal diseases (Brooker *et al.*, 2008).

Cold and hot extracts of *Azadirachta indica, Tridax precumbens, Carica papaya, Nicotiana tabacum* and *Aloe vera* were found to possess fungicidal properties against dry rot disease pathogens (*Fusarium oxysporum, Aspergillus niger, Rhizopus stolonifer* and *Penicilium oxalicum*) of yam tubers (*Dioscorea rotundata*) *in vitro*. Hot extract of *N. tabacum* was found as the most efficacious among the extracts (Taiga and Oufolaji, 2008).

Aspergilus flavus and *A. niger* were found to be sensitive against the crude methanol and flavonoid extracts of *Marchantia polymorpha* (Mewari and Kumar, 2008).

Cupressus benthamii and *Vetiveria zizanioides* extracts were effective in sporangial germination and disease reduction of *Phytophthora infestans* both in laboratory and greenhouse experiments (Goufo *et al.*, 2008).

Extracts of *Pinus roxburghii* and *Ranunculus* sp. inhibited *in vitro* conidial germination of *Botrytis elliptica* (Berk.) causing blight of lilium cultivars (Sharma and Dhancholia, 2008).

Acetone extracts from different parts of seven common invasive plant species occuring in South Africa (*Cestrum laevigatum, Nicotiana glauca, Solanum mauritianum, Lantana camara, Datura stramonium, Ricinus communis, Campuloclinium macrocephalum*) were studied as potential sources of antifungal agents for selected phytopathogenic fungi. All extracts exhibited antifungal activity. In addition, extracts of different parts of the same plant had varying degrees of inhibition. Generally, leaf extracts were more active than seed or flower extracts. Growth of *A. niger, Penicillium expansum* and *R. solani* was the most sensitive to all extracts tested. *L. camara* acetone leaf extract could be a viable option for controlling *F. oxysporum*. *C. macrocephallum* showed a high activity against *C. gleosporoides* and *F. oxysporum* (Mdee *et al.*, 2009).

Protease inhibitors isolated from *Acacia plumosa* Lowe seeds inhibited growth of *A. niger, Thielaviopsis paradoxa* and *Colleotrichum* sp.. Therefore, these potent antifungal agents may be of great interest as specific inhibitors to regulate proteolytic processes in fungi (Lopes *et al.*, 2009).

Methanolic extracts of *Barringtonia racemosa* L. showed excellent inhibitory activity against *Fusarium* sp., *Trichoderma kongii*, *Penicillium* sp., *Ganoderma tropicum*, *Ganoderma lucidum*, *Aspergillus* sp. and *Rhizopus* sp. in comparison with ethanolic and boiling water extracts. Phenolic acids and flavonoids were identified as active compounds (Hussin *et al.*, 2009).

The CH_2Cl_2 and MeOH extracts from leaves of *Piper caldense* containing caldensinic acid showed an antifungal activity against *Cladosporium cladosporoides* and *C. sphaerospermum* (Freitas *et al.*, 2009).

In the study of Anand and Bhaskaran (2009), the leaf extracts (10%) of *Abrus precatorius* (Gundumuthu) and *Aegle marmelos* (vilvum) demonstrated a high inhibition of spore germination and mycelial growth of *Colleotrichum capsici* and *Alternaria alternata*, the causal agents of fruit rot disease of chilli. In addition, the extract of *A. precatorius* was effective also in the pot culture experiment. However, it was not as efficient as the synthetic fungicide (carbendazim 0.1%).

An extract of *Yucca schidigera* showed similar efficency as resistance inducer [acibenzolar-S-methyl (ASM)] and sulphur to control an apple scab pathogen, *Venturia inequalis*, in seedling assays. Whereas yucca and sulphur gave significant inhibition of conidial germination *in vitro*, ASM did not inhibit germination. Histopathological studies showed that yucca extract primarily acted by inhibiting pre/penetration events and penetration itself. Therefore, yucca extracts probably acted by a direct fungitoxic effect. However, expression studies of two genes encoding the PR proteins, PR1 and PR8, in apple seedlings indicated that yucca extract may also affect plant defence as expression of both genes was up-regulated following yucca treatment (Bengtsson *et al.*, 2009).

Aqueous extracts of *Acacia nilotica, Achras zapota, Datura stramonium, Emblica officinalis, Eucalyptus globules, Lawsonia inermis, Mimusops elengi, Peltophorum pterocarpum, Polyathia longifolia, Prosopis juliflora, Punica granatum* and *Syringium cumini* have recorded antifungal activities against different *Fusarium* spp. (Satish *et al.*, 2009).

A leaf extract of *Datura metel* protected pear millet (*Pennisetum glaucum*) plants against downey mildew disease caused by *Sclerospora graminicola*. In addition, resistance provided by *D. metel* extract was demonstrated to be a systemic aquired resistance and was active at both early and later stages of plant growth (Devaiah *et al.*, 2009).

Dry cabbage (*Brassica oleracea*), garlic (*Allium sativum* L.) and alfaalfa (*Medicago sativa* L.) materials were effective in reducing the severity of disease caused by *P. capsici*, bothunder *in vitro* and *in vivo* conditions (Demirci and Solar, 2006).

Methanolic crude extracts from *Dolichos kilimandscharicus* and *Maerua subcordata* roots as well as *Phytolacca dodecandra* berries were screened *in vitro* for antifungal activity against *B. cinerea, F. oxysporum, Sclerotiorum rolfsii, Rhizoctonia solani, Botryosphaeria dothidea* and *Pythium ultimum*. *D. kilimandscharicus* showed the highest broad-spectrum antifungal activity inhibiting mycelial growth of *B. cinerea, B. dothidea* and *P. ultimum*. In

addition, its mycelial growth inhibitory activity was also confrontable with synthetic fungicides (Tegegne and Pretorius, 2007).

1.3.1.1 Novel plants for extracts to control fungal plant pathogens

New resources of plant-derived products are continuously being searched. Plant species that are tested for their antifungal properties are often those who have demonstrated their antifungal or antimicrobial properties in indigenous medicine and are supposed to contain some active compounds. Therefore, these plants are often traditionally used by local people but they are not grown intensively.

1.3.1.1.1 *Boerhavia* spp.

Boerhavia spp. is distributed in the tropical and subtropical regions of the Old and New World, in both hemispheres. Many species are characteristic of coastal habitats. The genus includes *B. coccinea* Mill., *B. erecta* L., *B. repens* L., *B. glabrata* Bl., *B. hualienense* Chen & Wu (Chen and Wu, 2007), *B. intermedia* M.E. Jones, *B. spicata* Choisy, *B. torreyana* S. Wats, and *B. wrightii* A. Gray (Spellenberg, 2000).


Boerhavia spp.

Boerhavia diffusa L. (*Nyctaginaceae*) is a shrub growing prostrate or ascending in habitats like grasslands, fields, fallow lands, wastelands or it is used as an ornamental plant. Medicinal attributes of the plant are found mainly in its roots, which are stout, fusiform and penetrating deeply in the soil, where it perennates (Singh, 2007). *B. diffusa* is known to have medicinal properties and its ethnobotanical use has a long history (Awasthi and Verma, 2006). It has digestive, diuretic, anti-infiammatory and antioxidant properties (Singh, 2007; Gacche and Dhole, 2006; Satheesh and Pari, 2003). In addition, Ashawat *et al.* (2006) reported photo protective activity of ethanolic extract of *B. diffusa*. Methanol extract from the roots exhibited spasmolytic activity (Borrelli *et al.*, 2006). *B. diffusa* contain also saponins, tannins, alkaloids, flavonoids and phenol glycosides (Ujowundu *et al.*, 2008; Maurya *et al.*, 2007; Edeoga and Ikem, 2002). In addition, it is rich in vitamins C, B3 and B2 and in some minerals such as Na, Ca and Mg (Ujowundu *et al.*, 2008). Chloroform extracts of the *B. diffusa* roots has shown to have antifungal properties (Agrawal and Srivastava, 2008) as well as the ether, ethyl acetate, ethyl alcohol and aqueous extract of aerial and root parts (Agrawal *et al.*, 2003; Agrawal *et al.*, 2004). Aqueous and ethanolic

extracts possess antibacterial activity (Adeyemi *et al.*, 2008; Sahni *et al.*, 2008; Aladesanmi *et al.*, 2007).

However, only few studies of *B. diffusa* extract efficiency in plant pathogen control have been made. Roots of *B. diffusa* contain basal proteins, which show high inhibitory activity against plant viruses. Moreover, root extract of this plant induce strong systemic resistance against TMV infection in tobacco susceptible host plant. In addition, such induced resistance can be further transfered in *in vitro* tobacco plants (Lohani *et al*., 2007). Also, *B. diffusa* leaf extract used as seed treatment reduced disease incidence of Bean Common Mosaic Virus (BCMV) under screenhouse and field conditions (Prasad *et al.*, 2007).

Recentlty, the RAPD markers has been used for the study of *Boerhavia* populations and association of the markers with leaf characteristics, flower colour and geographical locations has been made (Shukla *et al.*, 2003). In addition, Roy (2008) developed a shoot regeneration protocol for rapid multiplication of *B. diffusa* through *in vitro* culture of shoot tip and nodal explants. Such innovative processes in cultivating *B. diffusa* may support a wider diffusion of this plant species in crop protection use.

1.3.1.1.2 *Cordia* spp.

The genus *Cordia* (*Boraginaceae*) is diffused mainly in Central and South America and in Africa. The genus includes *C. leucocephalla* Moric, *C. curassavica* (Jacq) Roemer & Schultes, *C. gilettii* De Wild, *C. boissieri* A. DC., *C. bifurcata* Roem. & Schult., *C. inermis* (Mill.) I.M. Johnst., *C. pringlei* B.L. Rob., *C. piauhiensis* Fresenius., *C. leucomalloides* Taroda., *C. africana* Lam., *C. alliodora* (Ruiz & Pav.) Cham., *C. globosa* (Jacq.) Kunth and *C. latifolia* Roxb..

Many of the species are used traditionally in indigenous medicine, e.g. *C. curassavica* is used for treatment of gastrointestinal, respiratory and dermatological disorders (Hernandez *et al.*, 2006); *C. gilletii* root bark is traditionally used in Congo against malaria, diarrhea, wounds and skin diseases due to its antimicrobial and antioxidant properties (Kambu, 1990), that were studied by Okusa *et al.* (2007) on the extracts of root barks powder with n-hexane, dichlormethane, ethyl acetate, methanol and water. Other studies on the antibacterial activity of *Cordia* spp. have been made (Nakamura *et al.*, 1997; Ioset *et al.*, 1998; Lans *et al.*, 2000). Hernandez *et al.* (2006) have reported also antifungal activity of essential oil and extracts of *C. curassavica* against *Rhizoctonia solani*, *Aspergillus niger* and *Fusarium* spp.

Some active compounds have been isolated from genus *Cordia* such as pyrrolizidine alkaloids (Wassel *et al.*, 1087), terpenoids (Kuroyanagi *et al.*, 2003), flavonoids, lignans and

meroterpenoids naphtoquinones (Ioset *et al.*, 2000). The leaf essential oil of *C. leucocephala* is characterized by high percentages of sesquiterpenes with β-caryophyllene and bicyclogermacrene as main constituents (Diniz *et al.*, 2008). De Oliveira *et al.* (2007) have reported that chemical composition of essential oil of *Cordia* species varies a lot. Siddiqui *et al*,. (2006) isolated new aromatic compounds from the fruits of *C. latifolia*.

Recently, a genus *Cordia* have been studied also at a molecular level, e.g. microsatellite markers have been developed in *C. biffurcata* and *C. pringlei* (Spoon and Kesseli, 2008).

1.3.1.1.3 *Phyllanthus* spp.

The genus *Phyllanthus* (*Euphorbiaceae*) is distributed in tropical and subtropical regions; it is indigenous to India and it is also cultivated in China, Philippines, Cuba, Nigeria, and Guam. *Phyllanthus* spp. have a great diversity of growth forms including annual and perennial herbs, shrubs, climbers, aquatics and succulents plants. In spite of their variety, almost all *Phyllanthus* species express a specific type of growth called "phyllanthoid branching" in which the vertical stems bear deciduous, flower-bearing, plagiotropic stems. The leaves on the main axes are reduced to scales called "cataphylls", while leaves on the other axes develop normally (Webster and Grady, 1994).

The genus includes a large number of species, e.g. *P. niruri* L., *P. amarus* Schum. & Thonn., *P. tennelus* Roxb., *P. caroliniensis* Walt., *P. orbiculatus* L.C. Rich, *P. stipulatus* (Raf) Webster, *P. chacoensis* Morong, *P. klotzschianus* Müll. Arg., *P. claussenii* Müll. Arg., *P. heteradenius* Müll. Arg., *P. acuminatus* Vahl, *P. subemarginatus* Müll. Arg., *P. minutulus* Müll. Arg., *P. hypoleucus* Müll. Arg., *P. juglandifolius* Willd., *P. urinaria* L. and *P. grandyi* M.J. Silva & M.F. Sales.

P. niruri is an annual tropical herb, commonly found in coastal areas. It is wildely used as medicinal plant in Brazil (Sabir and Rocha, 2008). *P. niruri* shows several biological activities such as antioxidant, hepatoprotective, antiviral and antibacterial properties (Sarkar *et al.*, 2008, Shakila and Ponni, 2008; Harish and Shivanandappa, 2006; Thomas *et al.*, 1999). Water disinfection by alkaloid extracts of *P. niruri* shows bactericidal effect (Sunda *et al.*, 2008). The aqueous leaf extract of *Phyllanthus* sp. was effective in reducing the infection by Tomato Mosaic Virus (ToMV) (Deepthi *et al.*, 2007).

The active phytochemicals, flavonoids, alkaloids, terpenoids, lignans, polyphenols, tannins, coumarins and saponins, have been identified in various parts of *P. niruri* (Hassarajani and Mulchandani, 1990; Petchnaree *et al.*, 1986; Murugaiyah and Chan, 2007; Bagalkotkar *et al.*, 2006). Seeds of P. *niruri* contains linoleic, linolenic and ricinoleic acid (Ahmad *et al.*, 1981).

Recently, several studies aimed at imcreasing cultivation of *P. niruri*, for example by improving seedling characteristics like shoot and root length (Shakila and Ponni, 2008); a micropropagation protocol has been developed for rapid production of *P. niruri* plantlets using nodal segments of the mature plants as explants (Ong and Chan, 2006).

1.3.1.1.4 *Siparuna* spp.

Genus *Siparuna* (*Monimiaceae*) is widely spread in the Southern Hemisphere, mainly in tropical and subtropical regions of the Americas. Plants of *Monimiaceae* family are aromatic trees or shrubs bearing essential oils, often lemon scented, with opposite, simple, evergreen leaves (Hutchinson, 1967). The genus *Siparuna* includes 150 species (Philipson, 1993), e.g. *S. guianensis* Aubl., *S. apiosyce* De Candolle, *S. arianeae* V. Pereira, *S. dresslerana* T. Antonio, *S. gilgiana* Aubl., *S. griseo-flavescens* Perk., *S. nicaraguensis* Hemsl., *S. patelliformis* Aubl., *S. pauciflora* A. DC., *S. tonduziana* Perk, and *S. thecaphora* (Poepp. Et Endl.) A. DC.

Some of the species are used by local people in the treatment of gastrointestinal disorders, skin diseases, in the therapy of colds, fever, headache and rheumatism. They have tonic, stimulant, digestive and carminative properties (Peckolt and Peckolt, 1920; Corrêa, 1926, 1978). *S. guianensis* has shown antiprotozoal activity (Tempone *et al.*, 2005).

Siparuna sp. contains flavonoids, alkaloids, essential oils, terpenoids, lignans, and cinnamic acid derivates. The general composition of the essential oils from plants of the family *Monimiaceae* is a mixture of terpenoids and phenylpropanoids (Leitão *et al.*, 1999). Sesquiterpenoids were found in *S. pauciflora* (Jenett-Siems *et al.*, 2003), *S. thecaphora* (Vila *et al.*, 2002), and *S. macrotepala* (El-Seedi *et al.*, 1994). The major components of essential oils of *S. thecaphora* were germacene D, α-pinene, β-pinene, and β-caryophyllene (Ciccio and Gómez-Laurito, 2002). Kaempferol glycosides have been found in *S. apiosyce* (Leitão *et al.*, 2000). Alkaloids of *S. griseo-flavescens* (Lopez *et al.*, 1993), *S. tonduziana* (Lopez *et al.*,

1990), *S. pauciflora* (López *et al.*, 1988), *S. dresslerana*, *S. nicaraguensis*, *S. patelliformis* (Gerard *et al.*, 1986), and *S. gilgiana* (Chiu *et al.*, 1982) have been investigated.

S. guianensis is an important species of rain forest tree comunity in Brazil (Carvalho *et al.*, 2007). The main constituents of *S. guaianensis* leaf oil are decanoic acid, 2-undecanone (Fischer *et al.*, 2005), and epi-α-cadinol (Viana *et al.*, 2002). The fruit oil consists mainly of 2-undecanone, β-pinene, limonene (Fischer *et al.*, 2005), and terpinolene (Viana *et al.*, 2002).


S. guianensis

1.3.1.1.5 *Vitex* spp.

Vitex (*Verbenaceae*) is the genus of shrubs and trees, native of tropical, subtropical and warm temperate regions throughout the world. The genus includes 250 species, e.g. *V. agnus-castus* L., *V. cuneata* Schumach. & Thonn., *V. lucens* T. Kirk, *V. keniensis* Turill., *V. negundo* L., and *V. trifolia* L.

An important member of the genus is a chaste tree (*V. agnus-castus* L.), a shrub with natural habitat ranging from Southern Europe to Middle East (Baytop, 1984). For this species, a wide range of biological activities has been reported, e.g. seed extracts have proved to have repellent properties (Mehlhorn *et al.*, 2005). Fruits, flowers and leaves of *V. agnus-castus* contain phenolic acids and their derivates, flavonoids, tannins, iridoid glycosides, and diterpenoids (Proestos *et al.*, 2006 Saglam *et al.*, 2007; Hajdu *et al.*, 2007; Abel *et al.*, 1994). The main compounds of the essential oil are 1,8-cineole, sabinene, α-pinene, β-phellandrene and α-terpinyl acetate, trans-β-farnesene and bicyclogermacrene (Novak *et al.*, 2005; Sarikurkcu *et al.*, 2009; Zoghbi *et al.*, 1999; Sorensen and Katsiotis, 1999; Galletti *et al.*, 1996). The leaves differed from fruits in the concentrations of almost all compounds. The compositions of mature and immature fruits are equal. Two disticnt chemotypes (an α-pinene chemotype and α-terpinyl acetate chemotype) could be identified in *V. agnus-castus* (Novak *et al.*, 2005). Sarikurkcu *et al.* (2009) reported an excellent antioxidant activity of water extract of *V. agnus-castus* that can be due to the content of flavonoid casticin (Hajdu *et al.*, 2007). Pepeljnjak *et al.* (1996) reported antibacterial and antifungal activities of etheric and ethanolic extracts of *V. agnus-castus*.


V. agnus-castus

Recently, some studies on the innovative propagation methods of this endangered important medicinal plant have been made. Balaraju *et al.* (2008) developed a micropropagation method consisting of *in vitro* shoot induction and plant regeneration from a

mature apical meristem and nodal explants of *V. agnus-castus*. Chamandoosti (2007) studied a plantlet regeneration potential from seedling explants.

1.3.2. ANTAGONISTIC MICROORGANISMS

Mechanisms which biocontrol microorganisms use to weaken or destroy plant pathogens include their ability to parasitize the pathogens directly, production of antibiotics (toxins), their ability to compete for space and nutrients, production of enzymes that attack cell components of the pathogens, and induction of defence responses in the plants they surround. Although thousands of microorganisms have been shown to interfere with the growth of plant pathogens and to provide some protection from the diseases, strains of relatively few microorganisms have been registered and are available commercially for use so far (Agrios, 2005). These biocontrol methods are based on foliar spray, soil treatment, seed dressing, root protection of trees, stump treatment, treatment of pruning wounds and direct spraying of suspensions of natural hyperparasites onto fungal lesions (Vidhyasekaran, 2004).

1.3.2.1 Fungal antagonists

Biocontrol of soil-borne diseases

Coniothyrium minitans, *Gliocladium virens (*syn. *Trichoderma virens), Trichoderma harzianum* and *Trichoderma viride* are currently used as fungal biocontrol agents (BCAs) in soil and root microbiomes.

Coniothyrium minitans is a mycoparasite of sclerotia of *Sclerotinia* sp. Two products containing this BCA are available: Contans WG, in Germany and Switzerland, and KONI, in Hungary. Application must be made several weeks prior to planting crops to allow time for the sclerotia to be destroyed (Whipps and Lumsden, 2001).

The BCA *Gliocladium virens* has appeared on the market in two formulations, GlioGard™ and SoilGard™. These products target damping-off diseases of vegetable and ornamental plant seedlings caused by *Rhizoctonia solani* and *Pythium* spp. Application was confined to greenhouse or interior container use (Lumsden *et al.*,1996).

A commercial formulation of *Trichoderma harzianum* is sold as T-22 Planter Box™. This conidial formulation is designed for application to large-seeded crops such as maize, beans, cotton and soybeans, and can be applied to seeds already treated with fungicides (Harman and Björkman, 1998). Similar products using the same strain include a granular formulation used as a greenhouse soil amendment, which is called RootShield™, and contains

the entire thallus of *T. harzianum* colonized on clay particles (Harman and Björkman, 1998). It is claimed to control root diseases caused by *Fusarium, Rhizoctonia* and *Pythium* spp., but not *Phytophthora* spp. Another *T. harzianum* product available in the Czech Republic and Denmark for glasshouse use is Supresivit. It controls damping-off or root rots of ornamentals and forest-tree seedlings (Whipps and Lumsden, 2001).

Trichoderma viride is available as a BCA in India in a product named Ecofit. It controls root rot, seedling rot, damping-off, collar rot and Fusarium wilt in cotton, chick-pea, pigeonpea, bengal gram, groundnut, sunflower, soybean, tobacco and vegetables (Whipps and Lumsden, 2001).

Biocontrol of diseases of aerial plant parts

Ampelomyces quisqualis, Phlebiopsis (Peniophora) gigantea, Trichoderma harzianum, Trichoderma harzianum + Trichoderma polysporum are currently in use as fungal BCAs in aerial microbiomes.

Ampelomyces quisqualis, formulation AQ10, is the first biocontrol mycoparasite fungus developed specifically for controlling powdery mildew in strawberry, tomato, grape, tree fruit, and ornamentals (Dik *et al.*, 1998).

Phlebiopsis gigantea is a common wood-rotting saprotroph that prevents the colonization of freshly cut stumps of pine by the root-rotting fungus *Heterobasidion annosum*. It competes for the food with the pathogen. Commercial products containing oidia are available in the UK and in Finland as PG Suspension and Rotstop, respectively. *P. gigantea* is also available in other Scandinavian countries and Poland (Pratt *et al.*, 1999).

Strain T39 of *T. harzianum* has been used for greenhouse control of *Botrytis cinerea*. It is marketed as Trichodex™ in Europe and Israel. The strategy for best control involves alternating chemical and biological control treatments (Elad *et al.*, 1993).

A combination of *T. harzianum* and *T. polysporum*, sold as BINAB-T, is one of the oldest commercial biopesticide preparations still available. In Sweden and Denmark, it is used largely for the control of grey mould (*B. cinerea*) on strawberries and for the control of soil-borne pathogens. In Chile, it is used for the suppression of silver-leaf disease (*Chondrostereum purpureum*) and chlorotic leaf curl (*Eutypa*) in stone fruit and grapes, respectively (Whipps and Lumsden, 2001).

Biocontrol of post-harvest diseases

The yeasts *Candida oleophila* and *Cryptococcus albidus* are used to control postharvest diseases. Aspire™, a biocontrol product containing *C. oleophila* is registered in the USA and in Israel. The product is used to reduce rot diseases, green and blue mould, caused by *Penicillium digitatum* and *Penicillium italicum*, respectively. It is also efficacious against sour rot, caused by *Geotrichum candidum* (Wilson *et al.*, 1993; Droby *et al.*, 1998). *C. albidus* is used on pome fruits, especially apples and pears, against grey and blue mould caused by *B. cinerea* and *Penicillium expansum*, respectively (Whipps and Lumsden, 2001).

1.3.2.2 Bacterial Antagonists

Biocontrol of soil-borne diseases

Crown gall of pome, stone, and several small fruits (grapes, raspberries) and ornamentals caused by *Agrobacterium tumefaciens* can be controlled by nonpathogenic *Agrobacterium radiobacter* which produces a specific antibiotic. It is available as a commercial product Galltroll. Treatment of seeds such as cereals, sweet corn, and carrots with *Bacillus subtilis* or *Streptomyces* sp. has protected the plants against root pathogens (Agrios, 2005). In addition, *B. subtilis* induces resistance by activation of defence genes (Kilian *et al.*, 2000).

Pseudomonas rhizobacteria applied to seeds and roots of plants have resulted in less damping-off and soft rot, and in consistent increases in growth and yield in several crops. They are available as commercial products Kodiak and Dagger G. The most common soilborne diseases controlled by soilborne bacteria are damping-off and root rot diseases caused by the oomycetes *Pythium* and *Phytophthora* and by fungi *Rhizoctonia*, *Fusarium*, and *Gaeumannomyces* (Agrios, 2005).

Biocontrol of diseases of aerial plant parts

Numerous bacteria, most of them saprophytic gramnegative bacteria of the genera *Erwinia*, *Pseudomonas*, and *Xanthomonas* and a few of the gram-positive genera *Bacillus*, *Lactobacillus*, and *Corynebacterium*, are found on aerial plant surfaces. Some pathogenic bacteria, such as *Pseudomonas syringae* pv. *syringae*, *P. syringae* pv. *morsprunorum*, *P. syringae* pv. *glycinea*, *Erwinia amylovora*, and *E. carotovora*, also live on the surface of leaves before they infect and cause disease. In several cases, spraying leaf surfaces with preparations of saprophytic bacteria or with avirulent strains of pathogenic bacteria has

reduced considerably the number of infections caused by bacterial and fungal pathogens. However, none of these biological controls is used in practice to control any disease of aerial plant parts so far (Agrios, 2005).

Biocontrol of postharvest diseases

Pseudomonas bacteria protected lemons from Penicillium green mold and pear from various storage rots. *Pseudomonas syringae* is available as a commercial product Bio-Save. *Bacillus subtilis* protected the stone fruit from the brown rot caused by *Monilinia fructicola* (Agrios, 2005).

1.4 SCREENING METHODS OF ANTIFUNGAL ACTIVITY

Standardized screening methods are essential for successful identification of antifungal compounds. The methods should be rapid and should allow to examinate a large number of spores or detect any growth activity of mycelium. A preliminary screening should be made under *in vitro* conditions in laboratory. This type of bioassays enables to identify any potential antifungal activity of the tested extracts, and give the preliminary information on the efficient concentration and possible modes of action of the biofungicide. The next step consists in verifying the effectiveness of the novel biofungicide under in *in vivo* conditions.

Essential in all assays are the use of controls, comparison to commercial fungicides and also the use of different concentration of extracts (Uldahl and Knutsen, 2009). Nevertheless, screening methods can be in general influenced by various factors, e.g. chemical reactivity and purity of the tested extract, time, stage of growth of the fungus or plant, change in pH, oxidation of an active compound, medium composition, nature of environment, and scale of bioassay (Paxton, 1991).

1.4.1 LABORATORY SCREENING METHODS

Vegetatively growing cultures exposed to biological extracts are generally used in bioassays with fungi. The minimal inhibitory concentration (MIC) of extracts is usually determined through dilution methods (Rai and Mares, 2003), i.e. the extract is mixed with growth medium at various dilutions. *In vitro* screening methods for detecting antifungal

compounds can be categorized into (1) plate dish diffusion assays on agar medium (Langvad, 1999); (2) assays of fungal growth in liquid culture, which can be measured as increase in dry weight or increase in optical density caused by increased number or size of cells in the solution (Rai and Mares, 2003); and (3) whole tissue assays, in which the mycelial plug is transferred onto the tissue pieces of the plant tested for antifungal properties placed in Petri dish (Ross *et al.*, 2008).

The most common method is represented by plate dish diffusion assay. The fungal growth is estimated by measuring colonial mean radii of the fungus or inhibition zone compared to controls (Uldahl and Knutsen, 2009). One of such methods is poisoned plate technique, which consists of the mycelial radial growth inhibition on an agar medium mixed with extracts and inoculated with an agar plug from actively growing colonies of the fungus in a Petri plate. Alternatively, the poisoned plate can be inoculated with the spore suspension to effectuate a spore germination test (Abou-Jawdah *et al.*, 2002). Another diffusion method uses paper discs saturated with extracts and placed onto the agar surface in a Petri plate containing spores (Houdai *et al.*, 2004; Mahakhant, 1998; Soltani *et al.*, 2005). In the plate well method, spore suspensions are mixed with agar medium before plating in Petri dishes, where after wells are stamped in the agar, and the extract is added (Kellam *et al.*, 1988). A method of stable gradient technology, marketed as the Etest, uses a plastic strip establishing a continuous gradient of the test compound in surrounding agar (Cormican and Pfaller, 1996; Serrano *et al.*, 2003). An automatic image analysis method evaluates the viability and germination characteristics of fungal spores (Paul *et al.*, 1993). Another method is based on spore swelling and germination in liquid culture. This method allows screening of more extracts in a short time frame (Uldahl and Knutsen, 2009). In whole tissue assays, the mycelial plug is transferred onto the tissue pieces of the plant tested for antifungal properties placed in Petri dish (Ross *et al.*, 2008).

However, one of the most efficient means of screening for antifungal compounds is represented by thin-layer chromatography (TLC) bioautography. This method is simple, rapid, and allows the direct localization of active constituents in plant extracts (Hostettmann and Potterat, 1997). Antimicrobial activity is estimated by absorbing chemicals or extracts onto the surface of chromatographic plates and placing them directly in contact with the medium inoculated with fungal cultures (Wedge and Nagle, 2003). The bioautography methods are divided into (1) contact bioautography, (2) immersion bioautography, and (3) direct bioautography that is considered to be the most efficient technique. In direct bioautography, a developed TLC plate is dipped in the suspension of microorganisms growing in the broth or

this suspension is sprayed onto the plate (Horváth *et al.*, 2005). Then, the plate is incubated and microorganisms grow directly on it. The bacteria are killed by antibacterials to avoid contamination. The individuated chemical compounds with antifungal activity are often further examinated for antifungal properties by microtiter plate test (Scher *et al.*, 2004).

The antifungal activity of an extract can be determinated also by means of flow-cytometry. Green *et al.* (1993) have developed a flow cytometric assay for antifungal activity based on detection of increased permeability of the fungal cell membrane to propidium iodide (PI), a nucleic acid-binding fluorochrome largely excluded by intact cell membranes, following drug treatment. The flow-cytometric methods enable to identify and even separate living and dead fungal cells. The approximative spore size, granularity or shape can be also detected (Shapiro, 1995). This technique has several advantages such as, including short incubation time, higher precision in comparison with other screening methods, greater accuracy, and speed of analysis (Green *et al.*, 1994).

The screening for antifungal activity can be performed also with detached leaf method. In this method, the mycelial plugs or spore suspensions are applied to the detached leaves treated with plant extracts and placed in Petri dish (Gonfo *et al.*, 2008). Then, the diameter of the lesion caused by fungus is measured.

1.4.2 IN *VIVO* SCREENING METHODS

In vivo screening methods follow the preliminar tests in laboratory conditions with the aim to evaluate the efficacy of the antifungal extracts also in the natural growth conditions of cultural crops. This part of determination of antifungal compounds is a necessary step for a novel biofungicide registration as only this type of sreening proves real antifungal potential of the tested product. This screening includes growth chamber, greenhouse and field trials. It is particularly important that the conditions are favourable to the fungal development. Nevertheless, the plants tested cannot be stressed by other factors (e.g. water stress), apart the stress by fungal pathogen. In growth chamber, the growth conditions can be well controlled and the influence of external factors is minimized. However, the choice of crop spiecies for testing is limited by space. In the greenhouse, managing of growth conditions ideal for pathogen is more difficult. However, greenhouse tests are ideal for antifungal activity screening at any growth stage. For some crops, that are exclusively cultivated in the field, the field trials are necessary to establish the real functionality of biofungicide tested. In the field,

the timing of inoculation with pathogenic fungus is fundamental as each pathogen attacks the plant especially in some periods, when the conditions are the most favourable.

In *in vivo* experiments, both the choose of inoculation method and the inoculum concentration are important. The most common is an inoculation with a spore suspension; however, in some cases, the mycelial plugs are used for direct inoculation of wounded plant parts. The inoculum of air-borne pathogens is applied to aerial organs of the plant, while soil-borne pathogens are inoculated onto the soil surface or directly mixed with the substrate before transplanting. It is recommended to effectuate a pathogenity test prior to the antifungal screening to verify the virulence of the fungal pathogen and to determinate optimal inoculum concentration. The commercial fungicide should be used as a control for biocontrol agent activity screening where available. The ideal period and the way of application of the biofungicide to the plants should be also determined.

1.5 PLANT RESISTANCE MECHANISMS IN PLANT-PATHOGEN INTERACTIONS

Plants must continuously defend themselves against pathogen attack from fungi, bacteria, viruses, invertebrates and even other plants (Xing, 2007). Plant pathogens are generally divided into necrotrophs and biotrophs according to their lifestyles (Glazebrook, 2005; Plotnikova and Ausubel, 2007). Necrotrophs first destroy host cells to feed on the cell contents. On the contrary, biotrophs derive nutrients from living host tissues, often through specialized feeding structures (haustoria) that invaginate the host cell without disrupting it. Hemi-biotrophs or hemi-necrotrophs are the plant pathogens that display both lifestyles, depending on the stage of their life cycle.

The regulation mechanisms of any plant–pathogen interaction are complex and dynamic. Plant defence involves perception, transduction, and response signals as well as termination of signalling events. Many components of the perception systems and transduction pathways are now characterized and the underlying genes are known (Xing, 2007).

Although pre-existing barriers and chemical defences may hold an invader at bay for a short time, recognition of a pathogen attack is critical to a plant's overall survival strategy. Plants recognize many clues that they are under attack and respond accordingly with defensive strategies that are efficient in preventing and overcoming infection. Such "elicitors"

of plant defences include plant and fungal cell wall components, bacterial glycoproteins, viral capsid proteins, and microbial avirulence proteins. Avirulence proteins are produced by plant pathogens and are necessary for a pathogen to infect a plant. Plants have evolved a system to recognize avirulence proteins, which tells the plant that it is under attack and that it needs to do something about it. Recognition of avirulence proteins by plants forms the basis of "gene-for-gene" plant resistance, where the compatibility of the plant-pathogen interaction is decided based on the matching of dominant genotypes (*Fig. 1.39*). If a plant possesses the dominant resistance (R) gene corresponding to the pathogen's dominant avirulence gene (Avr), then the interaction is said to be incompatible, and no disease develops. Alternatively, if the plant possesses no matching R gene for a pathogen's avirulence gene, then the interaction is compatible and the infection proceeds. If we think of the pathogen's Avr proteins as a type of "key" that gains them access to a plant, then in an incompatible interaction, we can think of the plant's matching R gene as blocking access to the keyhole. In a compatible interaction, the keyhole is left unprotected, and the pathogen can simply let itself in and make itself at home.

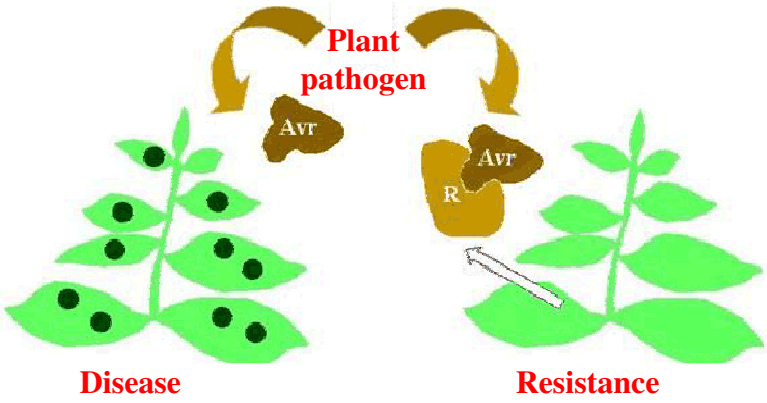


Fig. 1.39: Host-pathogen recognition.
If a plant has an appropriate *R* gene corresponding to the pathogen's *Avr* gene, then the plant is resistant and no disease develops. If, however, the plant does not have the matching *R* gene, the interaction may result in infection.

An R-Avr recognition event initiates a signaling cascade, which often results in a hypersensitive response and development of systemic resistance. The hypersensitive response is characterized by the collapse and death of tissue immediately surrounding the site of infection, often resulting in the appearance of brown spots on the leaf, which we sometimes recognize as a symptom of disease. This type of self-sacrifice by the plant is an attempt to contain the pathogen and prevent the infection from spreading throughout the plant.

The hypersensitive response may also be accompanied by the localized accumulation of another type of defensive secondary metabolites named phytoalexins in the area around the

lesion. Phytoalexins are similar to phytoanticipins in that they are low-molecular weight antimicrobials, but unlike phytoanticipins, phytoalexins are produced only during a pathogen attack, and tend to remain localized at the site of infection since they may actually be toxic to the plant. Dixon (2001) suspected that the broad non-host resistance that is the norm in plant-microbe interactions is often the result of the activity of natural products, although this is difficult to dissect genetically as several pathways may be involved.

Classically, two levels of resistance have been defined. Non-host resistance is where the entire plant species or genus is resistant and therefore not a host for the particular pathogen. Host resistance occurs where individuals within a species have developed genetically inherited ways of defending themselves against an organism that causes disease on other individuals within that plant species (Dickinson, 2003).

Mechanisms of resistance in plants can be subdivided into two categories, passive (constitutive) and active (induced) (*Fig. 1.40*).

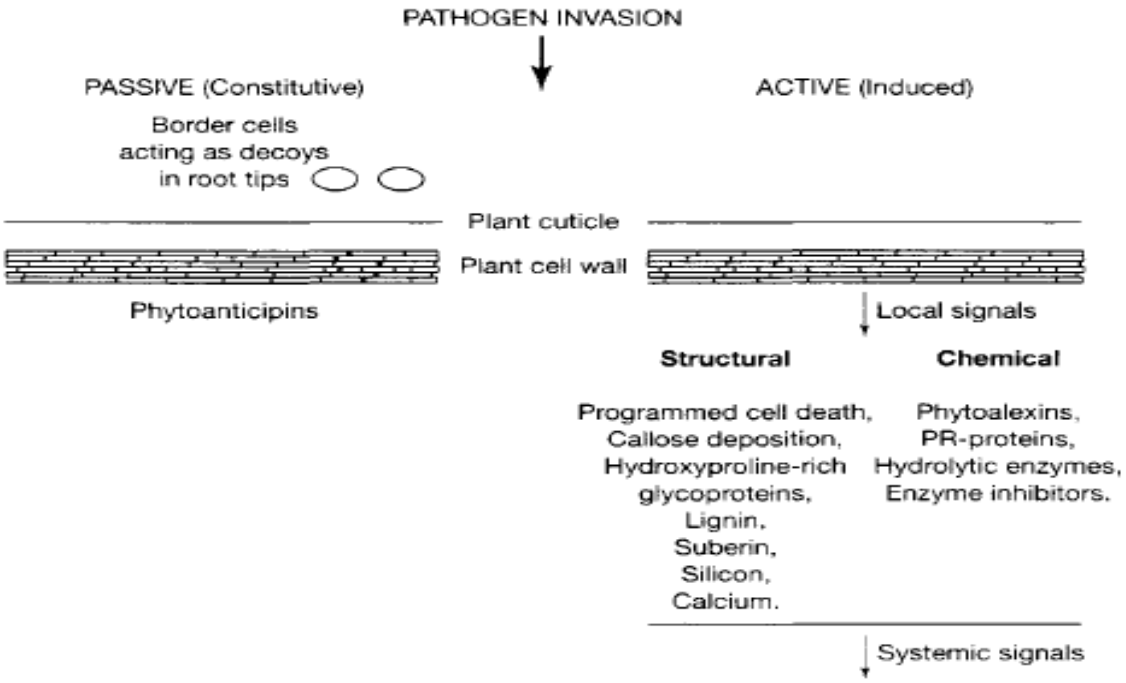


Fig. 1.40: Examples of passive and active defence mechanisms in plants (from Dickinson, 2003)

Passive mechanisms involve both structural elements, such as the cuticle and root border cells, and pre-formed antimicrobial chemical compounds within the plant termed phytoanticipins. These form the initial layers of protection against microbial attack (Dickinson, 2003). However, the most complex defence reaction involves turning on a cascade of genes involved in plant-pathogen interactions. The active and inducible defences include the hypersensitive response (local plant cell death), the production of reactive oxygen

species, the induction of specific gene expression within the plant, including genes involved in cell wall strengthening and/or repairing structural defences (forming of callose, hydroxylproline-rich proteins, etc.) and genes for biosynthesis of additional antimicrobial compounds, the induction of genes encoding pathogenesis related proteins, various hydrolytic enzymes and other defence-related proteins (Dickinson, 2003; Punja *et al.*, 2004; Dickinson, 2003).

In addition to the localized induction of defence responses, there are mechanisms that induce resistance in other parts of the plant through systemic signals, such as systemic acquired resistance (SAR). Another signaling molecule, jasmonic acid, is believed to cause another type of systemic resistance known as induced systemic resistance (ISR), in response to wounding from herbivores or infection by root-colonizing microbes. These induced responses put the plant on guard against a subsequent infection. Also SAR provides broad-spectrum protection against further infection lasting as long as several weeks. Furthermore, plants can signal to neighbouring plants through volatile compounds to enhance resistance in these plants (Dickinson, 2003).

1.5.1 INDUCED DEFENCE MECHANISMS

Plants have evolved several strategies to perceive the attacking pathogen and to translate this perception into an effective immune response (Jones and Dangl, 2006). In general, development of the disease depends on pathogen's ability to suppress the plant's immune response and the plant capability to recognize the pathogen as well as to activate efficient defence mechanisms. Defence responses in plants can be induced by various factors. The plant response depends on the nature of these factors. Some defences are induced by the presence of fungal spores on the leaf surface, whilst others require the pathogen to penetrate the surface before induction (Dickinson, 2003). Successful development of infection or its inhibition depends greatly on the events taking place at the host–pathogen interface during the establishment of infection (Plotnikova and Ausubel, 2007).

In the primary immune response, the plant immune system recognizes compounds of microbial pathogens, such as flagellin, chitin, glycoproteins, and lipopolysaccharides. These microbial components are referred to as pathogen-associated molecular patterns (PAMPs; Chisholm *et al.*, 2006; Jones and Dangl, 2006; Göhre and Robatzek, 2008; Nürnberger and Kemmerling, 2009). PAMPs activate pattern-recognition receptors (PRRs), which initiate various downstream signaling events resulting in the activation of a basal resistance called

PAMP-triggered immunity (PTI). When pathogen effector molecules that are transported into the host cell suppress PTI and promote virulence of the pathogen, it is called effector-triggered susceptibility (ETS). Plants with resistance (R) proteins, which recognize the attacker-specific effectors, result in a secondary immune response called effector-triggered immunity (ETI; Chisholm *et al.*, 2006; Jones and Dangl, 2006). Therefore, induction of defence mechanisms may be in response to non-specific elicitors, or may follow the classical gene-for-gene resistance model, the mechanism underlying host resistance. In this highly evolved form of inducible resistance, the product of a specific resistance gene in the plant is involved in recognition of a specific elicitor from the pathogen. These induced resistance responses have many similarities to the responses that occur when plants are wounded or undergo a natural processes such as abscission. This indicates that localized strengthening of structural defences and the production of anti-microbials are fundamental processes in plants (Dickinson, 2003).

Local signals are one of the first responses activated in many incompatible interactions prior to the induction of gene expression and protein synthesis is the production of ion fluxes, reactive oxygen species (ROS) or reactive oxygen intermediates (ROI), production of nitric oxide (NO) and phosphorylation cascades. Another defence response is **programmed cell death (PCD).** This hypersensitive response (HR) consists in localized death of plant cells. It is a temporally and spatially co-ordinated mechanism to limit the amount of host tissue lost to the pathogen, and one that restricts the ingress of biotrophic pathogens that require living cells as their source of nutrients. Other inducible defences are **induced structural barriers**. The first of these is cytoskeleton-based to fend off attack from potential pathogens prior to penetration, involving sensing of the developing pathogen on the surface. Formation of appositions referred to as papillae, consisting of callose (a β-1, 3-glucan polymer) and phenolics, on the inner surface of cell walls, along with deposition of hydroxyproline-rich glycoproteins such as extensin, phenolic compounds such as lignin and suberin, and minerals such as silicon and calcium also occurs, and these become cross-linked to form insoluble defensive structures and an additional barrier against pathogen invasion and ingress. Peroxidase enzymes also have a major role in cell wall strengthening and are induced by pathogen infection and elicitor treatment. In addition, plants are able to synthesize some chemical compounds (**phytoalexins**) *de novo* following pathogen attack. These antimicrobial compounds include a diverse array of low-molecular-weight secondary metabolites, and these generally act against a broad range of pathogens. They include terpenoid derivatives (e.g. sesquiterpenes); saponins; aliphatic acid derivatives; phenolics and phenylpropanoids (e.g.

isoflavonoids); nitrogencontaining organic compounds (e.g. alkaloids); and sulphur-containing compounds including inorganic elemental sulphur. Some of the genes encoding enzymes involved in the same pathways like secondary metabolites such as phenylalanine ammonia-lyases (PAL) and chalcone synthases (CHS) have been shown to be induced in association with the HR. Interestingly, plant species within the same families will tend to utilize the same chemical structures as antimicrobials, for example the sesquiterpenes are widely used in the *Solanaceae*. Many antimicrobial compounds are sequestered within plant cells as inactive precursors or are synthesized and accumulate in specialized vesicles that are then delivered to the site of microbial infection. Some antimicrobials may act synergistically with others. However, the pathogens can avoid the antimicrobials for example producing enzymes that degrade the compound. Other inducible defence responses include **pathogenesis-related proteins** (PR) and **other defence-related proteins**. The last ones include lectins, and the chitin-binding lectins common in cereal grains may bind to chitin in the cell walls of fungi and insects and retard their growth *in planta* allowing time for other more active defences to take effect. Some of the defence-related proteins appear to have roles in primary metabolism, indicating that a wide range of metabolic changes are induced as part of pathogen attack. As further evidence for the role of primary metabolism, it has been found that environmental conditions such as addition of exogenous nitrogen can influence whether plants become diseased. A further group of enzymes induced in plants as part of HR are the lipoxygenases. These may contribute to defence both through the production of volatile and non-volatile fatty-acid-derived secondary metabolites that are toxic to invading pathogens, and through induction of the signal molecules jasmonic acid and methyl (Dickinson, 2003).

1.5.2 PATHOGENESIS-RELATED PROTEINS

Various novel proteins are induced during pathogen attack, known collectively as the pathogenesis-related (PR) proteins. These proteins are expressed at low levels in healthy plants; certain isozymes are induced during pathogen attack both locally and systemically (Dickinson, 2003). The major criterion for being classified as a PR protein is that the protein should be novel and induced upon infection and should impede further pathogen progression, but not in all pathological conditions (Van Loon, 1990).

The proteins induced have been grouped into 17 PR classes, though not all are induced in all interactions or in all plant species. Most PRs and related proteins are induced through the action of the signaling compounds salicylic acid, jasmonic acid, or ethylene, and possess

antimicrobial activities *in vitro* through hydrolytic activities on cell walls, contact toxicity, and perhaps an involvement in defence signaling (Dickinson, 2003; van Loon *et al.*, 2006).

The biochemical role for many of these proteins has been determined. PR-1 proteins are the most common PR proteins detected in a large number of plant species. Some **PR-1** proteins have been shown to inhibit the growth of oomycetes. These proteins defend against invading pathogens, with a role in degrading the invading organism once it has already been contained. The chitinases (**PR-3, PR-4, PR-8, and PR-11**) are presumed to hydrolyze chitin in fungal cell walls. Glucanases (**PR-2**), proteinases (**PR-7**) and RNases (**PR-10**) presumably have similar roles as hydrolytic enzymes that will also have activity against bacteria and oomycetes. Some PR proteins have putative roles in combating pathogenicity factors. Protease inhibitors (e.g. **PR-6**) are produced to inhibit insect and microbial protease enzymes. The roles of the other PR-proteins are more diverse. The **PR-9** family comprises peroxidases, presumably involved in cell wall strengthening. The **PR-5** family of thaumatin-like proteins have homology to permatins that permeabilise fungal membranes, whilst the **PR-12** are defensins similar to antimicrobial compounds present in other organisms such as the insect defensins (Dickinson, 2003; Terras *et al.*, 1995; Van Loon and Van Strien, 1999). Thionins, cysteine-rich proteins, induced by pathogens are known as **PR-13** (Bohlmann *et al.*, 1988; van Loon, 1999). **PR-14** includes the lipid transfer proteins (LTPs) (Van Loon and Van Strien, 1999). A germin-like oxalate oxidase is considered as a **PR-15** protein (Zhang *et al.*, 1995). A barley oxalate oxidase-like protein was classified as **PR-16** protein (Wei *et al.*, 1998). The proteins **PR-17**, found in the plants of tobacco, wheat and barley, show a protease activity (Christensen *et al.*, 2002; Görlach *et al.*, 1996; Okushima *et al.*, 2000). Besides these 17 families, some unclassified PR proteins have also been described. Grenier and Asselin (1990) have identified **chitosanases** as pathogenesis-related proteins in barley, cucumber, and tomato leaves. Three chitosanase activities have been detected in spruce roots infected with Pythium sp. (Sharma *et al.*, 1993).

1.5.2.1 PR-1 proteins

The PR-1 family is often the most abundant and common group of proteins and is induced to very high levels upon infection (Punja *et al.*, 2004). They have been detected in rice, wheat, barley, corn, tomato, tobacco, and in several other plant species belonging to Graminaceae, Solanaceae, Amaranthaceae, and Chenopodiaceae (Ergon *et al.*, 1998; Fidantsef *et al.*, 1999; Cordelier *et al.*, 2003; Makandar *et al.*, 2006; Ménard *et al.*, 2004;

Rodrigues *et al.*, 2005; Seo *et al.*, 2003; Vidhyasekaran, 2002, 2004, 2007; Zabbai *et al.*, 2004).

1.5.2.2 PR-4 proteins

Two classes of PR-4 family have been recognized. Class I PR-4 proteins show similarity to hevein and wound-induced (WIN) proteins (Van Loon, 1999). This class has been purified from tobacco and the protein was localized intracellularly (Ponstein *et al.*, 1994b). The class II PR-4 proteins show similarity to acidic chitinases (Vidhyasekaran, 2008). In tomato, a PR-4 protein (P2), which is serologically related to PR-4 protein from tobacco, has been identified (Joosten *et al.*, 1990). The tomato P2 protein showed homology with the potato *win1* and *win2* gene products and with pre-pro-hevein from *H. brasiliensis* (Linthorst *et al.*, 1991).

1.5.2.3 PR-5 proteins

The PR-5 family or thaumatin-like proteins (TLPs), have a high degree of sequence similarity with each other and show immunological relationship to a sweet-tasting protein, thaumatin, which occurs in the fruit of the West African shrub, *Thaumatococcus danielli* (Cornelissen *et al.*, 1986; Pierpoint *et al.*, 1987). TLPs are not commonly detected in leaves of young healthy plants but rapidly accumulate to high levels in response to biotic or abiotic stresses. They are generally highly soluble proteins that are stable even at very low pH and are resistant to proteolysis. The extracellular TLPs are always acidic, whereas the vacuolar ones tend to be basic (Velazhahan *et al.*, 1999). Three subclasses of PR-5 proteins have been recognized in tobacco: the basic forms (osmotins), neutral forms (osmotin-like proteins, OLPs), and acidic (PR-S) proteins (Koiwa *et al.*, 1994). Although many TLPs are antifungal, their antifungal activity varies with the specific fungal genus (Vigers *et al.*, 1991; Abad *et al.*, 1996). At higher concentrations, TLPs can actively lyse fungal membranes, while at lower concentrations, they affect membrane permeability (Vigers *et al.*, 1992) which can cause leakage of cell constituents and increase the uptake of other antifungal compounds. The TLPs are induced in response to viral, fungal, and bacterial infections. They are also induced by nonpathogenic stress, such as osmotic stress, and by hormones and signal molecules such as abscisic acid (ABA), ethylene, SA, and jasmonic acid (JA) and by wounding (Velazhahan *et al.*, 1999). A cellulase elicitor induced *PR-5* gene in tobacco seedlings (Chang *et al.*, 1995).

1.5.2.4 PR-6 proteins

PR-6 family comprises proteinase inhibitors (PIs), the stable defence proteins found in seeds whose expression is developmentally regulated. They are also induced in leaves upon attack by pests or pathogens. The first such inhibitors were induced in tomato upon insect feeding (Hass *et al.,* 1982). Induction of PIs in plants in response to microbial attack has also been observed in many plant systems. Increases in trypsin and chymotrypsin inhibitory activities in tomato leaves infected by *Phytophthora infestans* were detected, and this induction was stronger in resistant lines when compared to the susceptible ones (Peng and Black, 1976). Pathogens and pests invading plant tissues rely on a set of proteinases as part of their virulence factors. These proteinases belong to four classes such as serine proteinases, cysteine proteinases, aspartic proteinases, and metalloproteinases. In parallel, plants have evolved genes encoding inhibitors that inactivate some of these proteinases and thus may reduce the ability of the pathogen or pest to digest host proteins, and therefore limit the availability of nitrogen source for the invader. The induction of subclasses of PIs in response to microbial infection has been reported in numerous plants (Heitz *et al.*, 1999). Serine proteinase inhibitors that inhibit trypsin and chymotrypsin contain several families: Kunitz family (Soybean trypsin inhibitor family), Bowman-Birk family, Barley trypsin inhibitor family, Potato inhibitor I family, Potato inhibitor II family, Squash inhibitor family, Ragi I-2-maize trypsin inhibitor family, and Serpin family. Cysteine proteinase inhibitors (phytocystatins) inhibit cysteine proteinases. Aspartic proteinase inhibitors inhibit cathepsin D and metalloproteinase inhibitors inhibit papain, cathepsin B, H, L (Koiwa *et al.*, 1997). Although most of these proteinase inhibitors are constitutively expressed, some of the serine proteinase inhibitors are induced because of infection by pathogens and they are considered as PR-6 proteins (Heitz *et al.*, 1999). Tomato inhibitor I (belonging to Potato inhibitor I family) is the type member of the PR-6 protein family (Green and Ryan, 1972). Tomato inhibitor II is also commonly induced in tomato because of stresses (Doares *et al.*, 1995).

1.5.3 SIGNALS INVOLVED IN TRANSCRIPTIONAL INDUCTION OF DEFENCE-RELATED GENES

PR proteins can be induced by natural signaling molecules like elicitors, SA, JA, and systemin, upon environmental stresses, insect feeding, or by chemical elicitors, and at different developmental stages of the plant (Van Loon, 1990). In addition, recently have been

studied other plant-derived substances such as plant extracts that may trigger induction of defence-related genes.

1.5.3.1 Biological induction

Elicitors are one of the natural signalling molecules that trigger biological induction of PR genes. They are derived from pathogens or from host-pathogen interactions and include oligosaccharides derived from chitin, glucan, and pectin, fungal cell wall derivatives, extracellular glycoproteins, polysaccharides, oligosaccharides, and harpins produced by pathogenic and nonpathogenic bacteria and fungi (Zhou, 1999). An elicitor (e.g. arachidonic acid from *Phytophthora infestans*) induces activation of the potato *PR* gene named *PR-10a* (Subramaniam *et al.*, 1997). Another class of elicitors is typified by the polypeptides encoded by avirulence *(avr)* genes of the pathogen. A pathogen containing a particular *avr* gene is recognized by the host plant that carries a corresponding resistance *(R)* gene and activates disease resistance in the host (Zhou, 1999).

Other biological inducer of *PR* genes is salicylic acid (SA), an endogenous signal molecule accumulated during fungal pathogenesis. SA is postulated to bind to a receptor which, in turn, may trigger the signal transduction cascade leading to the production of transcription factors regulating PR-protein expression or other defence proteins (Metraux, 2001). Inhibition of accumulation of salicylic acid inhibits expression of PR genes. This was shown in several *Arabidopsis* mutants and transgenic tobacco plants (Dangl *et al.*, 1996; Ryals *et al.*, 1996). SA induced mRNAs that encode for different PR proteins in tobacco such as PR-1, PR-2, PR-3, PR-4, PR-5 (Ward *et al.*, 1991). Also exogenous application of SA induces *PR* genes in many plants. Therefore, salicylic acid is the key signal in triggering transcription of some *PR* genes. H_2O_2 appears to be one of the important signals for SA accumulation (Leon *et al.*, 1995). Salicylic acid may also act as signal molecule in inducing other signals for induction of PR genes (Du and Klessig, 1997).

Jasmonic acid (JA) and methyl jasmonate (MeJA) are the other signal compounds synthesized from linolenic acid that are commonly involved in stress responses of plants. They systemically induce accumulation of PR proteins in plants during fungal pathogenesis. JA induces the accumulation of several polypeptides, proteinase inhibitors, and ribosome-inactivating proteins (RIP) in many plants (Vidhyasekaran, 1997). Jasmonic acid also induces PR-1, PR-3, PR-5, and PR-9 proteins/mRNAs in rice (Schweizer *et al.*, 1997), PR-1 proteins in tobacco leaves (Green and Fluhr, 1995) and in *Arabidopsis* (Penninckz *et al.*, 1996).

Jasmonate or methyl jasmonate induce expression of *PR-6* genes in various plants (Farmer and Ryan, 1992; Wasternack and Partheir, 1997). MeJA strongly induced the expression of the defensin gene *PDF1.2* in *Arabidopsis* (Penninckz *et al.*, 1996). The importance of jasmonic acid and methyl jasmonate in inducing PR proteins has been demonstrated by the correlation between their content and induction of PR proteins. When the concentration of endogenous jasmonates increased, the PR protein synthesis was activated (Lehmann *et al.*, 1995; Penninckz *et al.*, 1996). Ethylene response factors appear to play important roles in regulating jasmonate-responsive gene expression (Brown *et al.*, 2003). Jasmonic acid/methyl jasmonate may not induce all types of PR proteins and only specific proteins may be induced (Heitz *et al.*, 1999). Salicylic acid may act as an antagonist of jasmonic acid in inducing PR proteins and it may negatively regulate the function of jasmonates (Wasternack and Parthier, 1997).

Ethylene is another endogenous regulator of *PR*-protein gene expression and is considered as a stress-related phytohormone. For example, treatment of plants with ethylene causes an enhanced accumulation of PR proteins, including β -1,3 glucanases and chitinases (Ishige *et al.*, 1991; Xu *et al.*, 1994). Furthermore, ethylene induces basic PR-1 (Eyal *et al.*, 1992), two basic class I β-1,3-glucanases (Van de Rhee *et al.*, 1993), and PR-5 proteins (Koiwa *et al.*, 1994; Knoester *et al.*, 1998) in tobacco. It also induces a thionin (Epple *et al.*, 1997), defensins *Pdf1.2* (Penninckz *et al.*, 1996) and PR-1, PR-2, and PR-5 proteins in *Arabidopsis* (Lawton *et al.*, 1994). Ethylene-mediated induction of PR proteins in tobacco follows two pathways, one is light dependent and another not (Eyal *et al.*, 1992).

Systemin, a wound-inducible polypeptide detected in tomato leaves, trigger the synthesis of proteinase inhibitors I and II (Pearce *et al.*, 1991). Sometimes the signal inducing PR-protein expression might be a very simple compound, such as a short peptide (Gordon-Weeks *et al.*, 1991). There exists the possibility of synergism or antagonism among signal molecules on expression of *PR*-protein genes (Punja *et al.*, 2004).

1.5.3.2 Induction by chemicals

Exogenous application of chemicals, such as β-aminobutyric acid (BABA) or acibenzolar *S*-methyl (ASM), can induce accumulation of PR proteins, especially when challenged with pathogens (Siegrist *et al.*, 2000; Ziadi *et al.*, 2001; Silue *et al.*, 2002). Also other various chemicals, including 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH) were associated with production of PR proteins (Oostendorp *et al.*, 2001).

1.5.3.3 Induction by plant extracts

Several plant extracts controlled diseases by activating signal transduction systems. Milsana, a commercial product of leaf extracts from the giant knot weed *Reynoutria sachalinensis,* significantly reduced incidence of powdery mildew *(Sphaerotheca fuliginea)* in cucumber *(Cucumis sativus)*(Wurms *et al.*, 1999; Daayf *et al.*, 2000). In addition, Milsana treatment increased the levels of hydroxycinnamic acid, *p*-coumaric, caffeic, ferulic acids and *p*-coumaric acid methyl ester in the cucumber leaves. All of these phenolic compounds showed antifungal activity against common pathogens of cucumber, such as *Botrytis cinerea, Pythium ultimum,* and *P. aphanidermatum.* This suggests that the plant extract would induce synthesis of antifungal compounds and contribute to disease resistance (Daayf *et al.*, 2000). A pokeweed (*Phytolacca* spp.) antiviral protein (PAP) induces synthesis of pathogenesis-related proteins and a small increase in salicylic acid levels in tobacco (Smirnov *et al.*, 1997).

1.5.4 SYSTEMIC RESISTANCE MECHANISMS

Plants do not produce naturally antibodies against their pathogens, and most of their biochemical defences are inactive until they are mobilized by some signal transmitted from an attacking pathogen (Agrios, 2005). Responses to pathogen attack occur both in plant organ originally attacked (local response) and in distant, yet unaffected, parts (systemic response, *Fig. 1.41*) (Heil and Bostock, 2002). This generalized resistance may develop also in response to treatment with certain natural or synthetic chemical compounds (Agrios, 2005).

Systemic acquired resistance (SAR) is one form of inducible resistance that is activated throughout the whole plant after primary infection with a necrotizing pathogen and it is accompanied by increased levels of salicylic acid and pathogenesis-related proteins. The activated disease resistance is effective against both the inducing pathogen and other, unrelated pathogens (Agrios, 2005; Oostendorp *et al.*, 2001).

SAR has been demonstrated in many plant species, including cucurbits, bean, tomato and *Arabidopsis,* upon induction by bacteria, fungi and viruses. The spectrum of pathogens against which systemic resistance is effective varies among species and remains constant for each plant species. Also, the set of PR-proteins that are induced are highly plant specific. SAR is not effective against all pathogens (Dickinson, 2003; Kessmann *et al.*, 1994; Sticher *et al.*, 1997).

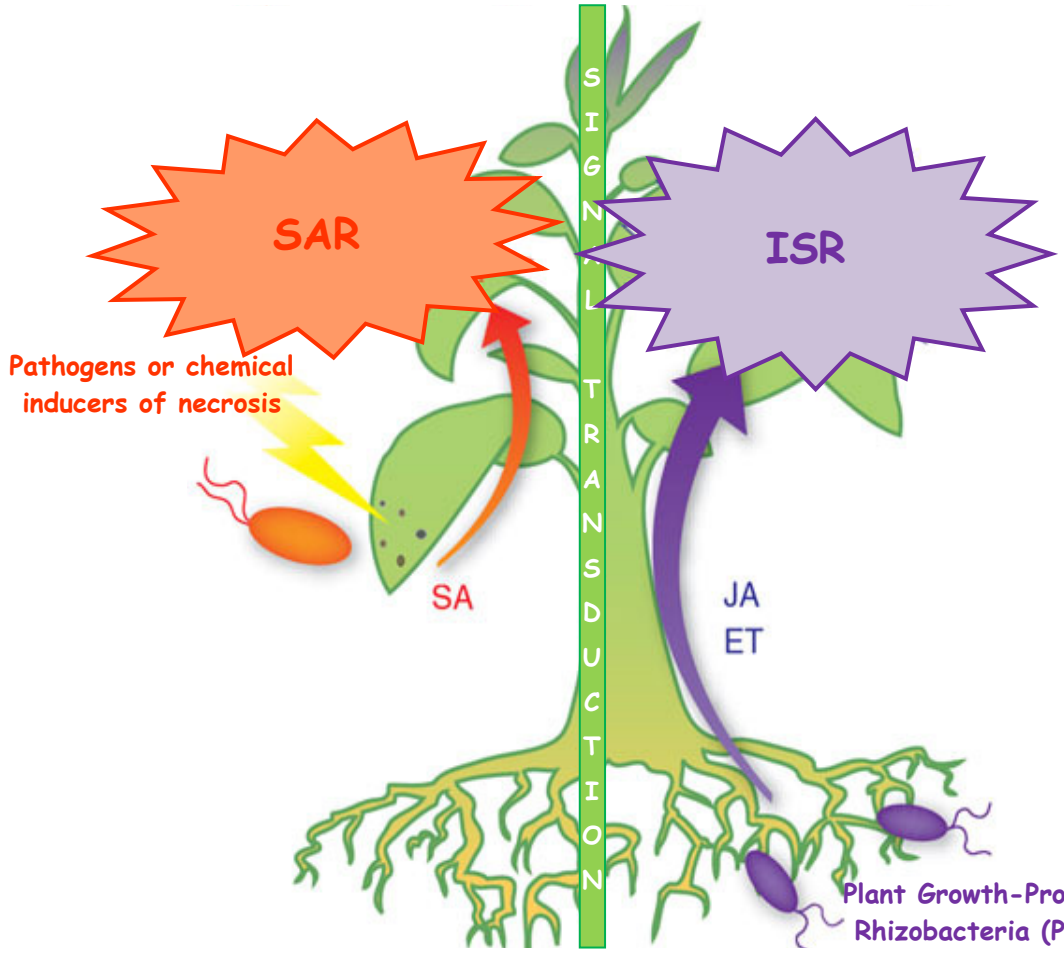


Fig. 1.41: Systemic resistance mechanisms in plants. Upon biotic or abiotic stresses mobile signal travels through the vascular system to activate defence responses in distal tissues using signal molecules: salicylic acid (SA), typical for Systemic acquired resistance (SAR); Jasmonate (JA) and ethylene (ET), typical for Induced systemic resistance (ISR).
[modified from Pieterse *et al.* (2009) and Dickinson (2003)]

Although SAR does not affect spore germination and appressorium formation, penetration is reduced drastically in systemically induced resistant tissue. The degree of SAR seems to be well correlated with the number of lesions produced on the induced leaf. However, SAR cannot be induced after the onset of flowering and fruiting in the host plant. It is characterized by the coordinate induction in uninfected leaves of inoculated plants. Products of several SAR genes, e.g., b-1,3-glucanases, chitinases, cysteinerich proteins related to thaumatin, and PR-1 proteins, have direct antimicrobial activity or are closely related to classes of antimicrobial proteins (Agrios, 2005).

Apart salicylic acid, several chemical compounds, e.g. salicylic acid, arachidonic acid, 2,6-isonicotinic acid (INA) and benzothiazole (BTH), may induce localized and systemic resistance in plants. These SAR-activating compounds induce expression of the same set of

SAR genes that are induced either by salicylic acid or by various infectious agents. In addition, they seem to prime or sensitize plants to respond faster and with additional defence reactions than those characteristic of SAR genes. Several other chemical compounds, such as the fungicides fosethyl-Al, metalaxyl, and triazoles, appear to have some resistance-inducing activity (Agrios, 2005).

The **induced systemic resistance (ISR)** is triggered by certain strains of nonpathogenic root-colonizing bacteria (rhizobacteria) rather than induction by phytopathogens. It is not dependent on SA accumulation in the plants but requires functioning jasmonic acid and ethylene (Dickinson, 2003). In the comparison with SAR, no defence-related proteins are detectable in plants with rhizobacteria-induced systemic resistance, in spite of a similar enhanced defensive capacity of the plants (van Loon *et al.*, 2006). However, application of higher levels of JA or ET induces production of antimicrobial peptides (defensins) (Pieterse *et al.*, 1998).

Nevertheless, there is an evidence of an overlap and also antagonism between the mechanisms regulating resistance in SAR and ISR. In addition, some necrotrophic pathogens induce a further form of systemic resistance, also via jasmonic acid and ethylene but independent on SAR and ISR (Dickinson, 2003).

Systemic resistance responses induced by either harmful or beneficial microorganisms or by treatment of plants with various natural or synthetic compounds are associated with a process called **priming** (*Fig. 1.42*). It means that plants develop enhanced capacity for faster and stronger activation of stress-inducible defence reactions upon second pathogen challenge rather than a direct induction of defence mechanisms (Beckers and Conrath, 2007). Such physiological condition in which plants are able to better and/or faster activate the defence responses to biotic or abiotic stress is called "primed state" of the plant. It is supposed that such sensitization is associated with accumulation of inactive signalling protein(s) in primed cells. Upon subsequent exsposure to stress, a second signalling event could hyperactivate the signalling protein(s) and then amplify signal transduction leading to more rapid and/or more intense activation of defence responses (Conrath *et al.*, 2006).

In *Arabidopsis,* priming was associated with SAR induced by previous infection with avirulent *Pseudomonas syringae* pv. tomato, that primed systemic tissues for potentiated activation of defence-related *PAL, PR-1, PR-2* and *PR-5* genes (Cameron *et al.*, 1999; Van Wees *et al.*, 1999; Kohler *et al.*, 2002).

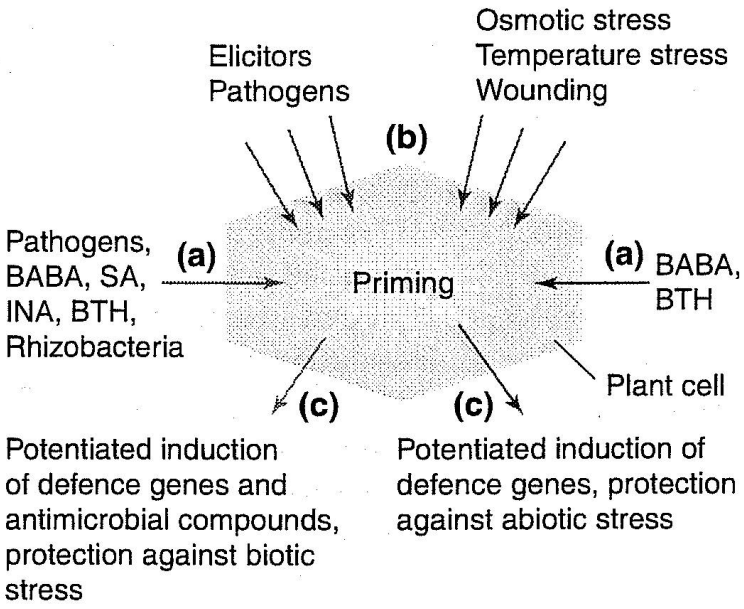


Fig. 1.42: Priming in induced resistance. (a) priming step, (b) challenge with biotic or abiotic stresses, (c) Potentiated response (from Conrath *et al.*, 2002)

Priming in beneficial plant-microbe interactions has been observed in *Dianthus* plants treated with plant growth-promoting rhizobacteria (PGPR) that mediated a faster increase in phytoalexin levels upon inoculation with pathogenic *Fusarium* sp. (Van Peer *et al.*, 1991). Colonization of tomato roots by micorrhizal fungi systematically protects the plant against *Phytophthora parasitica* without direct accumulation of PR proteins (Cordier *et al.*, 1998). Among the chemicals with priming potential, the most studied is β-aminobutyric acid (BABA), which is a potent inducer of resistance in plants against microbial pathogens (Jakab *et al.*, 2001; Cohen, 2002; Zimmerli *et al.*, 2000). The plant cells can be sensitized also by inhibition of steps in primary metabolism (Conrath *et al.*, 2006).

However the phenomenon of priming is studied intensively but its exact mechanism has yet not been elucidated. Hypothetically, the mechanism may vary among plant species and could depend on the priming agent. Priming might be an explanation of mode of action of many known or yet unexploited crop protective products.

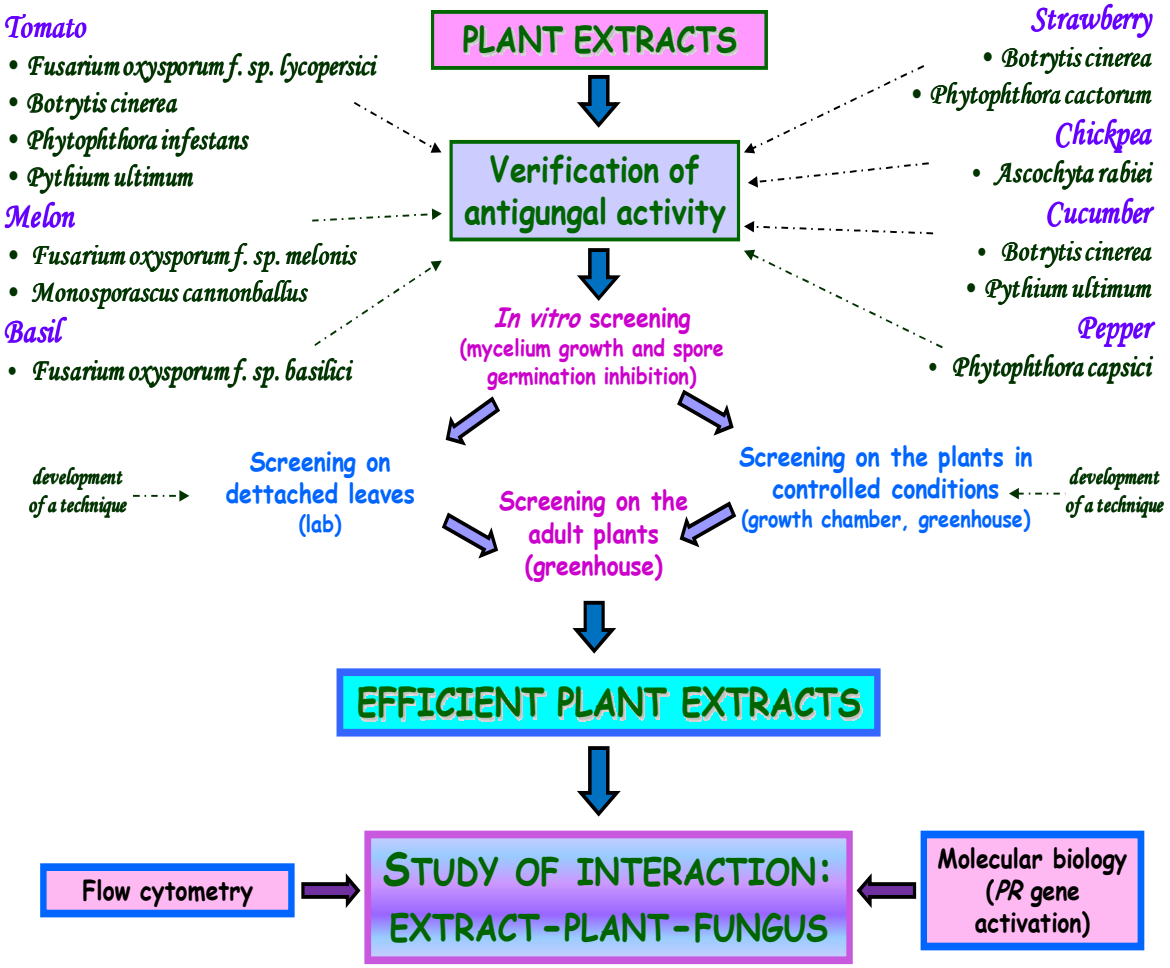
2

EXPERIMENTAL PART

2.1 INTRODUCTION

In the present thesis, the extracts of *Siparuna guianensis*, *Cordia leucocephala, Boerhavia diffusa*, *Vitex agnus-castus* and *Phyllanthus niruri* were screened for their antifungal activity both under *in vitro* and *in vivo* conditions. Consequently, the interactions of plant-fungus-extracts were studied at the molecular level with emphasis to pathogenesis-related protein gene activation.

The aim of the thesis was to found a functional natural plant extract for controlling fungal diseases in agricultural crops and to elucidate its mode of action. The strategy applied in the work is described in the following scheme.



2.2 ANTIFUNGAL SCREENING OF PLANT EXTRACTS

2.2.1 *SIPARUNA GUIANENSIS* EXTRACT / *BOTRYTIS CINEREA* AND *ASCOCHYTA RABIEI*

2.2.1.1 Abstract

Antifungal assays for the efficacy of *S. guianensis* extract in the control of *B. cinerea* and *A. rabiei* were performed both under *in vitro* and in *vivo* conditions. In mycelial growth inhibition tests, the most efficient concentration of the extract was 6 $ml \cdot l^{-1}$ controlling both fungi. In spore germination tests, the minimum inhibitory concentration which inhibited the spore germination completely was 6 $ml \cdot l^{-1}$ and 3 $ml \cdot l^{-1}$ for *B. cinerea* and *A. rabiei*, respectively. In detached tomato leaf assays, the most efficient extract concentration against *B. cinerea*, without causing phytotoxicity on leaves, was that of 6 $ml \cdot l^{-1}$. In greenhouse tests, the *S. guianensis* extract was efficient to control gray mold in cucumber leaves and young tomato plants at the concentrations of 3 $ml \cdot l^{-1}$ and 6 $ml \cdot l^{-1}$, respectively. However, the extract had no efficiency in controlling *B. cinerea* on strawberry, and young cucumber plants and fruits. The *S. guianensis* extract controlled well Ascochyta blight in chickpea at all concentrations tested.

These results give indications for a possible use of *S. guianensis* extract in plant disease management. Nevertheless, further studies on antifungal properties of *S. guianensis* extract should be performed under *in vivo* conditions for exact determination of dose and concentration to use for disease management in practice.

2.2.1.2 Introduction

Air-borne fungal plant diseases caused by *Botrytis* spp. and *Ascochyta* spp. can be destructive for many agricultural crops. Especially Botrytis diseases are probably the most common ones of vegetables, ornamentals, fruits, greenhouse and field-grown crops throughout the world; one of the most serious diseases is gray mold of strawberry (Agrios, 2005).

Although *B. cinerea* can be controlled by fungicides, the control is not always efficient because of its aggressiveness and widespread. In addition, *Botrytis* strains resistant to several systemic and even to some broad-spectrum fungicides have been found in various crops (Agrios, 2005). Genetic resistances to *B. cinerea* are not available up to now. Also, *Ascochyta rabiei*, the causal agent of the most destructive disease in many chickpea growing countries

(Basandrai *et al.*, 2007), is difficult to control. Even if several varieties resistant to the race 1 and race 2 of *A. rabiei* are available, their effectiveness varies depending on seasonal conditions (Davidson and Kimber, 2007) as well as on the effectiveness of chemical fungicides. Genetic resistances to the third race of the fungus are not available so far.

To resolve a problem of difficult management of these air-borne pathogens, it is proposable to search for novel antifungal compounds capable of controlling these diseases. Recently, many studies on various plant extract antifungal properties have been performed. Several plants have shown an *in vitro* antifungal activity against *B. cinerea* (*Tab. 2.2.1.1*).

Tab. 2.2.1.1: Plants tested for their antifungal activity against *B. cinerea in vitro*

Plants	**References**
wild marjoram (*Origanum syriacum* Sieb. Exs. Et. L.)	Abou-Jawdah *et al.* (2002)
garlic (*Allium* spp.)	Wilson *et al.* (1997); Curtis *et al.*(2004)
pepper (*Capsicum* spp.)	Wilson *et al.* (1997)
rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia genistoides*)	Coetze *et al.*(2008)
Orthosiphon stamineus Benth.	Hossain *et al.* (2007); Hossain *et al.* (2008)
Macaranga monandra	Salah *et al*. (2003)
Bazzania trilobata (L.) S.F. Gray	Scher *et al.* (2004)
Ruta graveolens L.	Oliva *et al.* (2003)
Rubus ulmifolius	Sisti *et al.* (2007)
paw-paw (*Carica papaya*) and bitter leaf (*Vernonia amygdalina*)	Ogwulumba *et al.*(2008)
D. kilimandscharicus	Tegegne and Pretorius (2007)
Metasequoia glyptostroboides	Bajpai and Kang (2009)
Nandina domestica Thunb.	Bajpai *et al.* (2009)
Silene armeria L.	Bajpai *et al.* (2008)
Cestrum nocturnum L.	Al-Reza *et al.* (2009)
wild rue (*Peganum harmala* L.)	Sarpeleh *et al.* (2009)
Atriplex inflata	Boughalleb *et al.*(2009)
rosemary (*Rosmarinus officinalis* L.)	Özcan and Chalchat (2008)
basil (*Ocimum* sp.)	Asgari *et al.*(2009)
Chelidonijum majus	Pârvu *et al.* (2008)
Verbena officinalis L.	Casanova *et al.* (2008)
Quillaja saponaria Mol.	Ribera *et al.* (2008)
Eugenia caryophyllata	Amiri *et al.*(2008)

Efficacy of some plant extracts against *B. cinerea* was confirmed also by *in vivo* assays, e.g. seaweed (*Ascophyllum nodosum*) extract on carrot plants that also induced higher transcript levels of some pathogenesis-related proteins (Jayraj *et al.*, 2008); powders, aqueous and ethanolic extracts of seeds and leaves of huamuchil (*Pithecellobium dulce*) (Bautista-Baños *et al.*, 2002), and *Thymus vulgaris* L. essential oil (Bhaskara Reddy *et al.*, 1997) on strawberry fruit; the methanol extract of stems of *Catalpa ovata* G. Don. containing an active dehydro-α-lapachone compound (Cho *et al.*, 2006); the formulated plant extract from the giant knotweed *Reynoutia sachaliensis* (Milsana) (Bardin *et al.*, 2008) on tomato plants; and the plant defence substances such as hydroxy fatty acids in cucumber (Hou and Forman, 2000).

Few reports have been made on antifungal activity of plant-derived products against *Ascochyta* spp. Chitinase from *Brassica juncea* showed to inhibit under *in vitro* conditions the growth of *A. rabiei* (Guan *et al.*, 2008). Tinivella *et al.* (2009) screened resistance inducers, commercially formulated microorganisms, non-formulated selected strains of different microorganisms and plant extracts, applied as dry or liquid seed treatments on naturally infested seeds, for their antifungal activity against Ascochyta blight of pea. Almost all seed treatments, apart the slight control by thyme oil and by a strain of *Clonostachys rosea*, turned to be ineffective in controlling Ascochyta infections. To our knowledge, there are no studies on the antifungal activity of crude plant extracts against *A. rabiei* in chickpea.

In the present study, the antifungal properties of *Siparuna guianensis* Aubl., an important tree species for example in Brazil (Carvalho *et al.*, 2007), were investigated. The genus *Siparuna* (*Monimiaceae*) is widely spread mainly in tropical and subtropical regions of the Americas. Plants of *Monimiaceae* family are aromatic trees or shrubs bearing essential oils, often lemon scented, with opposite, simple, evergreen leaves (Hutchinson, 1967). Some of the species is used by local people in the treatment of various dicorders and diseases. They have tonic, stimulant, digestive and carminative properties (Peckolt and Peckolt, 1920; Corrêa, 1926, 1978; Occhioni and Lyra, 1948). In addition, *S. guianensis* has shown antiprotozoal activity (Tempone *et al.*, 2005). The medicinal properties of *Siparuna* spp. are attributed to their content of


Siparuna guianensis

flavonoids, alkaloids, essential oils, terpenoids, lignans, and cinnamic acid derivates (Leitão *et al.*, 1999). The main constituents of *S. guaianensis* leaf oil are decanoic acid, 2-undecanone (Fischer *et al.*, 2005), and epi-α-cadinol (Viana *et al.*, 2002). The fruit oil consists mainly of 2-undecanone, β-pinene, limonene (Fischer *et al.*, 2005), and terpinolene (Viana *et al.*, 2002).

Till now, no study on antifungal activity of *S. guianensis* extract against fungal plant pathogens has been effectuated. The aim of the present research was to investigate antifungal properties of *S. guianensis* against *B. cinerea* and *A. rabiei* both under *in vitro* and *in vivo* conditions. In particular, *in vivo* plant extract efficiency against *B. cinerea* and *A. rabiei* was studied in strawberry, tomato, cucumber, and in chickpea, respectively.

2.2.1.3 Materials and methods

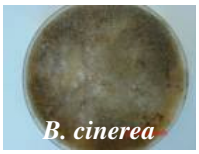
Plant material

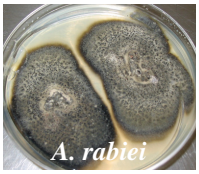
Crude methanol extract from roots of *S. guainanesis,* obtained by Soxhlet extraction technique (modified from Ehrman, 1994), was provided from AGROTECNOLOGIAS NATURALES S.L. (ATENS company, Spain).

For *in vivo* tests with *B. cinerea*, the strawberry cv. Marmolada (Azienda MAZZONI), the cucumber cv. Evergreen (ESASEM, Cesena), and the tomato cv. Superprecoce di Marmande (SGARAVATTI) have been utilized. The chickpea susceptible variety "Calia" has been considered for the artificial inoculations with *A. rabiei*.

Fungal pathogens

Isolates of *B. cinerea*, kindly provided by the fungal collection of the Tuscia University in Viterbo), and isolates of *A. rabiei* race 2 from ENEA collection were re-isolated from infected plant organs, after having verified their pathogenicity with appropriate tests. Pure cultures were *in vitro* maintained on potato dextrose agar (PDA, OXOID), and utilized for the experiments using 10-day-old cultures.





In vitro antifungal assays

The toxicity of plant extracts against the two fungi was performed according to Abou-Jawdah (2002) with a poisoned plate technique. Plant extracts sterilized by 0.2 µl filtration were added to PDA after autoclaving, when the temperature of the medium reached about 50°C, and then were mixed thoroughly. The final volume of extracts in 20 ml of PDA per

each Petri dish was adjusted to four different final concentrations (0.75, 1.5, 3.0, 6.0 $ml \cdot l^{-1}$). Unamended PDA plates served as controls.

Mycelial growth inhibition tests were performed placing, in the center of each plate, 3 pieces of 5 mm mycelial agar discs cut from the margin of actively growing fungal colonies. Colony diameter was measured after incubation at 20°C for 5 days, and mycelial development was observed for 14 days. All treatments were replicated 5 times. The percentage of inhibition was calculated comparing treated plates with control.

For **spore germination tests,** 200 µl of the spore suspension (10^6 $spore \cdot ml^{-1}$) were applied to PDA plates and incubated at 20°C for 18 hours. Then, a drop of 37% formaldehyde was added to the medium to inhibit further development of the germ tubes. A spore was considered germinated when the length of the germ tube equalled or exceeded that of the spore itself (Vicedo *et al.*, 2006). Two hundred spores were counted in each replicate. The percent inhibition was calculated according to Abbott's formula:

$$\frac{(\%\ \text{living in control} - \%\ \text{living in treatment})}{\%\ \text{living in control}} \times 100$$

The minimum inhibitory concentration (MIC) was established as the lowest concentration of tested extract that resulted in no visible spore germination or mycelial growth inhibition after 18 hours of incubation. Fungicide activity was considered when no fungal growth was observed in the plates and fungistatic activity was considered when fungal growth was delayed (Osorio *et al.*, 2010).

Screening of antifungal activity on detached leaves

The antifungal assays on detached tomato leaves were performed in Petri dishes containing perlite with two tomato leaves placed on the surface. The plant extracts at six different concentrations (6, 12, 24, 48, 96 and 192 $ml \cdot l^{-1}$) were sprayed onto the leaf surface in the amount of 100 or 200 µl and, then, the leaves were inoculated either with 5 mm-mycelial plugs or with 30 µl drops containing conidial suspension at the concentration of 10^6 $spores \cdot ml^{-1}$. Inoculated

Fig. 2.2.1.1: Evaluation scale of necrotic lesions

and uninoculated leaves treated with water, and uninoculated leaves treated with the extract were used as controls. All treatments were replicated 10 times. Each experiment was repeated at least twice. The development of necrotic lesions was evaluated according to a 0-11 scale (0=without lesions, 11=91-100% of the leaf surface covered with lesions; *Fig. 2.2.1.1*). In addition, the mycelial development was evaluated according to a 0-3 scale (0=without mycelium development, 3=maximum mycelium development).

In vivo antifungal assays

In the greenhouse tests with *Botrytis cinerea*, the treatments with *S. guianensis* extract at three different concentrations were carried out one day before inoculation; inoculated control treated with chemical fungicide (Switch, 0.8 $g \cdot l^{-1}$), inoculated control treated with distilled water, uninoculated control treated with extract at the highest concentration tested, and uninoculated control treated with distilled water were established. The plant aerial parts were inoculated with spore suspension at the concentration of $5 \cdot 10^5$ $spore \cdot ml^{-1}$ (*Fig. 2.2.1.2*). The experiments were carried out both on adult and young plants. The mix of peat and perlite (2:1), and perlite was used as a cultivation substrate for adult plants and young plants, respectively.

Fig. 2.2.1.2: Preparation of *B. cinerea* inoculum

The extract at the concentration of 0.75, 1.5 e 3.0 $ml \cdot l^{-1}$ was sprayed onto the adult strawberry plants (*Figs. 2.2.1.3, 2.2.1.4*) at the dose of 10-15 ml per plant. Each treatment consisted of 15 plants and was three times replicated. The treatments were weekly repeated for the total of 7 treatments. Strawberry plants were inoculated twice at the beginning of flowering and at the fruit ripening stage, 28 and 40 days after transplantation into 14 cm-diameter pots. The plants were kept in the greenhouse at a minimum temperature of 10°C and a maximum temperature of 30 °C, and high relative humidity (70%). The disease incidence and the percentage of the diseased leaves and fruits were valuated 24, 27, 32, 38, and 45 days after inoculation.

Fig. 2.2.1.3: Greenhouse experiment on strawberry

Fig. 2.2.1.4: Application of *S. guianensis* extract on strawberry plants

The extract at the concentration of 0.75, 1.5 e 3.0 $ml \cdot l^{-1}$ was sprayed onto adult cucumber plants (*Figs. 2.2.1.5, 2.2.1.6*) at the dose of 10-15 ml per plant. Two additional treatments with the extract at the concentration of 1.5 and 3.0 $ml \cdot l^{-1}$ have been added one day after inoculation. Each treatment consisted of six plants with three replications. The treatments were repeated weekly for the total of 5 treatments. Adult cucumber plants were inoculated at the beginning of flowering, 34 days after transplanting to the 20 cm-diameter pots. The plants were kept in the greenhouse at a minimum temperature of 16 ^{o}C and a maximum temperature of 33 ^{o}C, and high relative humidity (70%). The disease incidence and the percentage of the diseased leaves and fruits were evaluated 9, 16, 22, and 30 days after inoculation.

Fig. 2.2.1.5: Greenhouse experiment on adult cucumber plants

Fig. 2.2.1.6: Application of *S. guianensis* extract on cucumber plants

The extract at the concentration of 6, 12 e 24 $ml \cdot l^{-1}$ was sprayed onto the young plants of cucumber and tomato (*Fig. 2.2.1.7*) at the dose of 8 ml per plant. Each treatment consisted of 16 plants and was repeated three times. The treatment at the concentration of 24 $ml \cdot l^{-1}$ was additionally repeated also one day after inoculation. The plants were inoculated at three-leaf stage, 30 days after transplantation into 14 cm-diameter pots. The plants were kept in the greenhouse at a minimum temperature of 16 and a maximum temperature of 27 ^{o}C. High relative humidity was maintained at 70%, covering plants with plastic. The disease incidence was evaluated 14 days after inoculation according to a 0-4scale: (0) healthy plant, (1) 1-24% of plant diseased, (2) 25-49% of plant diseased, (3) 50-74% of plant diseased, (4) 75-100% of plant diseased.

Fig.2.2.1. 7: Greenhouse experiment on young cucumber (on left) and tomato plants (on right)

In two greenhouse tests with *A. rabiei*, the treatments with *S. guianensis* extract at three different concentrations (6,12, and 24 $ml \cdot l^{-1}$; 48, 96, and 192 $ml \cdot l^{-1}$) were effectuated one day before inoculation spraying young chickpea plants (*Fig. 2.2.1.8*) with the dose of 4 ml per plant. Each treatment consisted of 20 plants and was three times repeated. The treatment was repeated 7 days after inoculation. The inoculated control treated with distilled water, uninoculated control treated with extract at the highest concentration tested, and uninoculated control treated with distilled water were established. The plant aerial parts were inoculated with spore suspension at the concentration of $2 \cdot 10^5$ $spore \cdot ml^{-1}$ and $5 \cdot 10^5$ $spore \cdot ml^{-1}$ in the first and second experiment, respectively. The mix of sand and peat (2:1) was used as cultivation substrate. Chickpea plants were inoculated 21 days after sowing into polystyrene containers (58 x 32 x 6 cm) and then kept in the greenhouse inside transparent plastic

Fig. 2.2.1.8: Greenhouse experiment on chickpea

boxes at the temperature about 20°C and with 5 cm-strate of perlite in the bottom to maintain high relative humidit . Disease incidence was evaluated 14 days after inoculation according to a 0-5 scale (0=healthy plant; 5=plant dead) developed by Porta-Puglia *et al.*, (1996).

Statistical analysis

Data were subjected to one way analysis of variance (ANOVA). The follow up of ANOVA included Duncan's multiple range test ($P < 0.05$); in the figures, different alphabetic letters indicate significant differences among various treatments. Statistical elaboration of data was performed with the program SPSS 15.0 for Windows.

2.2.1.4 Results and discussion

In vitro antifungal assays

The MIC of *S. guianensis* extract was 6 $ml \cdot l^{-1}$ for both *B. cinerea* and *A. rabiei*. In addition, at this concentration and 5 days after inoculation, the extract inhibited the mycelial growth of *B. cinerea* (*Figs. 2.2.1.9, 2.2.1.11*) and *A. rabiei* (*Figs. 2.2.1.10, 2.2.1.12*) of 64 and 100%, respectively.

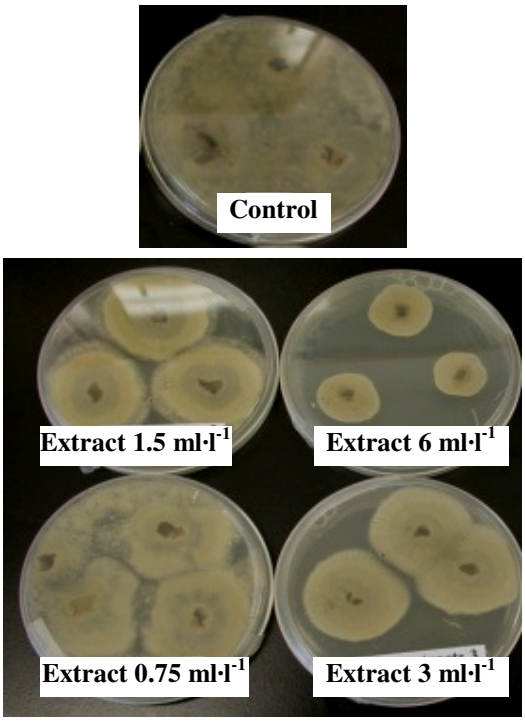


Fig.2.2.1.9: Inhibition of *B. cinerea* mycelial growth

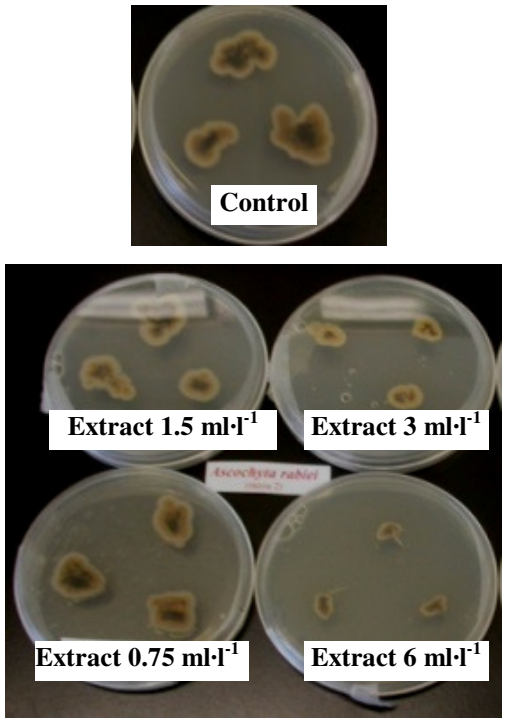


Fig. 2.2.1.10: Inhibition of *A. rabiei* mycelial growth

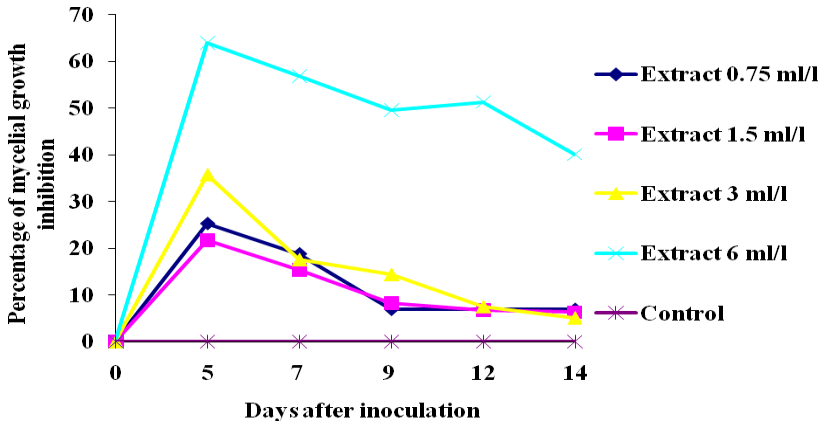


Fig. 2.2.1.11 : Percentage of *B. cinerea* mycelial growth inhibition over time

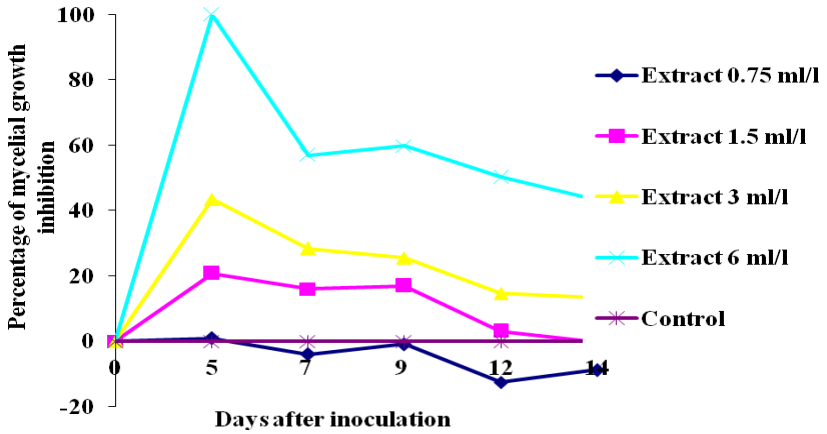


Fig. 2.2.1.12: Percentage of *A. rabiei* mycelial growth inhibition over time

The difference in percentage of inhibition of both fungi is in agreement with Haouala *et al.* (2008), who observed that the magnitude of inhibitory effects of fenugreek extract was species dependent. With the time, mycelial development increased suggesting that the inhibition effect of the plant extract is rather fungistatic than fungitoxic. A similar partial inhibition of *B. cinerea* mycelial growth by thyme oil and its fungistatic effect was reported by Bhaskara Reddy *et al.* (1998) who also observed a different percentage of inhibition in two fungal species that was correlated with their growth velocity. This was observed also in this study because *S. guianensis* extract was less effective against *B. cinerea* that grows faster in comparison with *A. rabiei*. Variation of fungal sensitivity depending on the species was also observed by Cho *et al.* (2006).

In the work of the thesis, spore germination was completely inhibited by *S. guianensis* extract at the concentration of 6 $ml \cdot l^{-1}$ and 3 $ml \cdot l^{-1}$ in *B. cinerea* and *A. rabiei*, respectively (*Fig. 2.2.1.13*). Also Bajpai *et al.* (2008) observed a detrimental effect on spore germination

depending on concentration as well as time-dependent kinetic inhibition of *B. cinerea* by *S. armeria* essential oil. Özcan and Chalchat (2008) reported also that the extent of fungal growth varied depending on the levels of essential oil of rosemary used in experiment.

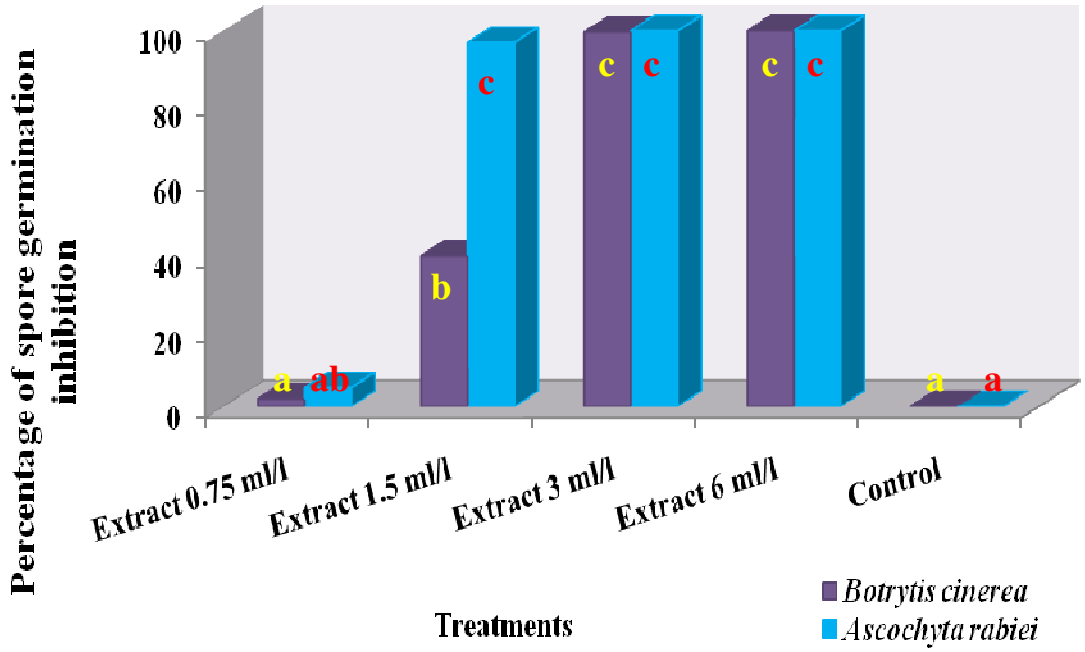


Fig. 2.2.1.13: Inhibition of spore germination of *B. cinerea* and *A. rabiei*

Moreover, irreversible ultrastructural changes in *B. cinerea* conidia by *C. majus* extract have been described by Pârvu *et al.* (2008). These changes could be responsible for fungicidal effect of *S. guianensis* extract on spore germination. In addition, Stuardo and San Martín (2008), with their experiments on fungal membrane integrity, showed that alkali treated saponins from quinoa (*Chenopodium quinoa* Willd) generate membrane disruption. Although petroleum ether extracts of various plants have been indicated as more efficient to control fungal pathogens respect to methanolic extracts Abou-Jawdah *et al.* (2002), our methanolic extracts of *S. guianensis* were highly effective in inhibiting especially *A. rabiei* growth.

Screening of antifungal activity on detached leaves

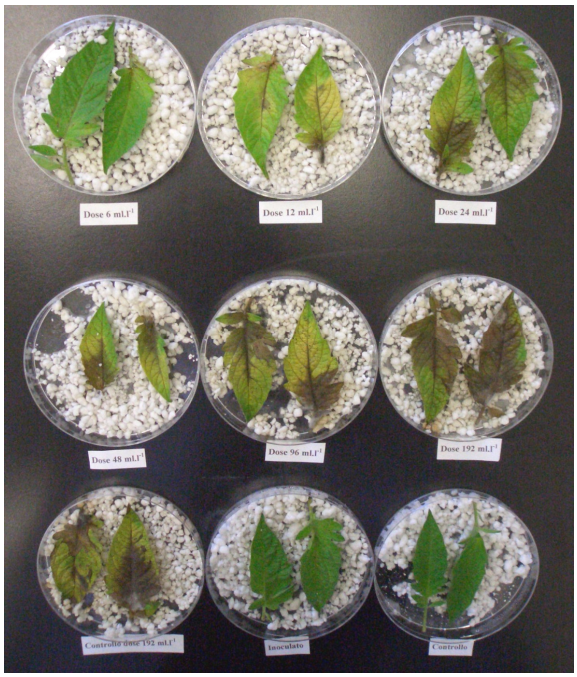


Fig. 2.2.1.14: Lesion development10 days after inoculation

S. guianensis extract applied to leaf surface showed its efficiency reducing the disease development only at the concentration of 6 ml·l^{-1}. A possible mode of action of fungicide applied to detached tomato leaves was described by Vicedo *et al.* (2006). *Botrytis* conidia, treated with adipic acid monoethyl ester (AAME) after inoculation on tomato leaves, were not able to attach and penetrate the leaf.

In our work, treatment with extract at high concentrations caused phytotoxicity on detached tomato leaves (*Fig. 2.2.1.14*), especially when applied in the amount of 200 µl per leaf. The lesions enlarged with the increase of concentration. The only extract treatments that did not cause the phytotoxicity was 6 ml·l^{-1}, and 3, 6, 12 ml·l^{-1}, when applied in the amount of 200 µl and 100 µl, respectively. The development of mycelium was more remarkable on the leaf surface treated with high concentrations of plant extract (96 ml·l^{-1} and 192 ml·l^{-1}). This can be explained by a higher susceptibility of the damaged tissues by high concentrations of plant extract to the fungal attack.

Therefore, the screening for antifungal activity of plant extracts by detached leaf method is suitable only when using low extract concentrations, which are not phytotoxic to leaf tissue. The experiments were influenced by concentration and dose of both extract and inoculum, as well as by the inoculation method and the developmental stage of plant and fungus. Inoculation with mycelial plug was faster in comparison with conidial suspension.

In vivo antifungal assays

In the greenhouse experiment, *S. guaianensis* extract were ineffective in controlling gray mold of strawberry. The percentage of diseased fruits in proportion to the total number of the


Symptoms of *B. cinerea* on strawberry fruit and leaf

fruits (*Fig. 2.2.1.15*) and to the total weight of harvested fruits (*Fig. 2.2.1.16*) increased with time and had a maximum at 38 days after inoculation. There was no significant difference between the reaction observed on the plant treated with the extract and that on the inoculated control. A chemical control was able to reduce fungal attack both in the fruit and in the leaves (*Fig. 2.2.1.17*), however it was not able to protect the plants completely. Also the uninoculated control was attacked gravely by *B. cinerra*. This indicates an excessive inoculum density in the greenhouse. Such results suggest rather to reduce the inoculum density by approaching it to the inoculum density occurring in naturally infested greenhouse. Also Blanco *et al.* (2006) observed a significant positive correlation between Botrytis fruit rot incidence and conidia in the air. Although gray mold in strawberry is very difficult to control ,especially by biological products, Ribera *et al.* (2008) reported a successful biocontrol of *B. cinerea* on strawberry fruit when pretreated with *Q. saponaria* extracts.

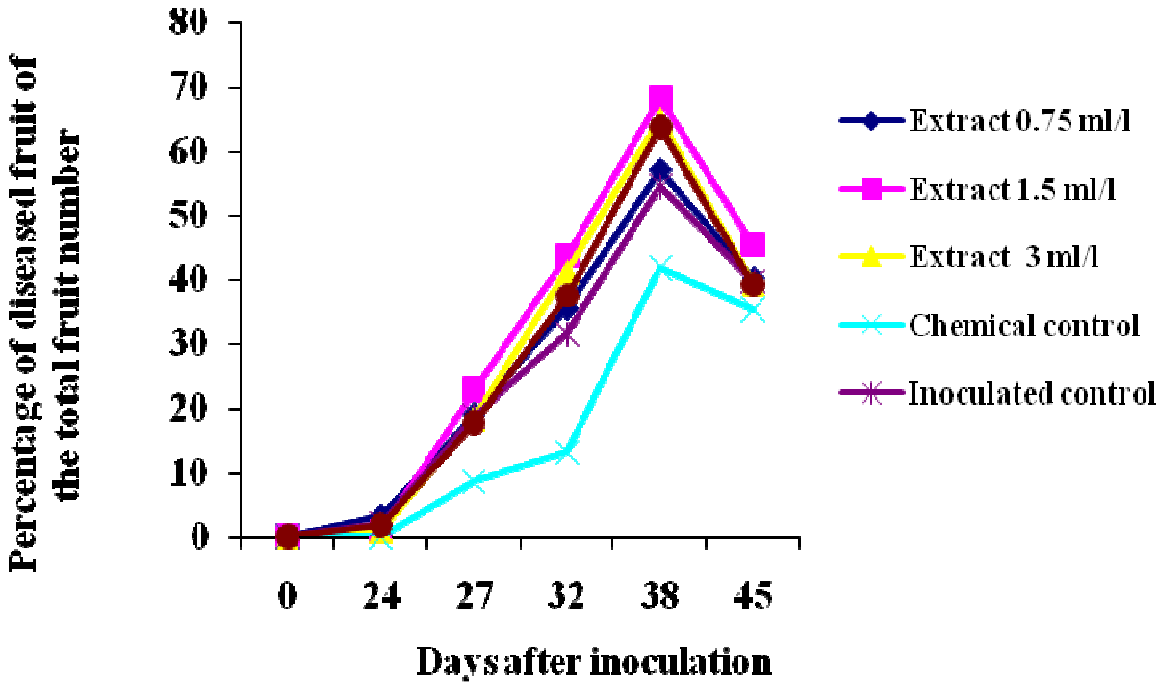


Fig. 2.2.1.15: Evaluation of *S. guaianensis* extract efficiency in control of *B. cinerea* in strawberry – percentage of a number of diseased fruits

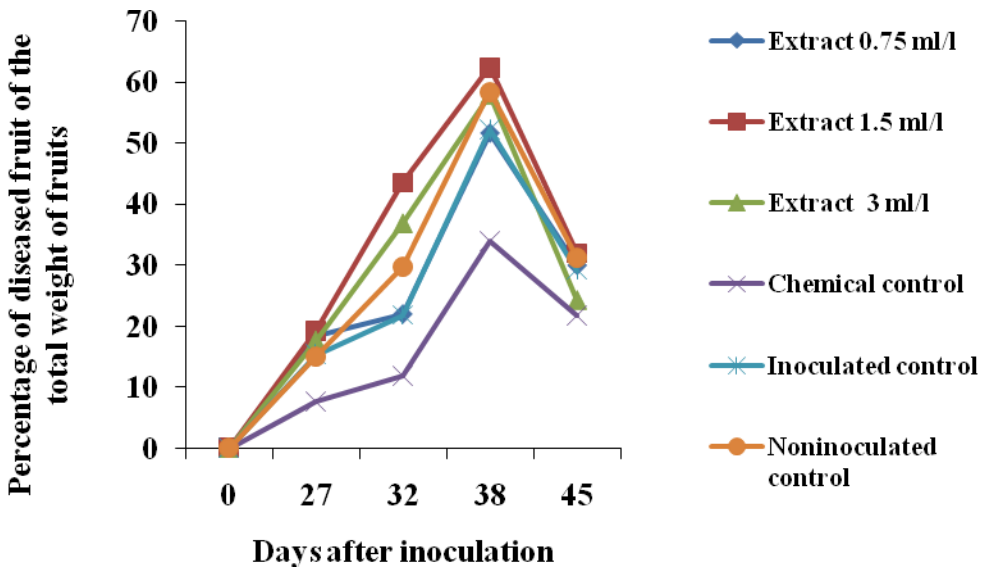


Fig. 2.2.1.16: Evaluation of *S. guaianensis* extract efficiency in control of *B. cinerea* in strawberry – percentage of diseased fruit of the total weight of harvested fruit

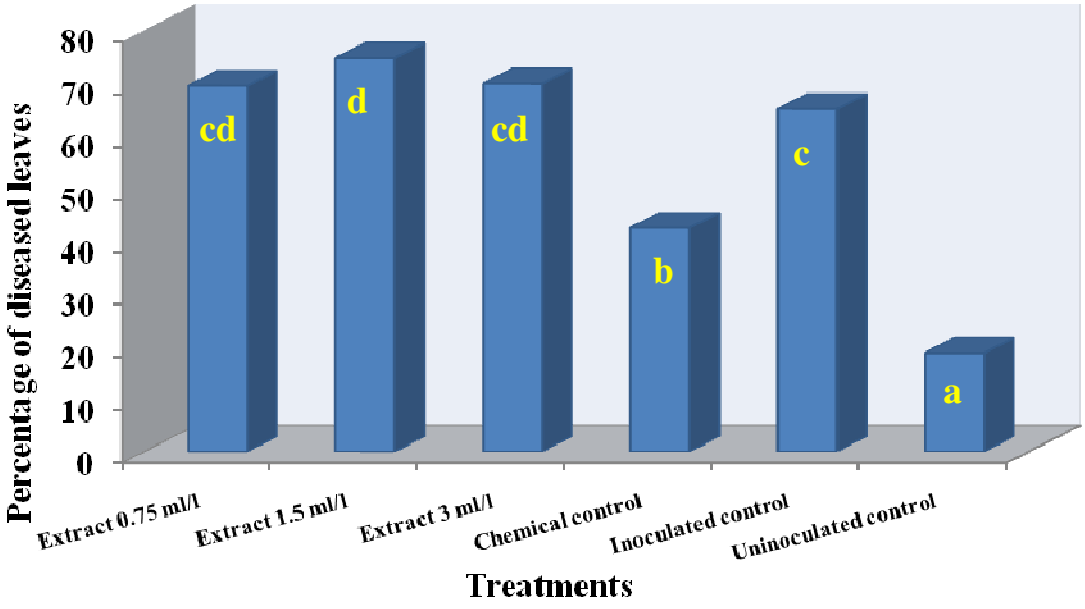
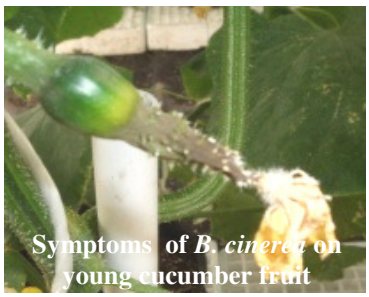


Fig. 2.2.1.17: Evaluation of *S. guaianensis* extract efficiency in control of *B. cinerea* on strawberry leaves


Symptoms of *B. cinerea* on cucumber leaves

S. guaianensis extract (3 $ml \cdot l^{-1}$) partially controlled the gray mold development on the leaves of adult cucumber plants in the greenhouse (*Fig. 2.2.1.18*). However, the commercial fungicide was more effective. There was no significant difference between the extract treatments applied before and after inoculation (*Fig. 2.2.1.19*). Ben-Shalom *et al.* (2003) observed higher efficacy of chitosan, when applied before inoculation. In addition, longer the chitosan stayed on the cucumber leaves before inoculation with *B. cinerea*, the lower was the disease percentage. In this case, chitosan acted as an elicitor that activated disease responses in plant. It is supposed that also some plant extacts can activate disease responses in plants, although their mode of action can be also direct at the contact with the fungus on the leaf surface. The gray mold was not controlled at all by the extract on young cucumber fruits. This could be explained by higher susceptibility of young fruit tissue to the fungus penetration in comparison with adult leaves or fruits. The results of the antifungal screening on adult plants suggest application of an higher extract concentration. Nevertheless, *S. guaianensis* extract had no effect neither in *Botrytis* control on young cucumber plants (*Fig. 2.2.1.20*).


Symptoms of *B. cinerea* on young cucumber fruit

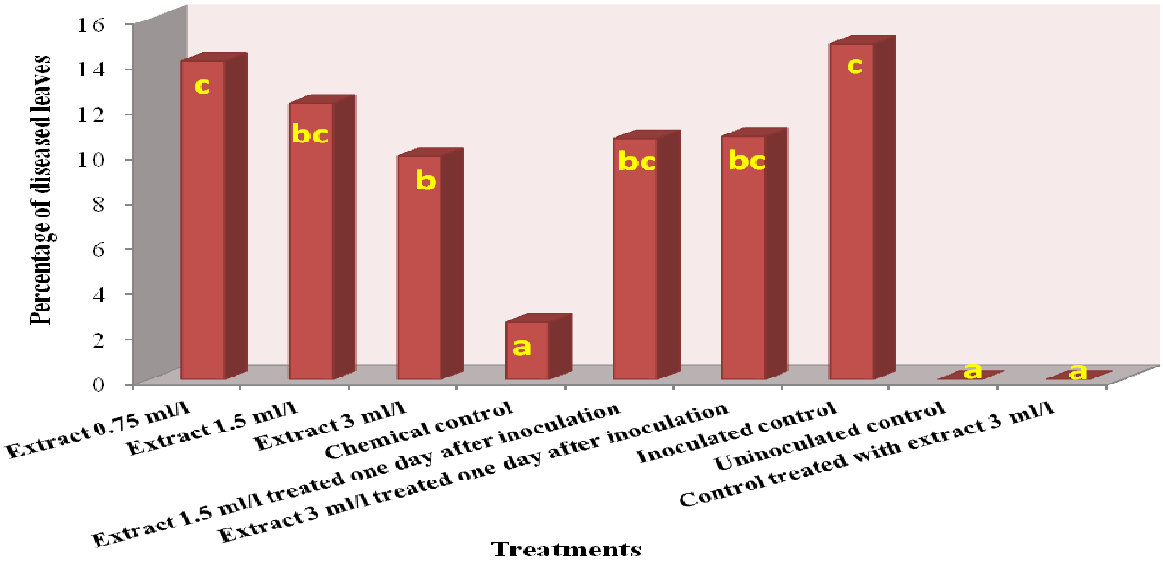


Fig. 2.2.1.18: Evaluation of *S. guaianensis* extract efficiency in control of *B. cinerea* on cucumber leaves

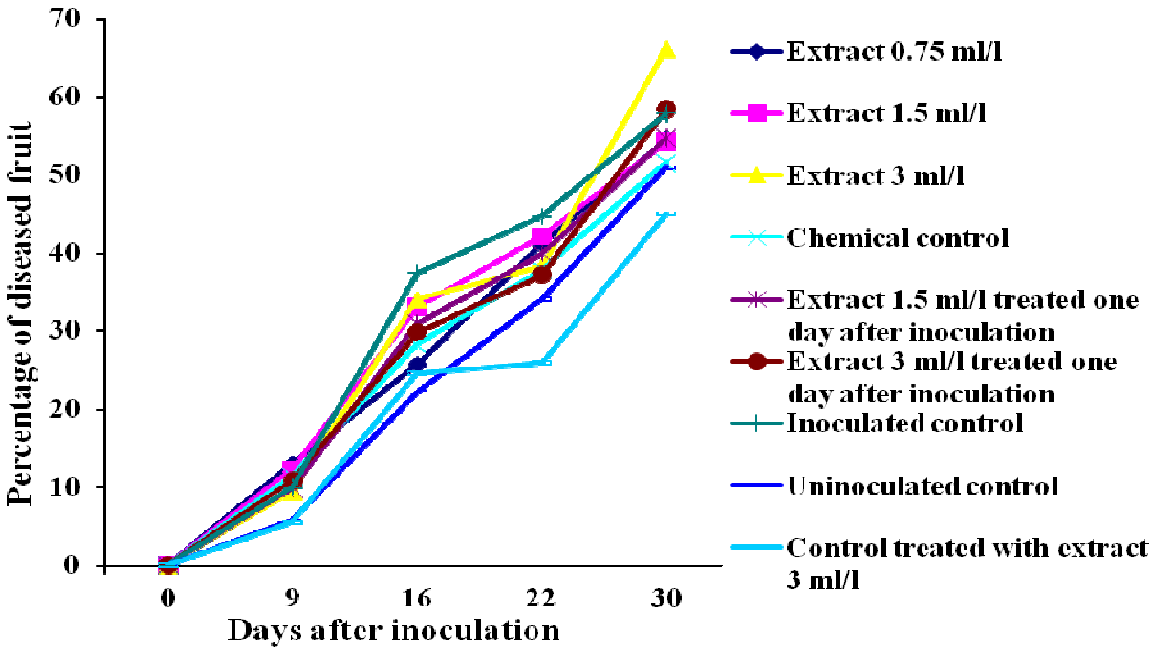


Fig. 2.2.1.19: Evaluation of *S. guianensis* extract efficiency in control of *B. cinerea* on cucumber fruit

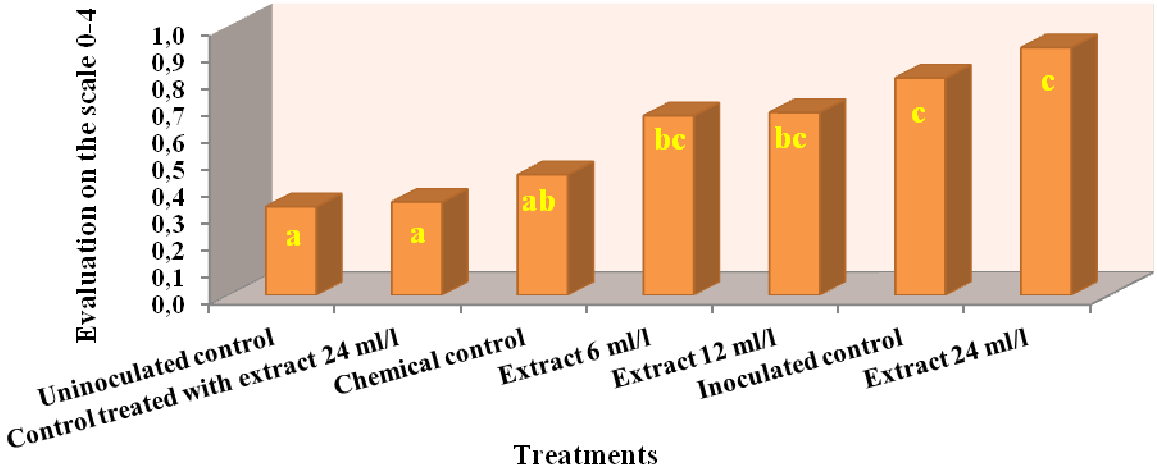


Fig. 2.2.1.20: Evaluation of *S. guaianensis* extract efficiency in control of *B. cinerea* on young cucumber plants

Botrytis disease was controlled by *S. guaianensis* extract at the lowest concentration tested (6 $ml \cdot l^{-1}$) in young tomato plants (*Fig. 2.2.1.21*). A moderate phytotoxicity was observed with the extract concentration of 12 and 24 $ml \cdot l^{-1}$ and within treatment with commercial fungicide. Such results suggest a higher suitability for use of plant extract that is more regardful of young plants than chemical fungicide. There were no differences between treatments applied before and after inoculation. Higher susceptibility to the fungus was observed when young tomato and cucumber plants were treated with higher concentrations of

plant extract. Also Ribera *et al.* (2008) reported a decrease of the leaf area damaged by *B. cinerea* in tomato, when pretreated with *Q. saponaria* extract.

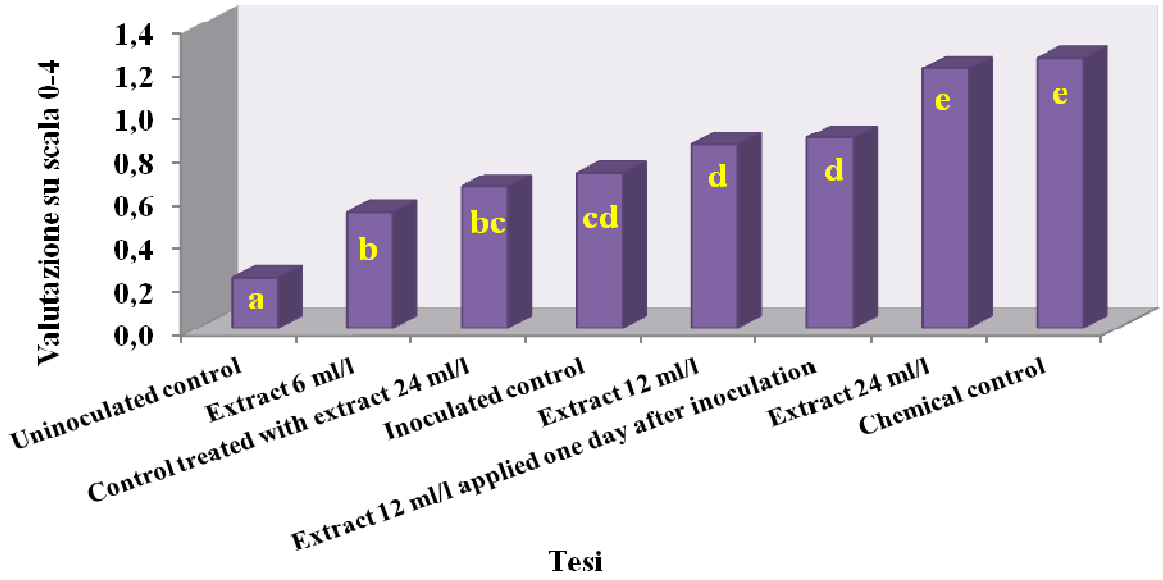
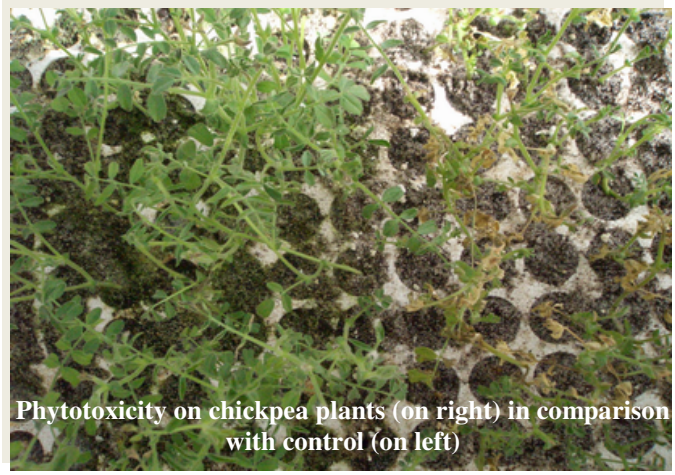


Fig. 2.2.1.21: Evaluation of *S. guaianensis* extract efficiency in control of *B. cinerea* on young tomato plants

In the first experiment, the extract of *S. guaianensis* reduced Ascochyta blight on chickpea at all concentrations (6,12 and 24 $ml \cdot l^{-1}$) in comparison with inoculated control (*Fig. 2.2.2.22*). However, the control of Ascochyta blight was only partial. This is in agreement with Tinivella *et al.* (2009) reporting a great difficulty to control efficiently *Ascochyta* spp. In the second experiment, when the concentrations of plant extract increased, the chickpea plants demonstrated some phytotoxicity. Therefore, despite the *S. guaianensis* extract is efficient in Ascochyta blight control, its use is possible only at low concentrations as the aerial plant organs are extremely sensitive to the treatments. The solution could consist in more frequent application of the plant extract at low concentration.



Phytotoxicity on chickpea plants (on right) in comparison with control (on left)

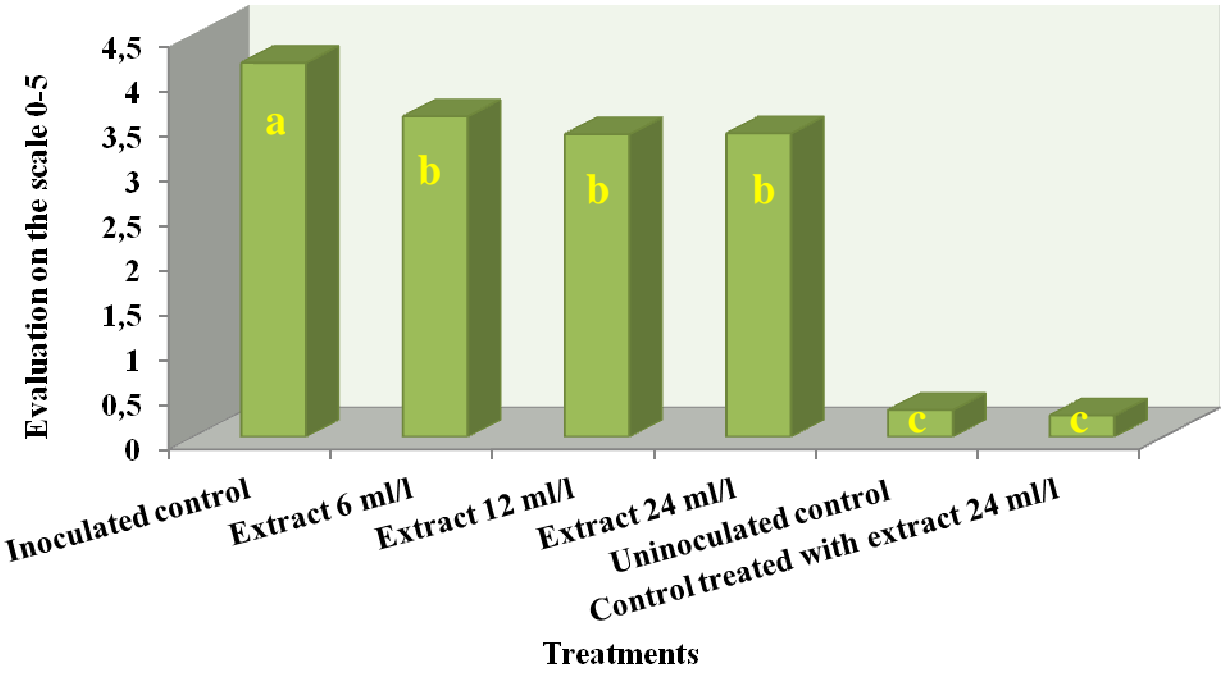


Fig. 2.2.2.22: Evaluation of *S. guaianensis* extract efficiency in control of Ascochyta blight on chickpea

2.2.1.5 Conclusions

S. guianensis extract showed antifungal activity both under *in vitro* and *in vivo* conditions. It had stronger inhibition effect against *A. rabiei* in comparison to *B. cinerea*. The results suggest a possible use of this plant extract as an alternative in disease management of these fungal pathogens. Nevertheless, additional experiments in various culture conditions are necessary to establish the exact dose and concentration of extract maximally efficient *in vivo* and not causing a phytotoxicity effect. The dose and concentration to use will vary in each crop and will depend on plant developmental stage.

2.2.2 *CORDIA LEUCOCEPHALA* EXTRACT / *FUSARIUM OXYSPORUM*

2.2.2.1 Abstract

Cordia leucocephala is a plant with known antifungal properties used in indigenous medicine. Its methanolic extract was tested for controlling *F. oxysporum* in basil, tomato and melon. In our *in vitro* tests, the minimum inhibitory concentration (MIC) of *C. leucocephala* extract was 7 $ml \cdot l^{-1}$ for *F. oxysporum* f. sp. *basilici*, and 14 $ml \cdot l^{-1}$ for *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *melonis*. Extract concentration of 7 $ml \cdot l^{-1}$ inhibited of 92 and 99 % spore germination of *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *melonis*, respectively. In tomato, the extract of *C. leucocephala* at all concentrations significantly reduced disease development in comparison with the inoculated control. Significant inhibitory effect on the Fusarium wilt in melon was observed after treatments by high concentrations of plant extract. In addition, its efficiency increased when applied immediately after inoculation. The plant extract did not control Fusarium wilt in basil. Therefore, the results indicated a possible use of *C. leucocephala* extract in disease management of tomato and melon. Further research should be performed to establish the application procedure of plant extract in practice.

2.2.2.2 Introduction

Fusarium vascular wilts are some of the most widespread and destructive diseases of many crops. *Fusarium oxysporum* f. sp. *basilici* causes sudden wilting and death in sweet basil under field and protected culture conditions; Fusarium wilt caused by *F. oxysporum* f. sp. *lycopersici* is one of the most common and damaging diseases of tomato; and *F. oxysporum* f. sp. *melonis* can devastate melon cultures both under greenhouse and field conditions. It can attack the plant at any developmental stage.

Currently, no efficient fungicide is available to control Fusarium wilt in basil. Also the availability of commercially acceptable resistant cultivars as well as availability of registered fungicides is limited (Minuto *et al.*, 1997). Reuveni *et al.* (2002) reported that the use of compost can induce protection against *F. oxysporum* f. sp. *basilici* and reduce the severity of the visual symptoms of Fusarium wilt. Also the arbuscular mycorrhizal fungus *Glomus mosseae* (Toussaint *et al.*, 2008), antagonistic *Fusarium* spp. (Minuto *et al.*, 1997), and Plantpro 45, an iodine-based compound (Adams *et al.*, 2003) confer a bioprotective effect against *F. oxysporum* f. sp. *basilici* under *in vivo* conditions. In tomato, biological control of

Fusarium wilt inoculating the plants with nonpathogenic strains of *F. oxysporum* or using antagonistic fungi, such as *Trichoderma* and *Gliocladium*, *Pseudomonas fluorescens*, and *Burkholderia cepacia* bacteria was successful. However, none of these methods have been used for the control of Fusarium wilt in practice so far (Agrios, 2005).

In the search for novel antifungal compounds, many studies of various plant extracts for antifungal properties under *in vitro* conditions have been performed (*Tab. 2.2.2.1*). In addition, commercial formulations of various plant extracts, such as pepper/mustard, cassia, clover, and neem showed their efficacy to control muskmelon wilt (Bowers and Locke, 2000).

Tab. 2.2.2.1: Plants tested for antifungal activity against *Fusarium* sp. *in vitro*

Fungi	**Plants**	**References**
Fusarium spp.	*Decalepis hamiltonii* (Wight&Arn)	Mohana *et al.*(2008)
	Rubus ulmifolius Schott	Sisti *et al.*(2008)
	Azadirachta indica	Wokoma and Nwaejike (2008)
	Cymbopogon spp.	Zida *et al.* (2008)
	Thalassia testudinum, Halodule wrighti, *Syringodium filiforme, Ruppia maritima*	Ross *et al* (2008)
	Vincetoxicum rossicum (Kleopow) Barbar.	Mogg *et al* (2008)
	Campuloclinium macrocephalu, Lantana camara	Mdee *et al.* (2009)
	Barringtonia racemosa L.	Hussin *et al.* (2009)
	Acacia nilotica, Achras zapota, Datura stramonium, Emblica officinalis, Eucalyptus globules, Lawsonia inermis, Mimusops elengi, Peltophorum pterocarpum, Polyathia longifolia, Prosopis juliflora, Punica granatum, Syringium cumini	Satish *et al.*(2009)
	Orthosiphon stamineus Benth	Hossain *et al.*(2008)
F. oxysporum	*Cymbopogon martini*	Sridhar *et al* (2003)
	Macaranga monandra	Salah *et al*. (2003)
	Azadirachta indica, Tridax precumbens, Carica papaya, Nicotiana tabacum, *Aloe vera*	Taiga and Oufolaji (2008)
	Dolichos kilimandscharicus, *Maerua subcordata*, *Phytolacca dodecandra*	Tegegne and Pretorius (2007)
	Metasequoia glyptostroboides Miki ex Hu	Bajpai and Kang (2009)
	Silene armetia L.	Bajpai *et al.* (2008)
	rosemary (*Rosmarinus officinalis* L.)	Özcan and Chalcht (2008)
	Wild marjoram (*Origanum syriacum* Sieb. Exs. Et. L.)	Abou-Jawdah *et al.* (2002)
F. oxysporum f. sp. *melonis*	rue (*Peganum harmala* L.)	Sarpeleh *et al.* (2009)


Cordia leucocephala

Plants of the genus *Cordia* (*Boraginaceae*), diffused in Central and South America as well as in Africa, are traditionally used in indigenous medicine, for example for gastrointestinal, respiratory and dermatological disorders (Hernandez *et al.*, 2006), and against malaria (Kambu, 1990). *C. gilletii* extracts of root barks powder with n-hexane, dichlormethane, ethyl acetate, methanol and water possess antimicrobial and antioxidant properties (Okusa *et al.*, 2007). Various studies on antibacterial properties of *Cordia* spp. have been performed (Nakamura *et al.*, 1997; Ioset *et al.*, 1998; Lans *et al.*, 2000; de Carvalho *et al.*, 2004). Hernandez *et al.* (2006) have reported also antifungal activity of essential oil and extracts of *C. curassavica* against *Rhizoctonia solani*, *Aspergillus niger* and *Fusarium* spp. The medicinal properties of *Cordia* sp. are derived by its content in some active compounds, e.g. pyrrolizidine alkaloids (Wassel *et al.*, 1087), terpenoids (Kuroyanagi *et al.*, 2003), flavonoids, lignans, and meroterpenoids naphtoquinones (Ioset *et al.*, 2000).

The aim of this study was to investigate antifungal properties of *C. leucocephala* that have not been reported so far. It is supposed to possess antimicrobial properties because of the high content in some active compounds such as sesquiterpenes with β-caryophyllene and bicyclogermacrene, as main constituents in the leaf essential oil (Diniz *et al.*, 2008), and because of the active compounds contained in roots of various *Cordia* species. Therefore, the root extract of *C. leucocephala* was investigated for its antifungal activity in the control of *F. oxysporum* f. sp. *basilici*, *F. oxysporum* f. sp. *lycopersici*, and *F. oxysporum* f. sp. *melonis* both under *in vitro* and *in vivo* conditions.

2.2.2.3 Materials and methods

Plant material

Crude methanol extract from roots of *Cordia leucocephala,* obtained by Soxhlet extraction technique (modified from Ehrman, 1994), was provided from AGROTECNOLOGIAS NATURALES S.L. (ATENS company, Spain).

Basil cv. Genovese (SEMIORTO), tomato cv. Superprecoce di Marmande (SGARAVATTI), and melon cv. Hale's Best Jumbo (ROYAL SLUIS) were used for *in vivo* tests.

Fungal pathogens

Isolates of *F. oxysporum* f. sp. *basilici* (SAIS), *F. oxysporum* f. sp. *lycopersici* (SAIS), and *F. oxysporum* f. sp. *melonis* 1,2W (ENEA) were re-isolated from infected plant organs. Pure cultures were maintained on potato dextrose agar (PDA, OXOID) and, after fungal multiplication, *in vitro* and *in vivo* experiments were carried out using 10-day-old cultures.

In vitro antifungal assays

The toxicity of plant extracts against *Fusarium* sp. was performed according to Abou-Jawdah (2002), using a poisoned plate technique. Plant extracts sterilized by 0.2 µl filtration were added to PDA after autoclaving, when the temperature of the medium reached 50°C, and then mixed thoroughly. The final volume of extract in 20 ml of PDA per each Petri dish was adjusted to four different final concentrations (1.75, 3.5, 7.0, 14.0 $ml \cdot l^{-1}$). Unamended PDA plates served as controls.

Mycelial growth inhibition tests were performed placing, in the center of each plate, 3 pieces of 5 mm mycelial agar discs cut from the margin of actively growing fungal colonies. Colony diameter was measured after incubation for 5 days, at 26°C, and mycelial development was observed for 14 days. All treatments were replicated 5 times. The inhibition percentage was calculated comparing treated plates with control.

For **spore germination tests**, 200 µl of the spore suspension (10^6 $spore \cdot ml^{-1}$) were applied to PDA plates and incubated at 26°C for 18 hours. Then, a drop of 37% formaldehyde was added to the medium to inhibit further development of the germ tubes. A spore was considered germinated when the length of the germ tube equaled or exceeded that of the spore itself (Vicedo *et al.*, 2006). Two hundred spores were counted in each replicate. The inhibition percent was calculated according to Abbott's formula:

$$\frac{(\%\ \text{living in control} - \%\ \text{living in treatment})}{\%\ \text{living in control}} \times 100$$

The minimum inhibitory concentration (MIC) was established as the lowest concentration of tested extract that resulted in no visible spore germination or mycelial growth at the 18th hour after treatment. Fungicide activity was considered when no fungal growth was observed in the plates and fungistatic activity was considered when fungal growth was delayed (Osorio *et al.*, 2010).

In vivo antifungal assays

The tests with *F. oxysporum* f. sp. *basilici* and *F. oxysporum* f. sp. *lycopersici* were performed in a growth chamber (*Fig. 2.2.2.1*). The experiments were carried out on plants of four- and two-leaf stage in basil and tomato, respectively. The plant roots were washed and cut to the length of 2 cm. Then the roots were immerged into the spore suspension ($5 \cdot 10^5$ spore·ml^{-1}) for 2 and 10 minutes for basil (*Fig. 2.2.2.2*) and tomato, respectively. The treatments with *C. leucocephala* extract at three different concentrations (7, 14 and 24 ml·l^{-1}, and 1.75, 3.5, and 7 ml·l^{-1} for basil and tomato, respectively) were carried out immediately after the inoculation and one day before inoculation for basil and tomato, respectively. The inoculated control treated with distilled water, uninoculated control treated with extract at the highest concentration tested, and uninoculated control treated with distilled water, were established. Each treatment (three replications) consisted of 17 and 15 plants of basil and tomato, respectively. The extract was weekly applied at the dose of 10 ml per plant for the total of 4 and 3 applications in basil and tomato, respectively. The treatment with extract at the concentration of 7 ml·l^{-1}, applied twice a week was added in the experiment with basil plants. The mix of peat (95%) and sand was used as a cultivation substrate for basil plants. A peat substrate was used for tomato plants. Plants were kept in the growth chamber at the temperature of 26^{o}C. Disease severity was assessed by means of a visual rating according to a 0-3 scale (*Fig. 2.2.2.3*): (0) no symptoms, (1) slight leaf epinasty and/or apex chlorosis, (2) leaf epinasty and plant stunting, (3) plant dead (Reuveni *et al.*, 2002). The evaluation of disease incidence was performed 20 and 13 days after inoculation in basil and tomato, respectively. In tomato, the disease incidence was evaluated three times a week for two weeks.

Fig. 2.2.2.1: Experiment on tomato plants in growth chamber

Fig. 2.2.2.2: Inoculation of basil plants

Fig. 2.2.2.3: Evaluation scale of Fusarium symptoms on basil plants

In 4 greenhouse tests with *F. oxysporum* f. sp. *melonis* 1,2W (*Fig. 2.2.2.4*), the treatments with *C. leucocephala* extract at three different concentrations, which varied in each experiment, were performed on melon first-true leaf plants. The substrate was inoculated with spore suspension ($5 \cdot 10^5$ spore$\cdot$ml^{-1}). The inoculated control treated with chemical fungicide (Bavistin®, 1ml$\cdot$l^{-1}), inoculated control treated with distilled water, uninoculated control treated with extract at the highest concentration tested, and uninoculated control treated with distilled water were established. Each treatment consisted of 12 pots (ø 15 cm) containing two plants per plot; it was three time replicated. The evaluation of disease symptoms was performed according to a 0-2 scale: (0) healthy plant, (1) stunting, chlorosis, epinasty, (2) plant dead.

Fig. 2.2.2.4: Greenhouse experiment on melon plants

In the first experiment, the melon plants were treated with the extract at the concentrations of 1.75, 3.5 and 7.0 ml$\cdot$l^{-1} at the dose of 6 ml per plant, one day before inoculation. The treatments with *Trichoderma* and mycorrhizal fungi amended and not amended with plant extract (7 ml$\cdot$l^{-1}) were added. A total of 5 treatments were weekly performed. The cultivation substrate was the mix of peat and perlite (2:1). The plants were kept in the greenhouse at a minimum temperature of 16°C and a maximum temperature of 31 °C, and a relative humidity about 65%. Disease symptoms were evaluated 21 days after inoculation and repeated twice a week for 17 days. Plant height was measured at the end of the experiment.

In the second experiment, the melon plants were treated with the extract at the concentrations of 1.75, 3.5 and 7.0 $ml \cdot l^{-1}$ at the dose of 10 ml per plant, one day before inoculation. A total of 4 treatments were weekly carried out. The cultivation substrate was the mix of peat and perlite (2:1). The plants were kept in the greenhouse at a minimum temperature of 16 ^{o}C and a maximum temperature of 26 ^{o}C, and relative humidity about 75%. The disease symptoms were weekly evaluated for two weeks since the 13rd day after inoculation.

In the third experiment, which was carried out in the growth chamber, the melon plants were treated with the extract at the concentrations of 14, 28, and 56 $ml \cdot l^{-1}$ at the dose of 10 ml per plant, one day before inoculation. The treatment with the extract (56 $ml \cdot l^{-1}$) immediately after inoculation was added. The treatments were weekly carried out for a total of 5 treatments. Peat was used as cultivation substrate. The plants were kept at the average temperature of 21 ^{o}C, and relative humidity about 75%. The disease symptoms were evaluated 18 and 24 days after inoculation according to the same scale previously described.

In the fourth experiment, melon plants were treated with the extract at the concentrations of 7, 14, and 28 $ml \cdot l^{-1}$ at the dose of 10 ml and 5 ml per plant one day before and after inoculation, respectively. The treatments were weekly performed for the total of 6 treatments. Peat was used as cultivation substrate. The plants were kept in the greenhouse at the average temperature of 26 ^{o}C, and relative humidity of 65%. The disease symptoms were evaluated 34 and 41 days after inoculation according to the same scale previously described.

Statistical analysis

Data were subjected to one way analysis of variance (ANOVA). The follow up of ANOVA included Duncan's multiple range test ($P < 0.05$); in the figures, different alphabetic letters indicate significant differences among various treatments. Statistical elaboration of data was performed with the program SPSS 15.0 for Windows.

2.2.2.4 Results and discussion

In vitro antifungal assays

The MIC of *C. leucocephala* extract was 7 $ml \cdot l^{-1}$ for *F. oxysporum* f. sp. *basilici*, and 14 $ml \cdot l^{-1}$ for *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *melonis*. In addition, at this concentration and after three days, the extract inhibited of 34 % the mycelial growth of *F. oxysporum* f. sp. *basilici* (*Figs. 2.2.2.5, 2.2.2.6*).

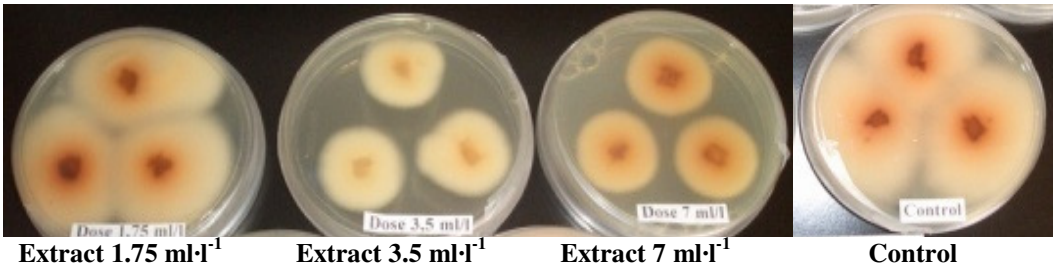


Fig. 2.2.2.5: Inhibition of *F. oxysporum* f.sp. *basilici* mycelial growth

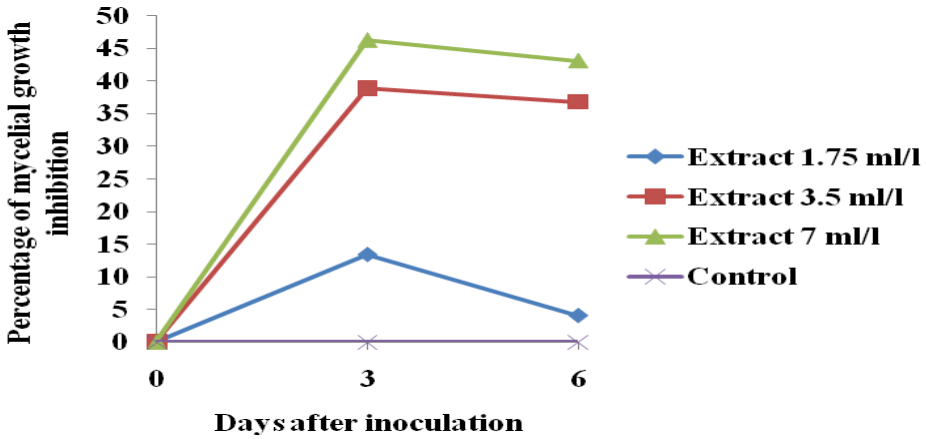


Fig. 2.2.2.6: Percentage of *F. oxysporum* f.sp. *basilici* mycelial growth inhibition over time

In the case of *F. oxysporum* f. sp. *lycopersici* (*Figs. 2.2.2.7, 2.2.2.9*) and *F. oxysporum* f. sp. *melonis* (*Figs. 2.2.2.8, 2.2.2.10*), mycelial growth was inhibited of 46 and 28 %, respectively, 5 days after inoculation. The variation in antifungal activity of plant extracts against different species of *Fusarium* was reported also by Satish *et al.* (2009). With the time, the inhibition effect of the extract diminished suggesting that the inhibition effect of the plant extract on mycelial growth is rather fungistatic than fungitoxic. A positive correlation between increasing extract concentration and percentage of fungal inhibition was observed (Özcan and Chalchat, 2008). Fawzi *et al.* (2009) reported not only the growth inhibition of *F. oxysporum* by plant extracts but also inhibition of its hydrolytic enzymes, β-glucosidase, pectin lyase, and protease. Therefore, this could be a possible explanation for the mode of action of some plant extracts.

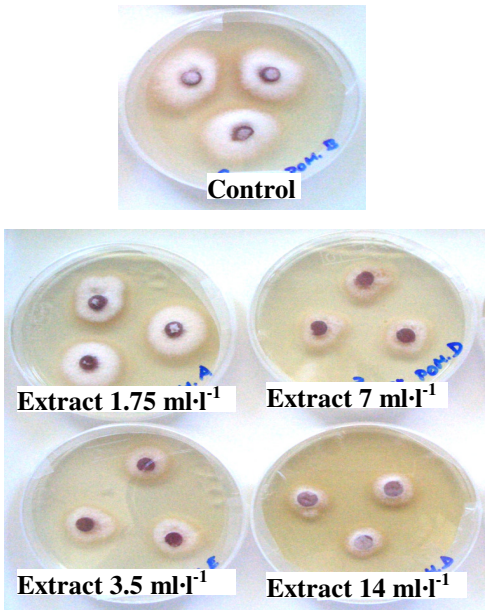


Fig.2.2.2.7: Inhibition of *F. oxysporum* f.sp. *lycopersici* mycelial growth

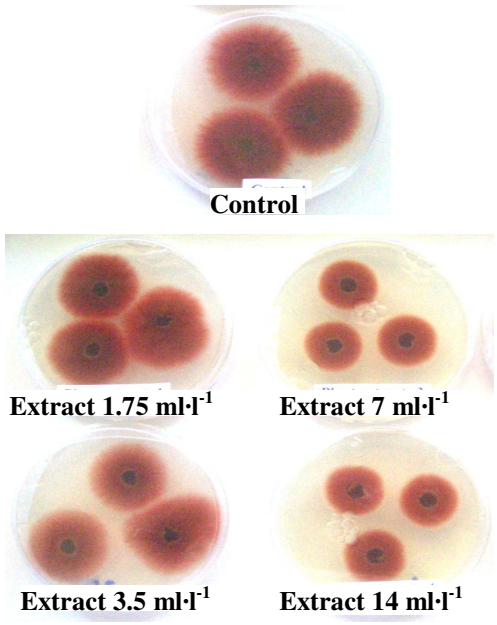


Fig. 2.2.2.8: Inhibition of *F. oxysporum* f.sp. *melonis* mycelial growth

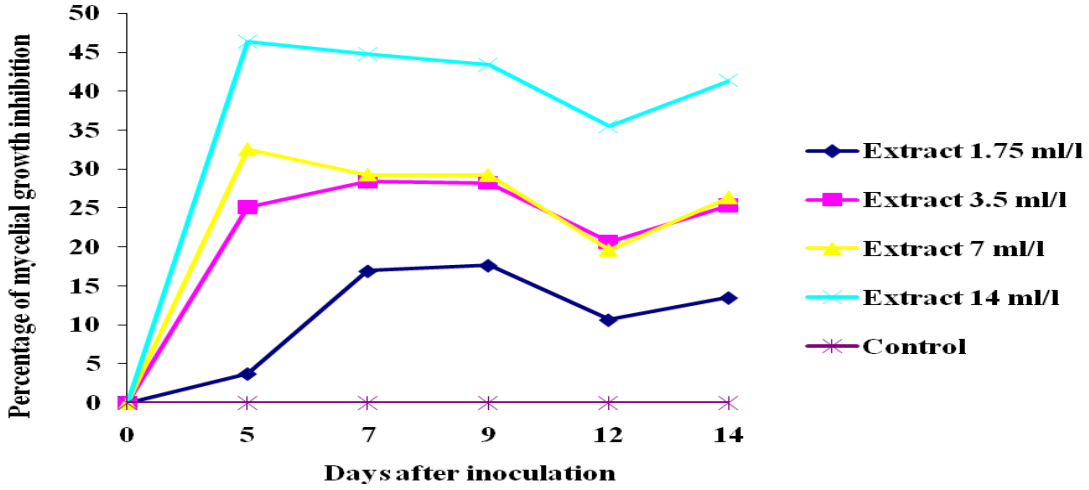


Fig. 2.2.2.9: Percentage of *F. oxysporum* f.sp.*lycopersici* mycelial growth inhibition over time

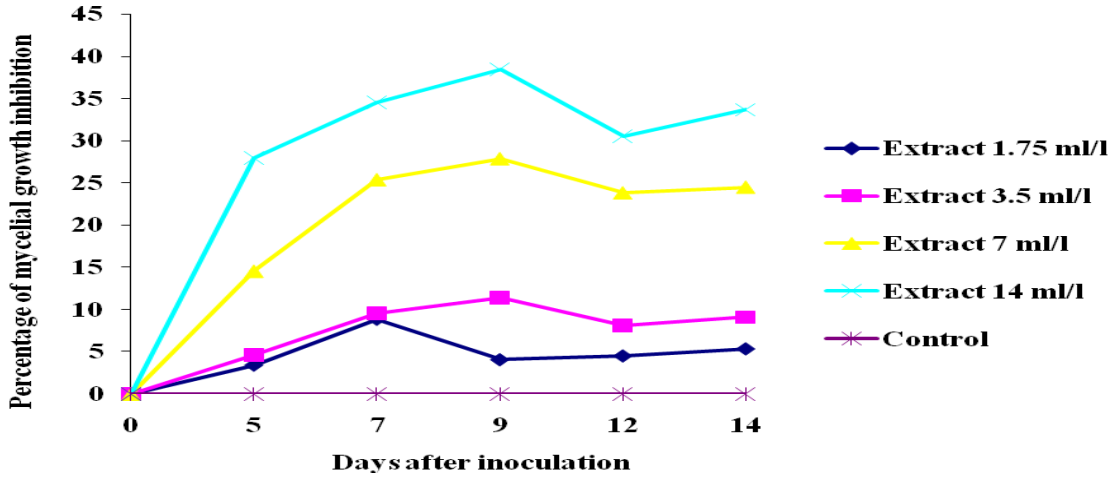


Fig. 2.2.2.10: Percentage of *F. oxysporum* f.sp.*melonis* mycelial growth over time

The spore germination was inhibited by the 7 ml·l^{-1} extract concentration of 92 and 99 % in *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *melonis* (*Figs. 2.2.2.11, 2.2.2.12*), respectively. Also Abou-Jawdah *et al.* (2002) observed higher inhibition of *F. oxysporum* spore germination than of mycelial growth upon plant extract treatment. Probably, spores are more sensitive in the liquid culture, in direct contact with the active molecules of plant extract, in comparison with hyphae on the agarose medium. The difference consists also in the ability of various fungal survival structures to adapt themselves to the environmental changes.

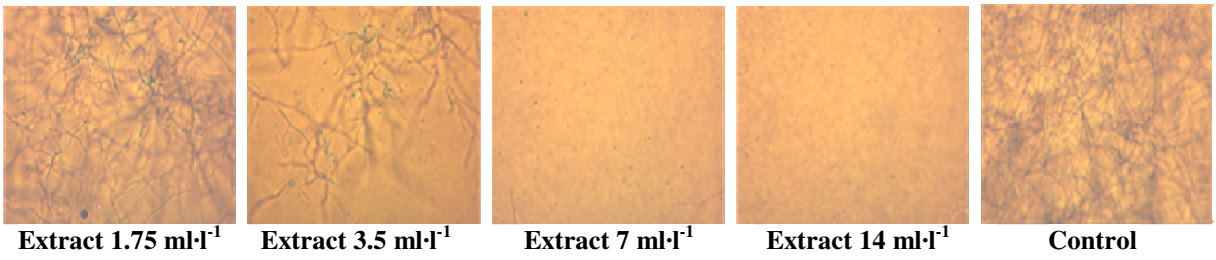


Fig. 2.2.2.11: Spore germination of *F. oxysporum* f.sp. *melonis* at different extract concentrations

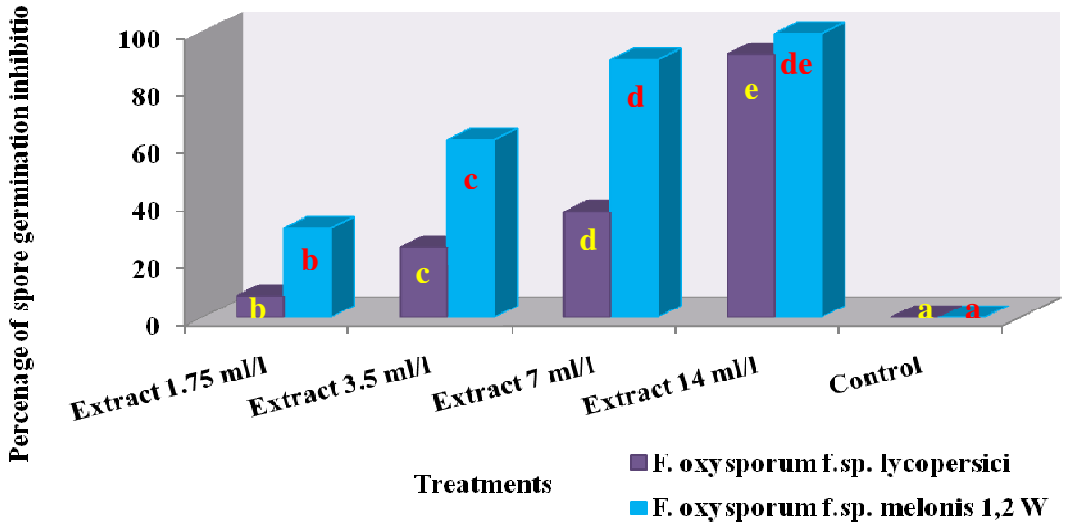


Fig. 2.2.2.12: Inhibition of spore germination of *F. oxysporum* f.sp. *lycopersici* and *F. oxysporum* f.sp. *melonis* 1,2 W

<u>*In vivo* antifungal assays</u>

In the *in vivo* tests for antifungal activity of *C. leucocephala* extract in basil, there was no significant difference among the treatments in comparison to inoculated control (*Fig. 2.2.2.13*). Moreover, the

extract at concentration of 28 $ml \cdot l^{-1}$ caused a phytotoxicity. Siddiqui *et al.* (2002) reduced a phytotoxicity of antifungal plant material on tomato plants that incorporating into the soil by addition of nitrogen.There was no difference among the extract treatments applied weekly and twice a week. These results confirmed a basil susceptibility to various chemicals, and suggested that lower concentrations of plant extract would be suitable for the control of the disease.

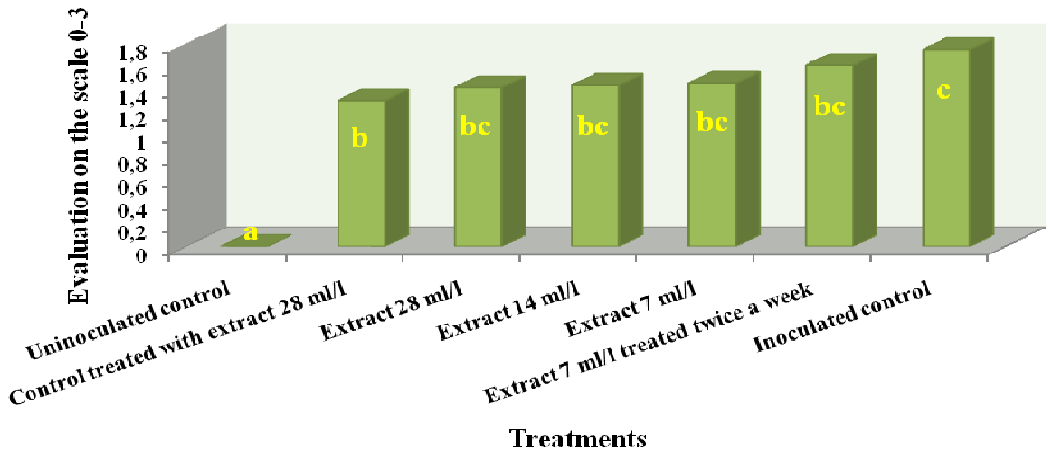


Fig. 2.2.2.13: Evaluation of Fusarium disease symptoms on basil plants

In tomato, the extract of *C. leucocephala* at all concentrations significantly reduced disease development in comparison with inoculated control (*Fig. 2.2.2.14*). In addition, there was no phytotoxicity caused by the plant extract. Similarly, Siddiqui (2002) observed that plant material such as shoots of Agremone mexicana incorporated into the soil had a marked effect on soilborne root-infecting F. solani and on growth pattern of tomato plants. They suggested that it can be due to the changed nutrient status of the soil or because of allelochemicals added to the soil through shoot material or products of microbial degradation. Furthermore, Shaukat and Siddiqui (2001) supposed that such suppression of the root-infecting fungi could be the result of structural and compositional changes either in fungal communities in the soil or in rhizosphere owing to the application of plant material. Therefore, also in the case of the inoculation of the plants by their immersion into inoculum suspension, the addition of plant extract in the soil suppressed further multiplication of fungi and subsequent infestation of the soil. Suppression of wilt development in the greenhouse and corresponding ability of various plant extracts to reduce populations of *Fusarium* in the soil was confirmed by Bowers and Locke (2000).

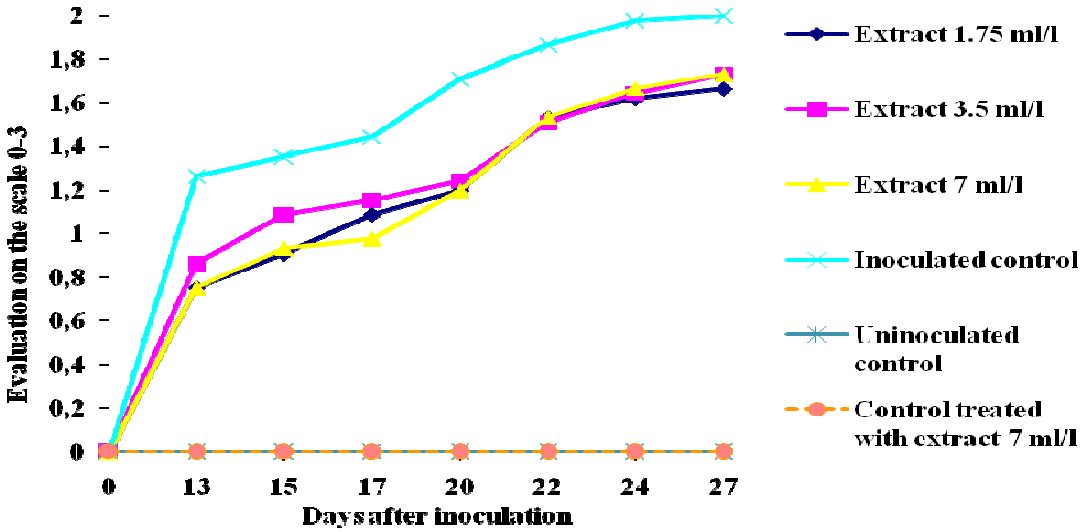


Fig. 2.2.2.14: Evaluation of Fusarium disease symptoms on tomato plants

Three experiments have been carried out on melon to determine the efficient concentration, dose and mode of application of *C. leucocephala* extract. In the first experiment, there was no significant difference in extract treatments (*Fig. 2.2.2.15*) and in plant height (*Fig. 2.2.2.16*). In the treatments with *Trichoderma* and mycorrhizal fungi, a positive correlation with plant height was observed. However, in combination with *C. leucocephala* extract, the positive effect of *Trichoderma* was significantly reduced; the difference between the effect of plant extract and that of mycorrhizal fungi, was not significant.

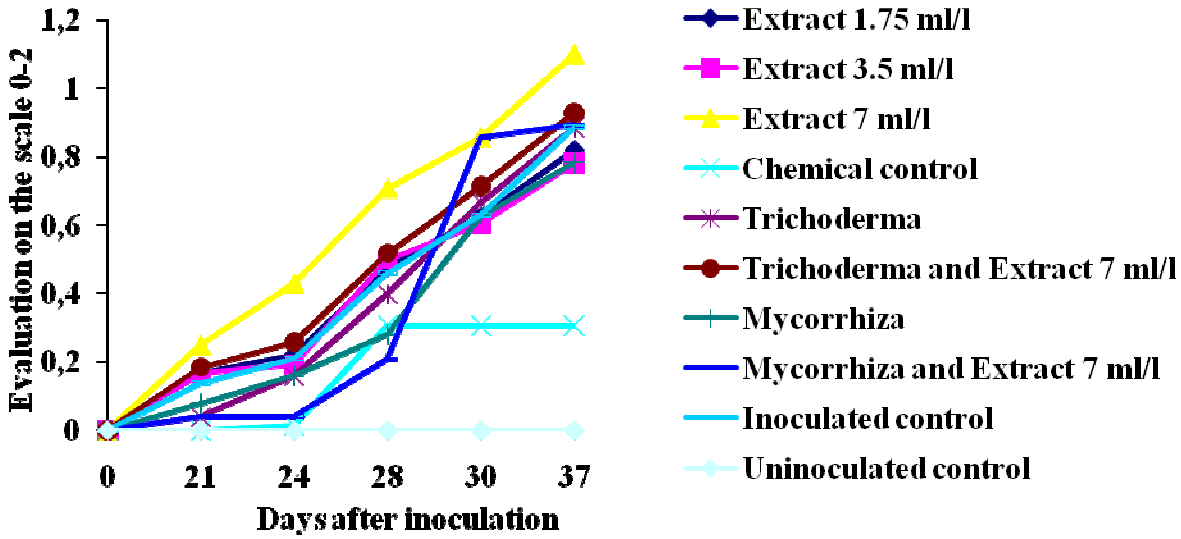


Fig. 2.2.2.15: Evaluation of Fusarium wilt on melon plants treated with plant extracts, *Trichoderma* and mycorrhizal fungi

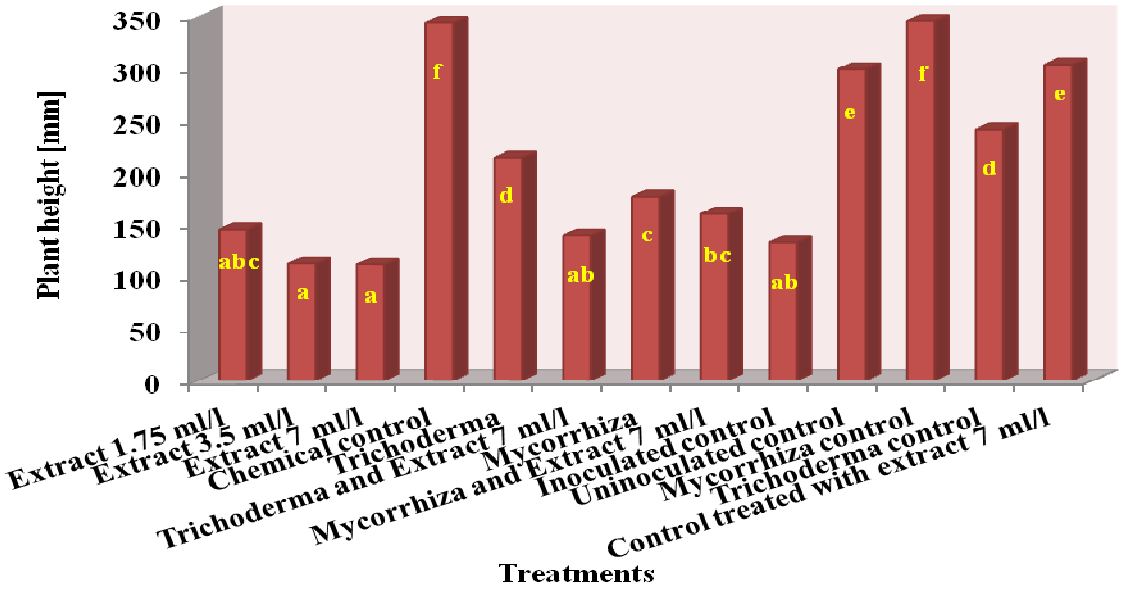


Fig. 2.2.2.16: Evaluation of plant height in melon plants affected by Fusarium wilt

In the second experiment, the treatment with *C. leucocephala* extract partially reduced disease symptoms at the concentration of 1.75 ml·l^{-1} in comparison with inoculated control 20 days after inoculation (*Figs. 2.2.2.17, 2.2.2.18*).

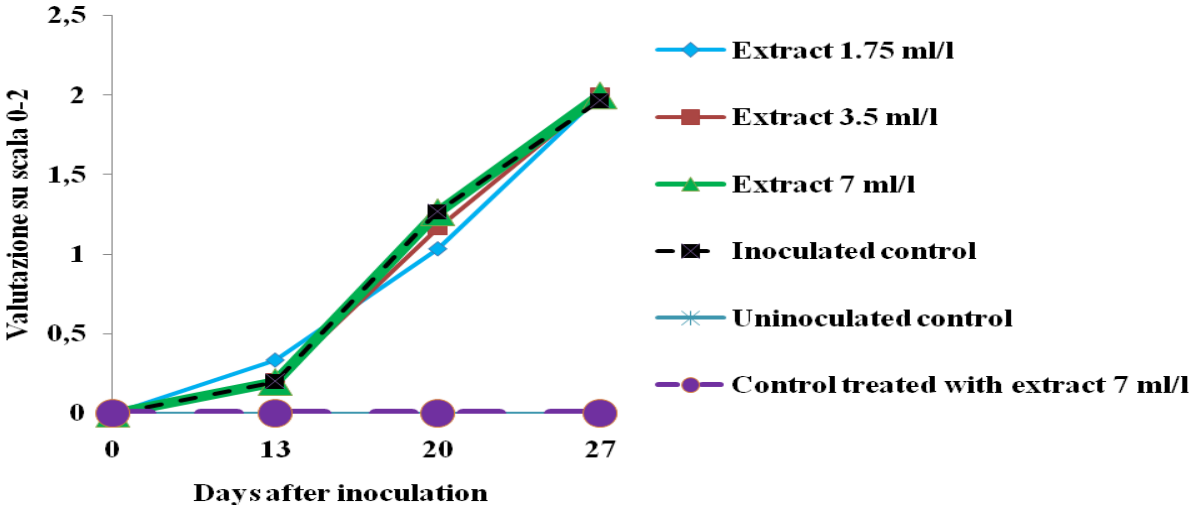


Fig. 2.2.2.17: Development of Fusarium wilt on melon plants in greenhouse test

Fig. 2.2.2.18: Comparison of disease symptoms in different treatments

In the third experiment, the significant inhibitory effect on Fusarium wilt in melon was observed with the treatments of elevated concentrations of plant extract (*Fig. 2.2.2.19*). In addition, its efficiency increased when applied immediately after inoculation. Such result indicates a direct inhibition effect on the fungus rather than an induction of disease mechanisms in plant. Also, Bowers and Locke (2000) observed significant decrease of population density of *F. oxysporum* f.sp. *melonis* after the treatment with plant extracts. They reported that the treatment of soil with plant extracts resulted in significant differences among extract concentrations, with significant interactions with time for both the population density experiments in the lab and disease development in the greenhouse. In the fourth experiment, the disease symptoms were reduced by the plant extract at the concentration of 7 $ml \cdot l^{-1}$ and 14 $ml \cdot l^{-1}$ (*Fig. 2.2.2.20*). However, a slight phytotoxicity was also observed on the plants treated with the extract at the concentration of 14 $ml \cdot l^{-1}$.

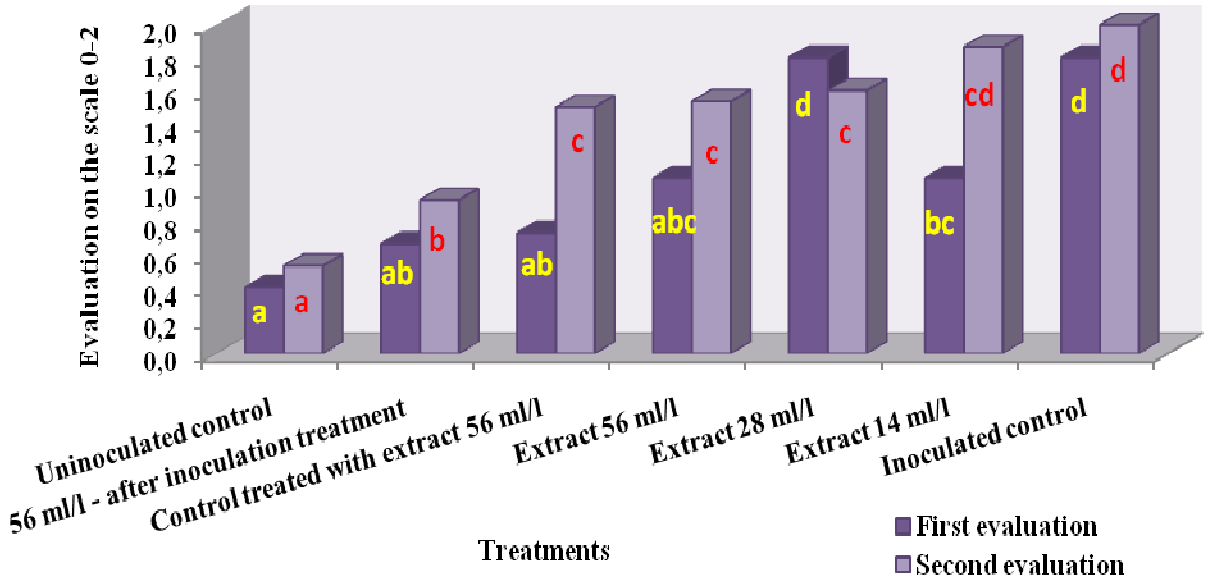


Fig. 2.2.2.19: Evaluation of Fusarium wilt on melon plants in growth chamber test

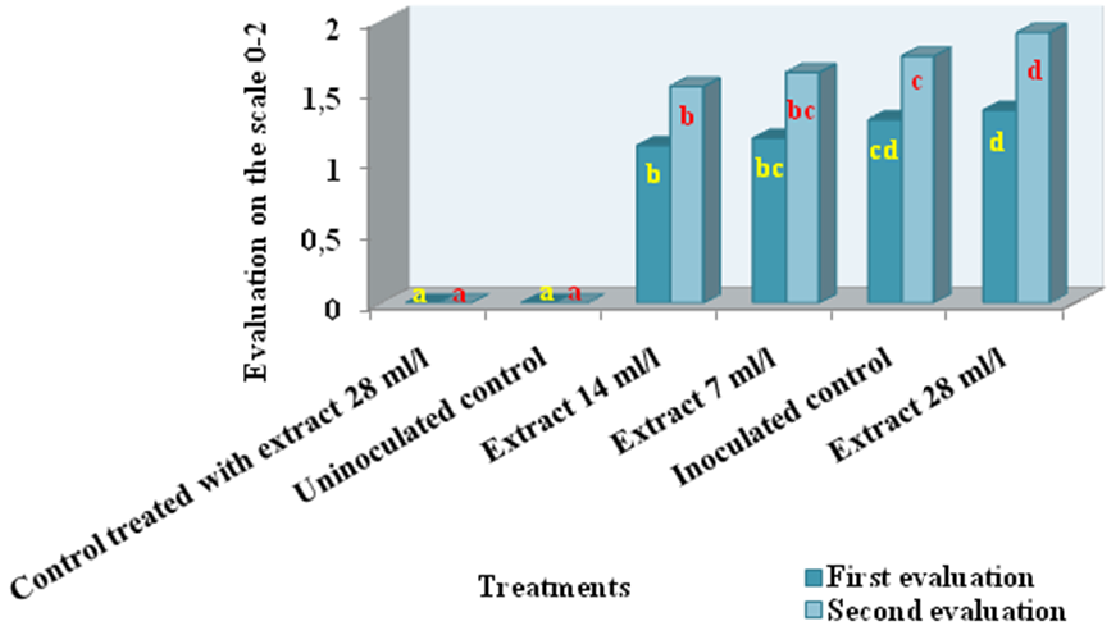


Fig. 2.2.2.20: Evaluation of Fusarium wilt in greenhouse test

2.2.2.5 Conclusions

The antifungal activity of *C. leucocephala* extract was proved under *in vitro* and *in vivo* conditions. The percentage of inhibition by plant extracts varied within three formae specialis of *F. oxysporum*. Although, the results indicated a possible use of this plant extract in disease management of Fusarium wilt in tomato and melon, further research should be performed to establish the application procedure of plant extract in practice.

2.2.3 *BOERHAVIA DIFFUSA* EXTRACT / *PHYTOPHTHORA SPP.*

2.2.3.1 Abstract

In this study, antifungal properties of *Boerhavia diffusa* L. (*Nyctaginaceae*) methanolic extract were investigated both under *in vitro* and *in vivo* conditions. The minimum inhibitory concentration of *B. diffusa* extract was 2.5 $ml \cdot l^{-1}$ for *P. infestans* and *P. cactorum*, and 10 $ml \cdot l^{-1}$ for *P. capsici*. The extract had fungitoxic effect on the mycelial growth of *P. infestans* and *P. cactorum*. *P. capsici* mycelial growth was inhibited of 90 % five days after inoculation. *B. diffusa* extract, at the concentration of 2.5 $ml \cdot l^{-1}$ and 5 $ml \cdot l^{-1}$, showed a moderate inhibition effect against *P. infestans* in tomato. The extract reduced disease symptoms of *P. capsici* in pepper at all concentrations. The concentration of 10 $ml \cdot l^{-1}$ showed the best efficacy in controlling the oomycete. As its efficiency was comparable even to that of the synthetic fungicide, *B. diffusa* extract can be considered as a potential valuable alternative to chemical control of this pathogen.

2.2.3.2 Introduction

Phytophthora spp. causes disease on many crops throughout the world. Some of the most destructive diseases are caused by *P. infestans*, the causal agent of late blight of potatoes and tomatoes both in the field and greenhouse. *P. capsici* has a wide host range and, for example, on pepper can cause very serious damages. Crown rot, caused by *P. cactorum*, is an important strawberry disease in temperate to subtropical regions (Maas, 2004).

Phytophthora spp. is difficult to manage. The most effective way to control this pathogen is represented by copper fungicides. However, the new strains of *P. infestans* and *P. capsici* are resistant to some of the systemic fungicides (metalaxyl) (Agrios, 2005; Ponti and Laffi, 1985; Cristinzio, 1993). No cultivars of tomato and potato are completely resistant to *P. infestans* (Crinò, 1993). Also in the case of *P. cactorum* there is the risk of resistance development against synthetic fungicides. Therefore, it is necessary to apply other alternatives of disease management. Recently, some plant derived products demonstrated antifungal activity against *Phytophthora spp.* (*Tab. 2.2.3.1*).

Tab. 2.2.3.1: Antifungal activity of some plants against *Phytophthora* spp.

Fungi	**Plants**	**References**
P. infestans	garlic	Curtis *et al.* (2004)
	Bazzania trilobata (L.) S.F. Gray	Scher *et al.* (2004)
	Cupressus benthamii, *Vetiveria zizanioides*	Goufo *et al.*(2008)
	Catalpa ovata G. Don.	Cho *et al.* (2006)
P. capsici	rosemary and lavender	Widmer and Laurent (2006)
	Orthosiphon stamineus Benth.	Hossain *et al.* (2007)
	Annemarrhena aphodeloides Bunge	Park *et al.*(2003)
	Cryptomeria japonica	Hwang *et al.* (2005)
	Metasequoia glyptostroboides	Bajpai and Kang (2009)
	Nandina domestica Thunb	Bajpai *et al.* (2008a)
	Silene armeria L.	Bajpai *et al.* (2008b)
	Cestrum nocturnum L.	Al-Reza *et al.* (2009)
P. cactorum	*Chenopodiaceae* wild species	Boughalleb *et al.* (2009)

In this study, antifungal properties of *Boerhavia diffusa* L. (*Nyctaginaceae*) were investigated. This shrub, distributed in the tropical and subtropical regions, is known to have medicinal properties found mainly in its root (Singh, 2007). Its ethnobotanical use has a long history (Awasthi and Verma, 2006). It has digestive, diuretic, anti-infiammatory and antioxidant properties (Singh, 2007; Gacche and Dhole, 2006; Satheesh and Pari, 2003). *B. diffusa* contains saponins, tannins, alkaloids, flavonoids, and phenol glycosides (Ujowundu *et al.*, 2008; Maurya *et al.*, 2007; Edeoga and Ikem, 2002). In addition, it is rich in vitamins C, B3 and B2 and in some minerals such as Na, Ca, and Mg (Ujowundu *et al.*, 2008).


B. diffusa

Some studies on *B. diffusa* antibicrobial activity have been performed. Chloroform extracts of the *B. diffusa* roots as well as the ether, ethyl acetate, ethyl alcohol and aqueous extract of aerial and root parts have shown to have antifungal properties (Agrawal and Srivastava, 2008; Agrawal *et al.*, 2003; Agrawal *et al.*, 2004). Aqueous and ethanolic extracts possess antibacterial activity (Adeyemi *et al.*, 2008; Sahni *et al.*, 2008; Aladesanmi *et al.*, 2007). However, only few studies of *B. diffusa* extract efficiency in plant pathogen control have been made. Roots of *B. diffusa* contain basal proteins which show high inhibitory activity against plant viruses. In addition, root extracts of this plant induce strong systemic resistance against TMV infection in tobacco susceptible host plant (Lohani *et al.*, 2007). Also *B. diffusa* leaf extract, used as seed treatment, reduced disease incidence of Bean Common Mosaic Virus (BCMV) under screenhouse and field conditions (Prasad *et al.*, 2007).

In the present study, methanolic extract of *B. diffusa* was screened for its antifungal properties against fungal plant pathogens such as *P. infestans*, *P. capsici* and *P. cactorum*. The experiments were carried out under *in vitro* and *in vivo* conditions.

2.2.3.3 Materials and methods

Plant material

Crude methanol extract from roots of *B. diffusa*, obtained by Soxhlet extraction technique (modified from Ehrman, 1994), was was provided from AGROTECNOLOGIAS NATURALES S.L. (ATENS company, Spain).

Tomato cv. Superprecoce di Marmande (SGARAVATTI), pepper Quadrato d'Asti Giallo selection Cubo (FOUR).

Fungal pathogens

Isolates of *P. infestans* (Tuscia University, Viterbo, Italy), *P. capsici* (University of Naples, Portici, Italy), and *P. cactorum* (MUCL 20873, from BCCM™, Belgium.) were re-isolated from infected plant organs. Pure cultures of *P. capsici* and *P. cactorum* were maintained on V8 medium (V8 juice 200 ml, plant agarose 15 g , $CaCO_3$ 2 g, distilled water up to the final volume of 1000 ml); for *P. infestans*, the V8 medium amended with boiled peas (V8 200 ml, agarose 15 g, $CaCO_3$ 0.5 g, 100 g of boiled peas, distilled water up to final volume of 1000 ml). *In vitro* experiments were carried out using 10-day-old cultures.

In vitro antifungal assays

The toxicity of plant extracts against *Phytophthora* sp. was performed according to Abou-Jawdah (2002), using a poisoned plate technique. Plant extracts sterilized by 0.2 µl filtration were added to V8 medium after autoclaving, when the temperature of the medium reached 50°C, and then mixed thoroughly. The final volume of the extract in 20 ml of V8 per each Petri dish was adjusted to four different final concentrations (2.5, 5, 10, 20 $ml \cdot l^{-1}$). Unamended V8 plates served as controls.

Mycelial growth inhibition tests were performed placing, in the center of each plate, 3 pieces of 5mm mycelial agar discs cut from the margin of actively growing fungal colonies. Colony diameter was measured after incubation for 5 days, at 27°C for *P. capsici*, at 21°C for *P. infestans,* and at 24°C for *P. cactorum*. Mycelial development was recorded at 11st and 13rd

day of culture for *P. infestans* and *P. cactorum*, respectively. All treatments were replicated 5 times. The percentage of inhibition was calculated comparing treated plates with control.

The minimum inhibitory concentration (MIC) was established as the lowest concentration of tested extract that resulted in no visible spore germination or mycelial growth after 20 hours of culture. Fungicide activity was considered when no fungal growth was observed in the plates, fungistatic activity was considered when fungal growth was delayed (Osorio *et al.*, 2010).

In vivo antifungal assays

The tests with *P. infestans* on tomato (*Fig. 2.2.3.1*) and *P. capsici* on pepper were performed in growth chamber. The experiments consisted of treatments with *B. diffusa* extract at three different concentrations (2.5, 5.0 and 10.0 $ml \cdot l^{-1}$), chemical control treated with Ridomil Gold (4 $g \cdot l^{-1}$ and 4 $kg \cdot ha^{-1}$ for tomato and pepper, respectively), inoculated control treated with distilled water, uninoculated control treated with extract at the highest concentration tested, and uninoculated control treated with distilled water.

Tomato plants at two-leaf stage (two weeks after sowing) were sprayed with a suspension containing $4 \cdot 10^4$ $sporangia \cdot ml^{-1}$ of *P. infestans*. The treatments with plant extracts and the other treatments were carried out one day after inoculation. The extract was applied weekly at the dose of 1.3 ml per plant for the total of 4 applications. Peat was used as a cultivation substrate. Plants were kept in the growth chamber at the temperature of 19-20 ^oC. Disease severity was assessed by means of a visual rating according to a 0-3scale (0=no symptoms - 3= plant dead. The evaluation of disease incidence was performed on 6th, 10 th and 17 th day after inoculation.

Fig. 2.2.3.1: Tomato plants inoculated with *P. infestans* in growth chamber experiment

The suspension of $2 \cdot 10^4$ $sporangia \cdot ml^{-1}$ of *P.* capsici was applied to the soil and to the collets of pepper plants at four-leaf stage (30 days after sowing). The treatments with plant extracts and other treatments were carried out immediately after inoculation. The extract was applied weekly at the dose of 6 ml per plant for a total of 4 applications. Mix of peat and sand (2:1) was used as a cultivation substrate. Plants were kept in the growth chamber at the

temperature of 27 °C. Disease severity was assessed by means of a visual rating according to a 0-5 scale (*Fig. 2.2.3.2*) (0=no symptoms, 1=apparently healthy with very small lesions beginning to form on stem, 2=slight stunting, very small lesions on stem, lower leaves wilted, 3=small lesions on stem, leaves wilted, plant stunted, 4=large lesions on stem, whole plant wilted, 5=stunted dead plant). The evaluation of disease incidence was performed at 6 th, 9 th, 12 th, and 16 th day after inoculation.

*Fig.2.2.3.2:*Evaluation scale of *P. capsici* disease symptoms on pepper plants

Statistical analysis

Data were subjected to one way analysis of variance (ANOVA). The follow up of ANOVA included Duncan's multiple range test ($P < 0.05$); in the figures, different alphabetic letters indicate significant differences among various treatments. Statistical elaboration of data was performed with the program SPSS 15.0 for Windows.

2.2.3.4 Results and discussion

In vitro antifungal assays

The MIC of *B. diffusa* extract is represented by 2.5 $ml \cdot l^{-1}$ for *P. infestans* and *P. cactorum*, and by 10 $ml \cdot l^{-1}$ for *P. capsici*. In addition, at this concentration the extract completely inhibited with a fungitoxic effect the mycelial growth of *P. infestans* (*Figs. 2.2.3.3, 2.2.3.5*) and *P. cactorum* (*Figs. 2.2.3.4, 2.2.3.6*). In the case of *P. capsici*, mycelial growth was inhibited of 90 % at the 5th day after inoculation (*Figs. 2.2.3.7, 2.2.3.8*). Also oil of *Metasequoia glyptostroboides* showed a remarkable antifungal effect on *P. capsici* spore germination along with increasing concentration (Bajpai and Kang, 2009) as well as the extract of *Cestrum nocturnum* showed time-dependent kinetic inhibition of *P. capsici* spores (Al-Reza *et al.*, 2009). However, Demirci and Dolar (2006) observed that onion, radish, garden cress, and lentil extracts stimulated the growth of *P. capsici* and, according to Linderman (1989), some plant pathogens increase their inoculum potential using organic materials as a source of energy. On the other hand, Widmer and Laurent (2006) observed a

greater inhibition of *Phytophthora* spore germination caused by natural plant extract respect to the same compounds contained in the extract but obtained synthetically. The difference in the inhibitory concentration of the same synthetic and natural compound can be explained by a slight difference in their chemical structure. These results show the importance of natural provenience of biofungicide.

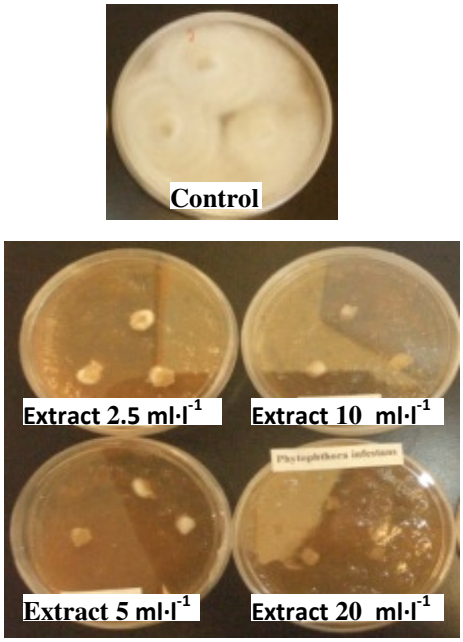


Fig. 2.2.3.3: Inhibition of *P. infestans* mycelial growth

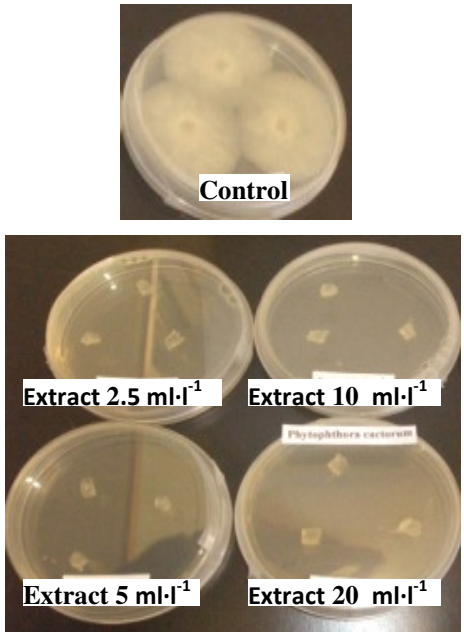


Fig. 2.2.3.4: Inhibition of *P. cactorum* mycelial growth

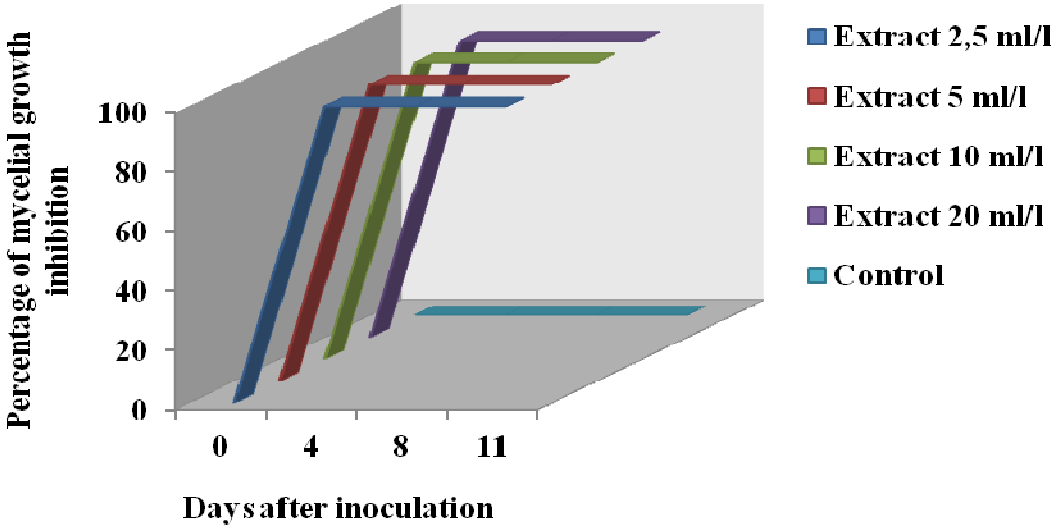


Fig. 2.2.3.5: P. infestans mycelial growth inhibition over time

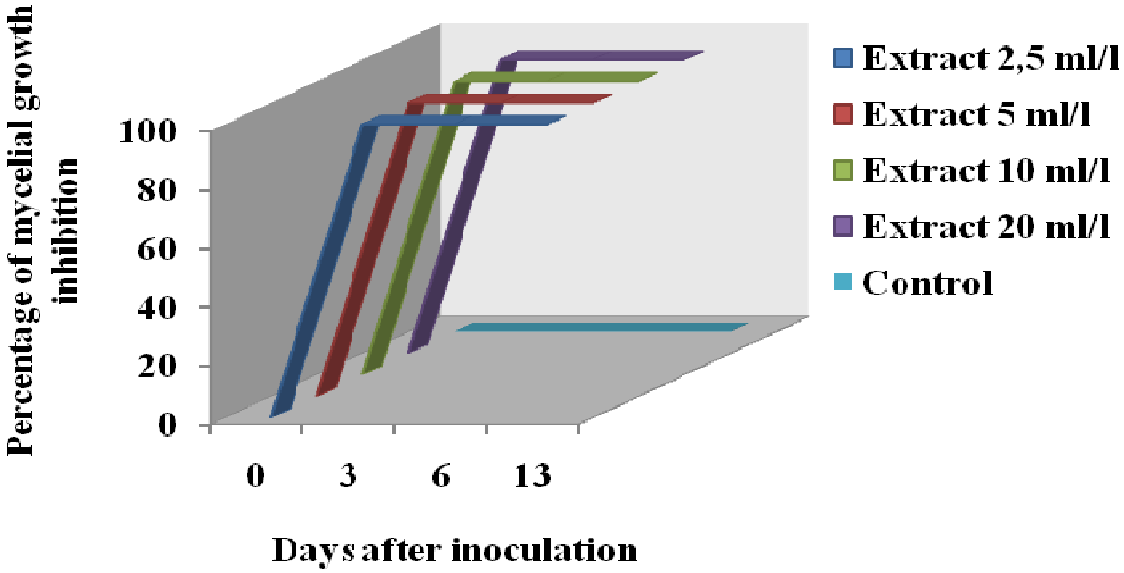


Fig. 2.2.3.6: P. cactorum mycelial growth inhibition over time

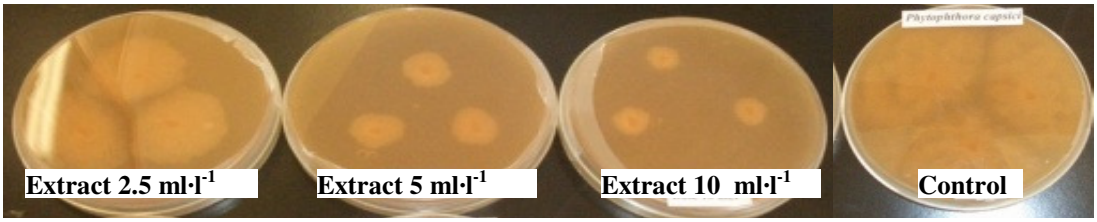


Fig. 2.2.3.7: Inhibition of *P. capsici* mycelial growth

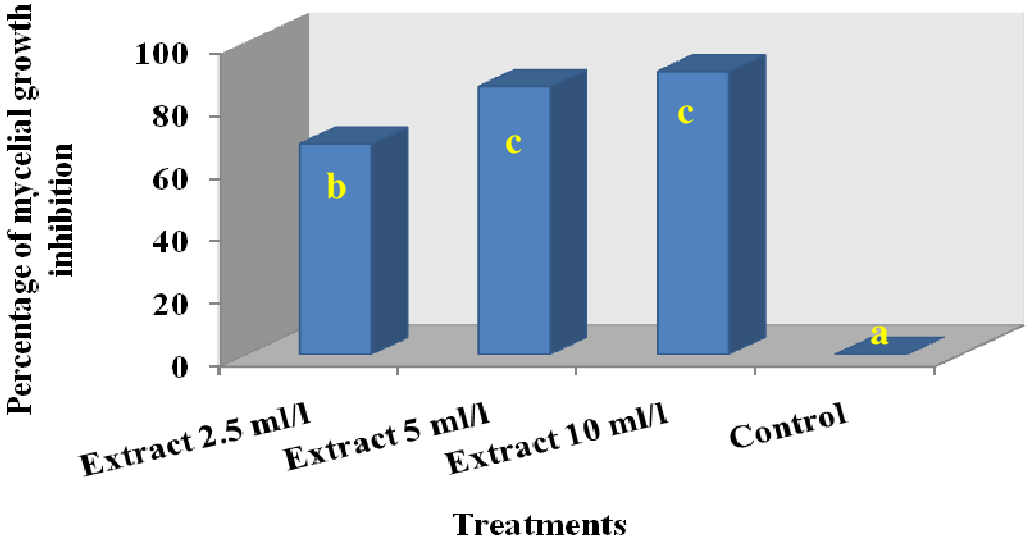
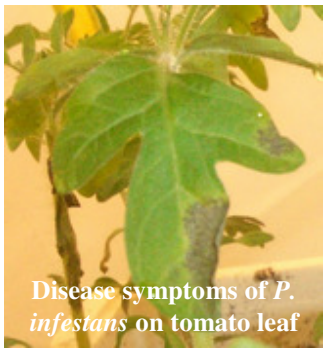


Fig. 2.2.3.8: Percentage of *P. capsici* mycelial growth inhibition

In vivo antifungal assays


Disease symptoms of *P. infestans* on tomato leaf

B. diffusa extract at the concentration of 2.5 ml·l^{-1} showed a moderate inhibition effect on *P. infestans* in tomato both 10 and 15 days after inoculation in comparison with the inoculated control. Also the extract at the concentration of 5 ml·l^{-1} slightly reduced disease symptoms of *P. infestans* in 15 days (*Fig. 2.2.3.9*). Curtis *et al.* (2004) reported that infection of tubers by *P. infestans* was more effectively reduced by prophylactic treatments with garlic extract than by post infection curative treatments. Therefore, further *in vivo* tests should be made to verify if treatments before inoculation are more effective even at lower concentrations of *B. diffusa* extract as it caused slight phytotoxicity at the concentration of 10 ml·l^{-1} after 15 days of treatments.

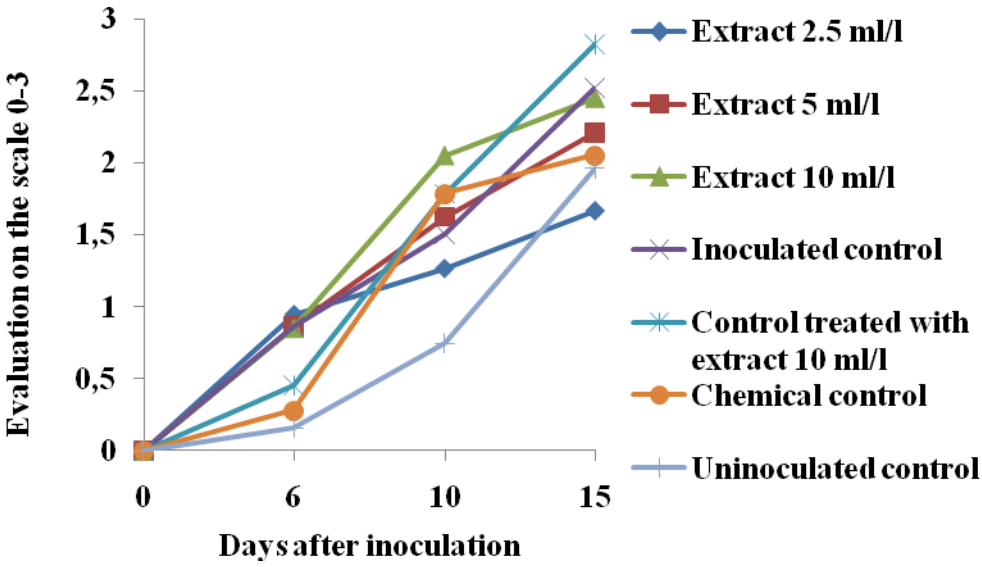


Fig. 2.2.3.9: Evaluation of *P. infestans* disease symptoms on tomato plants treated with *B.diffusa* extract

In pepper, *B. diffusa* extract inhibited *P. capsici* at all concentrations tested (*Fig. 2.2.3.10*). The extract at the concentration of 10 ml·l^{-1} showed the best effect in controlling *P. capsici*. In addition, its antifungal effect was comparable with that of the synthetic fungicide. In general, this means a great advantage for a biofungicide as it is often less effective in comparison with a commercial synthetic fungicide (Park *et al.*, 2003). Moreover, no phytotoxicity has been observed in pepper plants after treatment with *B. diffusa* extract and,

about this, Park *et al.* (2003) already supposed that plant drug used in traditional medicine has low biotoxicity to humans.

Widmer and Laurent (2006) expected that some degree of resistance by *Phytophthora* spp. could be developed also against the chemical compounds contained in plant extracts, as observed with synthetic fungicides. This confirms the continuous need of new sources of active compounds for plant defence control of the fungus.

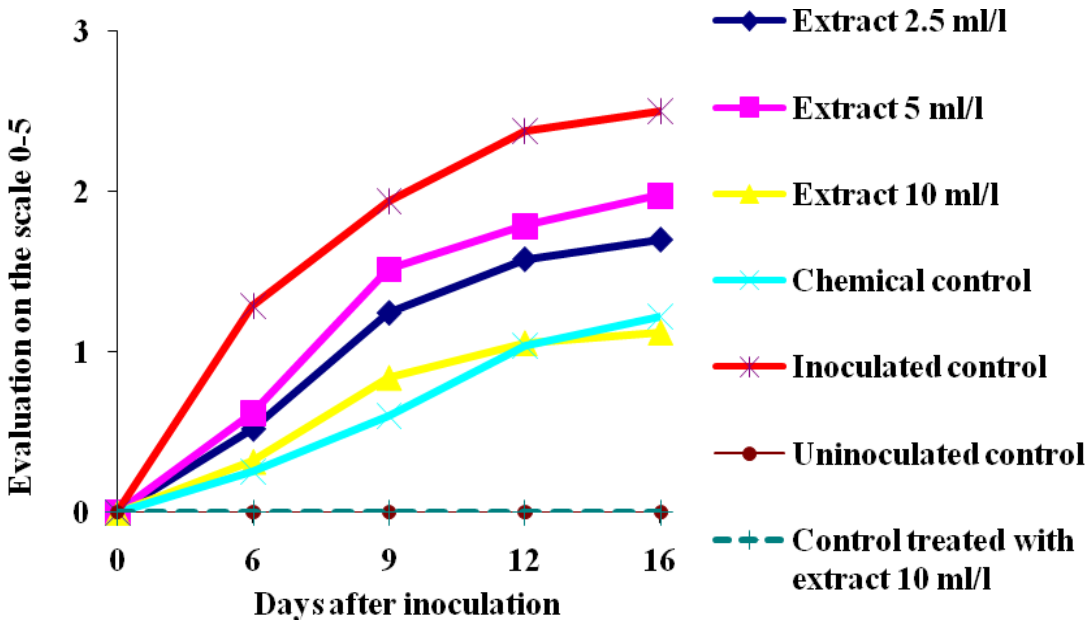


Fig. 2.2.3.10: Evaluation of *P. capsici* disease symptoms on pepper plants treated with extract

2.2.3.5 Conclusions

B. diffusa extract showed significant antifungal activity in controlling *P. capsici* that was comparable even to synthetic fungicide. Therefore, *B. diffusa* extract can be considered as an alternative to control this pathogen in young pepper plants under protected conditions. However, further experiments should be carried out to verify the efficacy of *B. diffusa* extract also under field conditions and in adult plants.

2.2.4 *VITEX AGNUS-CASTUS* EXTRACT / *PYTHIUM ULTIMUM*

2.2.4.1 Abstract

V. agnus-castus methanolic extract showed strong antifungal activity against *Pythium ultimum* under both *in vitro* and *in vivo* conditions. The extract at all concentrations delayed the mycelial growth of *P. ultimum* of 7 days in comparison with the control. The minimal inhibitory concentration was 2 $ml \cdot l^{-1}$. *V. agnus-castus* extract showed significant antifungal activity against *P. ultimum* also on tomato seedlings. Its efficacy was comparable with that of the synthetic fungicide. Therefore, *V. agnus-castus* extract can serve as a potential biological agent for controlling *P. ultimum*.

2.2.4.2 Introduction

Pythium ultimum is one of the most common causes of damping-off and seed rots of many agricultural crops. So far, no commercial varieties of plants resistant to *Pythium* are available (Agrios, 2005). Management of the *Pythium* diseases is primarily made through cultural practices and fungicides. However, *Pythium* attack on seeds or plantlets can be disastrous despite various control measures. Therefore, new ways of *Pythium* spp. management such as use of antagonists and various plant-derived products are continuously studied.

Some studies have been found on antagonistic activity of *Pythium oligandrum* against pathogenic *P. ultimum* (Martin and Hancock, 1987; Al-Hamdani *et al.*, 2007). Disease suppression by biosurfactant produced by *Pseudomonas koreensis* in tomato plants infected by *P. ultimum* was referred only to hydroponic cultivation system (Hultberg *et al.*, 2009).

A mycelial growth of *P. ultimum* was effectively inhibited *in vitro* by nyasol, an active compound of *Annemarrhena aphodeloides* Bunge rhizomes (Park *et al.*, 2003). A plant-derived coumarin found in plants such as clover, sweet woodruff, and grasses inhibited fungal growth of *Pythium* spp. suggesting that this compound can be used also for seed treatments to prevent fungal diseases (Brooker *et al.*, 2008). Hwang *et al.* (2005) evaluated the antifungal effect of wood vinegar of *Cryptomeria japonica* sapwood and its constituents against *P. splendens*. Acetone extracts from some invasive plant species exhibited an inhibitory activity against *P. ultimum* (Mdee *et al.*, 2009). Moreover, some authors reported the inhibitory activity of plant extracts against *P. ultimum* comparable with that of synthetic fungicides (Tegegne and Pretorius, 2007).


V. agnus-castus

In the present thesis, the antifungal activity of *Vitex agnus-castus* (*Verbenaceae*) (*Fig. 1*) was investigated. This shrub has a natural habitat ranging from tropical and subtropical to warm temperate regions. In this species, biological activity employed mainly in human medicine has been reported (Pearlstein and Steiner, 2008). Sarikurkcu *et al.* (2009) reported an excellent antioxidant activity of water extract of *V. agnus-castus* probably for its content of flavonoid casticin (Hajdu *et al.*, 2007). Seed extracts have showed to have repellent properties (Mehlhorn *et al.*, 2005). Pepeljnjak *et al.* (1996) reported antibacterial and antifungal activities of etheric and ethanolic extracts of *V. agnus-castus*.

Fruits, flowers and leaves of *V. agnus-castus* contain phenolic acids and their derivates, flavonoids, tannins, iridoid glycosides, and diterpenoids (Proestos *et al.*, 2006 Saglam *et al.*, 2007; Hajdu *et al.*, 2007; Abel *et al.*, 1994). The main compounds of the essential oil are 1, 8-cineole, sabinene, α-pinene, β-phellandrene and α-terpinyl acetate, trans-β-farnesene, and bicyclogermacrene (Novak *et al.*, 2005). Two distinct chemotypes (a α-pinene chemotype and a α-terpinyl acetate chemotype) could be identified in *V. agnus-castus* (Novak *et al.*, 2005).

To our knowledge, the antifungal activity of *V. agnus-castus* extract on fungal plant pathogens has not been investigated so far. In the present study, antifungal activity of *V. agnus-castus* methanolic extract against *P. ultimum* was studied both under *in vitro* and *in vivo* conditions. *In vivo* experiments were carried out on tomato inside a growth chamber with the emphasis to investigate the antifungal potential of this plant extract in comparison with that of the synthetic fungicide.

2.2.4.3 Materials and methods

Plant material

Crude methanol extract from stems of *V. agnus-castus,* obtained by Soxhlet extraction technique (modified from Ehrman, 1994), was provided from AGROTECNOLOGIAS NATURALES S.L. (ATENS company, Spain). For *in vivo* tests, tomato cv. Superprecoce di Marmande (SGARAVATTI) was used.

Fungal pathogens

Isolates of *P. ultimum* (MUCL 30159, from BCCM™, Belgium) were isolated from infected plant organs. Pure cultures were maintained on potato dextrose agar (PDA, OXOID), and the experiments were carried out using 10-day-old cultures.

In vitro antifungal assays

The toxicity of plant extracts against two fungi was performed with a poisoned plate technique according to Abou-Jawdah (2002). Plant extracts sterilized by filtration through 0.2 µl filter were added to PDA after autoclaving, when the temperature of the medium reached 50°C, and mixed thoroughly. The final volume of the extracts in 20 ml of PDA per each Petri dish was adjusted to four different final concentrations (2, 4, 8, 16 $ml \cdot l^{-1}$). Unamended PDA plates served as controls.

Mycelial growth inhibition tests were performed placing, in the center of each plate, 3 pieces of 5 mm mycelial agar discs cut from the margin of actively growing fungal colonies. Colony diameter was measured after incubation at 26°C for 3 days, and mycelial development was observed for 10 days. All treatments were replicated 5 times. The percentage of inhibition was calculated comparing treated plates with control. The minimum inhibitory concentration (MIC) was established as the lowest concentration of tested extract that resulted in no visible mycelial growth after 20 hours. Fungicide activity was considered when no fungal growth was observed in the plates and fungistatic activity was considered when fungal growth was delayed (Osorio *et al.*, 2010).

In vivo antifungal assays

In the growth chamber experiment on tomato (*Fig. 2.2.4.1*), the suspension containing 3-4 x 10^5 CFU of *P. ultimum* was applied to the substrate (sand:peat = 2:1), at the dose of 1 ml per seed, one day before sowing. The seedling containers were covered with the plastic to keep high relative humidity. The treatments with *V. agnus-castus* extract at three different concentrations (2, 4, 8 $ml \cdot l^{-1}$) and at the dose of 2.5 ml per seed were carried out one day after inoculation. The inoculated control treated with chemical fungicide (Previcur Energy, 3 $ml \cdot m^2$), inoculated control treated with distilled water, uninoculated control treated with extract at the concentration of 8 $ml \cdot l^{-1}$, and uninoculated control treated with distilled water were established. Each treatment consisted of 30 plants and was replicated three times. The treatments were repeated 14 days after sowing. The plants were kept at the temperature of

26°C and high relative humidity. Number of germinated seeds was recorded 7 and 14 days after inoculation.

Fig.2.2.4.1: Experiment of *V. agnus-castus* extract efficacy against *P. ultimum* on tomato plants in growth chamber

Statistical analysis

Data were subjected to one way analysis of variance (ANOVA). The follow up of ANOVA included Duncan's multiple range test ($P < 0.05$); in the figures, different alphabetic letters indicate significant differences among various treatments. Statistical elaboration of data was performed with the program SPSS 15.0 for Windows.

2.2.4.4 Results and discussion

In vitro antifungal assays

V. agnus-castus extract at all concentrations delayed the 7 day mycelial growth of *P. ultimum* in comparison with the control (*Figs. 2.2.4.2, 2.2.4.3*). The MIC was 2 $ml \cdot l^{-1}$. However, Haouala *et al.* (2008) reported that other *Pythium* species (*P. aphanidermatum*) were resistant when treated with fenugreek extracts. In addition, these authors also observed that aqueous plant extract lost their relative activity after one month of storage at environmental temperature up to 90 %, while the extracts stored at 4°C lost only 50 % of their activity. The results of the present study confirms that methanolic extract of *V. agnus-castus* maintained its antifungal activity at

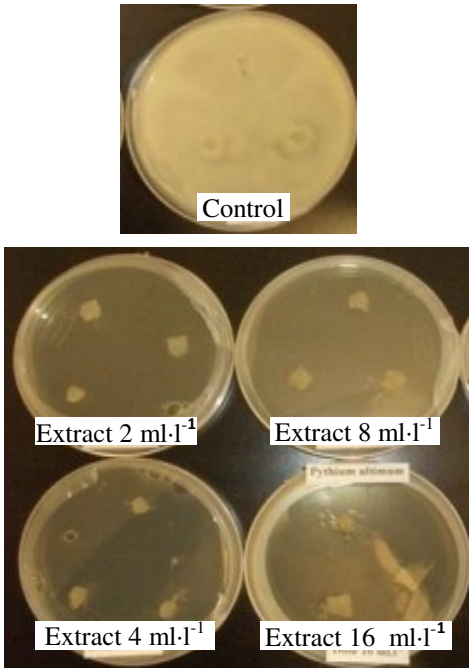


Fig. 2.2.4.2: Inhibition of *P. ultimum* mycelial growth

least for several months being stored at environmental temperature in the dark. The studies of Haouala *et al.* (2008) confirm that antifungal activity of fenugreek extract reside in its methanol fraction, as well as of the extract tested in the present study.

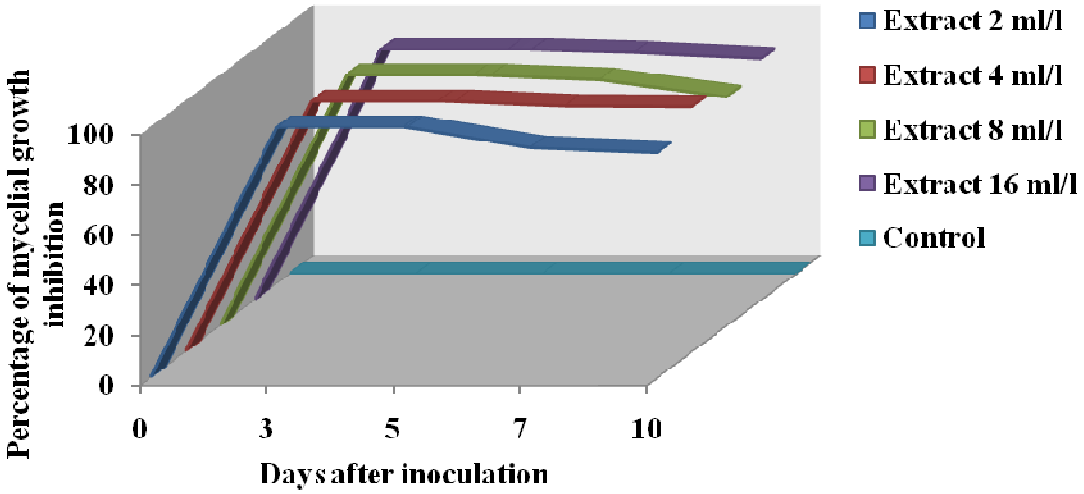


Fig. 2.2.4.3: Inhibition of *P. ultimum* mycelial growth over time

In vivo antifungal assays

V. agnus-castus extract at all concentrations showed significant antifungal activity against *P. ultimum* in tomato seedlings (*Fig. 2.2.4.4*). In addition, its efficacy was comparable with that of the synthetic fungicide. Moreover, no phytotoxicity by the plant extract was recorded in tomato plants. Also Zhang *et al.* (2009) reported that the extract of *Solidago canadensis* L. suppress *P. ultimum* growth both under *in vitro* and *in vivo* conditions on tomato seedlings. The authors suppose that this invasive plant species affects the oomycete through exudation of allelochemicals to the soil. In addition, Tohamy *et al.* (2002) reported difference of neem and garlic extract efficacy on soilborne pathogens in various plant age. Younger plants received better disease control than those treated at older developmental stage. Therefore, the efficacy of *V. agnus-castus* extract reported in the present thesis should be tested also at different developmental stages of plants to determine the concentrations of the plant extracts effective at these stages.

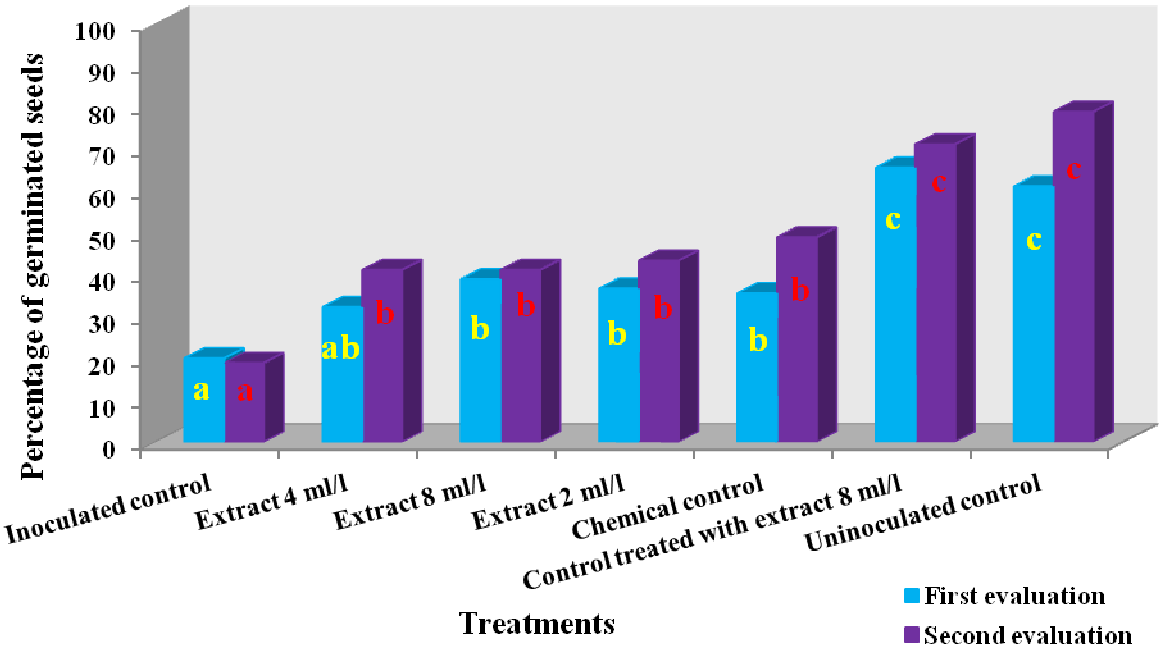


Fig. 2.2.4.4: Percentage of germinated tomato seeds after *P. ultimum* inoculation

2.2.4.5 Conclusions

The extract of *V. agnus-castus* showed significant antifungal activity in controlling *Pythium* damping off in tomato seedlings comparable to that of synthetic fungicide. The *V. agnus-castus* extract should be tested for *Pythium* control also in other crops to verify its efficacy in different culture systems and environments.

2.2.5 *PHYLLANTHUS NIRURI* EXTRACT / *MONOSPORASCUS CANNONBALLUS*

2.2.5.1 Abstract

A methanolic extract of a medicinal plant *Phyllanthus niruri* showed antifungal effect against *Monosporascus cannonballus* at all concentration tested. The concentration of 16 ml·l^{-1} was fungitoxic to the fungus. Minimum inhibitory concentration was 8 ml·l^{-1} and, at this concentration, the mycelial growth was inhibited of 81% at the 4th day after inoculation. The percentage of mycelial growth inhibition increased with the concentration of plant extract. *P. niruri* extract had no significant inhibition effect on *M. cannonballus* in melon plants under greenhouse conditions. The Monosporascus disease was partially controlled by mycorrhiza but the effect was not significant in comparison with that of plant extract treatments. The inhibition effect of the plant extract against mycorrhizal fungi was reported as the inhibition effect of mycorrhiza was slightly reduced by contemporary treatment with plant extract. An improvement of general health status of the plants was observed under the treatment with *P. niruri* extract at the concentration of 28 ml·l^{-1}. This suggests the possible use of *P. niruri* extract as a plant growth promoter. However, other experiments should be carried out to confirm the growth promoting potential of *P. niruri* plant extract, and to determine its minimum active dose.

2.2.5.2 Introduction

Root rot and vine decline caused by *Monosporascus cannonballus* has recently emerged as important problem in production of melons and watermelons. The disease occurs in areas with semiarid climate, saline and alkaline soils, and high temperatures in production season (Agrios, 2005). Management of *M. cannonballus* is difficult for its heat tolerance and resistant survival structures such as the ascospores (Martyn, 2002). In the same time, there is lack of genetic resistance in agricultural crops. Therefore, no effective control exists against the melon root rot and vine decline. The disease can be partially reduced by grafting, or by fungicides. Since methyl bromide has been phased out, there is the need of other control strategies. Apart crop rotations and integrated management practices, some biological control methods have recently been reported and, among them, particularly the use of antagonistic *Trichoderma* spp. (Bruton, 1998), hypovirulent isolate of *M. cannonballus* (Batten *et al.*, 2000), and plant extracts. Ethanolic extracts of *Senna alata* (L.) Roxb. root, stalk and green bean displayed high control potential of *M. cannonballus* under *in vitro* conditions (Viana *et*

al., 2008). Antifungal properties of water soluble, ethanolic and methanolic extracts from wild rue (*Peganum harmala* L.) were tested on *M. cannonballus* mycelial growth and spore germination. The maximum antifungal activity was detected in water soluble seed extract.


P. niruri

In the present thesis, antifungal activity of *Phyllanthus niruri* (*Euphorbiaceae*) was investigated. The genus *Phyllanthus* is distributed in tropical and subtropical regions (Webster and Grady, 1994). *P. niruri* (*Fig. 1*) is an annual tropical herb wildly used as medicinal plant in Brazil (Sabir and Rocha, 2008). This plant shows several biological activities as antioxidant, hepatoprotective, antiviral and antibacterial properties (Sarkar *et al.*, 2008, Shakila and Ponni, 2008; Harish and Shivanandappa, 2006; Thomas *et al.*, 1999; Sunda *et al.*, 2008). The aqueous leaf extract of *Phyllanthus* sp. was effective in reducing infection by Tomato Mosaic Virus (ToMV) (Deepthi *et al.*, 2007).

The active phytochemicals, flavonoids, alkaloids, terpenoids, lignans, polyphenols, tannins, coumarins, and saponins have been identified in various parts of *P. niruri* (Hassarajani and Mulchandani, 1990; Petchnaree *et al.*, 1986; Murugaiyah and Chan, 2007; Bagalkotkar *et al.*, 2006). Seeds of P. *niruri* contain linoleic, linolenic and ricinoleic acid (Ahmad *et al.*, 1981).

To our knowledge, the antifungal activity of *P. niruri* extract on fungal plant pathogens has not been investigated so far. In the present study, antifungal activity of *P. niruri* methanolic extract against *M. cannonballus* was studied both under *in vitro* and *in vivo* conditions. *In vivo* experiments were carried out on melon plants in the greenhouse.

2.2.5.3 Materials and methods

Plant material

Crude methanol extract from leaves and stems of *P. niruri*, obtained by Soxhlet extraction technique (modified from Ehrman, 1994), was provided from AGROTECNOLOGIAS NATURALES S.L. (ATENS company, Spain).

For *in vivo* tests, melon cv. Hale's Best Jumbo (ROYAL SLUIS) was used.

Fungal pathogens

Isolates of *M. cannonballus* (Tuscia University, Viterbo, Italy), kindly provided by the fungal collection of CRA-Plant Pathology Research Center of Rome (Italy), were re-isolated from infected plant organs. Pure cultures were maintained on potato dextrose agar (PDA, OXOID), and *in vitro* experiments were carried out using 28-day-old cultures with formed perithecia. For *in vivo* experiments, the fungus was grown on sterilized water-soaked pearl millet at the temperature of 31 °C for 28 days.


M. cannonballus mycelium grown on pearl millet

In vitro antifungal assays

Toxicity of plant extracts against *M. cannonballus* was performed according to Abou-Jawdah (2002) with a poisoned plate technique. Plant extracts sterilized by 0.2 µl filtration were added to PDA after autoclaving, when the temperature of the medium reached 50°C, and mixed thoroughly. The final volume of extract in 20 ml of PDA per each Petri dish was adjusted to four different final concentrations (2, 4, 8, 16 $ml \cdot l^{-1}$). Unamended PDA plates served as controls.

Mycelial growth inhibition tests were performed placing, in the center of each plate, 3 pieces of 5 mm mycelial agar discs cut from the margin of actively growing fungal colonies. Colony diameter was measured after incubation at 31°C for 4 days, and mycelial development was observed for 12 days. All treatments were replicated 5 times. The percentage of inhibition was calculated comparing treated plates with control. The minimum inhibitory concentration (MIC) was established as the lowest concentration of tested extract that resulted in no visible mycelial growth after 20 hours. Fungicide activity was considered when no fungal growth was observed in the plates and fungistatic activity was considered when fungal growth was delayed (Osorio *et al.*, 2010).

In vivo antifungal assays

In two greenhouse experiments on melon plants (*Fig. 2.2.5.1*), the inoculum of *M. cannonballus* was applied at the dose of 5 g of pearl millet per 1 l of the substrate per plant 20 days after sowing (one-leaf stage).

In the first screening, the treatments with *P. niruri* extract at the concentration 2 ml·m^2 was two time applied to the soil at the dose of 500 ml per plant, immediately after inoculation and 20 days after inoculation. Inoculated control treated with distilled water, uninoculated control treated with distilled water, and control treated with *Trichoderma* were established.

Fig. 2.2.5.1: Melon plants in greenhouse experiment

Each treatment consisted of 5 plants with three replications. The mix of peat and perlite (2:1) was used as a cultivation substrate. The plants were kept at a minimum temperature of 15^{o}C and a maximum temperature of 39^{o}C, and relative humidity about 65%. Root disease symptoms were evaluated 63 days after inoculation according to a 0-5 scale (0=roots without lesions, 5=dead plant; personal communication from Reda, R. and Aleandri, M.P.) (*Fig. 2.2.5.2*).

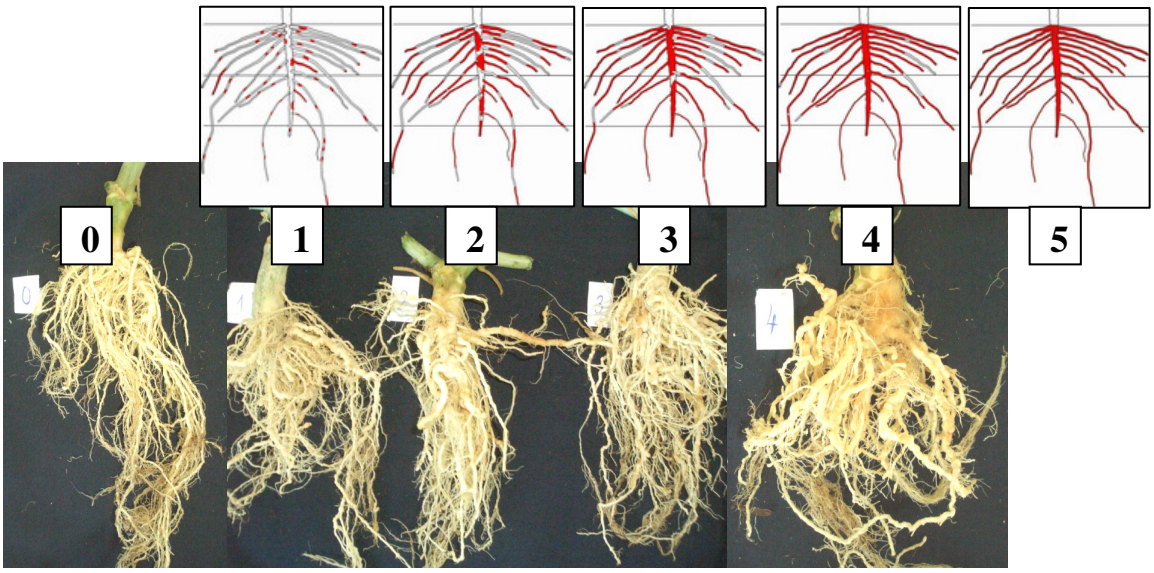


Fig. 2.2.5.2: Evaluation scale of melon root rot caused by *M. cannonballus*

In the second experiment, the treatments with *P. niruri* extract at three different concentrations (7, 14, 28 ml·l^{-1}) and at the dose of 500 ml per plant were carried out twice, immediately and 9 days after inoculation. Inoculated control treated with distilled water,

uninoculated control treated with extract at the concentration of 28 ml·l^{-1}, uninoculated control treated with distilled water, inoculated control treated with mycorrhizal fungi amended or unamended with extract (7 ml·l^{-1}) were established. Each treatment consisted of 5 plants and was three time replicated. The mix of sand and peat (95% and 5%) was used as a cultivation substrate. The plants were kept at a minimum temperature of 13°C and a maximum temperature of 41°C, and relative humidity about 65%. Root disease symptoms were evaluated 74 days after inoculation.

Statistical analysis

Data were subjected to one way analysis of variance (ANOVA). The follow up of ANOVA included Duncan's multiple range test ($P < 0.05$); in the figures, different alphabetic letters indicate significant differences among various treatments. Statistical elaboration of data was performed with the program SPSS 15.0 for Windows.

2.2.5.4 Results and discussion

In vitro antifungal assays

P. niruri showed antifungal effect against *M. cannonballus* at all concentration tested. However, only the concentration of 16 ml·l^{-1} was fungitoxic to the fungus. MIC was 8 ml·l^{-1}and, at this concentration, the mycelial growth was inhibited of 81% at 4th day after inoculation. The percentage of mycelial growth inhibition increased with the concentration of the plant extract (*Figs.2.2.5.3, 2.2.5.4*). In fact, use of different concentrations of plant extract influenced their antimicrobial activity as resulted in different experiments with the same extract (Somchit *et al.*, 2003). Viana *et al.* (2008) reported that *in vitro* efficacy of *S. alata* extract against *M. cannonballus* was comparable with that of synthetic fungicides. Some authors mentioned various factors influencing the efficacy of plant extract such as environmental conditions, plant provenience, extraction method, pH, and temperature (Ranganathan and Balajee, 2000).

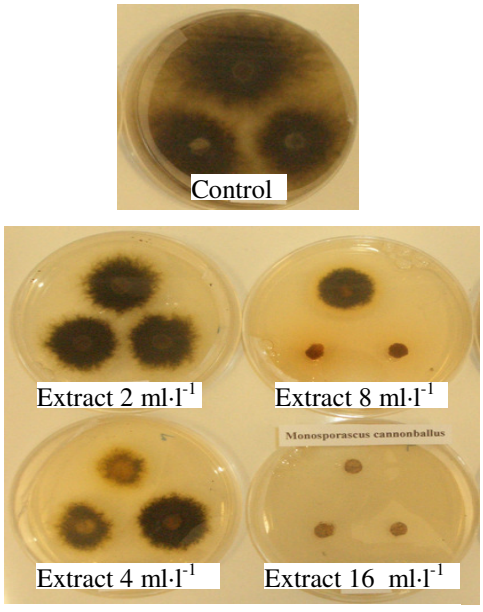


Fig. 2.2.5.3: Inhibition of *M. cannonballus* mycelial growth

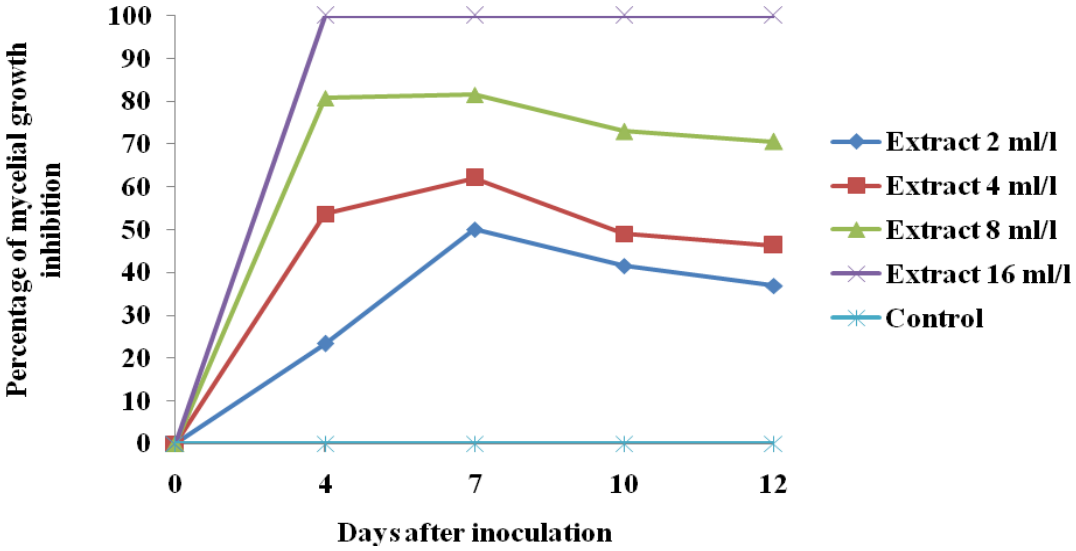
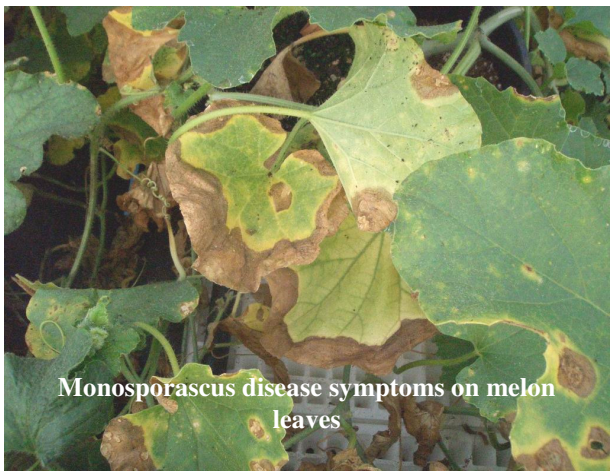


Fig.2.2.5.4: Curve of *M. cannonballus* mycelial growth inhibition

In vivo antifungal assays

Despite of a strong antifungal activity of *P. niruri* extract *in vitro*, there was no significant inhibition effect on Monosporascus infection on melon plants under greenhouse conditions (*Figs. 2.2.5.5, 2.2.5.6*). On the contrary, Batten *et al.* (2000) reported the efficiency of a hypovirulent isolate of *M. cannonballus* under greenhouse conditions while, under *in vitro* conditions, the avirulent isolate was not as efficent as a virulent strain in colonising melon roots. The explanation of the different results in efficacy of these biological agents may be the direct impact of the plant extract on the fungus under *in vitro* conditions, while there are many other factors influencing its efficacy under *in vivo* conditions. In the case of the fungal biocontrol agent, the extract functions better in its natural environment in the soil, where there are more favourable conditions for its growth.



Monosporascus disease symptoms on melon leaves

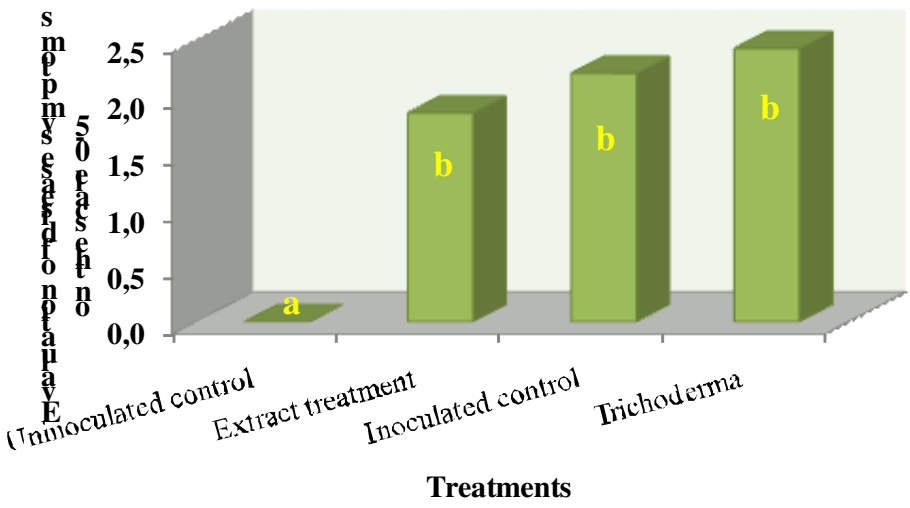


Fig.2.2.5.5: Evaluation of melon root rot caused by *M. cannonballus*

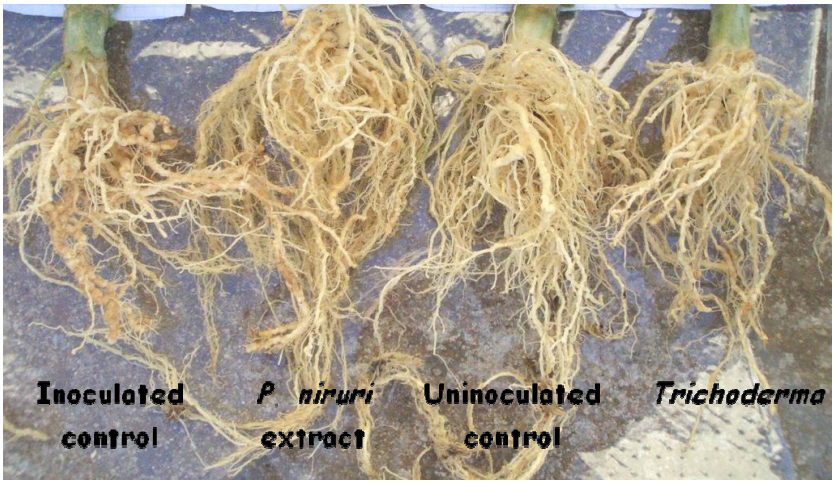


Fig. 2.2.5.6: Comparison of melon roots infected with *M. cannonballus*

Although antifungal activity against *M. cannonballus* on melon plants was not significant, an improvement of general health status of the plants was observed under the treatment with *P. niruri* extract at the concentration of 28 $ml \cdot l^{-1}$ (*Fig. 2.2.5.7*). This result suggested that the *P. niruri* extract may contain some substances favouring a better nutrient management of the plant and influencing positively its growth and health status. One of the substances favouring plant growth reported in the bibliography is for example brassinolide, a plant growth-promoting steroid isolated from *Brassica napus* pollen (Grove *et al.*, 1979).

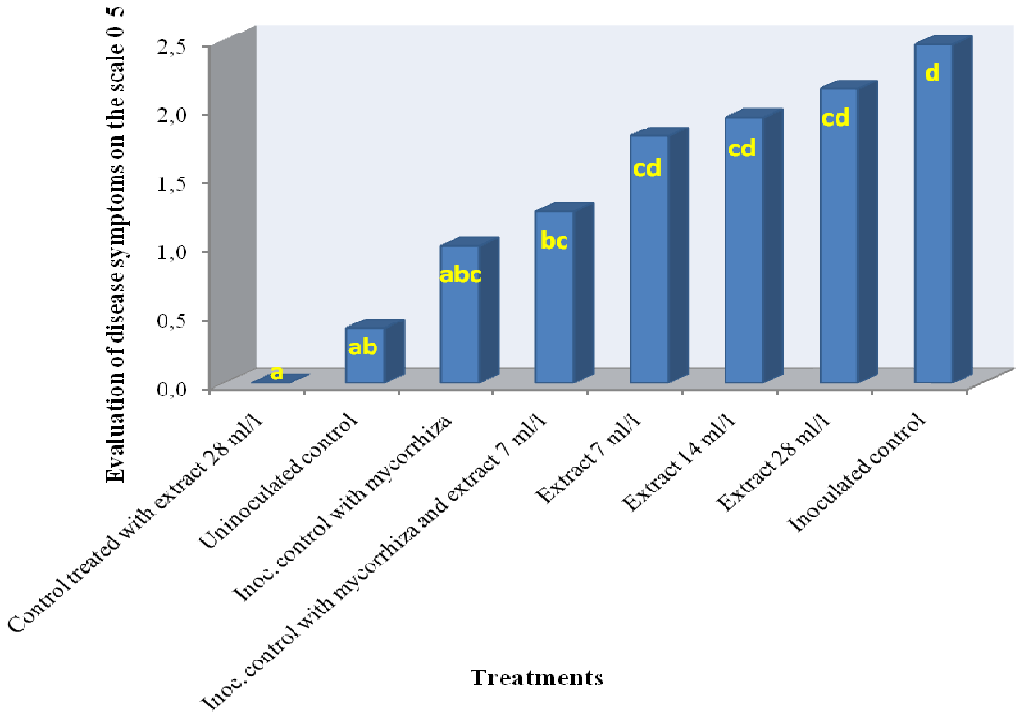


Fig. 2.2.5.7: Evaluation of Monosporascus disease symptoms on melon treated with plant extract and mycorrhiza

The Monosporascus disease was partially controlled by mycorrhiza but the effect was not significant in comparison with plant extract treatments. The inhibition effect of *M. cannonballus* by mycorrhiza was slightly reduced in the contemporary treatment with plant extract. This suggested that the extract has antifungal properties also against mycorrhizal fungi, so indicating its possible broad spectrum antifungal activity. This is an important observation as the synergism of various control methods in integrated disease management is often required.

2.2.5.5 Conclusions

P. niruri extract was fungitoxic against *M. cannonballus* at the concentration of 16 $ml \cdot l^{-1}$ under *in vitro* conditions. However, the extract did not control the disease in greenhouse conditions neither at higher concentration. In the greenhouse experiment the inhibition activity of the plant extract is probably influenced by many factors such as the type of substrate, temperature and humidity, in comparison with *in vitro* culture. The positive influence of *P. niruri* extract on plant growth suggests its possible use as a plant growth promoter. However, further research should be effectuated to confirm the possible employment of this plant extract as plant growth promoter. Further studies should be carried mainly on the minimal effective dose for application of plant extract.

2.3 INNOVATIVE METHODS TO STUDY PLANT/PATHOGEN/EXTRACT INTERACTIONS

2.3.1 DETERMINATION OF *CORDIA LEUCOCEPHALA* EXTRACT ANTIFUNGAL ACTIVITY BY FLOW CYTOMETRY

2.3.1.1 Abstract

The antifungal activity of *Cordia leucocephala* extract against *Fusarium oxysporum* f.sp *melonis* was evaluated by flow cytometry. Cytometry analysis of fungal development over time was verified also by microscope observations. It was possible to observe a fast effect of plant extract on spore vitality; at time 0, 14 $ml \cdot l^{-1}$ extract inhibited completely spore germination. Results indicate flow cytometry as a valuable method for screening of antifungal activity of plant extracts in early stages of fungal growth.

2.3.1.2 Introduction

Fungal plant pathogens represent a serious threat for cultivation of many vegetable crops. The management of fungal diseases is often difficult or not possible at all. Therefore, there is continuously a great demand for novel antifungal compounds. With increasing concern about biologically cultivated crops, alternative substances of natural origin acquire a great interest. The exploration of such compounds should be feasible and fast to enable the antifungal screening of many compounds in a short time. Nevertheless, the main methods currently used to determine antifungal activity are often time-consuming and less precise (Abou-Jawdah *et al.*, 2002; Hossain *et al.*, 2007; Cho *et al.* 2006; Bautista-Baños *et al.*, 2003). For this reason, innovative methods recently employed in antifungal screening (Wedge and Nagle, 2000; Wolfender, 2000; Scher *et al.*, 2004; Tabanca *et al.*, 2006; Green *et al.*, 1994) represent a great challenge.

One of such advanced techniques is represented by the flow cytometry that involves the optical analysis of individual microscopic particles (*Fig.2.3.1.1*) as they flow in a fluid stream past a sensing point (Doležel *et al.*, 2004; Bradner and Nevalainen, 2003). Flow cytometric assays for antifungal activity are based on detection of increased permeability of the fungal cell membrane to propidium iodide (PI) following drug treatment (Green *et al.*, 1994). It is also used in combination with fluorescein diacetate (FDA) to determine viability of eucaryotic cells. Living cells actively convert the non-fluorescent FDA into the green fluorescent compound "fluorescin", a sign of viability, while nucleus of membrane-

compromises cells fluoresce red, a sign of cell death (Jetti *et al.*, 2010). Up to now, the flow cytometric screening for antifungal activity was applied only to yeasts (Green *et al.*, 1994; O'Gorman *et al.*, 1991; Pore, 1990, 1991, 1992) but not to filamentous fungi. Although the protocol for screening of metabolic activity of filamentous fungi have been established (Bradner and Nevalainen, 2003), to our knowledge the screening for antifungal activity of plant extracts against fungal plant pathogens by flow cytometry have not been reported yet.

Therefore, the aim of this study was to investigate antifungal activity of *Cordia leucocephala* extract against *Fusarium oxysporum* f.sp. *melonis*, a causal agent of vascular wilt of melon, by flow cytometric analyses.

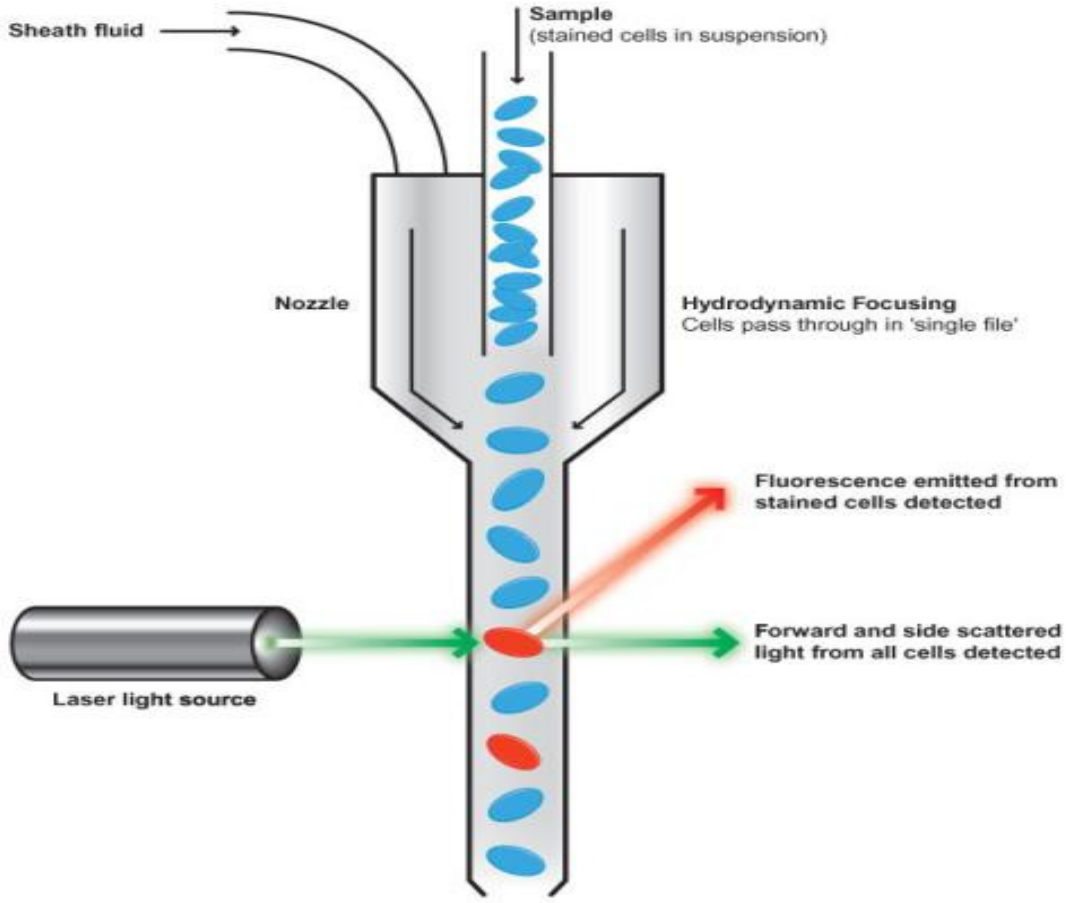


Fig.2.3.1.1: Scheme of cells running through the cytometer. The light scattered from the particles is detected by forward and side scatter. (www.abcam.com/technical, 2010)

2.3.1.3 Materials and methods

Plant extract

Crude methanolic extract from roots of *Cordia leucocephala*, obtained by Soxhlet extraction technique (modified from Ehrman, 1994), was provided from AGROTECNOLOGIAS NATURALES S.L. (ATENS company, Spain).

Fungal species

F. oxysporum f. sp. *melonis* 1,2W (ENEA) was isolated from infected plant organs. Pure cultures were maintained on potato dextrose agar (PDA, OXOID) and 10-day-old cultures were used for experiments.

Analyses of spore vitality

The observations on fluorescent microscope to verify the dynamics of fungal development over time preceded the cytometry analysis. In the same time the capacity of fungus (*F. oxysporum* f.sp. melonis 1,2 W) to absorb various fluorochromes was examined for choosing those optimal for the assay (PI and FDA).

Fresh conidia were harvested from Petri plates by washing with sterile water and filtered through sterile 40 µl filter to remove any residual mycelia. The conidial suspension was added to potato dextrose broth (PDB) and was adjusted to the final concentration of 10^6 spores·ml^{-1}. Eppendorf tubes containing 1ml of spore suspension were incubated in Thermomixer confort (Eppendorf, Milan, It) at 1400 rpm and 24°C. Germination of the conidia was monitored by light microscopy hourly for five hours, 100 spores were counted in each sample and the samples were submitted for staining.

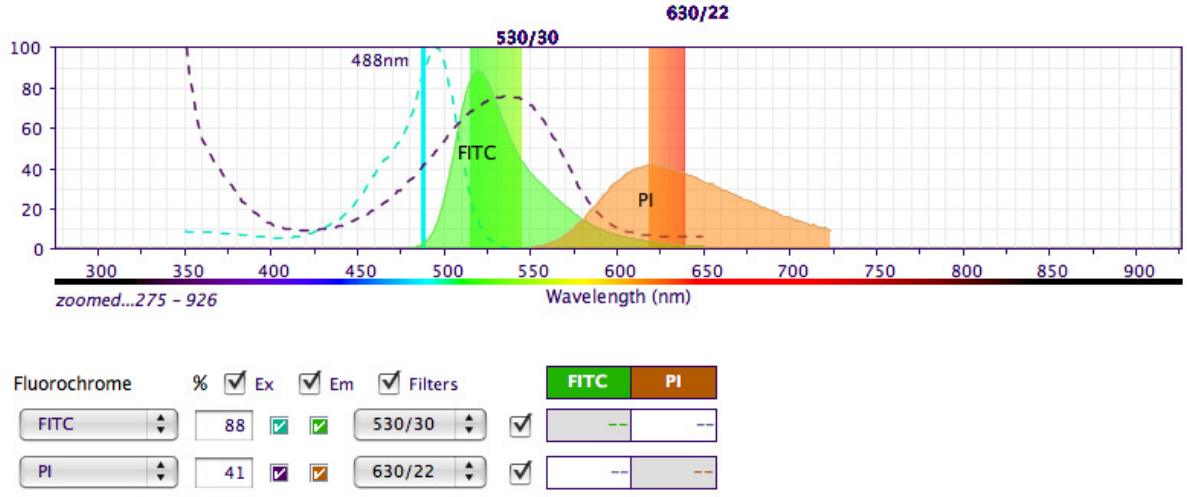


Fig. 2.3.1.2: The range of wavelength for fluorochrome excitation

FDA stock solution (1 mg·ml^{-1}) was prepared by dissolving FDA in an apolar solvent, acetone. The final concentration of FDA used for the experiments was 5 µg·ml^{-1}. Water soluble PI was used at the concentration of 10 µg·ml^{-1}. FDA and PI were added to each sample. The developed hyphae were observed also after fifteen hours to verify their vitality.

The same experimental scheme was used for the antifungal assays of *C. leucocephala* extract. The sample monitoring at 6 and 8 hours was added. The samples were amended with the plant extract and adjusted to three different concentrations (0.14, 1.4 and 14 ml·l^{-1}). Unamended samples served as a control.

Cytometry analysis

Data acquisition was performed using a FACAStarPLus (Becton Dickinson Mountain View, CA, USA) equipped with an argon ion laser with 100mW excitation light l=488 nm, that is able to excitate both fluorochromes. The optical filters for emission selection were: for green channel (FDA) band pass filter 530/15 nm, and 630/20 nm for orange channel (PI). The signals were separated using a dichroic filter of 560 nm. The suspensions flowed through a calibrated nozzle (Ø 100 µm) compatible with the conidial dimensions in the first phases of germination. Approximately 350 events were analyzed per second during analysis and the fluorescence intensity data were collected for 20.000 events in each sample. A contemporary emission of two fluorochromes and the signal relative to the diffracted light (Forward Light Scatter: FSC), index of the cellular morphology, was analyzed with the program for data acquisition and elaboration CellQuest Pro 4.2 (Becton Dickinson Mountain View, CA, USA). Biparametric cytograms were generated where the three parameters (Forward Light Scatter Channel - FSC, Green fluorescence - FL1 and Orange fluorescence - FL2) were coupled. The vital spore populations were detected by an elevated FCS signal with an high FL1 signal (FDA). Dead or damaged spores were detected by an orange fluorescence FL2 (PI) together with an FSC signal of lower intensity. The detection threshold in the FSC channel was set at a level just below the intensity of the lowest spore signals (at time zero). Debris (at time zero) that remained detectable in the FSC channel below the level of the lowest spore signals was removed from the analysis by electronic gating (Bradner and Nevalainen, 2003).

2.2.1.4 Results and discussion

The forward (size) and side scatter (granularity or shape) were used to track the early stages of growth (Shapiro, 1995; Bradner and Nevalainen, 2003) of *F. oxysporum* f.sp.

melonis 1,2 W upon *C. leucocephala* extract treatment in liquid culture (*Fig. 2.3.1.3*). Two supravital fluorescent stains provided evidence of metabolic activity. The initial samples at time zero were processed through the flow cytometer showing the region of ungerminated spores on the cytograms. After two-hour incubation, spores were observed by microscopy to have swollen as reflected in the increase of percentage of germinated spores. The hyphae development increased continuously as documented with the photos from microscope observations. The data obtained by flow cytometry and microscope observations correlated positively up to 5 hours of observations. At the end of 15 hours of incubation the hyphae increased significantly in size. Just 22% of spores remained ungerminated as documented by microscopic observations (*Tab. 2.3.1.1*), whereas the flow cytometry data shows 84 % of spores ungerminated. A possible explanation for disagreement of these values is that the spores enlarged the size which was not detectable by flow cytometry anymore as shown on the cytogram at 15 hours, and then the percentage of germinated spores (e.g. analysed particles) was reduced instead of increased. Therefore, the cytometry analysis is suitable only for early stages of fungal growth.

*Tab. 2.3.1.1:*Percentage of ungerminated spores detected by flow cytometer and counted on microscope

	Percentage of ungerminated spores	
Hours of culture	**flow cytometry**	**microscope**
0	99	100
1	96	99
2	94	98
3	90	95
4	82	81
5	70	71
15	84	22

In each antifungal experiment, untreated control cells were sampled first and then all samples were analyzed by plotting the data in a 2-parameter cytogram, with the *x* axis as the log value of FL1 (green fluorescence, FDA) and the *y* axis as the log value of FL2 (orange fluorescence, PI). The cytogram (*Fig. 2.3.1.4*) was divided into four quadrants; in the first quadrant the dead or damaged spores are visualized, the second quadrant contains the spores of low vitality, in the third quadrant are vital spores, and the fourth quadrant consists mainly of debris.

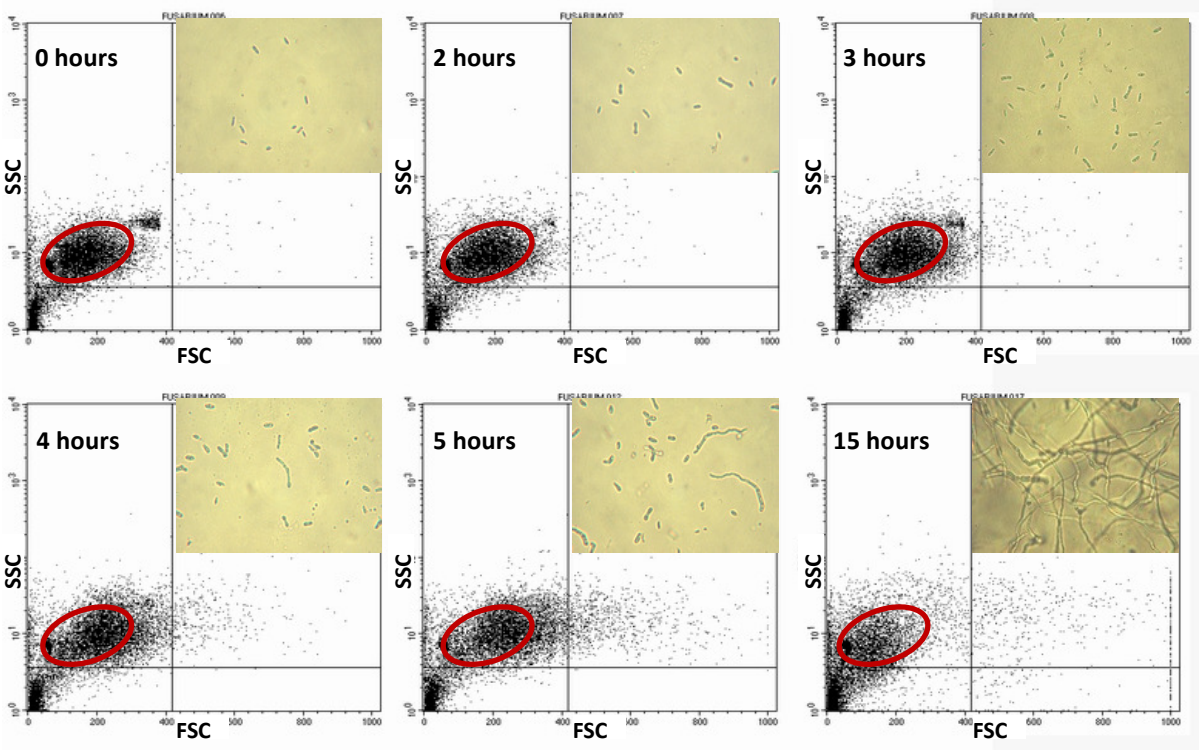


Fig. 2.3.1.3 : Dot plots (cytograms) showing the changes in *F. oxysporum* f.sp. *melonis* spores grown in liquid culture. All measurments are in arbitrary units (AU).

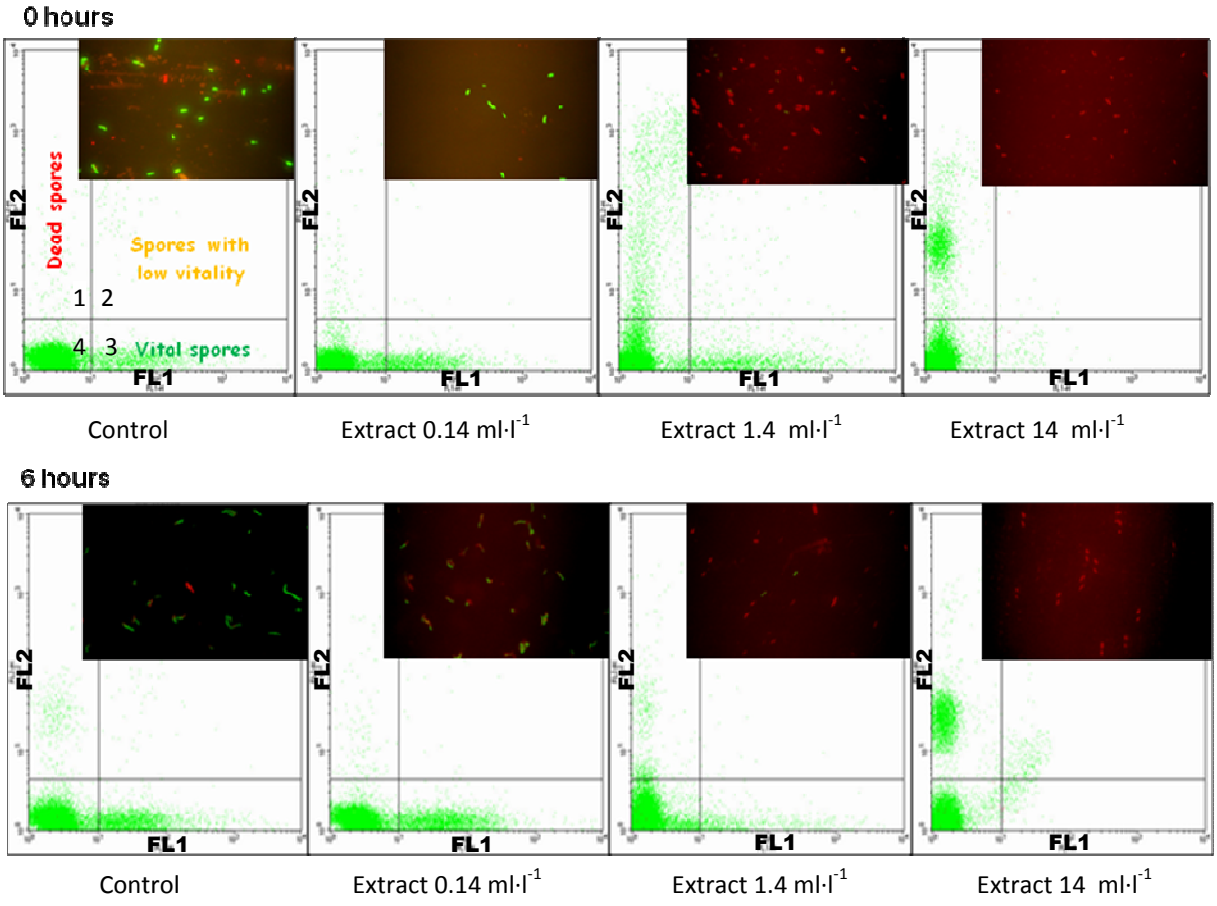


Fig. 2.3.1.4: Development of *F. oxysporum* f.sp. *melonis* spores after *C. leucocephala* extract treatment over time

The capacity of cytoplasmatic esterases to hydrolize the bond among the fluorescein molecule and the acetate groups is considered as a valid indication of the cellular vitality, enzymatic activity and cellular integrity. In fact, in the third quadrant of cytograms is possible to observe increasing spore vitality with the intensity of emitted fluorescent light by FDA. On the other hand, the use of PI enabled to identify the alterations in the cellular membrane function. The polar molecule of PI does not penetrate into undamaged cells, but to cells that lost their selective permeability for extracellular compounds. Although, some percentage of dead spores can be observed even in control treatment as is typical for natural fungal populations, it is possible to observe clearly the increasing number of dead or damage spores along with increasing extract concentration resulting in a significant fluorescence quantity emitted by the molecules of PI visible in the first quadrant. This effect was confirmed also by photos on fluorescence microscope. Moreover, the antifungal effect of *C. leucocephala* extract is evident immediately (at time 0) and its effect is maintained also after 8 hours of incubation.

The antifungal activity of plant extract was confirmed also analyzing data by cytograms with the *x* axis being the size (forward scatter) and the *y* axis being the log of the mean FDA fluorescence intensity (*Figs. 2.3.1.5, 2.3.1.6*). The extract at the concentration of 14 $ml \cdot l^{-1}$ reduced the spore vitality immediately at time 0 as is evident from the low fluorescence intensity of FDA in cytograms. Although, the vitality of spores at the extract concentrations 0.14 $ml \cdot l^{-1}$ and 1.4 $ml \cdot l^{-1}$ was comparable to untreated control and was maintained for the whole length of the experiment, in the culture containing the extract at 1.4 $ml \cdot l^{-1}$ is evident a smaller spore size as indicated by forward scatter values. These result suggest, that despite the plant extract at this concentration did not have a toxic effect on the fungus, it delayed or reduced the development of its hyphae anyway. These results also coincides with previous tests performed on *Fusarium* spp./*C. leucocephala* extract interaction by classical antifungal screening methods reported in this thesis.

As showed in this experiment for a rapid antifungal screening, flow cytometry (FCM) results can be obtained immediately, that is impossible with classical antifungal activity screening methods. In addition, previous reports on flow cytometry screening of antifungal activity on yeast cells showed an applicability of FCM analysis along 3.5 to 9 hours (Green *et al.*, 1994; O'Gorman *et al.*, 1991; Pore, 1990, 1991, 1992). Moreover, the cytometric method enables the use of minimal quantity of plant extract as it works with very small volumes at minimal concentrations. This can be a great advantage in the case when a plant extract is

available only in a small quantity as it is often problem with plant extracts obtained from *in vitro* cultures.

The results of this experiment are in agreement with Green *et al.* (1994) according to whom this technique has several advantages including shorter incubation time, higher precision in comparison with other screening methods, greater accuracy, and speed of analysis. In fact, the greatest advantage of this method is the screening of a broad range of conidial population of the fungus. In addition, each cell is monitored individually and not just as an average value for the whole polulation (Bradner and Nevalainen, 2003).

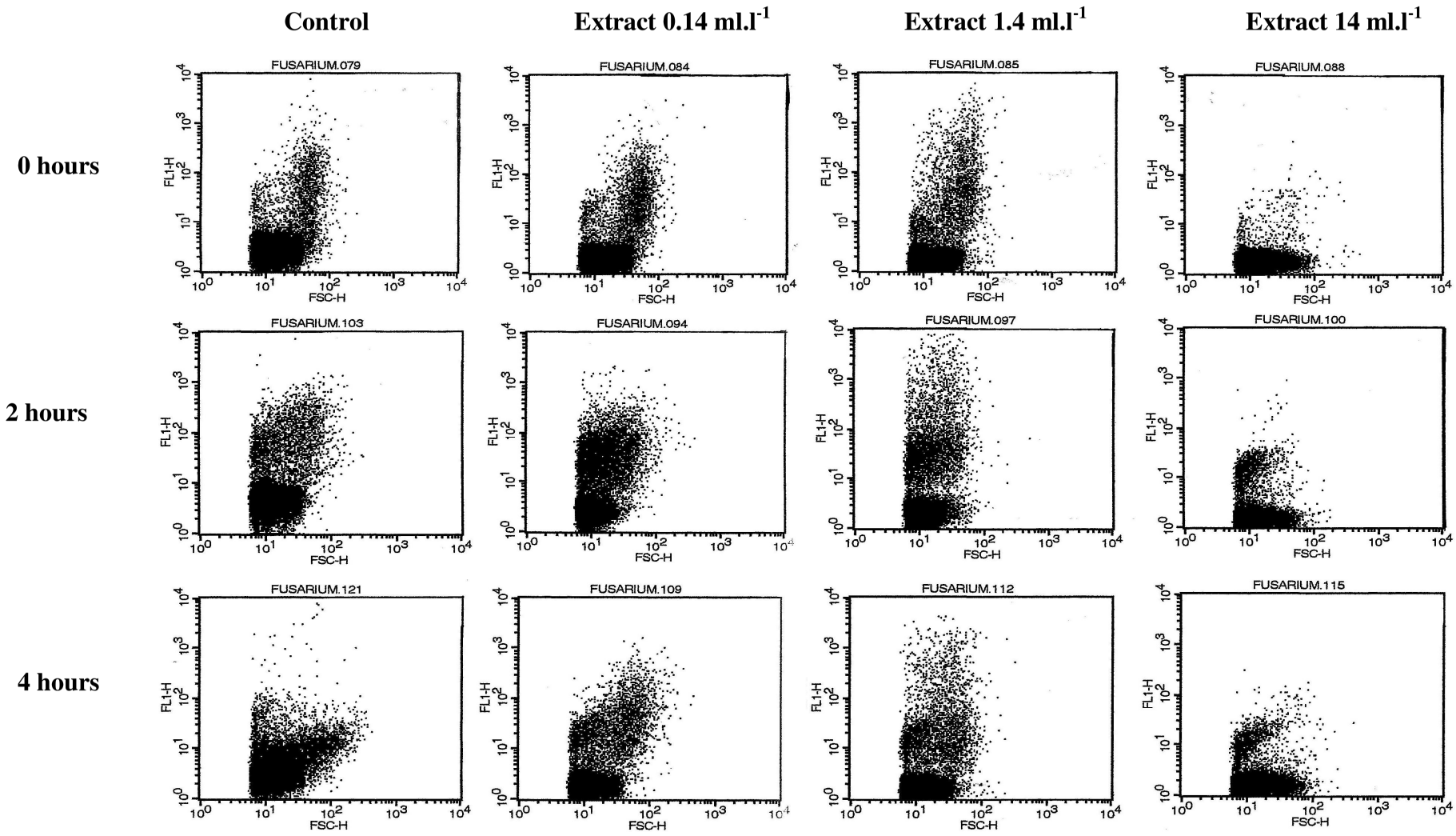


Fig. 2.3.1.5: Cytograms showing the effect of *C. leucocephala* extract on *F. oxysporum* f.sp. *melonis* at 0, 2, and 4 hours of culture. *x* axis is the size (forward scatter) and the *y* axis is the log of the mean FDA fluorescence intensity.

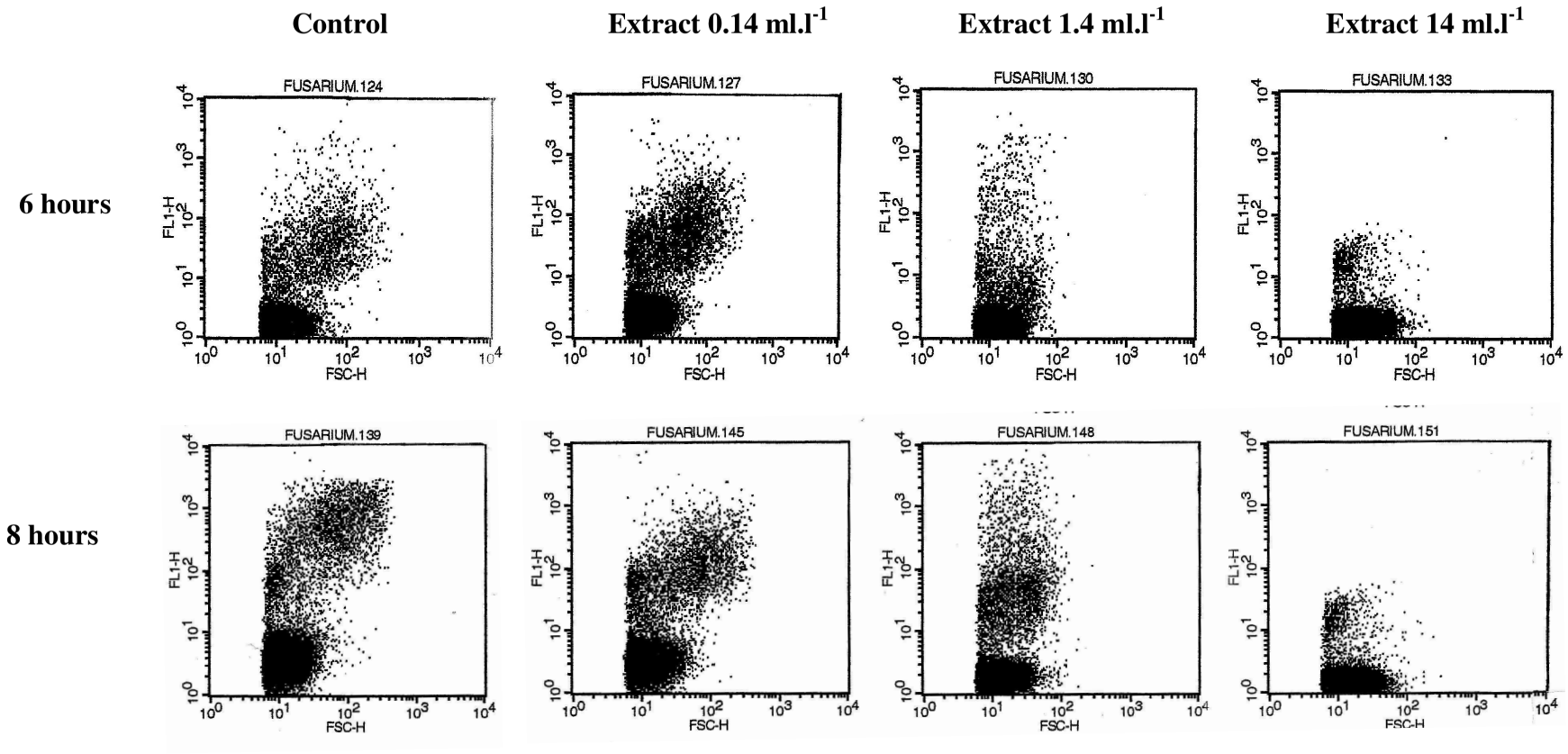


Fig. 2.3.1.6: Cytograms showing the effect of *C. leucocephala* extract on *F. oxysporum* f.sp. *melonis* at 6 and 8 hours of culture. *x* axis is the size (forward scatter) and the *y* axis is the log of the mean FDA fluorescence intensity.

2.3.1.5 Conclusions

Flow cytometry showed to be a valuable method for an early screening of antifungal activity of plant extracts. It was verified that the interaction of the tested fungus and the plant extract was well observable in early stages of fungal growth. The technique has many advantages in comparison with currently used methods of antifungal screening, especially in respect to an higher velocity and a better precision. In our opinion, flow cytometry has a great potential to be exploited for the screening of wide range of filamentous fungi and antifungal compounds.

2.3.2 MOLECULAR ANALYSIS OF *PR* GENE ACTIVATION BY TREATMENT WITH *VITEX AGNUS-CASTUS* EXTRACT AND *PYTHIUM ULTIMUM* INOCULATION IN TOMATO

2.3.2.1 Abstract

To determine the involvement of plant extract and pathogenic fungus in *PR* gene induction, tomato plants were treated with *Vitex agnus-castus* extract and/or inoculated immediately with *Pythium ultimum*. The expression of four *PR* genes was monitored at five time points within 48 hours after treatments and artificial inoculations. The treatments with plant extract resulted in significant activation of genes encoding PR-1 and PR-4 proteins already after three hours. The expression of *PR-1* and *PR-4* genes in the plants treated both with plant extract and fungus increased over time, while decreased in the plants treated just with plant extract. Twenty-four hours after treatments, *PR-1*, *PR-4*, and *PR-6* genes were induced very strongly and, at the 48th hour, they reached the maximum expression in plants treated both with plant extract and fungus. However, *PR-5* genes were not activated by *V. agnus-castus* extract at a significant level. Expression of three PR genes in tomato seedlings treated with *V. agnus-castus* extract and/or inoculated with *P. ultimum* indicates that the plant extract contributes to the activation of plant defence mechanisms. Apart the direct induction of *PR* genes, the extract acted also as a priming agent conferring to the plant enhanced defence responses upon pathogen inoculation. The activation of various *PR* genes suggests that the induction of disease responses by *V. agnus-castus* extract in tomato can be regulated by more signalling pathways.

2.3.2.2 Introduction

Plants are subjected to continuous stresses caused by pathogens or unfavourable environmental conditions. To protect themselves, they developed various defence mechanisms of mechanic or chemical type. These mechanisms may be either constitutive or induced by various stresses. In the induced defence response, the contact of plant with pathogen or other stresses triggers an immediate reaction in the plant at the molecular level that leads to activation of defence genes. Such defence genes encode the proteins involved in all possible plant defence mechanisms: from various chemicals with antimicrobial or antiinsecticidal properties to mechanical defences such as cell wall thickening.

The specific genes are activated only by certain stresses. There are for example genes inducible by chemicals, pathogens, or wounds. Some genes are induced by more types of

stresses, and some of them are probably highly specific responding just to a specific stress. These mechanisms are not elucidated yet because not all interactions between stresses and gene activation have been identified.

Induced responses in plant can be both local and systemic such as systemic acquired resistance (SAR) and induced systemic resistance (ISR). Systemic resistance responses induced by either harmful or beneficial microorganisms or by treatment of plants with various natural or synthetic compounds are associated with a process called "priming". It means that plants develop enhanced capacity for faster and stronger activation of stress-inducible defence reactions upon second pathogen challenge rather than a direct induction of defence mechanisms (Beckers and Conrath, 2007). Examples of priming can be represented by the colonization of tomato roots by mycorrhizal fungi (Cordier *et al.*, 1998) or by biocontrol agent *F. oxysporum* Fo47 (Aimé *et al.*, 2008) that systematically protects the plant against *Phytophthora parasitica*, and *Fusarium* sp. respectively, without direct accumulation of *PR* proteins. Among the chemicals with priming potential, the most studied involve β-aminobutyric acid (BABA) (Jakab *et al.*, 2001; Cohen, 2002; Zimmerli *et al.*, 2000), salicylic acid (SA), dichloroisonicotinic acid (INA), and benzothiadiazole (BTH) (Conrath *et al.*, 2002) as potent inducers of resistance in plants against microbial pathogens.

The pathogen attack triggers an activation of genes encoding proteins collectively known as pathogenesis-related (PR) proteins. These proteins have been grouped into 17 PR classes according to their biological activity and structural characteristics. Most PRs and related proteins are induced through the action of the signalling compounds salicylic acid (SA), jasmonic acid (JA), or ethylene (ET). However, many induction pathways still remain unknown. The PR proteins possess antimicrobial activities through hydrolytic activities on cell walls, contact toxicity, and involvement in defence signalling (Dickinson, 2003; Van Loon *et al.*, 2006).

The most common PR proteins detected in a large number of plant species are probably PR-1. Some PR proteins have been shown to inhibit the growth of oomycetes. The chitinases (PR-4) hydrolyze chitin in fungal cell walls. The PR-5 family of thaumatin-like proteins permeabilises fungal membranes. Proteinase inhibitors (PR-6) are produced to inhibit insect and microbial protease enzymes (Dickinson, 2003; Terras *et al.*, 1995; Van Loon and Van Strien, 1999).

Although in most *PR* genes is still unclear which gene of the PR family corresponds exactly to which protein, in tomato, the PR proteins were fully classified for many of nucleotide sequences (van Loon *et al.*, 2006) such as those for PR-1 (Tornero *et al.*, 1997),

PR-4 (Joosten *et al.*, 1990), PR-5 (Rep *et al.*, 2002) and PR-6 (Hass *et al.,* 1982; Gadea *et al.*, 1996). Various induced defence responses were largely reported in tomato upon pathogen attack. Application of microbes or their elicitors (Unger *et al.*, 2006; De Cal *et al.*, 2000; Cordier *et al.*, 1998; Perkovska *et al.*, 2007), and application of chemicals, e.g. methyl jasmonate (Yu *et al.*, 2009), and hexanoic acid (Vicedo *et al.*, 2009) induced disease resistance to *Botrytis cinerea*; salicylic acid (Mandal *et al.*, 2009), indol-3-acetic acid (IAA) (Sharaf *et al.*, 2004) and benzothiadiazole (BTH) (Benhamou and Bélanger, 1998) induced disease resistance to *Fusarium oxysporum* f.sp. *lycopersici*; BION® (Acibenzolar-S-methyl) and β-aminobutyric acid (BABA) induced disease resistance to *Phytophthora infestans* (Arici and Dehne, 2007).

Apart mycorrhizal microorganisms, there is the lack of studies on natural products such as plant extracts and their capacity to induce the defence responses in tomato. Medeiros *et al.* (2009) reported a defence gene expression induced by a coffee-leaf extract formulation (NEFID) against *Xanthomonas vesicatoria*.

Only a few reports exist of induced disease resistance to *Pythium* sp., a disastrous causal agent of tomato damping off. Hanafi *et al.* (2007) reported that plant growth promoting rhizobacteria (*Bacillus subtilis*) induced resistance to this pathogen, especially under saline conditions. The potential to induce disease response was reported in Milsana®, a commercial product of leaf extracts from the giant knot weed *Reynoutria sachalinensis*, which lead to accumulation of antifungal compounds effective against *P. ultimum* in cucumber leaves (Daayf *et al.*, 2000).

The aim of this study was to investigate *PR* gene activation after treatment with *Vitex agnus-castus* extract, a tropical shrub with known antifungal properties (Pepeljnjak *et al.*, 1996), and *Pythium ultimum* inoculation in tomato. In particular, activation of genes encoding PR-1, PR-4, PR-5 and PR-6 proteins was investigated in tomato seedlings.

2.3.2.3 Materials and methods

Plant material and treatments

Crude methanol extract from stems of *V. agnus-castus*, obtained by Soxhlet extraction technique (modified from Ehrman, 1994), was provided from AGROTECNOLOGIAS NATURALES S.L. (ATENS company, Spain).

Tomato seeds cv. Superprecoce di Marmande (SGARAVATTI) were sown into peat substrate and maintained at 25°C in the growth chamber. Extract of *V. agnus-castus* (8 $ml \cdot l^{-1}$)

at the dose of 2.5 ml per seedling at a cotyledonary stage was applied to the substrate and plant crown. One ml of the suspension containing 3-4 x 10^5 CFU of *P. ultimum* per plant was applied to the substrate and plant crown immediately after the extract treatment. The inoculated control treated with distilled water, uninoculated control treated with extract, and uninoculated control treated with distilled water, were established.

Twenty plant samples per treatment were collected 3, 6, 12, 24 and 48 hours after treatments, frozen immediately by liquid nitrogen, and stored at -80°C. All the materials used during the collection of plant samples for RNA preparation were treated with DEPC 0.1% (v/v) and autoclaved at 121°C for one hour.

RNA extraction

The plant tissue was frozen in liquid nitrogen and ground to a fine powder. The RNA extraction was performed from 50 mg of the powder with the kit "NucleoSpin® RNA Plant" (MACHEREY-NAGEL) following the manufacturer's instructions. The quantity of RNA obtained was determined by spectrophotometric reading at the wavelength of 260 nm considering that at this wavelength RNA concentration is 40µg·ml^{-1}. The contamination by proteins was evaluated by spectrophotometric reading at the wavelength of 280 nm calculating the ratio A_{260}/A_{280}. Under optimal conditions, the ratio should be approximately equal to 2. All the solutions and materials used during RNA preparation were treated with DEPC 0.1% (v/v) and autoclaved at 121°C for one hour.

Electrophoretic analysis of RNA

The RNA extracted was analysed on agarose gel 1.2% (w/v) with formaldehyde according to the protocol of Sambrook *et al.* (1989). The agarose was dissolved in buffer solution of MOPS 20 mM (pH 7.0), containing NaAc 8mM and EDTA 1 mM (RNA buffer), with formaldehyde added to reach the final concentration of 6.6 % (v/v). Thirty µl of RNA buffer, containing formaldehyde 6.6% (v/v) and formamide 50% (v/v), was added to RNA samples. The samples were kept at 65 °C for 15 minutes. Then, 1 µl of ethidium bromide 0.1 mg/l and 1 µl of the solution containing glycerol 50% (v/v), EDTA 1 mM, bromophenol blue 0.25% (w/v), and xylene cyanol 0.25 % (w/v) was added. Electrophoresis was performed in RNA buffer at 60 V for 90 minutes. At the end of the electrophoretic separation, the RNA was visualized with ultraviolet light.

cDNA synthesis and PCR (RT-PCR)

For RT-PCR reaction, the Reverse Transcriptase enzyme ImProm-II™ (PROMEGA) was used following the manufacturer's instructions. One µg of total RNA with oligo dT (final volume of 0.2 µM) were incubated at 70°C for 5 minutes, and then in the ice for 5 minutes. Then the reaction buffer containing $MgCl_2$ at the final concentration of 3 mM, dNTP at the final concentration of 125 µM, 1 µl of ImProm-II™ enzyme, and sterile RNA free water up to the final volume of 20 µl were added. The samples were incubated at 25 °C for 5 minutes, at 45 °C for one hour enabling cDNA synthesis, and finally at 70 °C for 15 minutes.

Amplification of cDNA was performed with the enzyme BIOTAQ™ (BIOLINE). The reaction mixture of the final volume of 25 µl was prepared following the manufacturer's instructions. The mixture contained 3.5 U of the enzyme, the reaction buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl of pH 8.8), dNTP at the final concentration of 2 mM, $MgCl_2$ at the final concentration of 2.5 mM, and the specific forward and reverse primers. The PCR reactions had the following program:

1 cycle	2 minutes at 95 °C (initial denaturation)
25 cycles	1 minute at 95 °C (denaturation)
	1 minute at 48°C (*PR-1*), 54 °C (*PR-4*), 59 °C (*PR-5*), 58 °C (*PR-6*), 54°C (*actin*) (annealing)
	30 seconds at 72 °C (extension)
1 cycle	5 minutes at 72 °C (final extension)

The specific primers were designed according to their known sequence (*Tab. 2.3.2.1*). To verify the quantity of RNA used for cDNA synthesis, cDNA was amplified with specific primers for *actin*, a gene expressed constitutively, according to a previously used protocol. The primers for *actin* were used also to identify an eventual contamination of RNA by genomic DNA.

Tab. 2.3.2.1: Primer sequences

Primer		Sequence	Accession number	Amplicon
***PR1b1* (*PR-1*)**	*for*	5'-GCACTAAACCTAAAGAAA-3'	Y08804	198 bp
	rev	5'-TAGTTTTGTGCTCGGGATGC-3'		
***PR-P2* (*PR-4*)**	*for*	5'-GCTACGAACGTTAGGGCAAC-3'	X58548	191 bp
	rev	5'-CTCAAGCATCTACCGCATGA-3'		
***PR-5x* (*PR-5*)**	*for*	5'-GTGACTTACACTTATGCTGCCACT-3'	AY093595	284 bp
	rev	5'-TGGTCCAAAGCATATTCAGCTAGG-3'		
***Proteinase inhibitor II* (*PR-6*)**	*for*	5'-TCCTTGCTCACCTACTTGTTCTTGG-3'	X94946	588 bp
	rev	5'-TTCCTTATGCTGTGGAAATACTTTG-3'		
Actin	*for*	5'-CCCAATTGAACACGGTATTGT-3'	BT013524	748 bp
	rev	5'-GGTGATTTCCTTGCTCATACG-3'		

Electrophoretic analysis of DNA

All the products of RT-PCR amplification were analyzed on agarose gel 1.2% (w/v) according to the protocol proposed by Sambrook *et al.* (1989). Agarose was dissolved in buffer Tris-acetate 40 mM and EDTA 1 mM (TAE). Ethidium bromide at the final concentration of 0.4 µg/ml was added to visualize DNA molecules with UV. Two µl of glycerol 40% was added to DNA samples. Electrophoresis was conducted in TAE at 100 V constant for 40 minutes. The dimension of DNA and its quantity were estimated comparing DNA electrophoretic mobility with that of molecular markers.

2.3.2.4 Results and discussion

To determine the involvement of plant extract and pathogenic fungus in *PR* gene induction, tomato plants were treated with *Vitex agnus-castus* extract and/or *Pythium ultimum*. The expression of four genes was monitored at five time points within 48 hours after treatments. DNA bands of activated *PR* genes were visualized on agarose gel (*Fig. 2.3.2.1*). In the control plants (C), treated only with the water, the significant activation of monitored *PR* genes was not observed for the whole duration of the experiment.

Expression analysis of *PR1b1* (*PR1*) gene

The treatment with *V. agnus-castus* extract resulted in a significant activation of *PR-1* gene already three hours after treatments, compared to the relative control. However, the gene induction was not observed in the inoculated plants without extract treatment (I). Therefore, it is supposed that the bands containing the DNA from plants treated both with *V. agnus-castus* extract and *P. ultimum* (TI) are visible on the agarose gel because of the plant extract presence

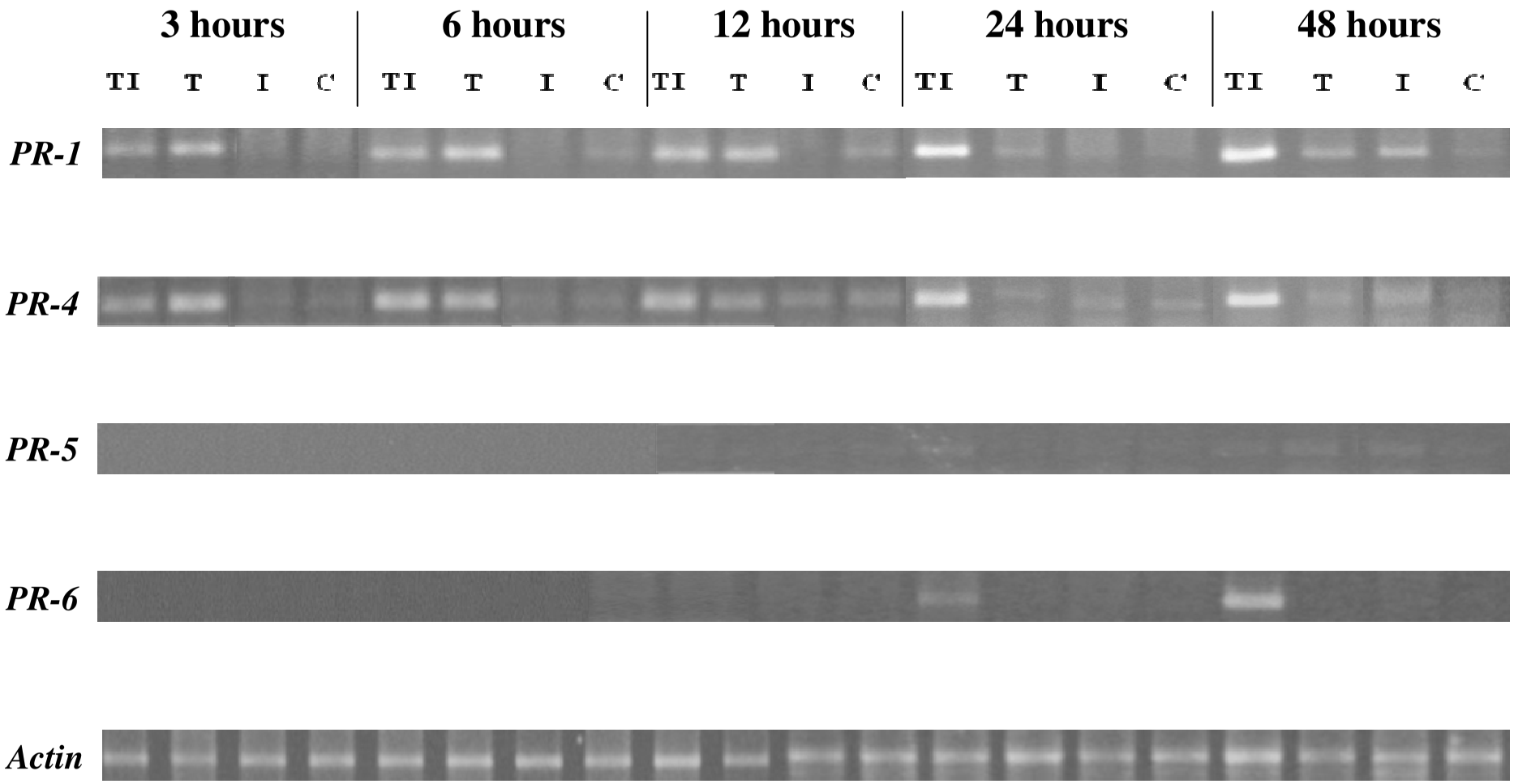


Fig.2.3.2.1: DNA bands of activated *PR* genes and *actin* in tomato seedlings visible on agarose gel over time. (TI) plants treated with *V. agnus-castus* extract and inoculated with *P. ultimum*, (T) plants treated with *V. agnus-castus* extract, (I) plants inoculated with *P. ultimum*, (C) control plants treated with distilled water.

in the treatment. At the 3rd hour, the band intensity was higher in the samples containing DNA of plants treated just with plant extract in comparison with those treated both with extract and fungus.

Six hours after treatments, the quantity of *PR-1* gene expression gradually increased. The two bands (T, TI) have stronger intensity and the difference between them seems to diminish, even if the band of the sample treated both with *V. agnus-castus* extract and *P. ultimum* remained less intensive.

Twelve hours after treatments, the *PR-1* gene expression seems to be equal in both samples (TI, I). It means that the levels of activated *PR-1* gene increased during six hours in the plants treated both with *V. agnus-castus* extract and *P. ultimum* while, in the samples from plants treated only with the extract, the *PR-1* gene expression remained unchanged or decreased insignificantly.

Twenty-four hours after treatments, the amount of DNA of *PR-1* gene remarkably increased in the plants treated both with the extract and the fungus. The expression of *PR-1* gene in the plants treated only with *V. agnus-castus* extract or only with *P. ultimum* was insignificant. However, Hong and Hwang (2002), detected numerous PR-1 proteins accumulated in tomato stem tissues already 24 hours after inoculation with the oomycete, *P. capsici*. The proteins were predominantly accumulated over oomycete cell walls and at the interface between host and oomycete cell walls. In addition, Benito *et al.* (1998) observed that the velocity of *PR-1* mRNA induction in tomato leaves infected by *Botrytis cinerea* was dependent on the temperature. Therefore, the *PR* gene expression by a pathogenic fungus is influenced probably by many factors such as environmental conditions and fungal species.

Fourty-eight hours after treatments, *PR-1* gene was activated also after *P. ultimum* infection. However, its expression was very low in the sample containing just inoculated plants in comparison with the samples from plants treated also by the *V. agnus-castus* extract (TI), where the amount of DNA continued to increase. This result is in agreement with Spletzer and Enyedi (1999), who reported expression of tomato *(PR)-1B* gene upon SA treatment within 24 hours, which was still evident after 48 hours. Activation of genes in the plants treated only with *V. agnus-castus* extract remained insignificant.

Although the *PR-1* activation by *V. agnus-castus* was stronger at 24 and 48 hours, the gene was expressed in smaller amounts also much earlier. Tornero *et al.* (1997) suggested that different mode of expression of various *PR-1* genes may be all complementary and necessary for the plant to acquire an efficient refractory state to resist pathogen attacks.

Expression analysis of *PR-P2* (*PR-4*) gene

Gene encoding PR-4 proteins was activated in the plants treated with *V. agnus-castus* extract (TI, T) already 3 hours after treatments, compared to relative control. Stronger gene activation was observed in the plants treated just with plant extract in comparison with the inoculated plants treated with the extract. Such results indicate a possible silencing or reduction of *V. agnus-castus* extract capacity to induce these genes in presence of *P. ultimum* at 3 hours after treatment. In *PR-4*, the difference between these two samples is more remarkable in comparison with the same samples of *PR-1* gene. Gene induction was not observed in the inoculated plants only.

At 6 hours, gene expression in plants treated both with *V. agnus-castus* extract and *P. ultimum* is significantly higher in comparison with the same sample at three hours. The expression of *PR-4* gene in the sample of plants treated only with *V. agnus-castus* extract visibly decreased. Interestingly, although the amount of amplified DNA increases in the sample treated with the extract and the fungus, the sample containing only inoculated plants do not show any significant band yet. It is supposed that the amount of amplified product would be the same in these two samples as the only inducing agent applied to the plants at the same dose, is the plant extract. Therefore, this response indicates some mechanisms which accelerate and enhances *PR-4* gene activation at this time point.

At 12 hours, *PR-4* gene expression in the plants treated both with *V. agnus-castus* extract and *P. ultimum* remained unchanged, while the amount of the activated gene significantly decreased in the plants treated only with the extract, being comparable with the water treated control.

Twenty-four hours after treatments, the *PR-4* gene expression significantly increased in the plants treated both with *V. agnus-castus* extract and the fungus. However, in comparison with *PR-1*, the increase of *PR-4* gene expression during the past six hours is less remarkable. The level of activated genes in the plants treated only with the extract is insignificant.

At 48 hours, *PR-4* gene is activated also by *P. ultimum*. However, as in *PR-1*, its quantity is significantly lower in comparison with the plants treated also by the extracts. Here, the quantity of activated *PR-4* gene increased remarkably and the increase is much stronger in comparison with *PR-1* gene. The level of activated genes in the plants treated only with the extract remained insignificant.

The results indicating the time necessary for activation of *PR-4* gene by *V. agnus-castus* extract in tomato are in agreement with Medeiros *et al.* (2009), who reported *PR-4*

gene expression in tomato leaves treated by coffee-leaf extract formulation (NEFID). Gene encoding PR-4 proteins were transcriptionally up-regulated and the corresponding enzyme activities were over-expressed as early as 24 hours post elicitation; they remained elevated for up to five days after NEFID exposure.

The results of simultaneous activation of genes *PR-1* and *PR-4*, especially at 24 and 48 hours, can be explained by the same transduction pathways of both genes. Therefore, as these genes use the same signal molecule, that is salicylic acid, the response of the plant to the treatments through the expression of these genes is similar. In fact, also Fiocchetti *et al.* (2006) reported the simultaneous expression of both genes in transgenic tomato upon SA-treatment. Increased transcription of PR genes was more intense for *PR-1* gene and less intense for *PR-4* gene. However, in the present study with *V. agnus-castus* extract, the activation of *PR-1* and *PR-4* genes was of the same significance, compared to relative controls.

Expression analysis of *PR-5x* (*PR-5*) gene

The expression of *PR-5* gene was not detected at all 3, 6 and 12 hours after any kind of treatment in any sample. Twenty-four hours after treatment, minimal gene expression can be observed only in plants treated with both *V. agnus-castus* extract and *P. ultimum*. At 48 hours, the band of a very low intensity is visible in the samples treated just with extract and just with fungus. However, the *PR*-5 gene activation in these samples is not significant in comparison with untreated plants. In the plants, treated with both extract and fungus, no activation was observed at 48 hours. However, in the study of Kavroulakis *et al.* (2006), *PR-1* and *PR-5* genes were expressed together in tomato plants grown on suppressive compost suggesting that two genes follow the same signalling pathway. The only difference in the expression of *PR-1* and *PR-5* genes was the location of transcripts, which were almost exclusively detected in the pericycle cells surrounding the root stele of the main and lateral roots in the case of *PR-1*, and in the phloem of root and stem tissues in the case of *PR-5*. Also Xu *et al.* (2003) detected simultaneous expression of *PR-1* and *PR-5* genes in tomato plants infected with Cucumber Mosaic Virus. In addition, Ishikawa *et al.* (2005) reported elevated expression of SAR marker genes *P4* (*PR1)* and *NP24* (*PR-5*) in tomato plants upon treatment with plant activators (validamycin A and validoxylamine A), the control agents against tomato fusarium wilt.

Although, *PR-5* gene is activated via salicylic acid pathway and is even used as a marker for this pathway, it did not give the same response under plant extract and fungus treatment as *PR-1* and *PR-4* genes, regulated by the same pathway. These results indicates

that, in this case, *PR-5* gene perhaps needs for its activation some other signal molecule different from SA that can be either synergic or not with SA. In fact, Kavroulakis *et al.* (2007) reported that *Fusarium solani* endophytic strain inoculating tomato roots, where the *PR*-5 gene expression was detected, requires ET- signalling pathway to confer resistance against soil-borne fungal pathogens. However, the signal molecule involved in *PR-5* transduction was not activated in plant by the application of both *V. agnus-castus* extract and *P. ultimum*, and no significant plant response was triggered.

If no gene activation was observed nor in the other three tested genes, the conclusion would be that *V. agnus-castus* extract do not cause any kind of stress to the plant, which would lead to *PR* gene activation. Nevertheless, as other *PR* genes showed remarkable levels of activation, the conclusion is that PR-5 protein simply does not participate on plant response to *V. agnus-castus* extract treatment.

Expression analysis of *Proteinase inhibitor II* (*PR-6*) gene

The activation of *PR-6* gene was not detected 3, 6 and 12 hours after treatment in any sample. In plants treated with both *V. agnus-castus* extract and *P. Ultimum*, the gene was activated 24 hours after treatment and gene expression remarkably increased 48 hours after treatments. The gene was activated neither by extract nor by fungus alone. The gene probably needs more time than 48 hours to be activated by *P. ultimum*.

These results clearly demonstrate that *PR-6* gene is not activated directly by an extract treatment but only after a challenge with the pathogen. Interestingly, *PR-1* and *PR-4* are activated directly by *V. agnus-castus* extract up to 12 hours after treatments but, at 24 hours, the direct activation disappear and the extract acts as a priming agent. The priming phenomenon was observed also by Aimé *et al.* (2008) in tomato roots and leaves upon treatment with protective *Fusarium* strain. These results confirm that priming might be the mode of action of some biocontrol agents. Factors such as plant history, elicitor and/or primer dose, and sensitivity of response being monitored may play a significant role in plant defence activation, because they may influent on whether defence response is activated directly or by priming (Paré *et al.*, 2005).

The different response of tested *PR* genes to *P. ultimum* application can be explained by different transduction pathways. For example, in the study of Hese *et al.* (2008), the expression of JA-responsive gene for the basic PR-6 protein was induced in tomato treated with *Pythium oligandrum*, but the SA-inducibe *PR1*(*P6*) gene was not up-regulated. These results indicated that, in the case of *P. oligandrum*, JA-dependent signalling pathway is

required for inducing resistance to *Ralstonia solanacearum*. On the contrary, *V. agnus-castus* extract used in this study acts via both SA- and JA-dependent pathways. Also Martinez De Ilarduya *et al.* (2003) reported the simultaneous activation of *PR-1* and *PR-6* (*proteinase inhibitors I and II*) genes in tomato by the same stress, aphid feeding. It means that some *PR* gene-inducing agents might involve both signalling pathways.

The expression of *PR-6* gene, which is regulated by JA-dependent signalling pathway (Hondo *et al.*, 2007) and activated by *V. agnus-castus* extract in tomato seedlings, as observed in the present study, is in agreement with the results of experiments previously reported in this thesis on the *V. agnus-castus* efficacy against *P. ultimum* in tomato seedlings. Kozlowski *et al.* (1999) suggested the methyl jasmonate involvement in induced disease resistance against *P. ultimum* in *Picea abies*. Therefore, JA-signalling pathways may play an important role in plant defence mechanisms against this pathogen.

2.3.2.5 Conclusions

Expression of *PR* genes in tomato seedlings after treatment with *V. agnus-castus* extract indicated that this plant extract contributes to the activation of plant defence mechanisms. Apart the direct induction of *PR* genes, the extract acted also as a priming agent conferring plant enhanced defence responses upon pathogen inoculation. The activation of various *PR* genes suggested that the induction of defence responses by *V. agnus-castus* extract in tomato can be regulated by more signalling pathways.

2.4 FINAL CONCLUSIONS

- All plant extracts tested showed antifungal activity against phytopathogenic fungi under *in vitro* conditions.

- Only in some interactions, plant extracts showed a significant efficiency for controlling fungal pathogens under *in vivo* conditions. They are *S. guianensis* / *B. cinerea* in tomato and cucumber, and *A. rabiei* in chickpea; *C. leucocephala* / Fusarium wilt in tomato and melon; *B. diffusa* / *P. capsici*; *V. agnus-castus* / *P. ultimum*.

- Under *in vivo* conditions, plant extracts were generally more effective against soil-borne pathogens.

- As the efficiency of the extracts of *B. diffusa* and *V. agnus-castus* was comparable to synthetic fungicide, they can be considered as a potential valuable alternative to chemical control of *P. capsici* and *P. ultimum*, respectively.

- Flow cytometry analysis enabled to observe the interaction fungus-plant extract in the early stages of fungal growth. The technique showed many advantages in comparison with other currently used methods of antifungal screening, especially characterized by higher velocity and precision. Therefore, flow cytometry has a great potential to be exploited for the screening of antifungal compound efficacy against filamentous fungi in the future.

- Expression of *PR* genes in tomato seedlings after treatment with *V. agnus-castus* extract indicated that this plant extract contributes to triggering of defence mechanisms in plant. Apart the direct induction of *PR* genes, the extract acted also as a priming agent conferring plant enhanced defence responses upon pathogen inoculation. The activation of various *PR* genes suggested that the induction of defence responses by *V. agnus-castus* extract in tomato can be regulated by more signalling pathways.

BIBLIOGRAPHY

Abad, L., D'Urzo, M., Liu, D., Narasimhan, M., Reuveni, M., Zhu, J., Niu, X., Singh, N., Hasegawa, P., Bressan, R., 1996. Antifungal activity of tobacco osmotin has specificity and involves plasma membrane permeabilization. *Plant Science*, 118: 11-23.

Abel, S., Oeller, P.W., Theologis, A., 1994. Early auxin-induced genes encode short-lived nuclear proteins. *Proc. Natl. Acad. Sci.* USA, 91, 326–330.

Abou-Jawdah, Y., Sobh, H., Salameh, A., 2002. Antimycotic activities of selected plant flora, growing wild in Lebanon, against phytopathogenic fungi. *Journal of Agricultural and Food Chemistry*, 50 (11): 3208-3213.

Adams, K.L., Cronn, R., Percifield, R., Wendel, J.F., 2003. Genes duplicated by polyploidy show unequal contribution to the transcriptome and organ-specific reciprocal silencing. Proceedings of the National Academy of Sciences USA, 100: 4649–4654.

Adeyemi, I.A., Omonigbehin, A.E., Stella, S., Oluwatosin, O., Jumoke, S., 2008. Antibacterial activity of extracts of *Alchornea cordifolia* (Schum and Thonn) Mull.Arg., *Boerhavia diffusa* (L) and *Bridellia micranthal* (Hoscht) Baill. used in traditional medicine in Nigeria on *Helicobacter pylori* and four diarrhoeagenic bacterial pathogens. *African Journal of Biotechnology*, 7 (20): 3764-3767.

Agrawal and Srivastava, 2008. Structural elucidation of bioactive principle of root of *Boerhavia diffusa* for its antifungal properties. *National Academy Science Letters*, 31 (7-8): 215-219.

Agrawal, A., Srivastava, S., Srivastava, J. N., & Srivastava, M. M. (2004). Inhibitory effect of the plant *Boerhavia diffusa* L. against the dermatophytic fungus microsporum fulvum. *Journal of Environmental Biology*, 25(3): 307-311.

Agrawal, N., Dasaradhi, P.V.N., Mohmmed, A., Malhotra, P., Bhatnagar, R.K., and Mukherjee, S.K. (2003). RNA interference: biology, mechanism, and applications. *Microbiol. Mol. Biol. Rev.* 67: 657-685.

Agrios, G.N., 2005. Plant Pathology. Elsevier Academic Press. San Diego, USA. 922 pp.

Ahmad, M.U., Husain, S.K., Osman, S.M., 1981. Ricinoleic acid in *Phyllanthus niruri* seed oil. *Journal of the American Oil Chemist's Society*, 58 (6): 673-674.

Aimé, S., Cordier, C., Alabouvette, C., Olivain, C., 2008. Comparative analysis of PR gene expression in tomato inoculated with virulent *Fusarium oxysporum* f.sp. *lycopersici* and the biocontrol strain *F. oxysporum* Fo47. *Physiological and Molecular Plant Pathology*, 73 (1-3): 9-15.

Akinbode, O.A., Ikotun, T., 2008. Efficacy of certain plant extracts against seed-borne infection of *Collectotrichum destructivum* on cowpea (*Vigna uniguculata*). *African Journal of Biotechnology*, 7 (20): 3686-3688.

Aladesanmi, A.J., Iwalewa, E.O., Adebajo, A.C., Akinkunmi, E.O., Taiwo, B.J., Olorunmola, F.O., Lamikanra, A., 2007. Antimicrobial and antioxidant activities of some Nigerian medicinal plants. *African Journal of Traditional, Complementary and Alternative Medicine,s* 4 (2): 173-184.

Alam, S.S., Bilton, J.N., Slawin, A.M.Z., Williams, D.J., Sheppard, R.N., Strange, R.N. Chickpea blight: Production of the phytotoxins solanapyrones A and C by *Ascochyta rabiei*, 1989. *Phytochemistry,* 28 (10): 2627-2630.

Al-Hamdani, A.M., Lutchmeah, R.S., Cooke, R.C., 2007. Biological control of *Pythium ultimum*–induced damping-off by treating cress seed with the mycoparasite *Pythium oligandrum*. *Plant Pathology*, 32 (4): 449 – 454.

Al-Reza, S.,M., Rahmanb,A., Ahmedb, Y., Kang, S.C., 2009. Inhibition of plant pathogens *in vitro* and *in vivo* with essential oil and organic extracts of *Cestrum nocturnum* L..*Pesticide Biochemistry and Physiology*, Article in press.

Alvarez, J. M., González-Torres, R., Mallor, C., & Gómez-Guillamón, M. L. (2005). Potential sources of resistance to fusarium wilt and powdery mildew in melons. *HortScien*ce, 40(6), 1657-1660.

Ament, H.A., Trigiano, R.N., 2004. *Oomycota*: The fungi-like organisms. In Trigiano, R.N., Windham, M.T., Windham, A.S. (Eds.), Plant Pathology, Concepts and Laboratory Excercises, CRC Press, Washington, USA., 300-316.

Amiri, A., Dugas, R., Pichot, A.L., Bompeix, G., 2008. *In vitro* and *in vivo* activity of eugenoil oil (Eugenia *caryophylata*) against four important postharvest apple pathogens. *International Journal of Food Microbiology,* 126 (1-2):13-19.

Anand, T., Bhaskaran, R., 2009. Exploitation of plant products and bioagents for ecofriendly management of Chilli fruit rot disease. *Journal of Plant Protection Research,* 49 (2): 195-203.

Anonymous 1998: 1998 Assessment of Alternatives to Methyl Bromide. Report of the Methyl Bromide Technical Options Committee, United Nations Environment Programme, Batchelor, T. (Ed.) Ozone Secretariat, (http://www.org.teap.org), pp. 354.

Arici, Ş.E., Dehne, H.W. 2007. Induced resistance against phytophthora infestans by chemical inducers BION and BABA in tomato plants. *Acta Horticulturae* 729: 503-507.

Asgari Marjanlu, A., Mostofi, Y., Shoeibi, Sh., Maghoumi, M. 2009. Effect of Basil (*Ocimum basilicum* L.) essential oil on gray mold control and postharvest quality of strawberry (cv. Selva). *Journal of Medicinal Plants*: 8 (29): 131-139+175.

Ashawat, M.S., Saraf, S., Saraf, S., 2006. Photo protective properties of *Boerhavia diffusa*. *Biosciences Biotechnology Research Asia,* 3 (1 A): 257-260.

Awade, A., De Tapia, M., Didierjean, L., Burkard, G., 1989. Biological function of bean pathogenesis-related (PR 3 and PR 4) proteins. *Plant Science,* 63 (2): 121-130.

Bagalkotkar, G., Sagineedu, S.R., Saad, M.S., Stanslas, J., 2006. Phytochemicals from *Phyllanthus niruri* Linn. and their pharmacological properties: A review. *Journal of Pharmacy and Pharmacology,* 58 (12): 1559-1570.

Bajpai, V.K., Kang, S.C., 2009. Antifungal Activity of Leaf Essential Oil and Extracts of *Metasequoia glyptostroboides* Miki ex Hu. *JAOCS, Journal of the American Oil Chemists' Society*: 1-10. Article in Press.

Bajpai, V.K., Shukla, S., Kang, S.C., 2008. Chemical composition and antifungal activity of essential oil and various extract of *Silene armeria* L., 99 (18): 8903-8908.

Bajpai, V.K.; Lee, T.J., Kang, S.C., 2009. Chemical composition and *in vitro* control of agricultural plant pathogens by the essential oil and various extracts of *Nandina domestica* Thunb., *Journal of the Science of Food and Agriculture*, 89 (1): 109-116.

Balaraju, K., Agastian, P., Preetamraj, J.P., Arokiyaraj, S., Ignacimuthu, S., 2008. Micropropagation of *Vitex agnus-castus*, (*Verbenaceae*). A valuable medicinal plant (2008). *In Vitro Cellular and Developmental Biology - Plant*, 44 (5): 436-441.

Banerji, K.D., Kumar, K., Saha, G.C., Mazumdar, A.K.D., 1985. Synthesis of some 2-(2'-chlorobenzoyl)coumaran-3-ones. *Journal of the Indian Chemical Society*, 62 (7): 531-533.

Bardin, M., Fargues, J., Nicot, P.C., 2008. Compatibility between biopesticides used to control grey mould, powdery mildew and whitefly on tomato. *Biological Control*, 46 (3): 476-483.

Basandrai, A.K., Basandrai, D., Pande, S., Sharma, M., Thakur, S.K., Thakur, H.L., 2007. A Development of Ascochyta blight (*Ascochyta rabiei*) in chickpea as affected by host resistance and plant age. *European Journal of Plant Pathology*, 119 (1): 77-86.

Batchelor, T.A., 2002. International and European Community controls on methyl bromide and the status of methyl bromide use and alternatives in the European Community. Proceedings of International Conference on Alternatives to Methyl Bromide. The Remaining Challenges, Seville, 5-8 March, 35-39.

Batten, J.S., Scholthof, K.-B.G., Lovic, B.R., Miller, M.E., Martyn, R.D., 2000. Potential for biocontrol of Monosporascus root rot/vine decline under greenhouse conditions using hypovirulent isolates of *Monosporascus cannonballus. European Journal of Plant Pathology*, 106 (7): 639-649.

Bautista- Baños, S., Hernandez-Polez, M., Diaz-Perez, J. C., Cano-Ochoa, C. F., (2000). Evaluation of the fungicidal properties of plant extracts to reduce *Rhizopus stolonifer* of "circula" fruit (*Spondias purpurea* L.) during storage. *Postharvest Biol Technol*, 20:99-106.

Bautista-Baños, S., García-Domínguez, E., Barrera-Necha, L.L., Reyes-Chilpa, R., Wilson, C.L., 2003. Seasonal evaluation of the postharvest fungicidal activity of powders and extracts of huamuchil (*Pithecellobium dulce*): action against *Botrytis cinerea, Penicillium digitatum*, and *Rhizopus stolonifer* of strawberry fruit. *Postharvest Biology and Technology* 29: 81-92.

Baytop, T., 1984. Therapy with medicinal plants in Turkey (Past and Present), No: 3255. Istanbul, Publications of Istanbul University.

Beckers, G.J., Conrath, 2007 U. Priming for stress resistance: from the lab to the field. *Current Opinion in Plant Biology*, 10 (4): 425-431.

Beever, R.E., Weeds, P.L., 2007. Taxonomy and Genetic Variation of *Botrytis* and *Botryotinia*. In Elad, Y., Williamson, B., Tudzynski, P., Delen, N. (Eds.), *Botrytis*: Biology, Pathology and Control. Springer, Dordrecht, The Netherlands, , 29-48.

Bell, J.A., Simpson, D.W., Harris, D.C., 1997. Development of a method for screening strawberry germplasm for resistance to *Phytophthora cactorum*. In: Van der Scheer, HATh, Leiten, P., Dijkstra, J. (Eds.) Proceedings of the 3rd International Strawberry Symposium. *Acta Horticulturae* 439: 175–9.

Bengtsson, M., Wulff, E.,m Lyngs Jørgensen, Pham, A., Lübeck, M., Hockenhull, J., 2009. Comparative studies on the effects of yucca extract and acibenzolar-S-methyl (ASM) on inhibition of *Venturia inaequalis* in apple leaves. *Eur J Plant Pathol* 124: 187-198.

Benhamou, N., Bélanger, R.R., 1998. Benzothiadiazole-mediated induced resistance to *Fusarium oxysporum* f. sp. *radicis-lycopersici* in tomato. *Plant Physiology*, 118 (4): 1203-1212.

Benito, E.P., Ten Have, A., Van'T Klooster, J.W., Van Kan, J.A.L., 1998. Fungal and plant gene expression during sznchronized infection of tomato leaves by *Botrytis cinerea*. *European Journal of Plant Pathology*, 104 (2): 207-220.

Ben-Shalom, N., , a, R. Ardia, R. Pintoa, C. Aki, b and E. Fallika, 2003. Controlling gray mould caused by *Botrytis cinerea* in cucumber plants by means of chitosan. *Crop Protection*, 22 (2): 285-290.

Bhaskara Reddy, M. V., Angers, P., Gosselin, A., Arul, J., 1997. Characterization and use of essential oil from *Thymus vulgaris* against *Botrytis cinerea* and *Rhizopus stolonifer* in strawberry fruits. *Phytochemistry*, 47 (8): 1515-1520.

Bhaskara Reddy, M.V., Arul, J., Ait-Barka, E., Angers, P., Richard, C. , Castaigne, F., 1998. Effect of chitosan on growth and toxin production by *Alternaria alternata* f. sp. *lycopersici. Biocontrol Science and Technology*, 8 (1): 33-43.

Blanco, C., De Los Santos, B., & Romero, F. (2006). Relationship between concentrations of *Botrytis cinerea* conidia in air, environmental conditions, and the incidence of grey mould in strawberry flowers and fruits. *European Journal of Plant Pathology*, 114(4): 415-425.

Bohlmann, H., Clausen, S., Behnke, S., Giese, H., Hiller, C., Reimann-Philipp, U., Schrader, G., Barkholt, V., Apel, K., 1988. Leaf-specific thionins of barley—a novel class of cell wall proteins toxic to plant pathogenic fungi and possibly involved in the defense mechanism of plants. *EMBO J*., 7:1559–1565.

Borrelli, F., Ascione, V., Capasso, R., Izzo, A.A., Fattorusso, E., Tagliatela-Scafati, O., 2006. Spasmolytic effects of nonprenylated rotenoid constituents of *Boerhavia diffusa* roots. *Journal of Natural Products*, 69 (6): 903-906.

Boughalleb, N., Trabelsi, L., Harzallah-Skhiri, F., 2009. Antifungal activity from polar and non-polar extracts of some *Chenopodiaceae* wild species growing in Tunisia. *Natural Product Research*, 23 (11): 988-997.

Bowers, J.H., Locke, J.C. 2000. Effect of botanical extracts on the population density of *Fusarium oxysporum* in soil and control of fusarium wilt in the greenhouse. *Plant Disease*, 84 (3):300-305.

Bowers, J.H., Sonoda, R.M., and Mitchell, D.J. 1990. Patho coefficient analysis of the effect of rainfall variables on the epidemiology of Phytophthora blight of pepper caused by *Phytophthora capsici*. *Phytopathology* 80: 1439-1446.

Bradner, J.R., Nevalainen, K.M.H., 2003. Metabolic activity in filamentous fungi can be analysed by flow cytometry. *Journal of Microbiological Methods*, 54: 193-201.

Brooker, N., Windorski, J., Bluml, E. 2008. Halogenated coumarin derivatives as novel seed protectants. *Communications in agricultural and applied biological sciences,* 73 (2): 81-89.

Brown, R.L., Kazan, K., McGrath, K.C., Maclean, D.J., Manners, J.M., 2003. A role for the GCC-box in jasmonate-mediated activation of the PDF1.2 gene of *Arabidopsis*. *Plant Physiol*.,132: 1020–1032.

Bruton, B.D., Conway, W.S., Gross, K.C., Zhang, J.X. , Biles, C.L., Sams, C.E., 1998. Polygalacturonases of a latent and wound postharvest fungal pathogen of muskmelon fruit. *Postharvest Biology and Technology*, 13 (3): 205-214.

Butt, T.M., Jackson, C., Magan, N., 2001. Introduction—fungal biological control agents: progress, problems and potential. In: T.M. Butt, C. Jackson and N. Magan (Eds.), Fungi as Biocontrol Agents. Progress, Problems and Potential, CABI Publishing, Wallingford, 1–8.

Cameron, R.K. , Paiva, N.L. , Lamb, C.J., Dixon, R.A., 1999. Accumulation of salicylic acid and *PR-1* gene transcripts in relation to the systemic acquired resistance (SAR) response induced by *Pseudomonas syringae* pv. tomato in *Arabidopsis*. *Physiological and Molecular Plant Pathology*, 55 (2): 121-130.

Carvalho, P.R., Machado Neto, N.B., Custódio, C.C., 2007. Salicylic acid in marigold seeds (*Calendula officinalis* L.) under different stresses [Ácido salicílico em sementes de calêndula (*Calendula officinalis* L.) sob diferentes estresses] *Revista Brasileira de Sementes*, 29 (1): 114-124.

Casanova, E., García-Mina, J.M., Calvo, M.I., 2008. Antioxidant and antifungal activity of *Verbena officinalis* L. leaves. *Plant Foods for Human Nutrition*, 63 (3): 93-97.

Chamandoosti, F. , 2007. Plantlet regeneration potential from seedling explants of vitegnus (*Vitex agnus castus*). *Pakistan Journal of Biological Sciences*, 10 (22): 4113-4117.

Chang, P.F.L., Cheah, K.T., Narasimka, M.L., Hasegawa, P.M., Bressan, R.A., 1995. Osmotin gene expression is controlled by elicitor synergism. *Physiol. Plant*. 95: 620–626.

Chen, S. , Wu, M. (2007). A taxonomical study of the genus *Boerhavia* (*Nyctaginaceae*) in Taiwan. *Taiwania*, 52(4): 332-342.

Chiocchetti A., Ghignone S., Minuto A., Gullino M.L., Garibaldi A., Migheli Q., 1999. Identification of *Fusarium oxysporum* f. sp. *basilici* isolated from soil, basil seed, and plants by RAPD analysis. *Plant Disease* 83 (6): 576-581.

Chisholm ST, Coaker G, Day B, Staskawicz BJ., 2006. Host-microbe interactions: shaping the evolution of the plant immune response. *Cell*.,124:803–814.

Chiu, S.Y.C., Dobberstein, R.H., Fong, H.H.S., Farnsworth, N.R., 1982. Oxoaporphine alkaloids from *Siparuna gilgiana*. *Journal of Natural Products*, 45 (2): 229-230.

Cho, J.Y., Choi, G.J., Son, S.W., Jang, K.S., lim, H.K., Lee, S.O., Sung, N.D., Cho, K.Y., Kim, J.-C., 2007. Isolation and antifungal activity of lignans from Myristica fragrans against various plant pathogenic fungi. *Pest Manag Sci,* 63: 935-940.

Cho, J.Y., Kim, H.J., Choi, G.J., Jang, K.S., Lim, H.K., Lim, C.H., Cho, K.Y., Kim, J.-C., 2006. Dehydro--lapachone isolated from *Catalpa ovata* stems: activity against plant pathogenic fungi. *Pest Management Science*, 62 (5): 414 – 418.

Christensen, J.H., Carter, T.R., Giorgi, F., 2002. PRUDENCE employs new methods to assess European climate change. EOS, AGU 83 (13) (26 March 2002).

Cicció, J. F., & Gómez-Laurito, J., (2002). Volatile constituents of the leaves of *Siparuna thecaphora* (*Siparunaceae*) from Turrialba, Costa Rica. *Revista De Biologia Tropical*, 50(3-4), 963-967.

Coetze, G., Marx, I.J., Pengilly, M., Bushula, V.S., Joubert, E., Bloom, M., 2008. Effect of rooibos and honeybush tea extracts against *Botrytis cinerea*. *South African Journal of Enology and Viticulture*, 29 (1): 33-38.

Cohen, R., S. Pivonia, Y. Burger, M. Edelstein, A. Gamliel, and J. Katan, J. 2000. Toward integrated management of Monosporascus wilt of melons in Israel. *Plant Dis.,* 84:496-505

Cohen, R.., Pivoia, S., Burger, Y., Edelstein, M., Gamliel, A., Katan, J., 2000. Toward integrated management of Monosporascus wilt of melons in Israel, *Plant Disease* 84: 496–505.

Cohen, Y. R., 2002. β-Aminobutyric Acid-Induced Resistance Against Plant Pathogens. *Plant Disease*, 86, 448–457.

Conrath, U., Beckers, G.J.M., Flors, V., Garcia-Agustin, P., Jakab, G., Mauch, F., Newman ,M.A., Pieterse, C.M.J., Poinssot, B., Pozo, M.J., Pugin, A., Schaffrath, U., Ton, J., Wendehenne, D., Zimmerli, L., Mauch-Mani, B., 2006. Priming: getting ready for battle. *Mol. Plant-Microbe. Interact.,* 19: 1062–1071.

Conrath, U., Pieterse, C.M.J, Mauch-Mani, B., 2002. Priming in plant-pathogen interactions. *Trends Plant Sci.* 7:210–16

Cordelier, S., de Ruffray, P., Fritig, B., and Kauffmann, S., 2003. Biological and molecular comparison between localized and systemic acquired resistance induced in tobacco by a *Phytophthora megasperma* glycoprotein elicitin, *Plant Mol. Biol.,* 51:109–118.

Cordier, C., Pozo, M. J., Barea, J. M., Gianinazzi, S., Gianinazzi-Pearson, V., 1998. Cell defense responses associated with localized and systemic resistance to phytophthora parasitica induced in tomato by an arbuscular mycorrhizal fungus. *Molecular Plant-Microbe Interactions*, 11(10): 1017-1028.

Cormican, M. G., Pfaller, M. A., 1996. Standardization of antifungal susceptibility testing. *Journal of Antimicrobial Chemotherapy*, 38(4): 561-578.

Cornelissen, B.J.C., Hooft van Huijsduijnen, R.A.M., Bol, J.F., 1986. A tobacco mosaic virus-induced protein is homologous to the sweet-tasting protein thaumatin. *Nature*, 321: 531-532.

Corrêa M.P., 1926. Diciona´rio das Plantas U´teis do Brasil e das Exo´ticas Cultivadas. Vol. I. *Imprensa Nacional*: 660,662.

Corrêa, M.P., 1978. Dicionário das Plantas Úteis do Brasil e das Exóticas Cultivadas. Vol. IV. Inst. Brasileiro de Desenv. Florestal, p. 41, 158, 646, 650, 656.

Crinò, P., 1993. *Phytophthora infestans*/Patata, pomodoro. In Crinò, P., Sonnino, A., Saccardo, F., Buiatti, M., Porta-Puglia, A., Surico, G., (Eds.), Miglioramento genetico delle piante per resistenza a patogeni e parassiti. Edagricole, Bologna, Italia, 310-335.

Cristinzio, G., 1993. *Phytophthora capsici*/Peperone. In Crinò, P., Sonnino, A., Saccardo, F., Buiatti, M., Porta-Puglia, A., Surico, G. (Eds.), Miglioramento genetico delle piante per resistenza a patogeni e parassiti. Edagricole, Bologna, Italia, 336-349.

Curtis, H., Noll, U., Störmann, J., Slusarenko, A. J., 2004. Broad-spectrum activity of the volatile phytoanticipin allicin in extracts of garlic (*Allium sativum* L.) against plant pathogenic bacteria, fungi and oomycetes. *Physiological and Molecular Plant Pathology*, 65(2), 79-89.

Daayf, F., Ongena, M., Boulanger, R., El-Hadrami, I., and Belanger, R. R., 2000. Induction of phenolic compounds in two cultivars of cucumber by treatment of healthy and powdery mildew-infected plants with extracts of *Reynoutria sachalinensis*. *J Chem Ecol*, 26:1579-1593.

Dangl, J.L., Dietrich, R.A., and Richberg, M.H., 1996. Death don't have mercy: cell death programs in plant–microbe interactions, *Plant Cell*, 8:1793–1807.

Davidson, J.A., Kimber, R.B.E. 2007. Integrated disease management of ascochyta blight in pulse crops *European Journal of Plant Pathology*, 119 (1): 99-110.

De Cal, A., Garcia-Lepe, R., & Melgarejo, P., 2000. Induced resistance by penicillium oxalicum against *Fusarium oxysporum* f. sp. *lycopersici*: Histological studies of infected and induced tomato stems. *Phytopathology*, 90(3), 260-268.

De Carvalho Jr., P.M., Rodrigues, R.F., Sawaya, A.C., Marques, M.O., Shimizu, M.T., 2004. Chemical composition and antimicrobial activity of the essential oil of *Cordia verbenacea* D.C., *Journal of Ethnopharmacology*, 95 (2004): 297–301

De Jesus Faria, T., Ferreira, R. S., Yassumoto, L., De Souza, J. R. P., Ishikawa, N. K., De Melo Barbosa, A., 2006. Antifungal activity of essential oil isolated from *Ocimum gratissimum* L. (eugenol chemotype) against phytopathogenic fungi. *Brazilian Archives of Biology and Technology*, 49(6): 867-871.

De Oliveira, J. C. S., Da Camara, C. A. G., & Schwartz, M. O. E., 2007. Volatile constituents of the stem and leaves of *Cordia* species from mountain forests of pernambuco (north-eastern brazil). *Journal of Essential Oil Research*, 19(5): 444-448.

Deepthi, N. , Madhusudhan, K.N. , Uday Shankar, A.C. , Kumar, H.B., Prakash, H.S., Shetty, H.S., 2007. Effect of plant extracts and acetone precipitated proteins from six medicinal plants against tobamovirus infection. *International Journal of Virology*, 3 (2): 80-87.

Demirci, F., Dolar, F. S., 2006. Effects of some plant materials on phytophthora blight (*Phytophthora capsici* leon.) of pepper. *Turkish Journal of Agriculture and Forestry*, 30(4): 247-252.

Devaiah, S.P., Mahadevappa, G.H., Shetty, H.S., 2009. Induction of systemic resistance in pearl millet (*Pennisetum glaucum*) against downy mildew (*Sclerospora graminicola*) by *Datura metel* extract. *Crop Protection*, 28 (9): 783-791.

Dickinson, M., 2003. Molecular Plant Pathology. BIOS Scientific Publishers, New York, USA, pp. 273.

Diniz, J.C., Viana, F.A., De Oliveira, O.F., Silveira, E.R., Pessoa, O.D.L., 2008. Chemical composition of the leaf essential oil of Cordia leucocephala moric from Northeast of Brazil. Journal of Essential Oil Research, 20 (6): 495-496.

Dixon R.A. 2001. Natural products and plant disease resistance. *Nature,* 411: 843-847.

Doares, S.H., Syrovets, F., Weiler, E.W., Ryan, C.A., 1995. Oligogalacturonides and chitosan activate plant defensive genes through the octadecanoid pathway. *Proc. Natl. Acad. Sci. U.S.A.*, 92:4095–4098.

Dong, H., Cohen, Y., 2001. Extracts of killed penicillium chrysogenum induce resistance against fusarium wilt of melon. *Phytoparasitica*, 29(5): 421-430.

Doughari, J.H., Nuya,S.P., 2008. *In vitro* antifungal activity of *Deterium microcarpum*. *Pak J Med Sci*, 24 (1): 91-95.

Droby, S., Cohen, L., Daus, A., Weiss, B., Horev, B., Chalutz, E., Katz, H., Keren-Tzur, M. and Shachnai, A. 1998. Commercial testing of Aspire™: a yeast preparation for the biological control of postharvest decay of citrus. *Biological Control* 12, 97–101.

Du, H. and Klessig, D.F., 1997. Role for salicylic acid in the activation of defense responses in catalase deficient transgenic tobacco. *Mol. Plant Microbe Interact.*, 10: 922–925.

Edeoga, H. O., Ikem, C. I., 2002. Tannins, saponins and calcium oxalate crystals from nigerian species of *Boerhavia* L. (*Nyctaginaceae*). *South African Journal of Botany*, 68(3): 382-385.

Ehrman, T., 1994. Chemical Analysis and Testing Task Laboratory Analytical Procedure, LAP-010. Standard Method for the Determination of Extractives in Biomass. In Standard Biomass Analytical Methods. National Renewable Energy Laboratory ("NREL"), Midwest Research Institute ("MRI"), Department Of Energy. http://cobweb.ecn.purdue.edu/~lorre/16/research/LAP-010.pdf.

Elad, Y., Zimmand, G., Zags, Y., Zuriel, S., Chet, I., 1993. Use of *Trichoderma harzianum* in combination or alternation with fungicides to control Cucumber grey mold (*Botrytis cinerea*) under commercial greenhouse condition. *Plant Pathol*. 42: 324-356.

Ellis, M. A., Wilcox, W. F., Madden, L. V., 1998. Efficacy of metalaxyl, fosetyl-aluminum, and straw mulch for control of strawberry leather rot caused by phytophthora cactorum. *Plant Disease,* 82(3): 329-332.

Elmer, W.H., 2001. Seeds as vehicles for pathogen importation. *Biol Invasions*, 3:263–271.

El-Seedi, H., Ghia, F., Torssell, K.B.G., 1994. Cadinane sesquiterpenes from *Siparuna macrotepala*. *Phytochemistry*, 35(6): 1495-1497.

Epple, P., Apel, K., and Bohlmann, H., 1997. Overexpression of an endogenous thionin enhances resistance of *Arabidopsis* against *Fusarium oxysporum. Plant Cell*, 9:509–520.

Ergon, A., Klemsdal, S.S., and Tronsmo, A.M., 1998. Interactions between cold hardening and *Microdochium nivale* infection on expression of pathogenesis-related genes in winter wheat. *Physiol. Mol. Plant Pathol.*, 53:301–310.

Erwin D.C. and O.K. Ribeiro, 1996. *Phytophthora* diseases worldwide. APS Press, St. Paul, MN, USA, 562 pp.

Eyal, Y., Sagee, O., and Fluhr, R., 1992. Dark induced accumulation of a basic pathogenesis-related (PR-1) transcript and a light requirement for its induction by ethylene, *Plant Mol.*, 19 (4): 589-599.

Faria, T. de J., Ferreira, R.S., Yassumoto, L., Pinto de Souza, J.R., Ishikawa, N.K., de Melo BarbosaI, A., 2006. Antifungal activity of essential oil isolated from *Ocimum gratissimum* L. (eugenol chemotype) against phytopathogenic fungi. *Braz. arch. biol. technol*., 49 (6): 867-871.

Farmer, E.E. and Ryan, C.A., 1992. Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors, *Plant Cell*, 4:129–134.

Fawzi, E.M., Khalil, A.A., Afifi, A.F., 2009. Antifungal effect of some plant extracts on *Alternaria alternata* and *Fusarium oxysporum*. *African Journal of Biotechnology*, 8 (11): 2590-2597.

Ferrari, V., 1998. Fusarium and root knot nematodes, two adversaries of melon which are difficult to control chemically. *Informatore-Agrario-Supplemento,* 54 (3): 48–50.

Ficcadenti, N., Sestili, S., Annibali, S., Campanelli, G., Belisario, A., Maccaroni, M., 2002. Resistance to *Fusarium oxysporum* f. sp. *melonis* race 1,2 in muskmelon lines nad-1 and nad-2. *Plant Disease*, 86(8): 897-900.

Fidantsef, A.L., Stout, M.J., Thaler, J.S., Duffey, S.S., and Bostock, R.M., 1999. Signal interactions in pathogen and insect attack: expression of lipoxygenase, proteinase inhibitor II, and pathogenesis-related protein P4 in the tomato, *Lycopersicon esculentum. Physiol. Mol. Plant Pathol*.,54:97–114.

Fiocchetti, F., Caruso, C., Bertini, L., Vitti, D., Saccardo, F., Tucci, M., 2006. Over-expression of pathogenesis-related protein gene in transgenic tomato alters the transcription patterns of other defenće genes. *Journal of horticultural Science and Biotechnology*, 81 (1): 27-32.

Fischer, D.C.H., Gualda, N.C.D.A., Bachiega, D., Carvalho, C.S., Lupo, F.N., Bonotto, S.V., Alves, M.D.O., Di Santi, S.M., Avila, P.E, Kirchgatter, Moreno, P.R.H., 2004. *In vitro* screening for antiplasmodial activity of isoquinoline alkaloids from Brazilian plant species. *Acta Tropica*, 92 (3): 261-266.

Freitas, G.C., Kitamura, J.O.S., Lago, J.H.G., Young, M.C.M., Guimarães, Kato, M.J., 2009. Caldensinic acid, a prenylated benzoic acid from *Piper caldense*. *Phytochemistry Letters,* 2: 119-122.

Gacche, R. N., Dhole, N. A., 2006. Antioxidant and possible anti-inflammatory potential of selected medicinal plants prescribed in the indian traditional system of medicine. *Pharmaceutical Biology*, 44(5): 389-395.

Gacche, R. N., Dhole, N. A., 2006. Antioxidant and possible anti-inflammatory potential of selected medicinal plants prescribed in the indian traditional system of medicine. *Pharmaceutical Biology*, 44(5): 389-395.

Gadea, J., Mayda, M. E., Conejero, V., Vera, P., 1996. Characterization of defense-related genes ectopically expressed in viroid-infected tomato plants. *Molecular Plant-Microbe Interactions*, 9(5): 409-415.

Galletti, G. C., Russo, M. T., Bocchini, P., 1996. Essential oil composition of leaves and berries of *Vitex agnus-castus* L. from Calabria, Southern Italy. *Rapid Communications in Mass Spectrometry*, 10(11): 1345-1350.

Gamliel, A., Katan, T., Yunis, H., & Katan, J., 1996. Fusarium wilt and crown rot of sweet basil: Involvement of soilborne and airborne inoculum. *Phytopathology*, 86(1): 56-62.

Garibaldi, A., Gullino, M.L., Minuto, G., 1997. Diseases of basil and their management, *Plant Dis.* 81 (2): 124–132.

Gerard, R.V., MacLean, D.B., Antonio, T.M., 1986. Examination of three Siparuna species for alkaloid content. *Phytochemistry*, 25 (9): 2155-2156.

Glazebrook, J., 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol,*. 43: 205-227.

Göhre, V., and Robatzek, S., 2008. Breaking the barriers: Microbial effector molecules subvert plant immunity. Annu. Rev. Phytopathol., 46: 189-215.

Gordon-Weeks, R., White, R.F., Pierpoint, W.S., 1991. Evidence for the presence of endogenous inducers of the accumulation of pathogenesis-related (PR-1) proteins in leaves of virus-infected tobacco plants and of an interspecific Nicotiana hybrid. *Physiological and Molecular Plant Pathology*, 38: 375-392.

Görlach, J., Volrath, S., Knauf-Beiter, G., Hengry, G., Beckhove, U., Kogel, K.-H., Oostendorp, M., Staub, T., Ward, E., Kessmann, H., and Ryals, J., 1996 Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. *Plant Cell*, 8:629–643.

Goufo, P., Teugwa, Mofor, C., Fontem, D.A., Ngnokam, D., 2008. High efficacy of extracts of Cameroon plants against tomato late blight disease. *Agronomy of Sustainable Development*, 28 (4): 567-573.

Green, L., Petersen, B., Steimel, L., Haeber, P., Current, W., 1994. Rapid determination of antifungal activity by flow cytometry. *Journal of Clinical Microbiology*, 32 (4): 1088-1091.

Green, R. and Fluhr, R., 1995. UV-B-induced *PR-1* accumulation is mediated by active oxygen species, Plant Cell, 7:203–212.

Green, T.R. and Ryan, C.A., 1972. Wound-induced proteinase inhibitor in plant leaves: a possible defense mechanism against insects, Science, 175:776–777.

Grenier, J., Asselin, A., 1990. Some pathogenesis-related proteins are chitosanases with lytic activity against fungal spores. *Molecular Plant Microbe Interactions,* 3: 401-7.

Grove, M. D., Spencer, G. F., Rohwedder, W. K., Mandava, N., Worley, J. F., Warthen, J. D., Steffens, G. L., Flippen-Anderson, J. L., and Cook, J. C., 1979. Brassinolide, a plant growth-promoting steroid isolated from brassica napus pollen. *Nature*, 281(5728):216-217.

Guan, Y., Ramalingam, S., Nagegowda, D., Taylor, P.W.J., Chye, M.-L., 2008. *Brassica juncea* chitinase BjCHI1 inhibits growth of fungal phytopathogens and agglutinates Gram-negative bacteria. *Journal of Experimental Botany*, 59 (12): 3475-3484.

Hajdú, Z., Hohmann, J. , Forgo, P. , Martinek, T. , Dervarics, M. , Zupkó, I. , Falkay, G. , Cossuta, D., Máthé, I., 2007. Diterpenoids and flavonoids from the fruits of *Vitex agnus-castus* and antioxidant activity of the fruit extracts and their constituents. *Phytotherapy Research*, 21 (4): 391-394.

Hanafi, A., Traoré, M. , Schnitzler, W.H. , Woitke, M., 2007. Induced resistance of tomato to whiteflies and pythium with the PGPR *Bacillus subtilis* in a soilless crop grown under greenhouse conditions. *Acta Horticulturae*, 747: 315-322.

Hancock, J.F., Maas, J.L., Shanks, C.H., Breen, P.J. and Luby, J.J. 1990. Strawberries (*Fragaria*). In: Moore, J.N. and Ballington Jr., J.R (Eds.), Genetic Resources of Temperate Fruit and Nut Crops, *Acta Horticulturae*, 290:491-546.

Haouala, R, Hawala, S., El-Ayeb, A., Khanfir, R., Boughanmi, N., 2008. Aqueous and organic extracts of *Trigonella foenum-graecum* L. inhibit the mycelia growth of fungi. *Journal of Environmental Sciences*, 20 (12): 1453-1457.

Harish, R., & Shivanandappa, T., 2006. Antioxidant activity and hepatoprotective potential of *Phyllanthus niruri*. Food Chemistry, 95(2), 180-185.

Hassarajani, S.A., Mulchandani, N.B., 1990. Securinine type of alkaloids from *Phyllanthus niruri*. *Indian Journal of Chemistry – Section B Organic Chemistry Including Medicinal Chemistr*y, 29 (9): 801-803.

Heil, M. and R. M. Bostock, 2002. Induced systemic resistance (ISR) against pathogens in the context of induced plant defenses. Annals of Botany, 89:502–512.

Heitz, T., Geoffroy, P., Fritig, B., and Legrand, M., 1999. The PR-6 family: Proteinase inhibitors in plant-microbe and plant-insect interactions. In Datta, S.K. and Muthukrishnan, S. (Eds.), Pathogenesis-Related Proteins in Plants. New York: CRC Press, 131-155.

Herman, R., Perl-Treves, R., 2007. Characterization and inheritance of a new source of resistance to *Fusarium oxysporum* f. sp. *melonis* race 1.2 in *Cucumis melo*. *Plant Disease*, 91 (9): 1180-1186.

Hernandez, T., Canales, M., Teran, B., Avila, O., Duran, A., Garcia, A.M., Hernandez, H., Angeles-Lopez, O., Fernandez-Araiza, M., Avila, G., 2006. Antimicrobial activity of the essential oil and extracts of *Cordia curassavica (Boraginaceae)*. *Journal of Ethnopharmacology*, 11: 137-141.

Hese, S., Takahashi, S., Takenaka, S., Nakaho, K., Arie, T., Seo, S., Ohashi, Y., Takahashi, H., 2008. Involvement of jasmonic acid signalling in bacterial wilt disease resistance induced by biocontrol agent *Pythium oligandrum* in tomato. *Plant Pathology*, 57 (5): 870-876.

Holz, G., Coertze, S., Williamson, B., 2007. The ecology of *Botrytis* on plant surfaces. In Elad, Y., Williamson, B., Tudzynski, P., Delen, (Eds.), *Botrytis*: Biology, Pathology and Control., N. Springer, Dordrecht, The Netherlands, 9-24.

Hondo, D., Hase, S., Kanayama, Y., Yoshikawa, N., Takenaka, S., Takahashi, H., 2007. The LeATL6-associated ubiquitin/proteasome system may contribute to fungal elicitor-activated defense response via the jasmonic acid-dependent signaling pathway in tomato. Molecular *Plant Microbe Interactions*, 20 (1), 72-81.

Hong, J.K., Hwang, B.K., 2002. Temporal and subcellular localization of *PR-1* proteins in tomato stem tissues infected by virulent and avirulent isolates of *Phytophthora capsici*. *Protoplasma*, 219 (3-4), 131-139.

Horváth Gy., Botz, L., Kocsis, B., Lemberkovics, É., Szabó, L. Gy, 2004. Antimicrobial natural products and antibiotics detected by direct bioautography using plant pathogenic bacteria. *Acta Botanica Hungarica*, 46 (1–2): 153–165.

Hossain, M.A., Ismail, Z., Rahman, A., 2008. Chemical composition and anti-fungal properties of essential oils and crude extracts of *Orthosiphon stamineus* Benth. *Industrial Crops and Products*, 27 (3): 328-334.

Hostettman, K. and Lea, J.P., 1987. Biologically active natural products. *Ann. Proc. Phytochem. Soc. Eur*. Oxford: Clarendon Press., 33-46.

Hostettmann, K. and Potterat, O., 1997. Strategy for the isolation and analysis of antifungal, molluscicidal and larvicidal agents from tropical plants. In "Phytochemicals for Pest Control", ACS Symposium Series vol. 658, (Hedin, P.A. (Ed.); American Chemical Society, Washinghton DC, 14-26.

Hou, C.T., Forman III, R.J., 2000. Growth inhibition of plant pathogenic fungi by hydroxy fatty acids. *Journal of Industrial Microbiology and Biotechnology*, 24(4), 275-276.

Houdai, T., Matsuoka, S., Matsumori, N., Murata, M., 2004. Membrane-permeabilizing activities of amphidinol 3, polyene-polyhydroxy antifungal from a marine dinoflagellate. *Biochimica Et Biophysica Acta - Biomembranes*, 1667(1): 91-100.

Hultberg, M., Alsberg, T., Khalil, S., Alsanius, B., 2009. Suppression of disease in tomato infected by Pythium ultimum with biosurfactant produced by Pseudomonbas koreensis. *BioControl*, 1-10. Article in Press.

Hussin, N.M., Muse, R., Ahmad, S., Ramli, J., Mahmood, M., Sulaiman, M.R., Shukor, M.Y.A., Rahman, F.A., Aziz, K.N.K., 2009. Antifungal activity of extracts and phenolic compoundsfrom *Barringtonia racemoca* L. (*Lecythidaceae*). *African Journal of Biotechnology*, 8 (12): 2835-2842.

Hutchinson, J., 1967. The Genera of Flowering Plants, Vol. II.Oxford University Press, London, p. 110.

Hwang, Y. , Matsushita, Y., Sugamoto, K., Matsui, T., 2005. Antimicrobial effect of the wood vinegar from *Cryptomeria japonica* sapwood on plant pathogenic microorganisms. *Journal of Microbiology and Biotechnology*, 15(5): 1106-1109.

Ioset, J., Marston, A., Gupta, M. P., & Hostettmann, K., 2000. Antifungal and larvicidal cordiaquinones from the roots of *Cordia curassavica*. *Phytochemistry*, 53(5): 613-617.

Ioset, J.R., Marston, A., Gupta, M.P., Hostettmann, K., 1998. Antifungal and larvicidal mesoterpenoid naphtaquinones and a naphtorixene from the roots of *Cordia linnaei*. *Phytochemistry,* 47: 729–734.

Ishige, F., Mori, H., Yamazaki, K., and Imaseki, H., 1991. The effect of ethylene on the coordinated synthesis of multiple proteins accumulation of an acidic chitinase and a basic glycoprotein induced by ethylene in leaves of azuki beans, *Vigna angulasis*, *Plant Cell Physiol*., 32:681–690.

Ishikawa, R., Shirouzu, K., Nakashita, H., Lee, H.-Y., Motoyama, T., Yamaguchi, I., Teraoka, T., Arie, T., 2005. Foliar spray of validamycin a or validoxylamine a controls tomato fusarium wilt. *Phytopathology*, 95(10):1209-1216.

Jakab, G., Cottier, V., Toquin, V., Rigoli, G., Zimmerli, L., Métraux, J. , 2001. β-Aminobutyric acid-induced resistance in plants. *European Journal of Plant Pathology*, 107(1): 29-37.

Jarvis, W.R., Epiedmiology. In:mJ.R. Coeley-Smith, K. Verhoeff, W.R. Jarvis (eds), The Biology of *Botrytis*. Academic Press:219-249.

Jayaraman Jayaraj, Ajith Anand, Subbaratnam Muthukrishnan, 2004. Pathogenesis-related proteins and their roles in resistance to fungal pathogens. In Punja, Z.K., Fungal Disease Resistance in Plants Biochemistry, Molecular Biology, and Genetic Engineering. Haworth Press, New York, USA.

Jayraj, J., Wan, A., Rahman, M., Punja, Z.K., 2008. Seaweed extract reduces foliar fungal diseases on carrot. *Crop Protection*, 27 (10): 1360-1366.

Jenett-Siems, K., Kraft, C., Siems, K., Jakupovic, J., Solis, P. N., Gupta, M. P., 2003. Sipaucins A - C, sesquiterpenoids from *Siparuna pauciflora*. *Phytochemistry*, 63(4), 377-381.

Jones, J.D.G., and Dangl, J.L., 2006. The plant immune system. *Nature* 444, 323-329.

Joosten, M.H.A.J., Bergmans, C.J.B., Meulenhoff, E.J.S., Cornelissen, B.J.C., de Wit, P.J.G.M., 1990. Purification and serological characterization of three basic 15 kD pathogenesis-related proteins from tomato, *Plant Physiol*., 94:585–591.

Kambu, K., 1990. Eléments de Phytothérapie Comparée: Plantes Médicinales Africaines. Centre de Recherche Pédagogique de Kinshasa, Kinshasa, p. 148.

Kavroulakis, N., Ntougias, S., Zervakis, G.I., Ehaliotis, C., Haralampidis, K., Papadopoulou, K.K., 2007. Role of ethylene in the protection of tomato plants against soil-borne fungal pathogens conferred by an endophytic *Fusarium solani* strain. *Journal of Experimental Botany*, 58 (14):3853-3864.

Kavroulakis, N., Papadopoulou, K.K., Ntougias, S., Zervakis, G.I., Ehaliotis, C., 2006. Cytological and other aspects of pathogenesis-related gene expression in tomato plants grown on a suppressive compost. *Annals of Botany*, 98 (3): 555-564.

Keinath ,A.P., 1994. Pathogenicity and host range of *Fusarium oxysporum* from sweet basil and evolution of disease control. *Plant Dis,* 78:1211–1215

Kellam, S.J., 1988. Results of a large scale screening programme to detect antifungal activity from marine and freshwater microalgae in laboratory culture. *J. Br. Phycol*., 23: 45-47.

Kessmann, H., Staub, T., Ligon, J., Oostendorp, M., & Ryals, J., 1994. Activation of systemic acquired disease resistance in plants. *European Journal of Plant Pathology*, 100(6): 359-369.

Knoester, M., Van Loon, L. C., Van Den Heuvel, J., Hennig, J., Bol, J. F., Linthorst, H. J. M., 1998. Ethylene-insensitive tobacco lacks nonhost resistance against soil-borne fungi. Proceedings of the National Academy of Sciences of the United States of America, 95(4): 1933-1937.

Kohler, A., Schwindling, S., & Conrath, U., 2002. Benzothiadiazole-induced priming for potentiated responses to pathogen infection, wounding, and infiltration of water into leaves requires the NPR1/NIM1 gene in *Arabidopsis. Plant Physiology*, 128(3): 1046-1056.

Koike, S. T., Gladders, P., and Paulus, A. O. 2007. Vegetable Diseases. A Color Handbook. Elsevier, Boston, MA.

Koiwa, H., Bressan, R.A., and Hasegawa, P.M., 1997. Regulation of protease inhibitors and plant defense, Trends Plant Sci., 2:379–384.

Koiwa, H., Sato, F., and Yamada, Y., 1994. Characterization of accumulation of tobacco *PR-5* proteins by IEF-immunoblot analysis, Plant Cell Physiol., 35:821–827.

Kozlowski, G., Buchala, A., & Métraux, J., 1999. Methyl jasmonate protects Norway spruce [*Picea abies* (L.) karst.] seedlings against *Pythium ultimum* trow. *Physiological and Molecular Plant Pathology*, 55(1): 53-58.

Kuroyanagi, M., Kawahara, N., Sekita, S., Satake, M., Hayashi, T., Takase, Y., 2003. Dammarane-type triterpenes from the brazilian medicinal plant cordia multispicata. Journal of Natural Products, 66(10): 1307–1312.

Langvad, F. (1999). A rapid and efficient method for growth measurement of filamentous fungi. *Journal of Microbiological Methods*, 37(1), 97-100.

Lans, C., Harper, T., Georges, K., Bridgewater, E., 2000. Medicinal plants used for dogs in Trinidad and Tobago. *Preventive Veterinary Medicine*, 45(3-4): 201-220.

Lawton, K.A., Potter, S.L., Uknes, S., and Ryals, J., 1994. Acquired resistance signal transduction in *Arabidopsis* is ethylene independent, *Plant Cell*, 6:581–588.

Lederer, W., Seemüller, E., 1992. Investigations on the predisposition of strawberry plants to crown rot (*Phytophthora cactorum*). Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz, 99(3):225-233.

Lehmann, J., Atzorn, R., Bruckner, C., Reinbothe, S., Leopold, J., Wasternack, C., and Parthier, B., 1995. Accumulation of jasmonate, abscisic acid, specific transcripts and proteins in osmotically stressed barley leaf segments, *Planta,* 197:156–162.

Leitão, G. G., Soares, S. S. V., De Barros, T., Brito, M., Delle Monache, F., 2000. Kaempferol glycosides from siparuna apiosyce. *Phytochemistry*, 55(6): 679-682.

Leitão, G.G., Simas, N.K., Soares, S.S.V., de Brito, A.P.P., Claros, B.M.G., Brito, T., de Barros, M., Delle Monache, F., 1999. Chemistry and pharmacology of monimiaceae: a special focus on *Siparuna* and *Mollinedia*. *J. Ethnopharmacol*. 65 (2): 87±102.

Leon, J., Lawton, M.A., and Raskin, I., 1995. Hydrogen peroxide stimulates salicylic acid biosynthesis in tobacco, *Plant Physiol*., 108:1673–1678.

Leonian, L.H., 1922. Stem and fruit blight of peppers caused by *Phytophthora capsici* sp. nov., *Phytopathology*, 12: 401-408.

Leroux, 2007: Chemical Control of *Botrytis* and its Resistance to Chemical Fungicides In Elad, Y., Williamson, B., Tudzynski, P., Delen, N. Springer (Eds.), *Botrytis*: Biology, Pathology and Control., Dordrecht, The Netherlands, 195-217.

Lilja, A.T., Parikka, P.K., Pääskynkivi, E.A., Hantula, J.I., Vainio, E.J., Vartiamäki, H. A., Hemmetty, A.H., Vestberg, M.V., 2006. *Phytophthora cactorum* and *Colletotrichum acutatum*: Survival and Detection. *Agriculturae Conspectus Scientificus*, Vol. 71(4): 121-128.

Linthorst, H.J.M., Danhash, N., Brederode, F.T., Van Kan, J.A.L., De Wit, P.J.G.M., and Bol, J.F., 1991. Tobacco and tomato *PR* proteins homologous to Win and Pro-Hevein lack the ‘‘Hevein’’ domain*, Mol. Plant Microbe Interact.*, 4:586–592.

Lohani, S., Jan, A., Verma, H. N., 2007. *In vivo* and *in vitro* resistance induction in tobacco by *Boerhaavia diffusa* systemic resistance inducing protein and transfer of induced resistance in in vitro tobacco plants. *Biotechnology*, 6(3): 389-392.

Lopes, J.L.S., Valadares, N.F., Moraes, D.I., Rosa, J.C., Araújo, H.S.S., Beltramini, L.M., 2009. Physico-chemical and antifungal properties of protease inhibitors from Acacia plumosa. *Phytochemistry* 70: 871-879.

Lopez, J. A., Laurito, J. G., Lin, F. -., Duah, F. K., Sharaf, M., Aly, Y., 1990. Alkaloids of *Siparuna tonduziana*. *Planta Medica*, 56(5), 492.

Lopez, J. A., Laurito, J. G., Lin, F., Sharaf, M., Wong, L. K., Schiff Jr., P. L., 1993. Alkaloids of *Siparuna griseo-flavescens*. *Planta Medica*, 59(1): 100.

Lopez, J.A., Aly, Y., Schiff Jr., P.L., 1988. Alkaloids of *Siparuna pauciflora*. *Planta Medica* 54: 552–553.

Louws, F.J., 2004. Phytophthora crown rot: widespread in Southern Region. Small Fruit Consortium: Pest Alert. http://www.smallfruits.org/SRSFC_News/Phytophthora0404.pdf.

Lumsden, R.D., Walter, J.F. and Baker, C.P., 1996. Development of *Gliocladium virens* for damping-off disease control. Canadian Journal of Plant Pathology 18, 463–468.

Maas, J.L., 2004. Strawberry disease management. In Naqvi, S.A.M.H., (Ed.) Diseases of Fruits and Vegetables Diagnosis and Management, Volume II. Springer Science USA, 441-483.

Mahakhant, A., Padungwong, P., Arunpairojana, V., Atthasampunna, P., 1998. Control of the plant pathogenic fungus *Macrophomina phaseolina* in mung bean by a microalgal extract. *Phycological Research*, 46(SUPPL.), 3-7.

Makandar, R., Essig, J. S., Schapaugh, M. A., Trick, H. N., Shah, J., 2006. Genetically engineered resistance to Fusarium head blight in wheat by expression of *Arabidopsis NPR1*. *Molecular Plant-Microbe Interactions*, 19(2), 123-129.

Mandal, S., Mallick, N., Mitra, A., 2009. Salicylic acid-induced resistance to *Fusarium oxysporum* f.sp. *lycopersici* in tomato. *Plant Physiology and Biochemistry*, 47 (7):642-649.

Martin, F.N., Hancock, J.G., 1987. The use of *Pythium oligandrum* for biological control of preemergence damping-off caused by *P. ultimum*, *Phytopathology,* 77: 1013–1020.

Martinez De Ilarduya, O., Xie, Q., Kaloshian, I., 2003. Aphid-induced defense responses in Mi-1.mediated compatible and incompatible tomato interactions. *Molecular Plant-Microbe Interactions*, 16 (8): 699-708.

Martyn, R. D., Miller, M. E., 1996. Monosporascus root rot and vine decline: An emerging disease of melons worldwide. *Plant Disease*, 80(7): 716-724.

Martyn, R.D. 2002. Monosporascus root rot and vine decline of melons. The Plant Health Instructor. DOI: 10.1094/PHI-I-2002-0612-01.)

Maurya, R., Sathiamoorthy, B., & Deepak, M., 2007. Flavonoids and phenol glycosides from *Boerhavia diffusa*. *Natural Product Research*, 21(2), 126-134.

Mdee, L.K., Masoko, P., Eloff, J.N., 2009. The activity of extracts of seven common invasive plant species on fungal phytopathogens. *South African Journal of Botany*, 75 (2): 375-379.

Medeiros, F.C.L., Resende, M.L.V., Medeiros, F.H.V., Zhang, H.M., Paré, P.W., 2009. Denefse gene expression induced by a coffee-leaf extract formulation in tomato. Physiological and Molecular Plant Pathology,

Mehlhorn, H., Schmahl, G., Schmidt, J., 2005. Extract of the seeds of the plant *Vitex agnus castus* proven to be highly efficacious as a repellent against ticks, fleas, mosquitoes and biting flies. Parasitol Res 95:363–365.

Ménard, R., Albar, S., de Ruffray, P., Jamois, F., Franz, G., Fritig, B., Yvin, J.-C., and Kauffmann, S., 2004. β-1,3-glucan sulfate, but not β-1,3-glucan, induces the salicylic acid signaling pathway in tobacco and Arabidopsis, *Plant Cell,* 16:3020–3032.

Mendes, M.E.E., 1989. Uso de substâncias no combate a pragas (monograph). LNETI-DTIQ, 4. Lisboa, pp 68.

Métraux , J.P., 2001. Systemic acquired resistance and salicylic acid: current state of knowledge. *Eur J Plant Pathol*, 107:13–18

Mewari, N., Kumar, P., 2008 Antimicrobial activity of extracts of *Marchantia polymorpha*. *Pharmaceutical Biology*, 46 (10-11): 819-822.

Minuto, A., Garibaldi, A., and Gullino, M. L., 1994. Biological control of Fusarium wilt of basil (*Ocimum basilicum* L.). *Proc. BCPC* 6D-18:811-816.

Minuto, A., Minuto, G., Migheli, Q., Mocioni, M., & Gullino, M. L., 1997. Effect of antagonistic *Fusarium* spp. and of different commercial biofungicide formulations on fusarium wilt of basil (*Ocimum basilicum* L.). *Crop Protection*, 16(8): 765-769.

Minuto, A., Mocioni, M., and Garibaldi, A. 1995. Preliminary trials on biological control of Fusarium wilt of basil. *Acta Hortic*., 382:173-177

Minuto, A., Mocioni, M., and Garibaldi, A., 1995. Preliminary trials on biological control of Fusarium wilt of basil. *Acta Hortic.* 382:173-177.

Mogg, C. , Petit, P. , Cappuccino, N. , Durst, T. , McKague, C. , Foster, M., Yack, J.E., Arnason, J.T., Smith, M.L., 2008. Tests of the antibiotic properties of the invasive vine *Vincetoxicum rossicum* against bacteria, fungi and insects. *Biochemical Systematics and Ecology*, 36 (5-6): 383-391.

Mohana, D.C., Raveesha, K.A. , Rai, K.M.L., 2008. Herbal remedies for the management of seed-borne fungal pathogens by an edible plant *Decalepis hamiltonii* (Wight & Arn). *Archives of Phytopathology and Plant Protection*, 41 (1): 38-49.

Molot, P.M. and Nourrisseau, J.G., 1974. Influence de quelques substances de croissance sur la sensibilité du fraisier aux attaques de *Phytophthora cactorum*. *Fruits*, 29:697-702.

Murugaiyah, V., Chan, K. L., 2007. Analysis of lignans from *Phyllanthus niruri* L. in plasma using a simple HPLC method with fluorescence detection and its application in a pharmacokinetic study. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, 852(1-2), 138-144.

Nakamura, N., Kojima, S., Lim, Y. A., Meselhy, M. R., Hattori, M., Gupta, M. P., 1997. Dammarane-type triterpenes from cordia spinescens. *Phytochemistry*, 46(6): 1139-1141.

Novak, J., Draxler, L., Göhler, I., Franz, C. M., 2005. Essential oil composition of *Vitex agnus-castus* - comparison of accessions and different plant organs. *Flavour and Fragrance Journal*, 20(2): 186-192.

Nürnberger, T., and Kemmerling, B., 2009. Pathogen-associated molecular patterns (PAMP) and PAMP-triggered immunity. *Annu. Plant Rev*., 34, 16-47.

Occhioni, A. and Lyra, M.E.M., 1948. Contribuic¸çaõ ao Estudo das Monimiaceas Medicinais Brasileiras. Arquivos do Jardim Botanico Vol. VIII.

Ogwulumba, S.I., Ugwuoke, K.I., Iloba, C., 2008. Prophylactic effect of paw-paw leaf and bitter leaf extracts on the incidence of foliar myco-pathogens of groundnut (*Arachis hypogaea* L.) in Ishiagu, Nigeria African *Journal of Biotechnology*, 7 (16): 2878-2880.

Okusa, P. N., Penge, O., Devleeschouwer, M., Duez, P., 2007. Direct and indirect antimicrobial effects and antioxidant activity of *Cordia gilletii* de wild (*Boraginaceae*). *Journal of Ethnopharmacology*, 112(3), 476-481.

Okushima, Y., Koizumi, N., Kusano, T., Sano, H., 2000. Secreted proteins of tobacco cultured BY2 cells: Identification of a new member of pathogenesis-related proteins. *Plant Molecular Biology*, 42(3), 479-488.

Oliva, A., Meepagala, K. M., Wedge, D. E., Harries, D., Hale, A. L., Aliotta, G., 2003. Natural fungicides from *Ruta graveolens* L. leaves, including a new quinolone alkaloid. *Journal of Agricultural and Food Chemistry*, 51(4): 890-896.

Ong, P. L., Chan, L. K., 2006. *In vitro* plant regeneration, flowering and fruiting of *Phyllanthus niruri* L. (euphorbiaceae). *International Journal of Botany*, 2(4), 409-414.

Oostendorp, M., Kunz, W., Dietrich, B., and Staub, T., 2001. Induced disease resistance in plants by chemicals, Eur. J. Plant Pathol., 107:19–28.

Osorio, E., Flores, M., Hernández, D., Ventura, J., Rodríguez, R., Ahuilar, C.N., 2010. Biological efficiency of polyphenolic extracts from pecan nuts shell (*Carya Illinoensis*), pomegranate husk (*Punica granatum*) and creosote bush leaves (*Larrea tridentata* Cov.) against plant pathogenic fungi. Industrial *Crops and Products*, 31 (1): 153-157.

Özcan, M.M, Chalchat, J.-C., 2008. Chemical composition and antifungal activity of rosemary (Rosmarinus officinalis L.) oil from Turkey. *International Journal of Food Sciences and Nutrition*, 59 (7-8): 691-698).

Pandey, B.K., Singh, U.S. and Chaube, H.S. 1987. Mode of infection of Ascochyta blight of chickpea caused by *Ascochyta rabiei*. *Journal of Phytopathology*. 119:88-93.

Paré, P.W., Farag, M.A., Krishnamachari, V., Zhang, H., Ryu, C.-M., Kloepper, J.W., 2005. Elicitors and priming agents initiate plant defense responses. *Photosynth Res*, 85: 149–159.

Park, H. J., Lee, J. Y., Moon, S. S., & Hwang, B. K., 2003. Isolation and anti-oomycete activity of nyasol from anemarrhena asphodeloides rhizomes. *Phytochemistry*, 64(5): 997-1001.

Park, I.-K., Kim, J., Lee, Y.-S., Shin, S.-C., 2008. *In vivo* fungicidal activity of medicinal plant extracts against six phytopathogenic fungi. *International Journal of Pest Management*, 54 (1): 63-68.

Pârvu, M., Pârvu, A.E., Crăciun, C., Barbu-Tudoran, L., Tămaş, M., 2008. Antifungal activities of *Chelidonium majus* extract on *Botrytis cinerea in vitro* and ultrastructural changes in its conidia. *Journal of Phytopathology*, 156 (9): 550-552).

Paul, G. C., Kent, C. A., Thomas, C. R., 1993. Viability testing and characterization of germination of fungal spores by automatic image analysis. *Biotechnology and Bioengineering*, 42(1): 11-23.

Paxton, J.D., 1991. Assay for antifungal activity. In: Hostemann, K. (ed.) ,Dey, P.M., Harborne, J.B. (series eds.) Methods in Plant Biochemistry 6. Academic Press, Harcourt Brace Jovanovich Publishers, London. p.33.

Pearce, G., Strydom, D., Johnson, S., Ryan, C. A., 1991. A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. *Science*, 253(5022): 895-898.

Peckolt, G., Peckolt, P. 1920. Histoória das Plantas Medicinais e Úteis do Brasilia Ed. Saõ Paulo, p. 146.

Peng, J. H., Black, L. L., 1976. Increased proteinase inhibitor activity in response to infection of resistant tomato plants by *Phytophthora infestans*. *Phytopathology* 66, 958-963.

Penninckz, I.A.M.A., Eggermont, K., Terras, F.R.G., Thomma, B.P.H.J., De Samblanx, G.W., Buchala, A., Metraux, J.P., Manners, J.M., and Broekaert, W.F., 1996. Pathogen-induced systemic activation of a plant defensin gene in *Arabidopsis* follows a salicylic acid independent pathway, *Plant Cell*, 8:2309–2323.

Pepeljnjak S., I. Kosalec, Z. Kalodera, D. Kustrak, 2003. Natural antimycotics from Croatian plants. In: Rai, M., D. Mares (eds.), Plant derived antimycotics. Current trends and future prospects. Hartworth Press. N. Y. 49-79 p.

Pepeljnjak, S., Antolic, A., Kuštrak, D., 1996. Antibacterial and antifungal activities of *Vitex agnus-castus* L. extracts. *Acta Pharmaceutica*, 46 (3): 201-206.

Perchepied, L., Pitrat, M., 2004. Polygenic inheritance of partial resistance to *Fusarium oxysporum* f.sp. *melonis* race 1.2 in melon. *Phytopathology* 94: 1331-1336

Perkovska, G. Y., Sergienko, V. G., Grodzinsky, D. M., Dmitriev, A. P., 2007. Disease resistance in tomato induced by an elicitor derived from cell walls of the fungal pathogen, *Botrytis cinerea*. *Acta Phytopathologica Et Entomologica Hungarica*, 42(2): 245-251.

Petchnaree, P., Bunyapraphatsara, N., Cordell, G.A., Cowe, H.J., Cox, P.J., Howie, R.A., Patt, S.L., 1986. X-ray crystal and molecular structure of nirurine, a novel alkaloid related to the securinega skeleton, from *Phyllanthus niruri* (*Euphorbiaceae). Journal of the Chemical society, Perkin Transactions* 1: 1551-1556.

Pettitt, T.R., Pegg, G.F.. 1994. The relationship between colonization and crown rot symptoms in strawberry plants infected with *Phytophthora cactorum*. *Annals of Applied Biology* 125, 267–277.

Phay, N., Higashiyama, T., Tsuji, M., Matsuura, H., Fukushi, Y., Yokota, A., and Tomita, F., 1999. An antifungal compound from roots of Welsh onion. *Phytochemistry*, 52:271-274.

Philipson, W.R., 1993. *Monimiaceae*. In: Kubtzki, K. (Ed.), The Families and the Genera of Vascular Plants, Vol. II. Springer-Verlag, Berlin, p. 426–437.

Pierpoint, W.S. and Shewry, P.R., 1987. Amino acid homologies suggest functions for pathogenesis related proteins, Oxford Surv. *Plant Mol. Cell. Biol*., 4:337–342.

Pieterse, C. M.J., Leon-Reyes, A., Van der Ent, S., Van Wees, S.C.V.M., 2009. *Nature Chemical Biology* 5: 308 – 316.

Pieterse, C.M.J., Van Wees, S.C.M., Van Pelt, J.A., Knoester, M., Laan, R., Gerrits, H., Weisbeek, P.J., and Van Loon, L.C., 1998. A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. *Plant Cell* 10, 1571-1580.

Plotnikova, J., Ausubel, F.M., 2007. Structural basis of plant–pathogen interactions. In Dyakov, Yu.T., Dzhavakhlya, D.K., Korpela, T. (Eds.), Comprehensive and Molecular Phytopathology. Elsevier B.V., Oxford, UK.

Ponstein, A.S., Bres-Vloemans, A.A., Sela-Buurlage, M.B., van den Elzen, P.J.M., Melchers, L.S., and Cornelissen, B.J.C., 1994. A novel pathogen- and wound-inducible tobacco (*Nicotiana tabacum*) protein with antifungal activity, *Plant Physiol*., 104:109–118.

Ponti, I., Laffi, F., 1985. Malattie crittogamiche delle piante ortive, L'informatore agrario, Verona, 1985.

Pore, R.S., 1990. Antibiotic susceptibility testing of Candida albicans by flow cytometry. *Curr. Microbiol*, 20: 323-328.

Pore, R.S., 1991. Ketoconazole susceptibility of yeasts by the FCST method. *Curr. Microbiol*, 23: 45-50.

Pore, R.S., 1992. Amphotericin B synergy testing by the FCST. *Curr. Microbiol*, 20: 171-177.

Porta-Puglia, A, Crinò, P., 1993. *Ascochyta rabiei*/cece. In Crinò, P., Sonnino, A., Saccardo, F., Buiatti, M., Porta-Puglia, A., Surico, G., (Eds.), Miglioramento genetico delle piante per resistenza a patogeni e parassiti. Edagricole, Bologna, Italia.

Porta-Puglia, A., Crinò, P., Mosconi, C., 1996. Variability in virulence to chickpea of an italian population of ascochyta rabiei. *Plant Disease*, 80(1), 39-41.

Prasad, H.P., Shankar, U.A.C., Kumar, B.H., Shetty, S.H., Prakash, H.S., 2007. Management of Bean common mosaic virus strain blackeye cowpeamasaic (BCMV-BICM) in cowpea using plant extracts. Archives of *Phytopathology and Plant Protection*, 40 (2): 139-147.

Proestos, C., Sereli, D., Komaitis, M., 2006. Determination of phenolic compounds in aromatic plants by RP-HPLC and GC-MS. *Food Chemistry*, 95(1): 44-52.

Rai, M., Mares, D., 2003. Plant Derived Antimycotics. Current Trends and Future Prospects. Haworth Press. Binghamtom, N.Y., USA, pp.587.

Ranganathan, S., Balajee, S. A. M., 2000. Anti-cryptococcus activity of combination of extracts of *Cassia alata* and *Ocimum sanctum*. *Mycoses*, 43(7-8): 299-301.

Reddy, M.V., and K.B. Singh. 1990. Relationship between ascochyta blight severity and yield losses in chickpea. *Phytopathol. Medit*. 31:59–66.

Rekah, Y., Shtienberg, D., Katan, J., 2000. Disease development following infection of tomato and basil foliage by airborne conidia of the soilborne pathogens *Fusarium oxysporum* f. sp. *radicis-lycopersici* and *F. oxysporum* f. sp. *basilici*. *Phytopathology*, 90(12): 1322-1329.

Rep, M., Dekker, H.L., Vossen, J.H., De Boer, A.D., Houterman, P.M., 2002. Mass spectrometric identification of isoforms of *PR* proteins in xylem sap of fungus-infected tomato. *Plant Physiol.* 130:904–17

Reuveni, R., Dudai, N., Putievsky, E., Elmer, W. H., & Wick, R. L., 1997. Evaluation and identification of basil germ plasm for resistance to *Fusarium oxysporum* f. sp. *basilicum*. *Plant Disease*, 81(9): 1077-1081.

Reuveni, R., Raviv, M., Krasnovsky, A., Freiman, L., Medina, S., Bar, A., 2002. Compost induces protection against fusarium oxysporum in sweet basil. *Crop Protection*, 21(7): 583-587.

Ribera, A., Cotoras, M., Zúñiga, G.E., 2008. Effects of extracts from in vitro-grown shoots of *Quillaja saponaria* Mol. on *Botrytis cinerea* Pers.*World Journal of Microbiology and Biotechnology*, 24 (9): 1803-1811.

Risser, G., Banihashemi, Z., Davis, D.W., 1976. A proposal nomenclature of *Fusarium oxysporum* f.sp. *melonis* races and resistance genes in *Cucumis melo*. *Phytopathology* 66: 1105-1106.

Risser, G., Rode, J.C., 1973. Selection pour la résistance à *Fusarium oxysporum* f.sp. *melonis*. In Risser. G. (Ed.) EUCARPIA. La Sélection du melon. INRA, Montfavet-Avignon, (FR), 37-39.

Ristaino, J. B., Larkin, R. P., & Campbell, C. L., 1993. Spatial and temporal dynamics of Phytophthora epidemics in commercial bell pepper fields. *Phytopathology*, 83(12): 1312-1320.

Ristaino, J. B., Parra, G., Campbell, C. L., 1997. Suppression of Phytophthora blight in bell pepper by a no-till wheat cover crop. *Phytopathology*, 87(3): 242-249.

Ristaino, J.B., 1991. Influence of rainfall, drip irrigation, and inoculum density on the development of Phytophthora root and crown rot epidemics and yield in bell pepper. *Phytopathology* 81:922-929.

Roberts, P.D., Adkins, S., Pernezny, K., Jones, J.B., 2004. Diseases of Pepper and their Management. In Naqvi S.A.M.H. (Ed.) Diseases of Fruits and Vegetables Diagnosis and Management Volume II. Springer Science USA, 333-387.

Rodrigues, F. Á, Jurick II, W. M., Datnoff, L. E., Jones, J. B., Rollins, J. A., 2005. Silicon influences cytological and molecular events in compatible and incompatible rice-magnaporthe grisea interactions. *Physiological and Molecular Plant Pathology*, 66(4): 144-159.

Ross, C., Puglisi, M.P., Paul, V.J., 2008. Antifungal defenses of seagrasses from the indian River Lagoon, Florida. Aquatic Botany, 88:134-141.

Ryals, J. A., Neuenschwander, U. H., Willits, M. G., Molina, A., Steiner, H., Hunt, M. D., 1996. Systemic acquired resistance. *Plant Cell*, 8(10): 1809-1819.

Sabir, S.M. , Rocha, J.B.T., 2008. Water-extractable phytochemicals from *Phyllanthus niruri* exhibit distinct in vitro antioxidant and in vivo hepatoprotective activity against paracetamol-induced liver damage in mice. *Food Chemistry*, 111 (4): 845-851.

Sağlam, H., Pabuçcuoğlu, A., Kivçak, B., 2007. Antioxidant activity of *Vitex agnus-castus* L. extracts. *Phytotherapy Research*, 21(11): 1059-1060.

Sahni, S., Sarma, B.K., Singh, K.P., 2008. Management of *Sclerotium rolfsii* with integration of non-conventional chemicals, vermicompost and *Pseudomonas syringae*. *World Journal of Microbiology and Biotechnology*, 24 (4): 517-522.

Salah, M. A., Bedir, E., Toyang, N. J., Khan, I. A., Harries, M. D., Wedge, D. E., 2003. Antifungal clerodane diterpenes from *Macaranga monandra* (L) muell. et arg. (*Euphorbiaceae*). *Journal of Agricultural and Food Chemistry*, 51(26): 7607-7610.

Sambrook, J., Fritsch, E.F., and Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual, 2nd edition. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press.

Sarikurkcu, C. , Arisoy, K. , Tepe, B. , Cakir, A. , Abali, G. , Mete, E., 2009. Studies on the antioxidant activity of essential oil and different solvent extracts of *Vitex agnus castus* L. fruits from Turkey. *Food and Chemical Toxicology*, 47 (10): 2479-2483.

Sarkar, M.K., Kinter, M., Mazumder, B., Sil, P.C., 2008. Purificationa and characterization of novel antioxidant protein molecule from *Phyllanthus niruri. Food chemistry*, Article in Press.

Sarpeleh, A., Sharifi, K., Sonbolkar, A. , 2009. Evidence of antifungal activity of wild rue (*Peganum harmala* L.) on phytopathogenic fungi [Nachweis der antifungalen Aktivität der Steppenraute (*Peganum harmala* L.) gegenüber pflanzenpathogenen Pilzen] Journal of Plant Diseases and Protection, 116 (5): 208-213.

Satheesh, M. A., Pari, L., 2003. Effect of red hogweed (*Boerhavia diffusa* L.) on plasma antioxidants in alloxan-induced diabetes. *Journal of Herbs, Spices and Medicinal Plants*, 10(4): 113-119.

Satish, S., Raghavendra, M.P., Raveesha, K.A., 2009. Antifungal potentiality of some plant extracts against *Fusarium* sp. *Archives of Phytopathology and Plant Protection*, 42 (7): 618-625.

Scher, J. M., Speakman, J. , Zapp, J., Becker, H., 2004. Bioactivity guided isolation of antifungal compounds from the liverwort *Bazzania trilobata* (L.) S.F. gray. Phytochemistry, 65(18): 2583-2588.

Schweizer, P., Buchala, A., Métraux, J., 1997). Gene-expression patterns and levels of jasmonic acid in rice treated with the resistance inducer 2,6-dichloroisonicotinic acid. *Plant Physiology*, 115(1): 61-70.

Seemüller, D.B. 1998. Crown Rot. In Maas, J.L., (Ed), Compendium of Strawberry Diseases. 2nd Edition. . APS Press. St. Paul, MN, USA).

Seo, S., Seto, H., Koshino, H., Yoshida, S., Ohashi, Y., 2003. A diterpene as an endogenous signal for the activation of defense responses to infection with tobacco mosaic virus and wounding in tobacco. *Plant Cell*, 15(4): 863-873.

Serrano, M. C., Morilla, D., Valverde, A., Chávez, M., Espinel-Ingroff, A., Claro, R., 2003. Comparison of etest with modified broth microdilution method for testing susceptibility of *Aspergillus* spp. to voriconazole. *Journal of Clinical Microbiology*, 41(11), 5270-5272.

Shakila, A., Ponni, C., 2008. Influence of seed treatment on germination and seedling vigour of *Phyllanthus niruri. Acta Horticulturae*, 782: 155-161.

Shapiro, H.M., 1995. Practical Flow Cytometry, 3rd ed. Alan R. Liss, New York.

Sharaf, E. F., Farrag, A. A., 2004. Induced resistance in tomato plants by IAA against *Fusarium oxysporum lycopersici*. *Polish Journal of Microbiology*, 53(2), 111-116.

Sharma, P., Børja, D., Stougaard, P., Lönneborg, A., 1993). *PR*-proteins accumulating in spruce roots infected with a pathogenic pythium sp. isolate include chitinases, chitosanases and β-1,3-glucanases. *Physiological and Molecular Plant Pathology*, 43(1): 57-67.

Sharma, V., Dhancholia, S. 2008. Evaluation of fungitoxicants, plant extracts and antagonists against Botrytis elliptica causing blight of lilium cultivars. Annals of Biology, 24 (1): 89-93.

Shaukat, S.S., Siddiqui, I.A., 2001. *Lantana camara* in the soil changes the fungal community structure and reduces impact of *Meloidogyne javanica* on mungbean. *Phytopathologia Mediterranea* 40 (2001): 245–252.

Shukla, N., Sangwan, N. S., Misra, H. O., Sangwan, R. S., 2003. Genetic diversity analysis in *Boerhavia diffusa* L. of different geographic locations in india using RAPD markers. *Genetic Resources and Crop Evolution*, 50(6): 587-601.

Siddiqui I.A., Shaukat S.S., Khan G.H., Zaki M.J., 2002. Evaluation of *Argemone mexicana* for control of root-infecting fungi in tomato. *Journal of Phytopathology* 150:321-329.

Siddiqui, B. S., Perwaiz, S., Begum, S., 2006. Two new abietane diterpenes from *Cordia latifolia*. *Tetrahedron,* 62(43): 10087-10090.

Siegrist, J., Orober, M., Buchenauer, H., 2000. Aminobutyric acid-mediated enhancement of resistance in tobacco to tobacco mosaic virus depends on the accumulation of salicylic acid. *Physiological and Molecular Plant Pathology* 56: 95–106.

Silué, D., Pajot, E., Cohen, Y., 2002. Induction of resistance to downy mildew (*Peronospora parasitica*) in cauliflower by DL-β-amino-n-butanoic acid (BABA). *Plant Pathology*, 51(1), 97-102.

Singh, A., *Boerhavia diffusa*: An-overexploited plant of medicinal importance in eastern Uttar Pradesh. *Current Science* 93 (4), p. 446.

Sisti, M., De Santi, M., Fraternale, D., Ninfali, P.m Scoccianti, V., Brandi, G., 2008. Antifungal activity of *Rubus ulmifolius* Schott standardized *in vitro* culture. *LWT* 41: 946-950.

Smirnov, S., Shulaev, V., Tumer, N. E., 1997. Expression of pokeweed antiviral protein in transgenic plants induced virus resistance in grafted wild-type plants independently of salicylic acid accumulation and pathogenesis-related protein synthesis. *Plant Physiol,* 114:1113-1121.

Soltani, N., Khavari-Nejad, R. A., Yazdi, M. T., Shokravi, S., Fernández-Valiente, E., 2005. Screening of soil cyanobacteria for antifungal and antibacterial activity. *Pharmaceutical Biology*, 43(5), 455-459.

Somchit, M. N., Reezal, I., Elysha Nur, I., Mutalib, A. R., 2003. *In vitro* antimicrobial activity of ethanol and water extracts of *Cassia alata. Journal of Ethnopharmacology*, 84(1), 1-4.

Sorensen, J. M., Katsiotis, S. T., 1999. Variation in essential oil yield and composition of Cretan *Vitex agnus castus* L. fruits. *Journal of Essential Oil Research*, 11(5): 599-605.

Spellenberg R.. 2000. Blooming "behavior" in five species of *Boerhavia (Nyctaginaceae)*. SIDA Contributions to Botany 19: 311-323.

Spletzer, M.E., Enyedi, A.J., 1999. Salicylic acid induces resistance to *Alternaria solani* in hydroponically grown tomato. *Phytopathology*, 89 (9): 722-727.

Spoon, T.R., Kesseli, R.V. 2008. Development of microsatellite markers in *Cordia bifurcata (Boraginaceae)* and cross-species amplification in *Cordia inermis* and *Cordia pringlei*. *Molecular Ecology Resources*, 8 (5): 989-992.

Sridhar, S. R., Rajagopal, R. V., Rajavel, R., Masilamani, S., Narasimhan, S., 2003. Antifungal activity of some essential oils. *Journal of Agricultural and Food Chemistry*, 51(26): 7596-7599.

Sticher, L., Mauch-Mani, B., Métraux, J. P., 1997. Systemic acquired resistance. Annual review of phytopathology, 35:235-70

Stuardo, M., San Martín, R, 2008. Antifungal properties of quinoa (*Chenopodium quinoa* Willd) alkali treated saponins against Botrytis cinerea. *Industrial Crops and Products*, 27 (3): 296-302.

Subramaniam, R., Després, C., Brisson, N., 1997. A functional homolog of mammalian protein kinase c participates in the elicitor-lnduced defense response in potato. *Plant Cell*, 9(4): 653-664.

Sunda, W.G., Hardison, D.R. 2008. Contrasting seasonal patterns in dimethylsulfide, dimethylsulfoniopropionate, and chlorophyll a in a shallow North Carolina estuary and the Sargasso Sea Aquatic Microbial Ecology, 53 (3): 281-294.

Suresh, G., Narasimhan, N. S., Masilamani, S., Partho, P. D., Gopalakrishnan, G., 1997. Antifungal fractions and compounds from uncrushed green leaves of *Azadirachta indica*. *Phytoparasitica*, 25(1): 33-39.

Tabanca, N., Demirci, B., Baser, K. H. C., Aytac, Z., Ekici, M., Khan, S. I., 2006. Chemical composition and antifungal activity of salvia macrochlamys and salvia recognita essential oils. *Journal of Agricultural and Food Chemistry*, 54(18), 6593-6597.

Tabanca, N., Demirci, B., Crockett, S. L., Başer, K. H. C., Wedge, D. E., 2007. Chemical composition and antifungal activity of *Arnica longifolia*, *Aster hesperius*, and *Chrysothamnus nauseosus* essential oils. *Journal of Agricultural and Food Chemistry*, 55(21): 8430-8435.

Taiga, A., Olufolaji, D.B. 2008. *In vitro* screening of selected plant extracts for fungicidal properties against dry rot fungal of yam tubers (*Dioscorea rotundata*) in Kogi State, *Nigeria Research Journal of Biotechnology*, 3 (SPEC. ISS.):309-311.

Tegegne, G., Pretorius, J.C, 2007. *In vitro* and *in vivo* antifungal activity of crude extracts and powdered dry material from Ethiopian wild plants against economically important plant pathogens. *BioControl* 52: 877-888.

Tempone, A. G., Treiger Borborema, S. E., De Andrade Jr., H. F., De Amorim Gualda, N. C., Yogi, Á, Salerno Carvalho, C., 2005. Antiprotozoal activity of Brazilian plant extracts from isoquinoline alkaloid-producing families. *Phytomedicine*, 12(5): 382-390.

Terras, F. R. G., Eggermont, K., Kovaleva, V., Raikhel, N. V., Osborn, R. W., Kester, A., Rees, S. B., Vanderleyden, J., Cammue, B. P. A., Broekaert, W. F., 1995. Small cysteine-rich antifungal proteins from radish: their role in host defence. *Plant Cell* 7, 573–588.

Thomas, E., Shanmugam, J., Rafi, M. M., 1999. *In vitro* antibacterial activity of certain medicinal plants of kerala. *Biomedicine*, 19(3), 185-190.

Tinivella, F. , Hirata, L.M. , Celan, M.A. , Wright, S.A.I. , Amein, T., Schmitt, A., Koch, E., Van Der Wolf, J.M., Groot, S.P.C., Stephan, D., Garibaldi, A., Gullino, M.L., 2009. Control of seed-borne pathogens on legumes by microbial and other alternative seed treatments. European Journal of Plant Pathology, 123 (2): 139-151.

Tohamy, M.R.A., Aly, A.Z., Abd-El-Moity, T.H., Atia, M.M., Abed-Rl-Moneim, M.L., 2002. Evaluation of some plant extracts in control damping-off and mildew diseases of cucumber. *Egypt J. Phytopathol*., 30 (2): 71-80.

Tornero, P., Gadea, J., Conejero, V., Vera, P., 1997. Two PR-1 genes from tomato are differentially regulated and reveal a novel mode of expression for a pathogenesis-related gene during the hypersensitive response and development. Molecular Plant-Microbe Interactions, 10 (5): 624-634.

Toussaint, J.-P., Kraml, M., Nell, M., Smith, S.E., Smith, F.A., Steinkellner, S., Schmiderer, C., Vierheilig, H. and Novak, J., 2008. Effect of *Glomus mosseae* on concentrations of rosmarinic and caffeic acids and essential oil compounds in basil inoculated with *Fusarium oxysporum* f.sp. *basilici*. Plant Pathology, 57 (6): 1109-1116.

Trigiano, R.N., Baird, R.E., Jeffers, S.N., 2004. Laboratory Exercises with the Oomycetes. In Trigiano, R.N., Windham, M.T., Windham, A.S. (Eds.), Plant Pathology, Concepts and Laboratory Excercises, , CRC Press, Washington, USA.

Ujowundu, C.O., Igwe, C.U., Enemor, V.H.A., Nwaogu, L.A., Okafor, O.E., 2008. Nutritive and anti-nutritive properties of *Boerhavia diffusa* and *Commelina nudiflora* leaves. *Pakistan Journal of Nutrition*, 7 (1): 90-92.

Uldahl, S.A., Knutsen, G., 2009. Spore swelling and germination as a bioassay for the rapid screening of crude biological extracts for antifungal activity Journal of Microbiological Methods, 79 (1): 82-88.

Unger, C., Wilhelm, I., Jünger, R., Thalmann, R., 2006. Evidence of induced resistance of tomato plants against phytophthora infestans by a water extract of dried biomass of *Penicillium chrysogenum*. *Journal of Plant Diseases and Protection*, 113(5): 225-233.

Van de Rhee, M. D., Lemmers, R., Bol, J. F., 1993. Analysis of regulatory elements involved in stress-induced and organ-specific expression of tobacco acidic and basic β-1,3-glucanase genes. *Plant Molecular Biology*, 21(3): 451-461.

Van Loon LC, Bakker PAHM. 2006. Induced systemic resistance as a mechanism of disease suppression by rhizobacteria. In Siddiqui, Z.A., (Ed.), *PGPR*: *Biocontrol and Biofertilization*, Dordrecht: Springer, 39–66.

Van Loon, L.C., 1990. The nomenclature of pathogenesis-related proteins. *Physiol M1 Plant Pathol* 37: 229-230.

Van Loon, L.C., 1999. Occurrence and properties of plant pathogenesis-related proteins, Pathogenesis-Related Proteins in Plants (S.K. Datta and S. Muthukrishnan, eds.), CRC Press, Boca Raton, 1–19.

Van Loon, L.C., Rep, M., and Pieterse, C.M.J., 2006. Significance of inducible defense related proteins in infected plants. *Annu. Rev. Phytopathol.* 44, 135-162.

Van Loon, L.C., Van Strien, E.A., 1999. The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiol. Mol. Plant Pathol*. 55:85– 97.

Van Peer, R., Neimann, G.J. and Schippers, B.M., 1991. Induced resistance and phytoalexin accumulation in biological control of Fusarium wilt of carnation by Pseudomonas sp. strain WCS417r. *Phytopathology* 81: 728–734.

Van Wees, S.C.M., Luijendijk, M., Smoorenburg, I., Van Loon, L.C., and Pieterse, C.M.J., 1999. Rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis* is not associated with a direct effect on expression of known defense-related genes but stimulates the expression of the jasmonate-inducible gene Atvsp upon challenge. Plant Mol. Biol. 41: 537-549.

Velazhahan, R., Datta, S.K., Muthukrishnan, S., 1999. The PR-5 family: thaumatin-like proteins.-In: Datta, S.K, Muthukrishnan, S. (ed.): Pathogenesis-Related Proteins in Plants. CRC Press, Boca Raton, 107–129.

Vestberg, M., 2006: *Phytophthora cactorum* and *Colletotrichum acutatum*: Survival and detection. *Agriculturae Conspectus Scietitificus* 71 (4): 121-128.

Viana, F. A., Andrade-Neto, M., Pouliquen, Y. B. M., Uchoa, D. E. A., Sobral, M. M. S. Z., De Morais, S. M., 2002. Essential oil of siparuna guianensis aublet from the amazon region of Brazil. *Journal of Essential Oil Research*, 14(1), 60-62.

Viana, M.G., de Albuquerque, C.C, de Medeiros, E.V., Viana, F.A., de Silva, K.M.B., 2008. Evaluation of the fungicide potential of ethanolic extracts of Senna alata against Monosporascus sannonballus. *Ciencia e Agrotecnologia*, 32 (5): 1387-1393.

Vicedo, B., De La O Leyva, M., Flors, V., Finiti, I., Del Amo, G., Walters, D., 2006. Control of the phytopathogen botrytis cinerea using adipic acid monoethyl ester. *Archives of Microbiology*, 184(5), 316-326.

Vicedo, B., Flors, V., De La O Leyva, M., Finiti, I., Kravchuk, Z., Real, M.D., García-Agustín, P., 2009. Hexanoic Acid-Induced Resistance Against *Botrytis cinerea* in Tomato Plants. 22 (11): 1455-1465.

Vidhyasekaran, P. (2002). Bacterial Disease Resistance in Plants: Molecular Biology and Biotechnological Applications, Haworth Press, Binghamton, NY, p. 520.

Vidhyasekaran, P. , 2004. Concise Encyclopedia of Plant Pathology, Haworth Press, Binghamton, NY, pp. 619.

Vidhyasekaran, P., 1997. Fungal pathogenesis in plants and crops. Marcel Dekker Inc., New York, pp. 553.

Vidhyasekaran, P., 2007. Handbook of Molecular Technologies in Crop Disease Management, Haworth.

Vidhyasekaran, P., 2008. Fungal Pathogenesis in Plants and Crops Second Edition Molecular Biology and Host Defense MechanismsTaylor & Francis Group, LLC, pp. 509.

Vigers, A. J., Roberts, W. K., Selitrennikoff, C. P., 1991. A new family of plant antifungal proteins. *Molecular Plant-Microbe Interactions* 4(4): 315-323.

Vigers, A. J., Wiedemann, S., Roberts, W. K., Legrand, M., Selitrennikoff, C. P., Fritig, B., 1992. Thaumatin-like pathogenesis-related proteins are antifungal. *Plant Science*, 83(2), 155-161.

Vila, R., Iglesias, J., Canĩigueral, S., Santana, A. I., Solís, P. N., Gupta, M. P., 2002. Chemical composition and biological activity of the leaf oil of *Siparuna thecaphora* (poepp. et endl.) A.DC. *Journal of Essential Oil Research*, 14(1), 66-67.

Ward, E.R., Uknes, S.J.,Williams, S.C., Dincher, S.S.,Wiederhold, D.L., 1991. Coordinate gene activity in response to agents that induce systemic acquired resistance. *Plant Cell* 3:1085–94

Wassel, G., El-Menshawi, B., Saeed, A., Mahram, G., Reich, J., 1987. New sources of pyrrolizidine alkaloids: genus *Cordia* and *Schismus*. *Scientia Pharmaceutica* 55: 166-193.

Wasternack, C., Parthier, B., 1997. Jasmonate-signalled plant gene expression. *Trends in Plant Science* 2, 302-307.

Webster, J., Grady L., 1994. Classification of the *Euphorbiaceae*. *Annals of the Missouri Botanical Garden* 81 (1): 3–32.

Webster, J., Weber, R.W.S., 2007. Introduction to fungi. Cambridge University Press, New York.

Wedge. D. E., Nagle, D.G., 2000. A new 2D-TLC bioautography method for the discovery of novel antifungal agents to control plant pathogens. *J Nat Prod* 63:1050-1054.

Wei, Y., Zhang, Z., Andersen, C. H., Schmelzer, E., Gregersen, P. L., Collinge, D. B., 1998. An epidermis/papilla-specific oxalate oxidase-like protein in the defence response of barley attacked by the powdery mildew fungus. *Plant Molecular Biology*, 36(1): 101-112.

Weltzien, H.C., Kaack, H.J., 1984. Epidemiological aspects of chickpea Ascochyta blight. In: M.C. Saxena, K.B. Singh (eds), Ascochyta blight and winter sowing of chickpeas. Nijhoff and Junk Publ. (for ICARDIA), The Hague, Netherland, 35-44.

Whipps, J.M., Lumsden, R.D., 2001. Commercial Use of Fungi as Plant Disease Biological Control Agents: Status and Prospects. In: T.M. Butt, C. Jackson and N. Magan (Eds.), Fungi as Biocontrol Agents. Progress, Problems and Potential, CABI Publishing, Wallingford, 9-22.

White, D., Chen, W., 2007. Towards identifying pathogenic determinants of the chickpea pathogen *Ascochyta rabiei*. *European Journal of Plant Pathology* 119:3-12.

Widmer, T. L., Laurent, N., 2006. Plant extracts containing caffeic acid and rosmarinic acid inhibit zoospore germination of *Phytophthora* spp. pathogenic to theobroma cacao. *European Journal of Plant Pathology*, 115(4): 377-388.

Wilson, C. L., Solar, J. M., El Ghaouth, A., Wisniewski, M. E., 1997. Rapid evaluation of plant extracts and essential oils for antifungal activity against botrytis cinerea. *Plant Disease*, 81(2): 204-210.

Wojdyla, A. T., 2000. Influence of some compounds on development of *Sphaerotheca pannosa* var. *rosae. J Plant Protect Res,* 40:106-121.

Wokoma, E.C., Nwaejike, C., 2008. Antifungal effects of solvent extracts of neem leaves on seed pathogens of groundnut. *Asian Journal of Microbiology, Biotechnology and Environmental Sciences*, 10 (4):725-731.

Wurms, K., Labbe, C., Benhamou, N., and Belanger, R. R., 1999. Effects of Milsana and benzothiadiazole on the ultrastructure of powdery mildew haustoria on cucumber. *Phytopathology,* 89:728-736.

Xing,T., 2007. Signal transduction Pathways and disease resistant genes and their applications to fungal disease control. In Punja Z., De Boer, S.H., Sanfaçon, H. (Eds.), Biotechnology and Plant Disease management, CAB International.

Xu Y., Chang P.F.L., Liu D., Narasimhan M.L., Raghothama K.G., Hasegawa P.M., Bressan R.A., 1994. Plant defense genes are synergistically induced by ethylene and methyl jasmonate. *Plant Cell 6*, 1077-1085.

Xu, P., Blancaflor, E.B., Roossinck, M.J., 2003. In spite of multiple defense responses, tomato plants infected with Cucumber mosaic virus and D satellite RNA succumb to systemic necrosis. *Molecular Plant-Microbe Interactions*, 16 (6): 467-476.

Yu, M., Shen, L., Fan, B., Zhao, D., Zheng, Y., Sheng, J., 2009. The effect of MeJA on ethylene biosynthesis and induced disease resistance to *Botrytis cinerea* in tomato. *Postharvest Biology and Technology*, 54 (3): 153-158.

Yucel, S., 1995. A study on soil solarization and combined with fumigant application to control Phytophthora crown blight (*Phytophthora capsici* leonian) on peppers in the east mediterranean region of Turkey. *Crop Protection*, 14(8): 653-655.

Zabbai, F., Jarosch, B., Schaffrath, U., 2004. Over-expression of chloroplastic lipoxygenase RCI1 causes PR1 transcript accumulation in transiently transformed rice. *Physiological and Molecular Plant Pathology*, 64(1): 37-43.

Zamora-Natera, F., García-López, P., Ruiz-López, M., Salcedo-Pérez, E., 2008. Composition of alkaloids in seeds of *Lupinus mexicanus* (*Fabaceae*) and antifungal and allelopathic evaluation of the alkaloid extract [Composición de alcaloides en semillas de *Lupinus mexicanus* (*Fabaceae*) y evaluación antifúngica y alelopática del extracto alcaloideo]. *Agrociencia*, 42 (2): 185-192.

Zhang Ziguo, Collinge, D. B., Thordal-Christensen, H., 1995. Germin-like oxalate oxidase, a H_2O_2-producing enzyme, accumulates in barley attacked by the powdery mildew fungus. *Plant Journal*, 8(1): 139-145.

Zhang, S., Jin, Y., Tang, J., Chen, X., 2009. The invasive plant *Solidago canadensis* L. suppresses local soil pathogens through allelopathy. *Applied Soil Ecology*, 41 (2): 215-222.

Zhou, J.M., 1999. Signal transduction and pathogenesis-induced *PR* gene expression. In: Datta, S.K., Muthukrishnan, S. (eds) Pathogenesis-Related Proteins in Plants. 1st edition. CRC Press LLC, Boca Raton, 195-20.

Ziadi, S., Barbedette, S., Godard, J. F., Monot, C., Le Corre, D., Silué, D., 2001. Production of pathogenesis-related proteins in the cauliflower (*Brassica oleracea* var. *botrytis*)-downy mildew (*Peronospora parasitica*) pathosystem treated with acibenzolar-S-methyl. Plant Pathology, 50(5): 579-586.

Zida, D.a b , Tigabu, M.a , Sawadogo, L.b , Odén, P.C.a 2008. Initial seedling morphological characteristics and field performance of two Sudanian savanna species in relation to nursery production period and watering regimes Forest Ecology and Management, 255 (7): 2151-2162.

Zimmerli, L., Jakab, G., Métraux, J., Mauch-Mani, B., 2000. Potentiation of pathogen-specific defense mechanisms in *Arabidopsis* by β-aminobutyric acid. Proceedings of the National Academy of Sciences of the United States of America, 97(23): 12920-12925.

Zoghbi, M. D. G. B., Andrade, E. H. A., Maia, J. G. S., 1999. The essential oil of *Vitex agnus-castus* L. growing in the amazon region. Flavour and Fragrance Journal, 14(4): 211-213.

ACKNOWLEDGEMENTS

The greatest thanks belong to Dr. Paola Crinò, my tutor, who always encouraged me and trusted in me. In these three years, she always helped me in all possible ways, being my guide and transferring me her precious knowledge. She was almost like a 'second mum' to me and I will never be able to thank her enough for her kindness, willingness, and friendliness.

Sincere thanks to Dr. Giuseppe Colla, my tutor, who enabled me to carry out this wonderful work, and always helped me in my research activities and supervised my greenhouse experiments.

I am very grateful to Prof. Francesco Saccardo for giving me the opportunity to carry out this PhD thesis and also for his precious advices and support during these three years.

Many thanks to Dr. Sergio Lucretti for introducing me to flow cytometric analyses, and for collaborating and supervising me in this part of my work. I am grateful to him also for reviewing this part of my thesis.

Many thanks to Dr. Luca Nardi for assisting me to plan the cytometric experiment.

Many thanks to Prof. Carla Caruso for giving me the opportunity to carry out the molecular analysis in her lab.

Many special thanks belong to Dr. Silvia Proietti, who introduced me to the molecular analyses, always patiently supervised my work giving me precious advices and always kindly helped me. I am grateful to her also for reviewing the molecular part of my thesis.

Many thanks to Dr. Anna Ciancolini for helping me kindly in my research activities.

Many thanks to Mrs. Giovanna Ciccotti for her technical support during the experiments; and to other persons from ENEA, who made me staying in a friendly environment.

Many thanks to Mr. Giuseppe Bocchino for his technical support during the greenhouse experiments in Viterbo.

This thesis was realised thanks to financial support from AGROTECNOLOGIAS NATURALES S.L.

Many sincere thanks to Šárka Machková, who made me feel in Italy like at home, helping me in every kind of situation and encouraging me in my life and work, being the most precious friend during these years spent in Viterbo.

Many thanks to my wonderful friends Barča, Jovanka, Alena, Irena, and Caterina, who accompanied me during these years, and encouraged me all the time.

Special thanks to Nilde for receiving me with favour and making me feel like at home with her splendid kindness.

Many affectionate thanks to Augusto, who always helped me and encouraged me, making cheerful even difficult periods.

Many thanks to all of my family that was close to me all the time.

Special thanks to my parents who always trusted in me and supported me unconditionally.