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"Biological control of post-harvest diseases on apple by using plant essential oils and *Trichoderma* culture filtrates"

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Abstract

Pure essential oils have been purified and chemically characterized from cinnamon, clove and black pepper. All these oils have antifungal properties and when applied to apple fruits in post harvest can efficiently control *Botrytis cinerea*, *Alternaria alternata* or *Penicillium expansum*. In addition, application of these compounds or *Trichoderma* culture filtrate can induce systemic resistance mechanisms in apple fruit. This has been confirmed through phytoalexins and proteomic analysis. The accumulation of the phytoalexins scopoletin, umbelliferone, and scoparone was induced by the three essential oils or *Trichoderma* CF. Proteomic analysis indicated many changes in protein production caused by the treatment with the oils, *Trichoderma* CF and the pathogens. The differential spots (TOTD) obtained were up to 166 in clove plus *P. expansum* treatment. Twenty nine of the most interesting differentially expressed proteins were further analysed by MALDI-TOF MS. The protein groups identified included: pathogenesis-related proteins belonging to the PR-10 sub-family, some antimicrobial proteins, enzymes involved in the biosynthesis of antimicrobial compounds pathway and ribosomal proteins. The most interesting essential oil was the one extracted from black pepper because it was not phytotoxic on tomato seedlings and apple fruits at all the tested concentrations and showed a good disease control activity. A new bio-formulate for post-harvest application, based on a combination of three different active ingredients (black pepper oil, *Trichoderma* CF and a natural ISR-inducing agent also used as emulsifier) was designed and successfully tested as a synergistic mixture.

Introduction

1. Apple fruits post-harvest diseases control

Apple (*Malus domestica* Borkh.) is a member of Rosaceae family and considered one of the most economically important fruit tree crop of temperate zones (Martinelli *et al.,* 2008). Italy ranks as the sixth producer of apple fruits after China, USA, India, Turkey and Poland (http://faostat.fao.org, 2012) with approximately 2,411,200 tons/year. Apples are one of the most widely consumed fruits, due in part to their wide-range of beneficial effects on human health. A high intake of apples has been shown to prevent a variety of chronic diseases and reduce the risk of lung cancer, asthma, type-2 diabetes, thrombotic stroke, and ischemic heart disease (Hansen *et al.,* 2009; Chai *et al.,* 2011). These benefits are associated with the large content of structural cell walls and polysaccharides (Sun-Waterhouse *et al.,* 2008), as well as various phytochemical antioxidants (Devic *et al.,* 2010; Lee *et al.,*2003; McGhie *et al.,* 2005).

Apple production has a great economic importance but it is affected by several pre- and post-harvest diseases. Despite of the use of modern storage facilities, losses from 5 to 25% of apples and pears are still being recorded. Fungal pathogens such as *Botrytis cinerea*, *Penicillium expansum* and *Alternaria alternata* are responsible for the main economical losses. After harvest, apples are stored at low temperature (0-1°C) to maintain quality and to minimize spoilage. However, development of fungal diseases, caused mainly by *P. expansum* and *B. cinerea*, cannot be avoided. Control measures are still principally based on the protection of fruits from pre- and post-harvest infection by using fungicide treatment. However, in the context of consumer reluctance to accept chemical residues in food and of public concern for environmental safety, there is an increasing demand to develop alternative disease control methods. This becomes a critical issue with respect to the de-registration of effective and widely used fungicides and the development of fungicide-resistant strains of post-harvest pathogens. In this regard, biological control has been developed as a valid alternative. Biocontrol is generating a great enthusiasm as a key player in sustainable agriculture although the relevance of biological control agents (BCAs) in plant disease management appears to be limited until now. Post-harvest biological control could be considered as particularly promising because: 1) the application sites are limited to the harvested commodities and the BCA is not spread into the environment, 2) the environmental conditions are defined and stable in storage rooms, 3) the harvested commodities could be considered high value crops. BCAs are mainly targeting the

post-harvest diseases that develop through wounds in the fruits (such as *B. cinerea*, *P. expansum*, *A. alrernata*, *Rhizopus spp.*). However, latent infections are not reported to be controlled by BCAs. The most widely used are yeasts followed by bacteria, with only a few species belonging to mycelial fungi been applied. Table 1 lists the most common biopesticides (formulated BCAs) for post-harvest diseases already on the market. Three bacteria and four yeasts have acquired full registration. Among the bacteria, *Pseudomonas syringae* strains specifically target post-harvest diseases, while a *Bacillus subtilis* strain is reported as and effective BCA also against pre-harvest diseases. Formulations based on *P. syringae* strains may be considered the oldest on the market. The *B. subtilis* product is the only one to be registered both in USA and Europe.

Table 1: Commercially available biological control products to manage post-harvest fruit diseases.

Microorganism(s) contained	Product trade name	Fungal disease target	Crop	Manufacturer or distributor/ countries with registration
Bacillus subtilis QST713	Serenade/ rhapsody/ Sereande garden	Powdery mildew, downy mildew, Cercospora leaf spot, early blight, late blight, brown rot, fire blight, and others	Cucurbits, grapes, hops, vegetables, peanuts, pome fruits, stone fruits, and others	AgraQuest (CA, USA)/ European community, USA, Canada
Pseudomonas syringae ESC-10	Bio-Save 10LP	*B. cinerea, Penicillium* spp., *Mucor pyroformis, Geotrichum candidum*	Pome fruit, citrus, cherries, and potatoes	EcoScience Corp. (Longwoord, FL)/USA
Pseudomonas syringae ESC-11	Bio-Save 110	*B. cinerea, Penicillium* spp., *Mucor pyroformis, Geotrichum candidum*	Pome fruit, citrus, cherries, and potatoes	EcoScience Corp. (Longwoord, FL)/USA
Aureobasidium pullulans	Boni protect	*B. cinerea*	Pome fruit	Bio-Protect GmbH belonging to Bio-Firm from BIOMIN (Austria)/Germany, In preparation for Europe
Cryptoccocus albidus	Yield plus	*B. cinerea and P. expansum*	Apple and Pear	Anchor Yeast (South Africa) belonging to Lallemand group/South Africa
Metschnikowia fructicola	Shemer	P. digitatum, P.italicum, P.expansum,B. cinerea, Rhizopus stolonifer, Aspergillus niger, Fusarium and Sclerotinia sclerotium	Citrus, pome and stone fruits, grapes, strawberries and sweet potatoes	Agrogreen, belonging to Bayer group/Israël, in preparation for Europe
Canida oleophila strain O	Nexy	*B. cinerea, Penicillium* spp.	Pome fruits	Lesaffre- Bionext (France)/ Europe

Integrated Pest Management (IPM) is an ecologically safe method aimed at minimizing the undesirable side effects of agrochemicals thus protecting the environment and the human health (Roth *et al.,* 2007; Smilanick *et al.,* 1995, 1997). In this contest, alternative methods for plant disease control, including the use of microbes, their metabolites and products derived from plants, are highly desirable considering that the number of biological products available on the market for post-harvest diseases control is still very limited (Tab.1).

2. *Trichoderma spp.* as biocontrol agents

Trichoderma spp. are asexually reproducing fungi that are often the most frequently isolated from soil. They are very useful microbes, by producing beneficial effects on crops and they may have naturally sustained the agricultural yields that have supported the human population over the millennia (Lorito *et al.,* 2010). Many strains have been exploited as biocontrol agents (BCAs) successfully used worldwide as biopesticides and biofertilizers. It is typical of several species to be active producers of secondary metabolites with antibiotic activity (Sivasithamparam and Ghisalberti 1998; Harman 2000; Harman *et al.,* 2004; Walters *et al.,* 2005; Lorito *et al.,* 2006; Woo *et al.,* 2006). Various *Trichoderma*–based formulations are available commercially for crop production (Harman 2000). For instance, *T. harzianum* strain T-39, the active ingredient of the commercial product Tricodex™, induces resistance towards *B. cinerea* in tomato, tobacco, lettuce, pepper and bean plants, with a symptom reduction ranging from 25 to 100% (Abadi 2008). Moreover, *Trichoderma spp.* function as biocontrol agents for a wide range of economically important aerial and soil borne plant pathogens (Brunner *et al.,* 2005). The biocontrol mechanism of *T. harzianum* is a complex process mediated by the secretion of extracellular enzymes, such as chitinases, glucanases and proteinases, as well as secondary metabolites (Vinale *et al.,* 2006). The mechanisms that *Trichoderma spp.* use to antagonize phytopathogenic fungi include competition, colonization, antibiosis and direct mycoparasitism (Harman 2006, 2011; Howell 2003). This antagonistic potential serves as the basis for effective biological control applications against a wide spectrum of plant pathogens (Harman *et al.,* 1991; Lorito *et al.,* 2010).

The colonization of the root system by rhizosphere competent strains of *Trichoderma* results in increased development of root and/or aerial systems and crop yields (Bae *et al.*, 2011; Chacon *et al.,* 2007; Kubicek *et al.,* 1998; Yedidia *et al.,* 2003). *Trichoderma* has also been described as being involved in other biological activities such as the induction of plant systemic resistance (Shoresh *et al.,* 2010; Tucci *et al.*, 2011) and antagonism against plant pathogenic nematodes (Jegathambigai *et al.*, 2008; Sharon *et al.*, 2001). Some strains of *Trichoderma* have also been noted to be aggressive biodegraders in their saprophytic phases, in addition to acting as competitors to fungal pathogens, particularly when nutrients are a limiting factor in the environment (Worasatit *et al.*, 1994). The molecular cross-talk that occurs between the fungal BCA and the plant is important for producing the desired beneficial effects. Somehow the plant is able to sense, possibly by the detection of the released fungal compounds, that *Trichoderma* is not a hostile presence (Woo and Lorito 2007). Molecules produced by *Trichoderma* and/or its metabolic activity also have potential for

applications to promote plant growth (Chacon *et al.*, 2007; Vinale *et al.*, 2008a; 2008b; Yedidia *et al.*, 1999). The induction of systemic resistance (ISR) observed *in planta* determines an improved control of different classes of pathogens (mainly fungi and bacteria), which are spatially and temporally distant from the *Trichoderma* inoculation site. This phenomenon has been observed in many plant species, both dicotyledons (tomato, pepper, tobacco, cotton, bean, cucumber) and monocotyledions (corn, rice). For example, *Trichoderma* induces resistance towards *B. cinerea* in tomato with a symptom reduction ranging from 25 to 100% (Tucci *et al.*, 2011). Moreover, *Trichoderma* determined an increased production of defence-related plant enzymes, including various peroxidases, chitinases, β-1,3-glucanases, and hydroperoxide lyase involved in the lipoxygenase pathway (Harman *et al.*,2004; Howell *et al.*, 2000; Yedidia *et al.*,1999).

Trichoderma is able not only to produce toxic compounds with a direct antimicrobial activity against pathogens, but also to generate molecules that stimulate the plant to produce its own defence metabolites. In fact, the ability of *T. virens* to induce phytoalexins accumulation and localized resistance in cotton has been reported (Hanson and Howell 2004). In cucumber, root colonization by strain T-203 of *T. asperellum* caused an increase in the leaf level of phenolic glucoside, where the corresponding aglycones (phenolic glucosides with the carbohydrate moieties removed) are strongly inhibitory of a range of bacteria and fungi (Yedidia *et al.*, 2003).

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

Extensive testing of *T. harzianum* strain T22 conducted for the registration of this biocontrol agent in the USA by the Environmental Protection Agency (EPA) has found that the CWDEs do not have a toxic effect on humans and animals (ED50 and LD50), and that they do not leave residues. These enzymes can be used as single or mixed combinations of CWDEs having a high antifungal effect, and obtained from fermentation in inducing conditions, also by over-expressing the encoding genes in modified *Trichoderma* or other microbes. They can be considered as a potential improvement

over the use of the living microorganism in commercial formulations because are easily characterized, resist desiccation, are stable at temperatures up to 60 °C, and are active over a wide range of pH and temperatures in the agricultural environment.

3. Property and application of plant essential oil extracts

Plant essential oils are hydrophobic concentrated mixtures principally containing volatile aromatic compounds and generally extracted by distillation. They are used in perfumes, cosmetics, soaps and other products, for flavouring food and drink, and for adding scents to incense and household cleaning products. Essential oils are also popular nowadays due to aromatherapy, a branch of alternative medicine claiming that essential oils and other aromatic compounds have curative effects. In the last decades, scientific studies associated beneficial properties (antioxidant, anti-inflammatory, antiviral, antibacterial, stimulators of central nervous system, etc.) of several plants and herbs to some of the compounds found in the extracted essential oil. For example, the valerian-extracted valerenic acid, a sesquiterpenoid compound, and its derivatives (acetoxyvalerenicacid, hydroxyvalerenic acid, valeranone, valerenal) are recognized as relaxant and sedative; lavender extract is used as antiseptic and anti-inflammatory for skin care; menthol is derived from mint and is used in inhalers, pills or ointments to treat nasal congestion; thymol, the major component of thyme essential oil is known for its antimicrobial activity; limonene and eucalyptol appear to be specifically involved in protecting the lung tissue. Therefore, essential oils have become a target for the recovery of natural bioactive substances useful for human treatments.

Essential oils are composed of lipophilic substances, containing the volatile aroma components of the vegetal matter, which are also involved in the defence mechanisms of the plants. The essential oil represent a small fraction of plant composition, and is comprised mainly by monoterpenes and sesquiterpenes, and their oxygenated derivatives such as alcohols, aldehydes, ketones, acids, phenols, ethers, and esters. The amount of a particular substance in the essential oil composition varies from really high proportions (e.g. around 80–90% w/w of α-limonene present in orange essential oil) to traces. Nevertheless, components present in traces are also important, since all of them are responsible for the characteristic odour, flavour and bioactive properties. Thus, it is important that the extraction procedure applied to recover essential oils from plant matrix maintains the natural proportion of its original components. Essential oils could be obtained from roots and

rhizomes (such as ginger), leaves (mint, oregano and eucalyptus), bark and branches (cinnamon, camphor), flowers (jasmine, rose, violet and lavender) and fruits and seeds (orange, lemon, pepper, nutmeg). In general, essential oil represents less than 5% of the vegetal dry matter. Although essential oils are present in all organs, their composition may vary when different parts of the plant are used as raw material. Other factors, such as cultivation, soil and climatic conditions as well as harvesting time, can also determine the composition and quality of the essential oil (Celiktas *et al.,* 2007).

The main compounds of plant essential oils are terpenes, which are also called isoprenes since derived from isoprene (2- methyl-1,3-butadiene, chemical formula C5H8). Main hydrocarbon terpenes present in plant essential oil are monoterpenes (C10), which may constitute more than 80% of the essential oil, and sesquiterpenes (C15). They can present acyclic as well as mono-, bi- or tricyclic structures. Terpenoids are derived from these hydrocarbons, for example by oxidation or just reorganization of the hydrocarbon skeleton. Terpenoids present in essential oils have a wide variety of chemical organic properties, acting as alcohols, aldehydes, ketones, acids, phenols, ethers, or esters.

Plant essential oils have antimicrobial activity against a variety of plant pathogens and pests. Several studies have explored the potential of essential oils as antifungal agents (Abd-Alla *et al.,* 2001; Abdolahi *et al.,* 2010; Cowan, 1999; Grane and Ahmed 1988; Kurita *et al.,* 1981; Wilson *et al.,* 1997). Most of them have been reported to inhibit postharvest fungi *in vitro* (Hidalgo *et al.,* 2002; Kordali *et al.,* 2005).The use of these volatile compounds has attracted an increasing interest in recent years. Also, the antifungal activity of essential oils from oregano and thyme showed significant efficacy in apple fruits infected with *B. cinerea* and *P. Expansum* (Lopez-Reyes *et al.,* 2010). In addition, the antifungal activity of clove oil in apples was evaluated against *B. cinerea*, *M. fructigena*, *P. expansum* and *P. Vagabunda* (Amiri *et al.,* 2008). Moreover, because of plant essential oils have low mammalian toxicity, are biodegradable, multifunctional, non-persistent in the environment and cheap to produce, the possibility of developing their use in crop protection is considered an attractive possibility (Abdolahi *et al.,* 2010). For example, essential oils of cinnamon and clove are known to have potent antibiotic activity and their application for controlling postharvest diseases has been suggested (Feng and Zheng 2007; Kishore *et al.,* 2007).

4. Chitosan: an antifungal, ISR inducer and emulsifier compound

Chitosan is a high molecular weight carbohydrate polymer found in nature but also manufactured from chitin. It is a natural cationic polyelectrolyte formed by N-acetyl-D-glucosamine units with β (1–4) glycosidic bounds (Pinotti *et al.,* 2001). Chitosan has excellent emulsifying properties and has received increased attention in terms of commercial applications in both the food (Li and Xia 2011) and plant protection (Badawy and Rabea 2011; Romanazzi 2010; Walker 2004) industries. This has elicited a large number of research papers and patents related to chitosan (Badawy and Rabea 2011). Commercial products based on chitosan are available and they have been shown effectiveness when dissolved in acid solution. Chitosan has a double mechanism of action: it reduces the development of decays caused by fungi, and induces resistance response in the plant tissues (Romanazzi 2010). Moreover, chitosan has been proven to control numerous pre- and post-harvest diseases on various horticultural commodities. It has been reported that both soil and foliar plant pathogens among fungi, bacteria and virus can be controlled by chitosan applications (Bautista-Banos *et al.,* 2006). Perdones *et al.,* (2012) reported that the use of chitosan-based films (alone or in combination with other natural compounds such) was effective in controlling the decay of cold-stored strawberries. In addition to the direct antifungal effect, chitosan can also activate several biological processes in the treated plant, including the accumulation of chitinases, proteinase inhibitors and pathogenesis-related proteins (PR proteins), the synthesis of phytoalexins , the increase of cell wall lignification (El Ghaouth *et al.,* 1994) and callose formation (El Hadrami *et al.,* 2010). Pre- and post-harvest chitosan treatment of table grapes, strawberries and sweet cherries reduces the decay in the field and during storage (1%) (Romanazzi 2010).

5. Proteomic analysis

Biological sciences are experiencing an extensive revolution based on the use of ‘omics tools. Proteome-wide functional classification using bioinformatics approaches is becoming an important method for revealing unknown protein functions (Muturi *et al.,* 2010). Proteomic involves the use of biological, biochemical, genetic and other techniques to simultaneous study thousands of proteins: It allows to catalogue, decipher the structure, speculate on the interaction and function of a large group of proteins produced in specific systems or associated to a studied process (Pandey and

Mann 2000; Gorg *et al.,* 2004). Moreover, comparative proteomics, based on two-dimensional electrophoresis (2-DE) coupled to tandem mass spectrometry, has the potential to screen many metabolic pathways simultaneously for alterations at the protein level. Nowadays, comparative proteomic is becoming attractive to plant biologists as the availability of nucleotide sequences increases, providing new opportunities for protein identification. The accumulation of nucleic acid data, in parallel to the advancements in sequencing technologies, has permitted the development of better performing methods for the analysis of protein content also with non-model plants (Bianco *et al.,* 2013). In recent years, research on *Arabidopsis thaliana* demonstrated that proteomic is a very powerful tool for studying molecular mechanisms of plants. Although many methods have been reported, optimized protocols for the preparation of protein extracts suitable for 2-DE have mainly been developed for young vegetative plant tissues that have high protein content and low amounts of contaminants. However, it is very difficult to obtain high quality protein suitable for 2-DE analysis from fruits, because of the low protein content and the presence of extraction-interfering substances such as pigments, carbohydrates, polyphenols, polysaccharides and starch (Song *et al.,* 2006).

6. Induction of systemic resistance (ISR) in the plant

Induced resistance (IR) is a state of enhanced defensive capacity developed by a plant reacting to specific biotic or chemical stimuli (Van Loon *et al.,* 1998). In the last decade, it has become clear that elicitation of ISR is a widespread phenomenon caused by a variety of non-pathogenic microorganisms including biological control agents. Induced resistance depends on the recognition of a stress by the plant. This generates a cascade of events, eventually leading to the expression of defence mechanisms that include physical barriers and/or metabolites and proteins interfering with the spread of the invading microorganism. IR may be expressed locally as well as in uninfected parts of the plant (ISR). In this case, the initial recognition event also leads to the production of an endogenous systemically translocated signal that can activate resistance mechanisms in remotely located tissues. Research on ISR- is focused on determining the factors involved in this process, in order to identify novel molecules that are useful for diagnostics, selective crop breeding, bio-pesticide and biotechnological product development (Harman *et al.,* 2004, Kuc 2001; Bakker *et al.,* 2003). Biocontrol agents and their metabolites can induce systemic resistance (ISR) as observed *in*

planta, thus improving the control of different classes of pathogens. This phenomenon has been observed in many plant species, both dicotyledons and monocotyledions (Abadi 2008; Sticher *et al.,* 1997). Terry and Joyce (2004) indicated that induction of natural disease resistance (NDR) in harvested horticultural crops by using physical, biological and/or chemical elicitors should considered as one of the preferred strategies for disease management. In the post-harvest research field, studies indicated that also fruits respond to stresses by activating the production of phytoalexins and pathogenesis-related proteins (PR proteins) (Ben-Yehoshua 2003). Inducible defence-related proteins have been described in many plant species upon infection with oomycetes, fungi, bacteria, viruses or insect attack. They have been classified into 17 families (Van Loon *et al.,* 2006). PRs are defined as proteins coded by the host plant, that are induced specifically in pathological or stress conditions, do accumulate both locally and systemically in the infected plant, and are associated with the development of systemic acquired resistance (SAR) (Van Loon *et al.,* 1999). Several PR proteins have direct antimicrobial activity against plant pathogens (Van Loon *et al.,* 1997).

Plants synthesize an enormous variety of metabolites that can be classified into two groups based on their function: primary metabolites, which participate in nutrition and vital metabolic processes within the plant, and secondary metabolites (also referred to as natural products), which influence ecological interactions between plants and their environment (Rodriguez-Concepcion and Boronat 2002). Phytoalexins are antimicrobial secondary metabolites produced *ex-novo* by the plants as a defence mechanism in response to biotic and abiotic stresses (Afek and Sztejnberg 1988; Ahmed 2011; Beerhues 2011). They are extensively studied as promising plant disease-controlling molecules (Gonzalez-Lamothe *et al.,* 2009). Phytoalexins accumulation and their role in fruit resistance to pathogens have been studied in banana, capsicum and citrus (Ben-Yehoshua *et al.,* 1992; Ahmed 2011). In apple tree, the biosynthesis of biphenyl and dibenzofuran phytoalexins has been confirmed (Beerhues 2011), but the accumulation of phytoalexins in apple fruits has been very poorly investigated so far.

Aim of the work

Essential oils of plants and herbs are important natural sources of bioactive substances. In fact, there are increasing scientific evidences that support the use of essential oil against human and plant diseases.

The BCA *Trichoderma* and its metabolites are widely used as biological tools for plant protection against different types of stresses.

The aim of the present work was to design a new bio-formulation based on a synergistic mixture of plant essential oils and *Trichoderma* culture filtrates (CF) for the control of post-harvest disease of pome fruits.

The resulting commercially applicable product could be used against major post-harvest pathogens, produced cheaply, applied easily and safely with no harms for humans and the environment.

To reach this goal we used a variety of techniques in order to achieve:

1. Extraction of essential oils from several plants, selected (*in vitro* and *in vivo*) on the base of toxic effects on important post-harvest pathogens of apples (*P. expansum*, *B. cinerea* and *A. alternata)*, as well as on the fruit.
2. Production of *Trichoderma* culture filtrates (CF) having a strong antifungal activity (*in vitro* and *in vivo*) against main post-harvest pathogens (*P. expansum*, *B. cinerea* and *A. alternata*).
3. Induction of mechanisms of disease resistance in apple fruits by using the selected essential oils and the *Trichoderma* culture filtrates, as determined by phytopathological assays and molecular analyses of plant response (i.e. tests of phytoalexins and PR-proteins accumulations).
4. Designing of a new bio-formulation made of a synergistic combination of plant extracts and fungal metabolites (CF), eventually potentiated by using additional natural products acting as adjuvants with multiple beneficial effects.

Materials and Methods

1. Essential oils extraction, analysis and phytotoxicity

1.1. Essential oils extraction using steam distillation

We used cleaner production of essential oils by using the simple steam distillation method without using any solvents only water steam. Every 100 gm from cinnamon (trunk bark), cloves (dried flower buds), and black pepper (dried fruits) had been ground using WARING blinder HGBSSE, USA for 3 minutes. The distillation method was done using 400 ml water for 100 gm plant material. The mixture was allowed to stand overnight at room temperature for the hydrolysation and then hydrodistilled using a stationary distillatory (Falc distiller, Italy) at 100 °C. The distillate containing water and the essential oil was collected into several separatory funnels, and allowed to stand for 2 h to separate the oil from the water phase. The water phase was discarded.

1.2. Gas chromatography-mass spectrometry (GC-MS) characterization

Resolution gas-chromatographic (HRGC) analyses of essential oils and identification peaks with mass spectrometer detector (MS) were performed. 5 µl of the essential oils were dissolved using 95 µl of ethyl acetate, then 1 µl from this mixture was injected into the gas chromatograph. Perkin Elmer Autosystem XL gas chromatograph equipped with a fused silica capillary column SP 2380 (Supelco, Bellefonte, USA) 100 m X 0.25 mm; 0.20 µm film thickness, was used. The column was held at 100°C for 5 min after injection, heated at 3 °C min^{-1} to 165 °C, held at 165 °C for 10 min, and then heated at 3 °C min^{-1} to 260 °C and held at the final temperature for 28 min. The injector temperature was initially set at 50 °C for 0.1 min, increased at 400 °C min^{-1} up to 260 °C and held for 10 min. Split ratio was 1:60, and gas carrier (H2) flow was set at 20 cm s^{-1}, FID temperature was set at 260 °C (Romano *et al.,* 2010).

1.3. Phytotoxicity of cinnamon, clove and black pepper essential oils

Tomato seed germination and root elongation test is used to assess the essential oils toxicity. Water agar media contains 0.05%, 0.025%, 0.012%, 0.006%, 0.003%, 0.0015% and 0.0008% from each oil was applied in Petri dish (90 mm). After media solidification sterilized tomato seeds (agitated for 30 min in sodium hypochloride "NaClO", 1 % as chlorine) were put in one row and each treatment had 15 replicates. The Petri dishes were then wrapped with Parafilm then were maintained for 7 days at 25 °C under dark conditions on horizontal position. Root elongation was recorded after seven days from incubation.

2. *In vitro* antifungal activity of cinnamon, clove and black pepper essential oils

P. expansum was friendly donated from Dr. Simona Sanzani Department of Plant Protection and Applied Microbiology, Faculty of Agriculture, University of Bari, Italy. While *B. cinerea* and *A. alternata* was friendly donated from Dr. Michelina Ruocco, Italian National Research Center (CNR), Napoli, Italy.

The obtained fungi were used for pathogenesity test. The healthy apple fruits (*Malus domestica*, cv. golden delicious) were disinfected in sodium hypochloride (NaClO, 0.2 % as chlorine) for 5 min and rinsed under tap water, dried at room temperature and punctured with a sterile needle at the equatorial region (3 mm depth, 3–4 mm wide, 3 wounds per fruit). Wounds were inoculated by spore suspension (20 µl 3 x 10^5 spores/ml) from 15 days old cultures. The pathogens were reisolated after 7 days from the artificially diseased fruits using potato dextrose agar Petri dishes.

2.1. Antifungal activity of pure essential oils by direct application

The pathogens plugs were placed at the centre of Petri dishes containing one-fifths of PDA. Ten microlitre of the essential oil (25%, 10%, and 5% concentrations diluted by water then agitated for 30s using vortex at 1800 rpm were applied on the top of each plug. The untreated pathogens plugs considered as controls. The pathogen growth was measured after 3 days as colony diameter. Each treatment was replicated three times.

2.2. Volatile antifungal activity of pure essential oils

This assay was done according to Wilson *et al.,* (1997) with some modificatuions. Rapid assay using microtiter plates was used to determine the volatile fungicidal activity of the essential oils on spore

germination of the pathogens. Four µl of 100%, 50%, 25%. and 12.5% concentrations from the essential oils (diluted by mineral oil) were used as different treatments. The oil was applied on the attached parafilm for multi-well plate lid. In order to precisely align the oil droplet, the parafilm was attached to the top of the multi-well plate lid by pressing, so that an imprint of the top of the wells appeared on the parafilm. The oil droplets were then placed in the centre of each well imprint. After the oils were pipetted onto parafilm, the plate lid was placed over the multi-well plate with the parafilm down. Microtiter wells were inoculated with 200 µl of the pathogen spore suspension (3 x 10^5 spores/ml). The untreated pathogens spores suspension considered as controls. Each treatment was replicated three times. Subsequent changes in optical density following spore germination in the wells was measured after 48 h using Thermo Sientific Multiscan FC microtiter reader by the program ScanIt for Multiscan FC 2.5.1. The results were recorded as spore germination inhibition (% of control).

3. Post-harvest biocontrol of apple decay disease caused by *Alternaria alternata*, *Penicillium expansum* or *Botrytis cinerea*

Essential oils and *Trichoderma* culture filtrate preparation:

Two concentrations of the essential oils were done:-

1- 10% of essential oil emulsion (10% essential oil, 89% sterilized water and 1% Tween 20; Sigma, USA).
2- 5% of essential oil emulsion (5% essential oil emulsion, 94.5 % sterilized water and 0.5% Tween 20).

The two concentrations were prepared from each essential oil. All the resultant emulsions were shaken for 30 s before application to ensure a homogeneous essential oil mixture.

Trichoderma harzianum strain TMik was grown, left to sporulate and maintained on potato dextrose agar (PDA). Spores were collected by using 10 mL of distilled water with 0.1% Tween-20. *T. harzianum* TMik was grown in two-step liquid cultures. For inoculation fungus disks (PDA Perti dishes) used to inoculate 200mL of potato dextrose broth (PDB) in a 300-mL Erlenmeyer flask. Mycelia were grown at 25° C on a shaker at 120 rpm for 72 h. The mycelium, harvest by filtration through Whatman 3MM paper and washed with deionized water, was immediately used to inoculate a 500 mL flask containing 300 mL defined synthetic media (SM) containing per liter: 680mg

KH_2PO_4, 870mg K_2HPO_4, 1g NH_4NO_3, 200mg KCl, 200mg $CaCl_2$, 200mg $MgSO_4*7H_2O$, 2mg $FeSO_4$, 2mg $MnSO_4$ and 2mg $ZnSO_4$) with supplementation of glucose 1% (w/v) as carbon source. To develop inexpensive substrates for the growth of *T. harzianum* and effective inducers for enzymatic production we used (1%) burley spent grain (BSG). The flasks were incubated at 25°C on a rotary shaker at 120 rpm for 96 h. All the above growth conditions were chosen in order to determine whether extracellular metabolites in culture broth were produced *in vitro* and, possibly, responsible for biological control of *T. harzianum* TMik against *P. expansum, B. cinerea*, and *A. alternata*.

3.1 Direct inhibition of post-harvest pathogens on apple fruits

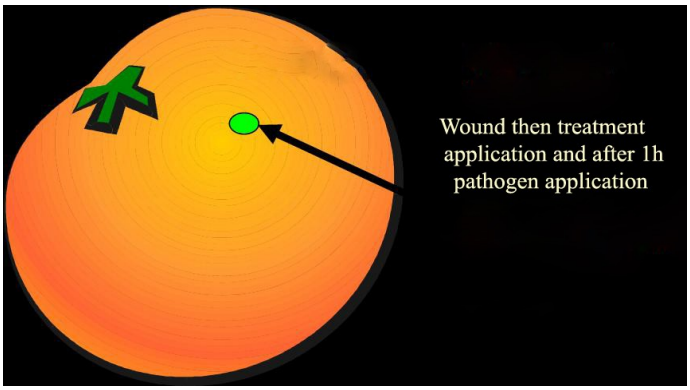


Fig. 1. Direct application of the treatments, the wounded fruits were treated with 10 µl (10 % and 5%) of cinnamon, clove, black pepper oils, or *Tricoderma* CF (100%), and after 1 h were inoculated with 20 µl of *P. expansum, B. cinerea,* or *A. alternata* spore suspensions (3 x 10^5 spores/ml).

Apple fruits obtained from the market were disinfected in sodium hypo-chloride (NaClO, 0.2 % as chlorine) for 5 min and rinsed under tap water, dried at room temperature and punctured with a sterile needle at the equatorial region (3mm depth, 3-4 mm wide, 3 wounds per fruit). Ten µl of the essential oils (10 % and 5 % concentrations) and of *Trichoderma* filtrate were dropped for each wound. The pathogens suspensions (20 µ1 3 x 10^5 spores/ml) were applied after one hour from the treatments application in the same wound as described in (Fig. 1). Then the fruits were packed in growth chamber at 25 °C 60 % humidity and the decayed lesions were measured after 6 days. The inoculated fruits with the pathogens considered as controls. Each treatment was replicated three times, and the experiment repeated twice.

3.2 Induction of systemic resistance in apple fruit

The fruits were sterilized, punctured as described in (3.1). Then, the pathogens suspensions were applied in a new wounds after 24 h from the treatments application, this wound is far away from the first one (treatments) by one centimetre as described in (Fig. 2).

After that, the fruits were packed in growth chamber at 25 °C 60 % humidity and the decayed lesions were measured after 6 days. Each treatment was replicated three times, and the experiment repeated twice.

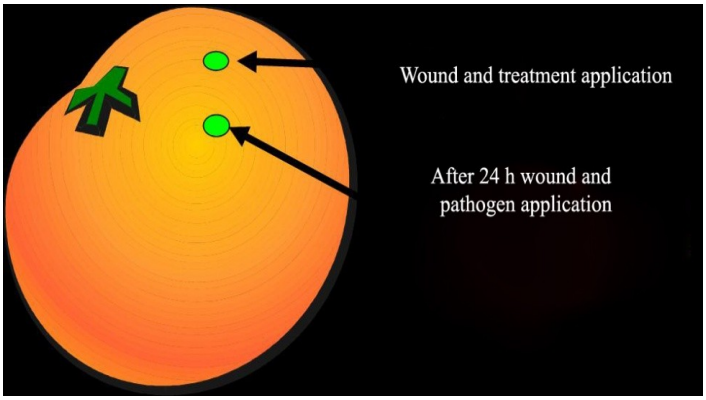


Fig. 2. Indirect application of the treatments, the fruits wounds were treated with 10 µl (10 % and 5% concentrations) of cinnamon, clove, black pepper oils, or *Trichoderma* CF (100%). After 24 h the fruits were inoculated with 20 µl of *P. expansum, B. cinerea,* or *A. alternata* spore suspensions (3 x 10^5 spores/ml) at one centimetre distance from the treatment application.

4. Biofungicide formulation of black pepper essential oil and *Trichoderma* filtrate by using chitosan as emulsifier

Powdered chitosan and acetic acid 98% purity was obtained from SIGMA Chemical Co. (St. Louis, MO). The essential oils and *Trichoderma* CF were obtained as described before. Distilled water was used for the preparation of all solutions. The stock buffer solution was prepared by dispersing 100 mM acetic acid in water and then adjusting the pH to 3 using 1 M HCl. The emulsifier solution was prepared by dispersing 2 % w/v powdered chitosan into stock buffer solution and agitated over night. The emulsion (mixture) was prepared by mixing of 1.25 % v/v black pepper oil and 50 % v/v *Trichoderma* CF with 48.75 % v/v emulsifier solution and homogenizing for 60s by using vortex. The emulsifier solution was used in order to create stable emulsion without droplet aggregation.

4.1. Synergistic interactions between black pepper essential oil, *Trichoderma* culture filtrate and chitosan

In vivo antifungal activity and synergistic effect of black pepper oil, *Trichoderma* CF and chitosan separately and all the possible combinations between them were tested in apple fruit infected by *P. expansum*. Limpel's formula was used to asses the synergetic effect of combinations between black pepper oil, *Trichoderma* filtrate, and chitosan. Limpel's formula is $Ee = X+Y-(XY/100)$, in which *Ee* is the expected effect from additive responses of two inhibitory agents and X & Y are the percentages of inhibition relative to each agent used alone. Thus, if the combination of the two agents produces any value of inhibition greater than *Ee* then synergism exists.

The used emulsions were (1)- 1.25 % v/v black pepper oil (2)- 50 % v/v *Trichoderma* filtrate (3)- 48.75 % v/v emulsifier solution (4) 1.25 % v/v black pepper oil + 50 % v/v *Trichoderma* filtrate (5) 1.25 % v/v black pepper oil + 48.75 % v/v emulsifier solution (6) 50 % v/v *Trichoderma* filtrate + 48.75 % v/v emulsifier solution (7) the mixture of the three contents components. All the resultant emulsions were diluted by water except the mixture and shaken by vortex for 30 s before application to ensure a homogeneous essential oil mixture. Apple fruits were sterilized and punctured as shown in (Fig. 1), the fruits were sub-emerged in the emulsions and after 1 h inoculated by the pathogens. Non sub-emerged fruits were used as control. *P. expansum* pathogen suspension (20 µl 3 x 10^5 spores/ml) was applied after one hour from the treatments application in the same wound. Then the fruits were packed in growth chamber at 25 °C 60 % humidity and the decayed lesions were measured after 6 days. The inoculated fruits with the

pathogen considered as controls. Each treatment was replicated three times, and the experiment repeated twice.

4.3. Induction of systemic resistance in apple fruit

As described before in the direct application (3.1), the indirect application experiment design was the same. But, in the indirect application the pathogens suspensions were applied in a new wounds after 24 h from the treatments application, this wound is far away from the first one (treatments) by one centimetre as described in (Fig. 2).

5. Biochemical characterization of induced resistance in apple fruits caused by plant essential oils or a *Trichoderma* culture filtrate

5.1 Phytoalexins detection and quantification

The samples quantified by HPLC using SHAMAZU LC-10AD Liquid chromatography and 10 µl of the samples were injected to Phenomenex, Prodigy 5µ ODS3 100A, 4.6 X 250 mm diameter column after filtration by 0.45 µm syringe.

The mobile phase A was made of HPLC water plus containing 0.1 % trifloroacetic acid (TFA); the mobile phase B was made of acetonitrile containing 0.1 % TFA. The elution gradient to obtain correct separation of the three deferent phytoalexins at solvent flow 0.5 ml/min was: 0-8 min, 80% (A) and 20% (B); 8-10 min, 65% (A) and 35% (B); 10-30 min, 55% (A) and 45% (B); 30-38 min, 45% (A) and 55% (B); 38-40 min, 35% (A) and 65% (B); 40-45 min, 94% (A) and 6% (B). The phytoalexins were detected using a UV detector, SPD- M10A SHAMAZU Diode Array Detector at extension and emission of wavelengths of 228, 324, and 341; respectively. The phytoalexins were identified by comparison to the retention time of authentic calibration standards and quantified by peal area comparison using standard curves. Phytoalexins concentrations were calculated against authentic standards of scoparone, scopoletin, and umbilliferone ranging from 20-100 µg/ml.

5.2. The apple fruit proteome during the interaction with pathogen, essential oils and/or *Trichoderma* culture filtrate

5.2.1. Fruits application and protein extraction of apple fruits using phenol extraction method

Apple fruits were sterilized, punctured as shown in (Fig. 3). Ten µl of the pathogens suspensions (20 µ1, 3 x 10^5 spores/ml) were dropped in the centre wound; and 10 µl of essential oils (10 %) and of *Trichoderma* were dropped in the other four wounds that surround the centre wound. The distance between the centre wound and the other wounds is 2 centimetres. The control consisted of unwounded fruits, wounded fruits, wounded fruits treated with 1% tween, wounded fruits treated with the pathogen. Each treatment was replicated two times and each replicate consisted of two fruits.

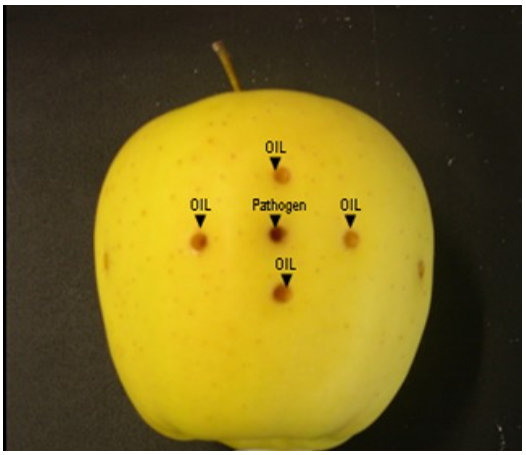


Fig. 3. Apple fruit central wound was inoculated with 20 µl of the pathogen suspension (3 x 10^5 spores/ml), then 10 µl of 10 µl (10 % concentration) of cinnamon, clove, black pepper oils, or *Trichoderma* CF (100%) were dropped in the other four wounds surrounding the central one.

5.2.2. Protein extraction, two dimensional electrophoresis (2-DE) and MALDI- TOF MS analysis

Phenol-extracted proteins were done after 3 days from application accoeding to (Delaplace *et al.,* 2006). Approximately 2 g of apple fruit peels (fresh weight, FW) were collected from distance between the centre wound (pathogen) and the treated wounds (treatment). Then, homogenized using liquid nitrogen and incubated on ice for 10 min with 4 mL of extraction buffer (0.7 M

sucrose, 50 mMEDTA, 0.1 M KCl, 10 mM thiourea, 0.5 M Tris, pH 7.5 with 2 mM PMSF and 50 mM DTT added sequentially). The homogenate was centrifuged (15 min, 13 0006 g, 4°C) and the supernatant was subsequently extracted (by vortexing at 1800 rpm) for 10 min with 5 mL of pH 8.0 buffered phenol at room temperature (RT). After centrifugation (10 min, 6000 g, 4°C), the phenol phase was re-extracted with 5 mL of extraction buffer during 10 min (RT) and centrifuged again using the same parameters. The buffer phase was removed. The proteins contained in the phenol phase were precipitated overnight at –20 °C by the addition of 20 mL of 0.15 M ammonium acetate in methanol and then centrifuged at 20 000 g for 20 min at 4°C. The pellet was washed twice with 4 mL of 0.1 M cold ammonium acetate in methanol and once with 10 mM DTT cold acetone. The washed pellet was air-dried for 30 min at RT and then solubilized in 200 µL of a rehydration buffer modified from (5 M urea, 2 M thiourea, 2% w/v CHAPS, 2% w/v 3-(4-heptyl)phenyl-3-hydroxy-propyl-dimethylammonio-propanesulfonate (C7BzO), 20 mM DTT, 5 mM tris(2-carboxyethyl) phosphine hydrochloride (TCEP-HCl)) during 45 min at RT before storage at -80 °C. The rehydration buffer volume was added in one step to thoroughly wet the inner walls of a 2 mL eppendorf tubes and dissolve the protein pellet using vortex 20 min.

Protein concentration was measured using the Bio-Rad protein assay dye reagent consisting of phosphoric acid and methanol (Bio-Rad Laboratories, Hercules, CA, USA) BSA was used as a standard. The protein concentration was expressed as µg/ml.

The purity and overall quality of protein extracts were evaluated with Laemmli buffer SDS-PAGE. Protein (1 mg) was suspended in 15 µL of loading buffer and transferred to Bio-Rad Mini-Protean TGX Gel. The protein marker which used was Precision plus protein standards BIO-RAD with molecular weight range (250, 100, 75, 50, 37, 25, 20, 15, and 10) kDs. Electrophoresis was conducted using a Bio-Rad mini-Protean™ II apparatus (Bio-Rad Laboratories) at 100 V for 1.5 h. The gel was then stained using EZBlue Gel Staining Reagent (Sigma, USA) overnight and destained with water according to the manufacturer instructions.

Isoelectric focusing and two dimensional electrophoresis (2-DE). Isoelectric focusing (IEF) was carried out by using 7 cm immobilised-pH-gradient (IPG) strips (Bio-Rad, Richmond, CA, USA) with a pH 4-7. IEF was performed on a Protean IEF Cell (BioRad), using 7cm ReadyStrip IPG strips with a linear pH gradient of 4–7 (BioRad). Protein samples (750 mg for preparative gels), were loaded onto strips and soaked in rehydration buffer (final volume 150 µL) as passive

rehydration over night at room temperature, the rehydration buffer containing 5 M urea, 2 M thiourea, 2% w/v CHAPS, 2% w/v 3-(4-heptyl)phenyl-3-hydroxy-propyl-dimethylammonio-propanesulfonate (C7BzO), 20 mM DTT, 5 mM tris (2-carboxyethyl) phosphine hydrochloride (TCEP-HCl)) and 0.2% v/v carrier ampholyte. Prior to the second dimension, the gel strips were equilibrated in 6 M urea, 20% w/v glycerol, 2% w/v SDS, 50 mM Tris-HCl pH 8.8, and 2% w/v DTT for 10 min, followed by 10 min in the same buffer containing 2.5% w/v iodoacetamide. Electrophoresis in the second dimension was carried out using a Protean apparatus (BioRad) and 12% polyacrylamide gels (7 cm) in 25 mM Tris pH 8.3, 0.192 M glycine and 0.1% w/v SDS, with 100 V applied for 1.5 h. Two replicates for each sample were done. Gels were stained overnight with EZBlue Gel Staining Reagent and destained with deionised water 5h. Each protein extract was run on duplicate gels. Gel images were acquired by a GS-800 Imaging Densitometer (Bio-Rad) and analysed with the PD-Quest software. Image files were recorded by using a red filter (wavelength 595-750 nm) and a resolution of 36,3 x 36,3 microns. The signal intensity of each spot was determined in pixel units (Optical Density, O.D.) and normalized to the sum of the intensities of all the spots included in the standard gel. Each spot is indicated with the SSP number (Standard Spot Number) assigned by PD-Quest software. Spot detection and matching between gels were performed. For quantitative analysis, after normalization of the spot densities against the whole-gel densities, the percentage volume of each spot was averaged for two different gels performed.

MALDI-TOF MS analyses were performed as described by (Talamo *et al.,* 2003) using Voyager DE-Pro spectrometer (PerSeptive BioSystems, Framingham, MA, USA) equipped with an N2 laser (λ = 337 nm). The protein spots were excised from gels and digested with bovine trypsin. Tryptic digested peptides were re-suspended in 10 ml of a 1% acetic acid solution. The samples were mixed (1:1) with a matrix of a saturated a-cyano-4-hydroxycinnaminic acid (HCCA) solution [10 mg/ml acetonitrile (ACN) / 0.2% trifluoroacetic acid (TFA), 70/30] (SIGMA). Then, 1ml of the mixture was deposited on the MALDI (matrix-assisted laser desorption/ionisation) plate and allowed to dry under vacuum.

Peptide mass spectra were obtained on a Voyager-DE Pro MALDI-TOF (time of flight) mass spectrometer (Applied Biosystem, Foster City, CA, U.S.A.) equipped with a 337 nm laser and delay extraction, operated in positive-ion reflector mode for the mass range between 890 and 3500 Da. Mass calibration was performed with the ions from human adrenocorticotropic hormone - ACTH

(fragment 18-39) (Sigma) at 2465,1989 Da, and Angiotensin III human (MP Biomedicals, Irvine, CA, USA) at 931,5154 Da as internal standards. Peptide mass fingerprint (PMF) data were matched to the National Centre Biotechnology Information (NCBI) non-redundant database entries against proteins from plant or all species, using Mascot software (Matrix Science, London, UK) (http://www.matrix-science.com). The following search parameters were applied: one missed cleavage by trypsin, mass tolerance: ± 1 kDa, alkylation of cysteine by carbamidomethylation was set as variable modification. The Mascot program compares theoretical and experimental peptides values derived by virtual hydrolysis of proteins present in the database with specific proteolytic agent, and then supplies a list of hypothetical candidates with the probability that the peptides found belong to that species. Similarities between the peptide fragmentation of apple protein clones and known proteins were determined. Moreover, Matrix science database (www.matrixscience.com/home.html) was used to determine if the known proteins were homologous to the analysed protein spots.

Results

1. Essential oils extraction, analysis and phytotoxicity

1.1. Essential oils extraction using steam distillation

One hundred grams of cinnamon dried bark, dried flower buds of clove, and dried fruits of black pepper used for extraction in yielded, by a steam distillation method, 0.9 g, 8.35 g and 1.63 g of pure oil respectively.

1.2. Gas chromatography-mass spectrometry (GC-MS) characterization

Five microliters of each extracted essential oil were dissolved in 95 µl of ethyl acetate and 1 µl of this solution was analysed by gas chromatography mass spectrometry (GC-MS) to obtain the molecular profile (Tab. 2). We found 6 different molecules in cinnamon oil of which the main was the aromatic monoterpene cinnamaldehyde (73.2 %). In clove oil, only 4 different molecules were detected and the predominant one was the phenolic compound eugenol (55.1%). The black pepper was the most complex mixture: it contained 16 different molecules of which the most abundant was the sesquiterpene caryophyllene (21.4 %). Notably, cinnamaldehyde and eugenol were exclusively present in cinnamon and clove essential oil, while caryophyllene was identified in all the three essential oils analysed.

Table 2. GC-MS analysis of the essential oils extracted from cinnamon, clove, or black pepper. In the table are reported only the compounds ≥ 1%.

Major components of the essential oils assessed by GC-MS			
Compound	Cinnamon oil (%)	Clove oil (%)	Black pepper oil (%)
Eugenol	3,62	55,11	-
Cinnamaldehyde	73,24	-	-
1,6-Octadien-3-ol, 3,7-dimethyl	1,13	-	-
Caryophyllene	6,28	33,12	21,4
à-Caryophyllene	1,15	3,43	1,4
2-Propen-1-ol, 3-phenyl-, acetate	6,95	-	-
Phenol, 2-methoxy-4-(2-propenyl)-, acetate	-	5,77	-
Bicyclo[3.1.0]hexane, 4-methyl-1-(1-methylethyl)	-	-	2,35
1R-à-Pinene	-	-	7,19
à-Phellandrene	-	-	11,12
Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene	-	-	9,45
á-Myrcene	-	-	1,88
à-Phellandrene	-	-	2,77
Tricyclo[2.2.1.0(2,6)]heptane, 1,3,3-trimethyl	-	-	10,85
Benzene, 1-methyl-2-(1-methylethyl)	-	-	1,79
Limonene	-	-	15,53
Copaene	-	-	1,67
Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)	-	-	1
Naphthalene, decahydro-4a-methyl-1-methylene	-	-	1,86
Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl	-	-	1,29
Cyclohexene, 1-methyl-4-(5-methyl-1-methylene-4-hexenyl)	-	-	1,15

1.3. Phytotoxicity of cinnamon, clove and black pepper essential oils

In order to determine the phytotoxicity level of the essential oils, tests were conducted by seeding tomato on water agar added with 0.05%, 0.025%, 0.012%, 0.006%, 0.003%, 0.0015% and 0.0008% of each extract. Root elongation was recorded after seven days from incubation. The most phytotoxic oil extract was that from cinnamon, which inhibited root elongation by 55% when used at 0.0015% and 95% at 0.006%. The maximum cinnamon oil concentration that didn't produce phytotoxicity on tomato seedlings was 0.0008%. Clove oil inhibited root elongation by 34% when used at 0.0015% and 97% when applied at 0.012%. The maximum clove oil concentration that

didn't produce phytotoxicity on tomato seedlings was 0.0008%. Pepper oil was not phytotoxic: it didn't compromise the root elongation at any of the tested concentrations (Fig. 4).

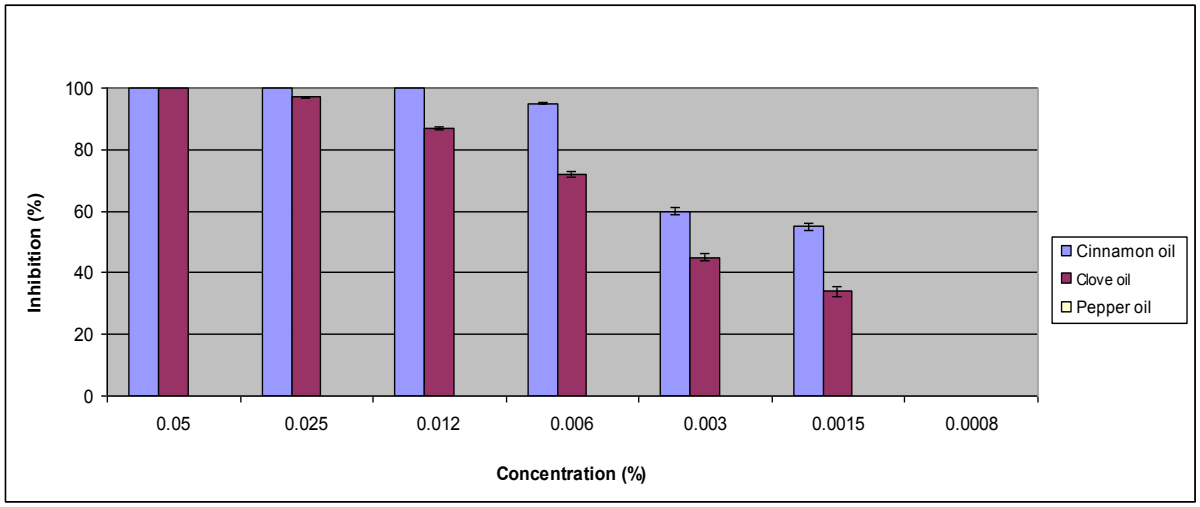


Fig. 4. Effect of cinnamon, clove and black pepper essential oils on tomato root elongation. Tomato seeds were sown on water agar media containing the essential oils at different concentration (0.05%, 0.025%, 0.012%, 0.006%, 0.003%, 0.0015% and 0.0008%).

2. *In vitro* antifungal activity of cinnamon, clove and black pepper essential oils

Two different assays were performed in order to test the antifungal activity of the essential oils extracted from cinnamon, clove, or black pepper.

2.1 Antifungal activity of pure essential oils by direct application

Petri dishes containing 1/5 diluted PDA were inoculated in the centre with the pathogen plugs (*P. expansum*, *B. cinerea*, or *A. alternata*) then 10 µL of a dilution from the essential oils where added directly on the top of the growing colonies. Cinnamon essential oil showed the stronger antifungal activity against the three pathogens: 80% of radial growth inhibition (RGI) was obtained when it was applied at 5% concentration and 100% of RGI when used at 10%. Black pepper essential oil was more active in controlling *A. alternata* and *B. cinerea* than of *P. expansum* radial growth, in fact 80 % of RGI for *A. alternata* and *B. cinerea* was obtained at 5% concentration, while *P. expansum* was inhibited by only 20% of in the same condition (Figs. 5, 6 and 7).

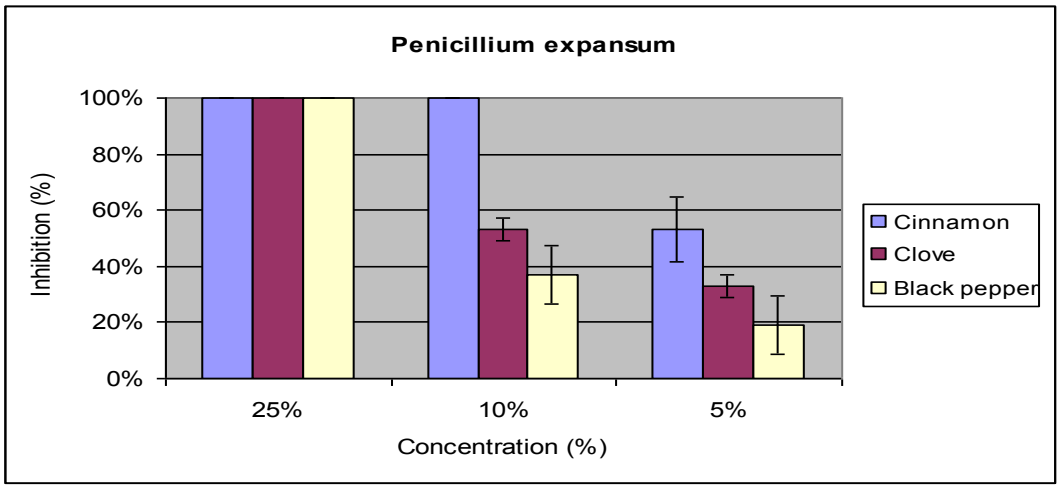


Fig. 5. Radial Growth Inhibition (RGI) of *P. expansum* colony by plant essential oils (cinnamon, clove, or black pepper), 25%, 10%, and 5% were used as dose and the fungal colony diameter was measured 3 days after the inoculation.

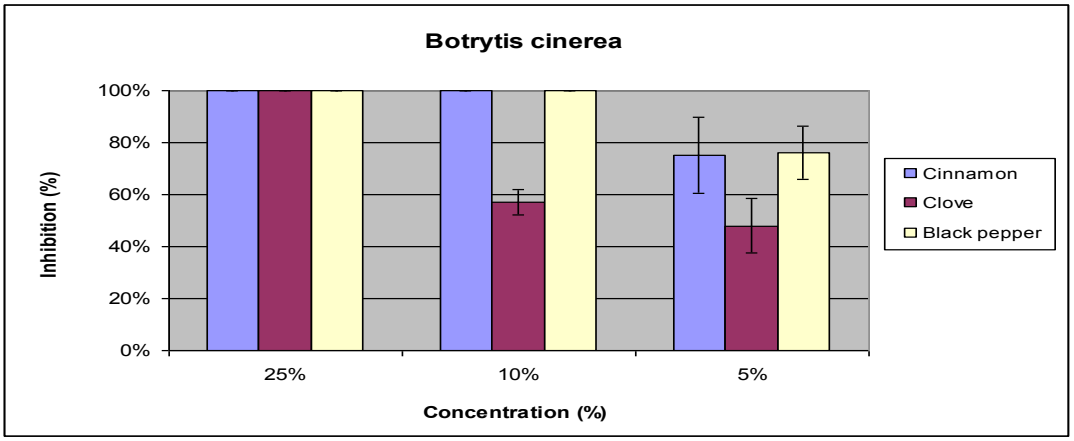


Fig. 6. Radial Growth Inhibition (RGI) of *B. cinerea* colony by plant essential oils (cinnamon, clove, or black pepper), 25%, 10%, and 5% were used as dose and the fungal colony diameter was measured 3 days after the inoculation.

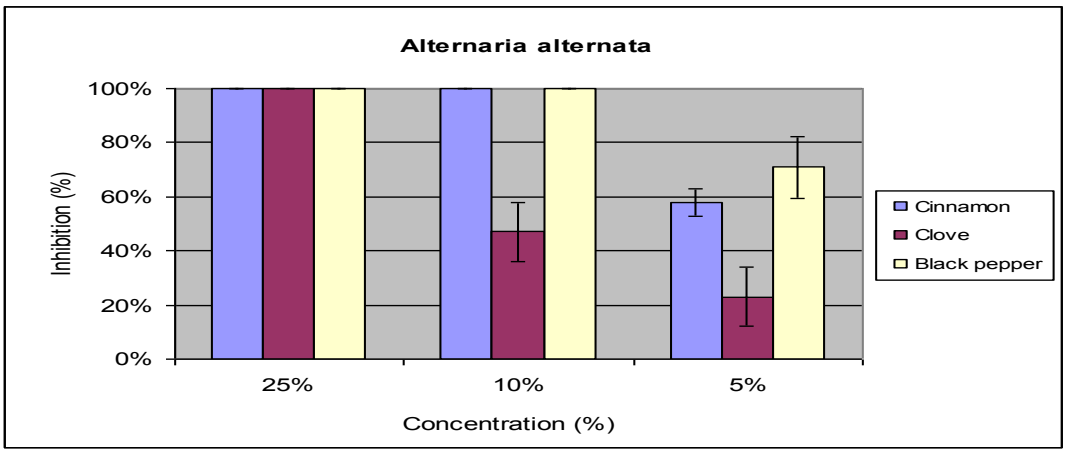


Fig. 7. Radial Growth Inhibition (RGI) of *A. alternata* colony by plant essential oils (cinnamon, clove, or black pepper), 25%, 10%, and 5% were used as dose and the fungal colony diameter was measured 3 days after the inoculation.

2.2 Volatile antifungal activity of pure essential oils

To test the volatile antifungal activity of the three essential oils, a fast assay based on the optical density changes caused by spore germination in liquid media was performed. Four microliters of the essential oils diluted (100%, 50%, 25% and 12.5%) in mineral oil were used for treatments.

The volatile component of cinnamon oil generated a Spore Germination Inhibition (SGI) of 55% on *B. cinerea*, 44% on *A. alternata* and 16% on *P. expansum* (Fig. 8). Similar results were obtained with the volatile component of clove oil (Fig. 9), while the volatile component of pepper had a stronger antifungal activity against the three tested pathogens (Fig. 10).

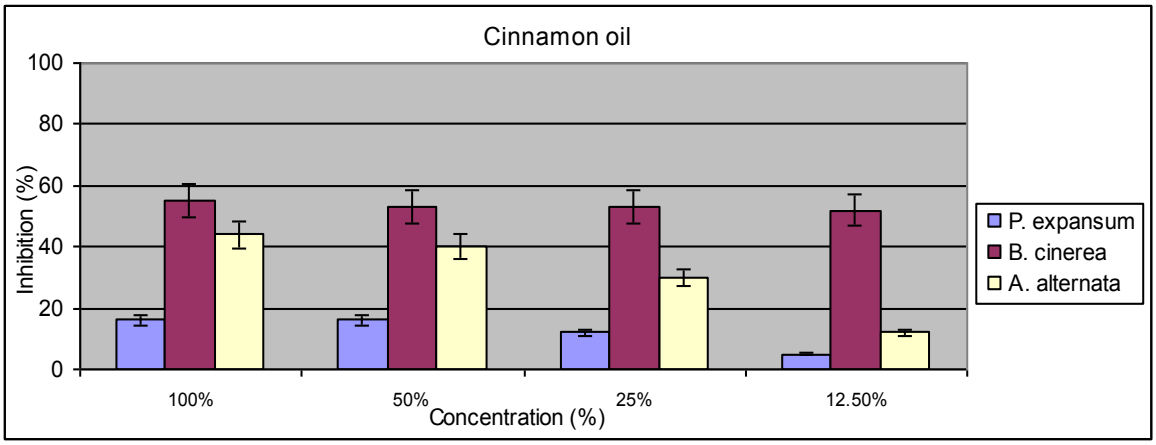


Fig. 8. Spore Germination Inhibition (SGI) of *P. expansum, B. cinerea,* or *A. alternata* by the volatile fraction of 100%, 50%, 25% and 12.5% cinnamon essential oil after 48 hours of interaction with pathogen spore suspension.

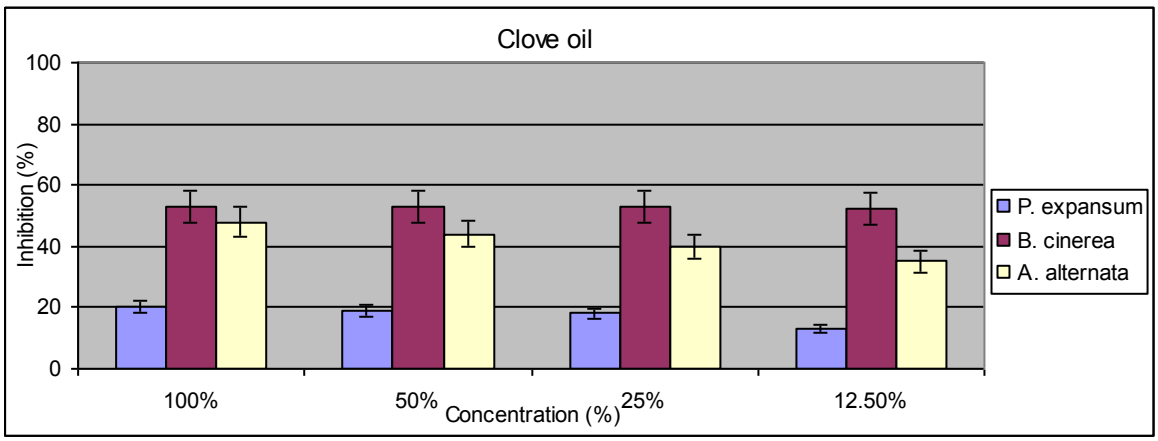


Fig. 9. Spore Germination Inhibition (SGI) of *P. expansum, B. cinerea,* or *A. alternata* by the volatile fraction of 100%, 50%, 25% and 12.5% clove essential oil after 48 hours of interaction with pathogen spore suspension.

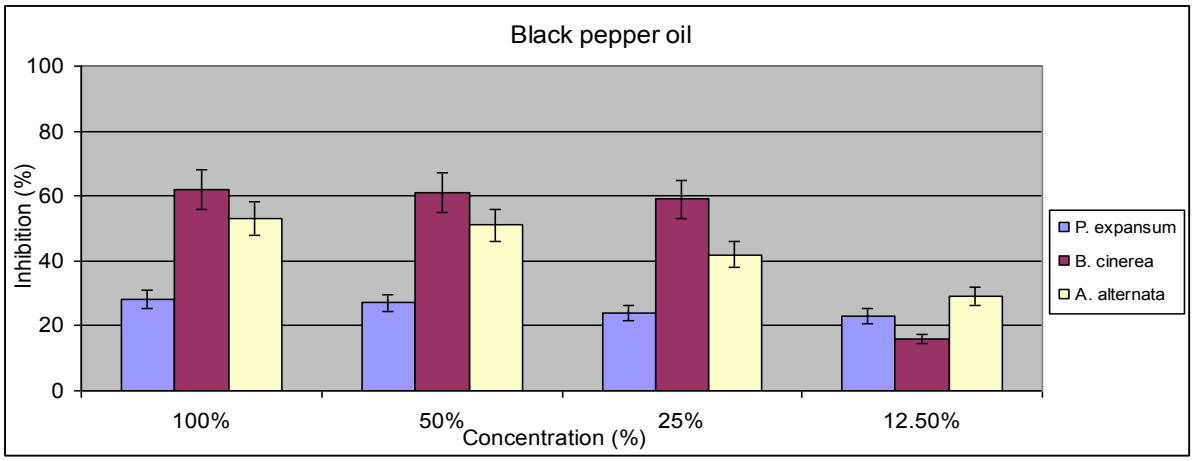


Fig. 10. Spore Germination Inhibition (SGI) of *P. expansum, B. cinerea,* or *A. alternata* by the volatile fraction of 100%, 50%, 25% and 12.5% black pepper essential oil after 48 hours of interaction with pathogen spore suspension.

3. Post-harvest biocontrol of apple decay disease caused by *Alternaria alternata*, *Penicillium expansum* or *Botrytis cinerea*

The efficacy of the three essential oils and a culture filtrate (CF) from the starin MK1 of *T. harzianum* in controlling the development of the pathogens *P. expansum, B. cinerea,* or *A. alternata* on apple fruits has been tested *in vivo*. Direct pathogen inhibition and systemic resistance induction in has been tested.

3.1 Direct inhibition of post-harvest pathogens on apple fruits

To assess the direct effect of the three essential oils or *Trichoderma* CF on the disease development, apples were wounded and loaded with 10 µl of the essential oils or *Trichoderma* CF. Twenty microliters of spore suspension of the pathogen (3 x 10^5 spores/ml) were inoculated after one hour in the same wound were the treatment was applied. The diameter of the decayed lesions was recorded after six days. Pathogens development was completely blocked on apple fruits treated with cinnamon oil 10%. *B. cinerea* development was also strongly impaired by the treatments with cinnamon oil 5% and clove oil (10% and 5%). Black pepper oil and *Trichoderma* CF were the less active in controlling the decay of apple fruits reaching no more than a 11% of disease development inhibition (Figs. 11 and 12).

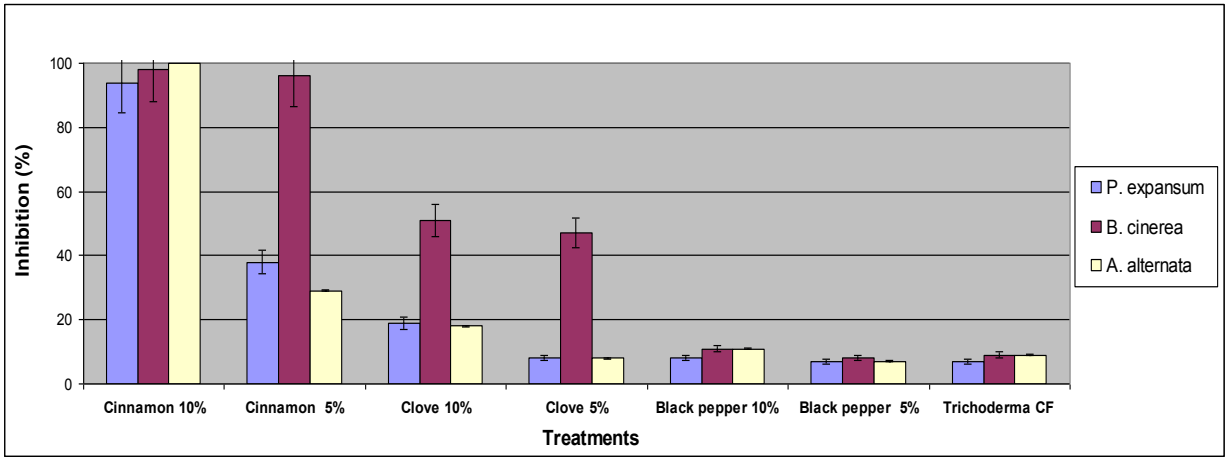


Fig. 11. Direct effect of plant essential oil and *Trichoderma* CF application on decay disease development of apple fruits 6 days from treatments. The wounded fruits were treated with 10 µl (10 % and 5%) of cinnamon, clove, black pepper oils, or *Tricoderma* CF (100%), and after 1 h were inoculated with 20 µl of *P. expansum, B. cinerea,* or *A. alternata* spore suspensions (3 x 10^5 spores/ml).

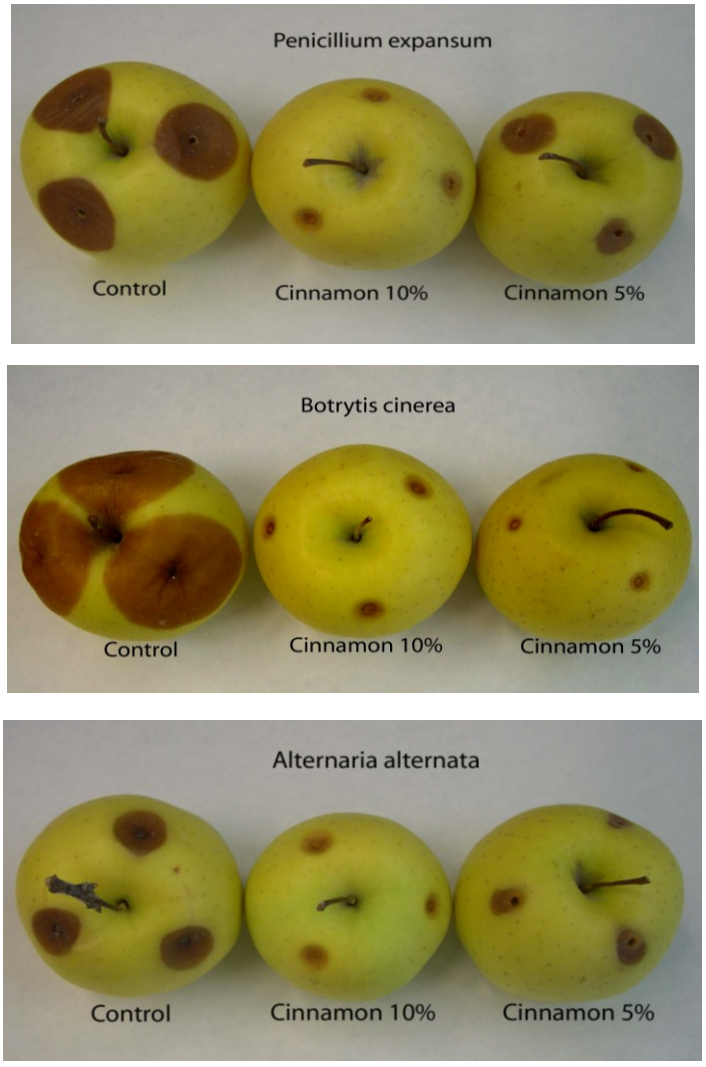


Fig. 12. Effect of cinnamon oil treatments on apple fruit decay development (direct effect on the pathogen).

3.2 Induction of systemic resistance in apple fruit

To verify if the application of essential oils or *Trichoderma* CF induces systemic resistance in apple, the fruits were first treated with the compound and then inoculated with the pathogen in a new wound after 24 h. A good induction of resistance, with up to 29 % reduction of disease development compared with the untreated control, was obtained against *P. expansum* and *B. cinerea* by using cinnamon and clove oils (10% and 5%). *Trichoderma* CF and black pepper oil induced lower level of systemic resistance (up to 8% of disease reduction compared with the control) against the three tested pathogens (Fig. 13).

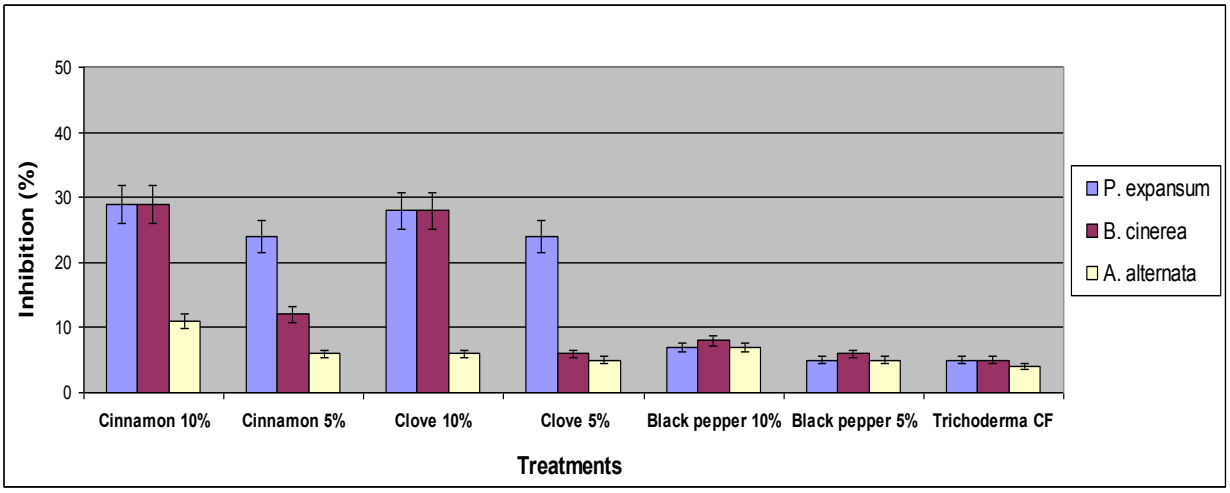


Fig. 13. Effect of essential oil and *Trichoderma* CF on decay disease development of apple fruits by induced systemic resistance. The fruits wounds were treated with 10 µl (10 % and 5% concentrations) of cinnamon, clove, black pepper oils, or *Trichoderma* CF (100%). After 24 h the fruits were inoculated with 20 µl of *P. expansum, B. cinerea,* or *A. alternata* spore suspensions (3 x 10^5 spores/ml) at one centimetre distance from the treatment application, and the data collected after 6 days.

4. Synergistic interactions between black pepper essential oil, *Trichoderma* culture filtrate and chitosan

A combination of essential oil, *Trichoderma* CF and chitosan (used as emulsifier) has been assayed in order to verify the possibility to develop a bio-formulation containing more components acting synergistically with different mechanisms of action synergic between them. Black pepper oil, *Trichoderma* CF and chitosan have been chosen: the first for its antifungal and decay control activities and lack of phytotoxicity, the second for the known direct and indirect antimicrobial activity and the third for its emulsifier property. Apple fruits were sterilized and wounded, then submerged in the prepared emulsions containing the three components in the following ratio: black pepper oil 1.25 % v/v, *Trichoderma* CF 50 % v/v, chitosan 48.75 % v/v, alone or in all the possible combinations. The emulsifier solution (chitosan) was prepared by dispersing 2 % w/v powdered chitosan in 100 mM acetic acid. After 1 h the fruits were inoculated by using the pathogen suspension (20 µl of 3 x 10^5 spores/ml). The incidence of decay, on treated apple, was recorded after six days from pathogen application (Fig. 14).

A synergistic effect in controlling the disease development was found when black pepper oil or *Trichoderma* CF were mixed with chitosan (decay development was reduced by 35% and 22% respectively), but the stronger activity was shown by all the three components applied together. In this case the inhibition of apple decay caused by *P. expansum* was reduced up to 50% (Fig. 14).

In the case of induction of systemic resistance, any of the mixture of the tested compounds showed a synergistic interaction (Fig.14).

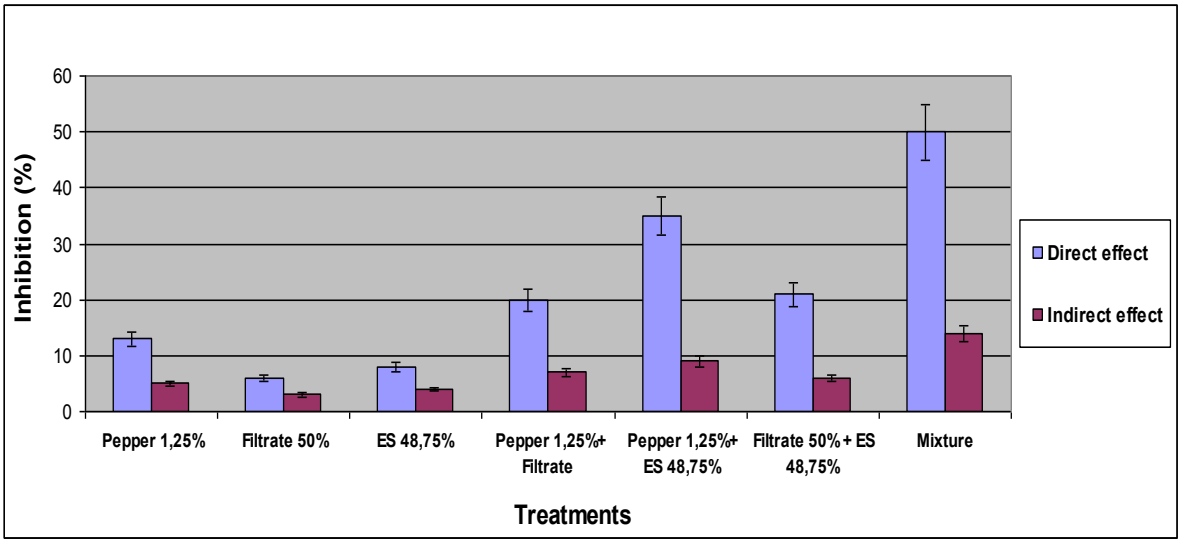


Fig. 14. Inhibition of apple decay by black pepper oil (1.25%), *Trichoderma* CF (50%) and chitosan (48,75%), applied alone and in combination. Disease development was recorded 6 days after *P. expansum* application. Direct effect was tested by submerging the wounded fruits in the compounds and inoculating them after 1 h with 20 µl of *P. expansum* spore suspension (3 x 10^5 spores/ml). Induction of systemic resistance (indirect approach) was tested by inoculating the pathogen at a different sites of the treatment.

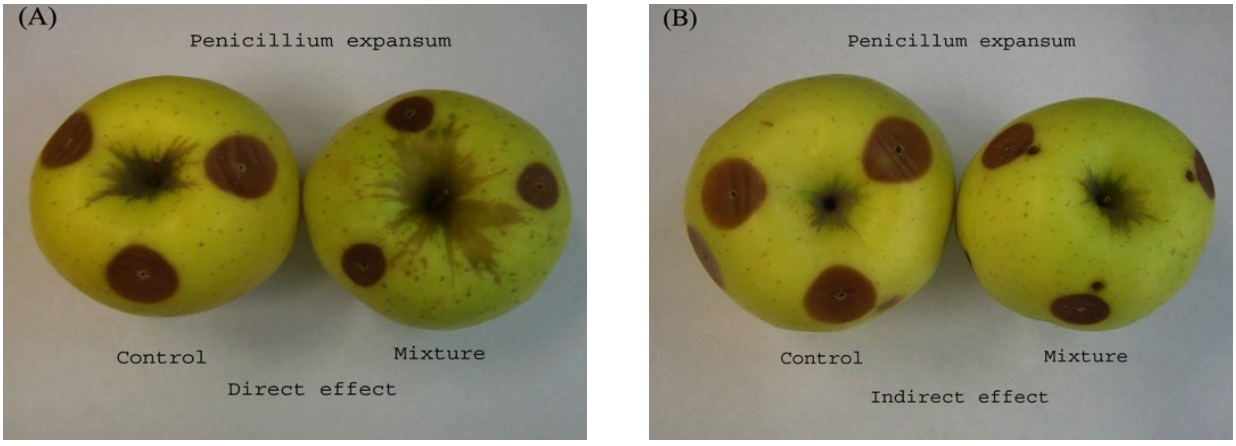


Fig. 15. The direct (A) and indirect effect (B) of treatments with the biocontrol mixture (black pepper oil 1.25 % + *Trichoderma* CF 50 % + ES 48.75 %) on *P. expansum* development on apple fruits.

5. Biochemical characterization of induced resistance in apple fruits caused by plant essential oils or a *Trichoderma* culture filtrate

Induction of resistance in plant is the result of a complex modification of the molecular, biochemical and cellular status of the plant. Several are the metabolites and proteins characterized in plant and know to be involved in these changes. Application of the three essential oils (black pepper, cinnamon, or clove) and of *Trichoderma* CF induced systemic resistance in apple fruits. In order to study the molecular mechanisms involved in the observed induction of resistance in apple fruit, proteome changes and phytoalexin accumulation have been analysed.

5.1. Phytoalexins detection and quantification

By using ethyl acetate, organic extracts were prepared from apples previously (24 and 72 hours) wounded and treated with each of the essential oils (black pepper, cinnamon, clove) or *Trichoderma* CF. The organic extracts were examined by HPLC analysis to monitor the presence of the phytoalexins scopoletin, umbelliferone, and scoparone.

The organic extracts were analysed by using HPLC (SHAMAZU LC-10AD Liquid chromatography "Phenomenex, Prodigy 5µ ODS3 100A, 4.6 X 250 mm diameter column") and the three phytoalexins were detected using florescent light detector. The retention time for the standards corresponding to scopoletin, umbelliferone, and scoparone was 19.3, 19.7, and 22.5 min respectively. The concentration of the three phytoalexins in the samples was calculated by interpolation with the standard curve. The accumulated levels of the phytoalexins were determined 24 and 72 hours after the treatments application. Cinnamon and clove oil treatments induced the three phytoalexins 24 and 72 hours after the treatments (p.t.), while black pepper and *Trichoderma* CF induced umbelliferone and scoparone 24 hours p.t. and scopoletin and scoparone 72 hours p.t. (Fig. 16-17).

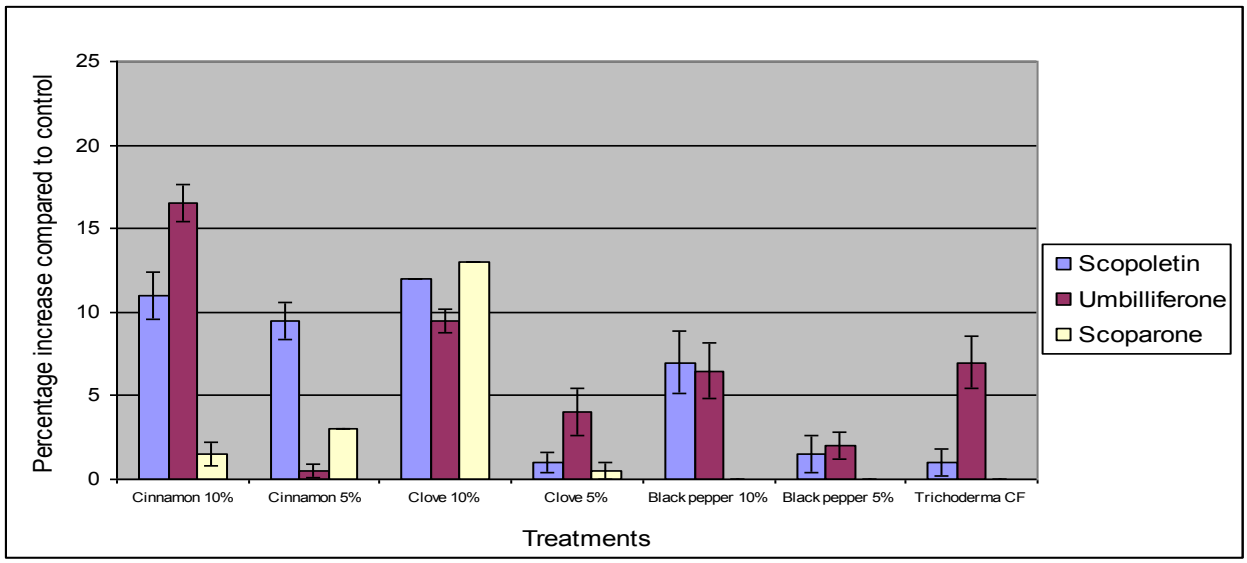


Fig. 16. Increasing ratio of the phytoalexins scopoletin, umbelliferone, and scoparone in apple fruits 24 hours after treatment with black pepper, cinnamon, clove or *Trichoderma* CF as compared with the untreated control.

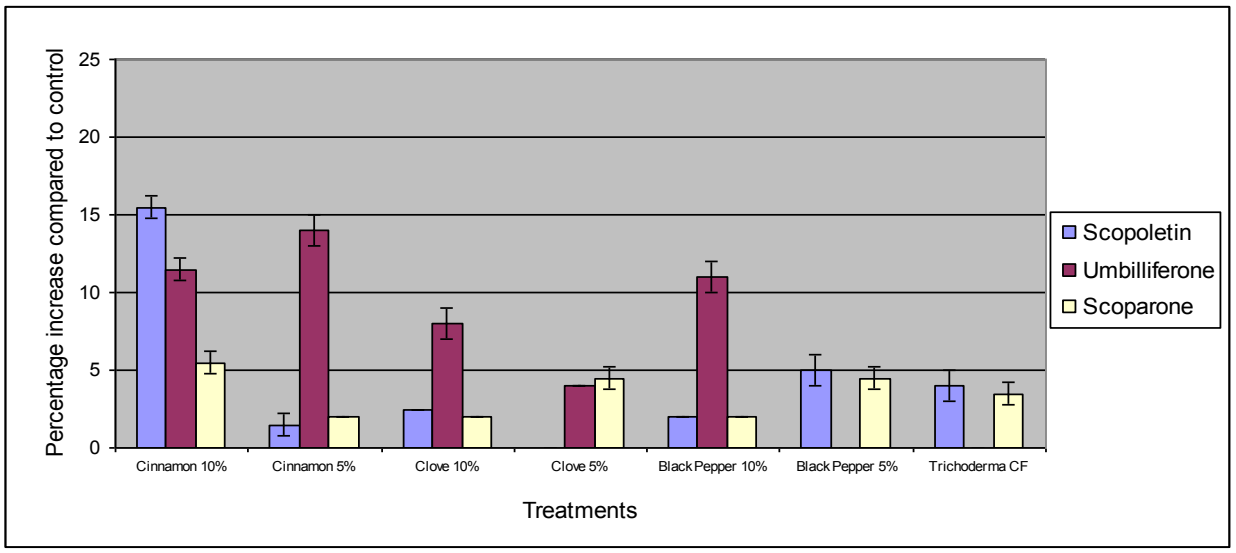


Fig. 17. Increasing ratio of the phytoalexins scopoletin, umbelliferone, and scoparone in apple fruits 72 hours after treatment with black pepper, cinnamon, clove or *Trichoderma* CF as compared with the untreated control.

5.2. The apple fruit proteome during the interaction with pathogen, essential oils and/or *Trichoderma* culture filtrate

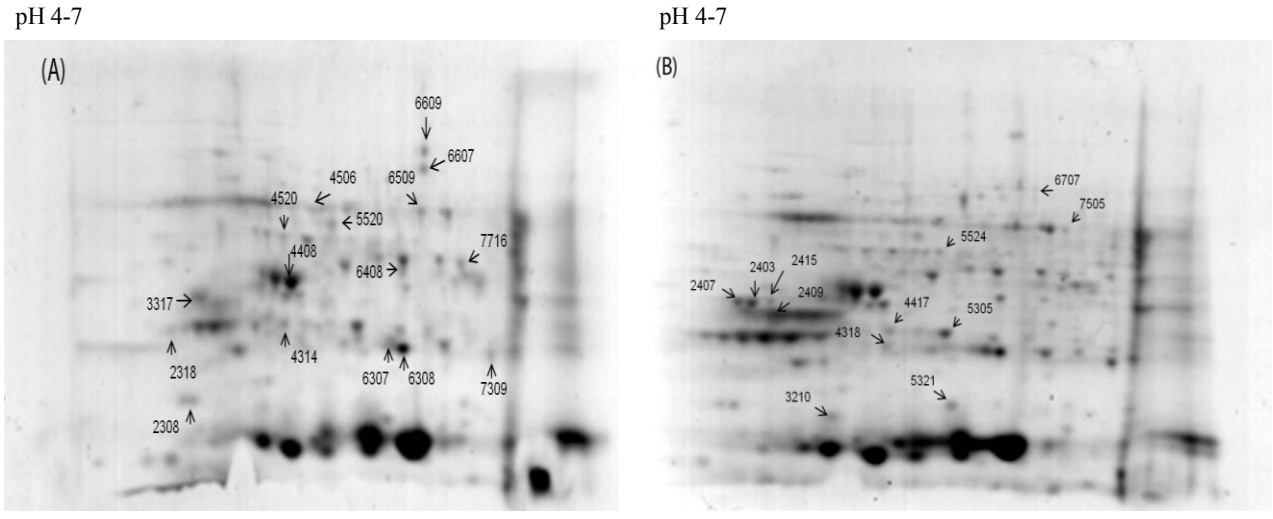
Induction of systemic resistance in apple fruits by using essential oil extracts or *Trichoderma* CF has been assessed by *in vivo* tests (Fig. 3). Biotic and abiotic stresses can strongly modulate the proteome of specific tissues.

5.2.1 Protein extraction and two-dimensional electrophoresis (2-DE) analysis

Proteins of apple fruits were extracted from 21 different interactions (Tab. 3). Apples used for this experiment were treated in the same way as for *in vivo* disease control experiments. In detail, samples to be extracted were collected at sites distant 2 cm from the centre of the wound (where pathogen and/or molecules were applied) 3 days after treatments. Total proteins from apple tissues were obtained by using a phenol extraction method. We used this method followed by ammonium acetate/methanol precipitation because apple fruit tissue is recalcitrant to protein extraction for 2DE analysis. By using this method we obtained a good yield of protein (2 mg of total protein from 2 gr of fresh tissue) and avoided the interference caused by pigments, carbohydrates, polyphenols, polysaccharides, and starch. The passive rehydration and isoelectric focusing (IEF) was carried out by using 7 cm immobilised-pH-gradient strips with a pH gradient 4-7. IEF was performed on a Protean IEF Cell (first-dimensional electrophoresis), and the second dimension (2D) electrophoreses were run by using SDS-PAGE (8–12%); all the gels were stained with Brilliant Blue Comassie (CBB) (Fig. 18).

Table 3. Proteomic analysis experimental design.

Apple treatments	
1- Unwounded fruit (control)	12- Wounded + Clove 10% + *B. cinerea*
2- Wounded + Tween 1%	13- Wounded + Clove 10% + *A. alternata*
3- Wounded + *Penicillium expansum*	14- Wounded + Black pepper 10%
4- Wounded + *Botrytis cinerea*	15- Wounded + Black pepper 10% + *P. expansum*
5- Wounded + *Alternaria alternata*	16- Wounded + Black pepper 10% + *B. cinerea*
6- Wounded + Cinnamon 10%	17- Wounded + Black pepper 10% + *A. alternata*
7- Wounded + Cinnamon 10% + *P. expansum*	18- Wounded + *Trichoderma* culture filtrate (CF)
8- Wounded + Cinnamon 10% + *B. cinerea*	19- Wounded + *Trichoderma* CF + *P .expansum*
9- Wounded + Cinnamon 10% + *A. alternata*	20- Wounded + *Trichoderma* CF + *B. cinerea*
10- Wounded + Clove 10%	21- Wounded + *Trichoderma* CF + *A. alternata*
11- Wounded + Clove 10% + *P. expansum*	



(Cinnamon) (*Trichoderma* culture filtrate)

Fig. 18. Two-dimensional electrophoreses gels (SDS-PAGE gradient 8–12%) of protein extracted from apple tissue. Panel A: proteins obtained from apple treated with cinnamon oil (10%). Panel B: proteins obtained from apple treated with *Trichoderma* CF (100%). Spot numbering refers to Table 5 showing protein identification as obtained by MALDI-TOF MS analysis

5.2.2 Differential protein spots obtained from the PD-Quest analysis of 2D gels

Two dimensional gel analyses performed with the PD-Quest software (Bio-Rad) was found to be useful to generate the 2D maps from the various conditions of interaction, and perform quantitative and qualitative analysis of the differential protein spots. The obtained gels allowed the separation of hundreds of proteins and provided a representative picture of the proteome, as shown in (Tab. 4).

Table 4. Changes occurring in the proteome of apple fruit tissue when different interaction conditions (pathogen, essential oil or *Trichoderma* CF) were compared.

Treatments	**TOT**	**DTOT**	**ON**	**OFF**	**INCR**	**DECR**
1- Tween 1%	89	43	3	6	10	24
2- *Penicillium expansum*	140	63	4	5	46	8
3- *Botrytis cinerea*	133	107	59	21	26	1
4- *Alternaria alternata*	180	75	8	5	58	4
5- Cinnamon 10%	134	55	1	3	19	32
6- Cinnamon 10% + *P. expansum*	195	80	11	22	0	47
7- Cinnamon 10% + *B. cinerea*	153	60	9	3	46	2
8- Cinnamon 10% + *A. alternata*	143	65	20	23	0	22
9- Clove 10%	114	89	18	45	24	2
10- Clove 10% + *P. expansum*	204	166	63	46	53	4
11- Clove 10% + *B. cinerea*	129	105	34	52	9	10
12- Clove 10% + *A. alternata*	224	136	47	45	18	26
13- Black pepper 10%	246	152	59	47	35	11
14- Black pepper 10% + *P. expansum*	175	139	41	56	37	5
15- Black pepper 10% + *B. cinerea*	164	128	29	54	20	25
16- Black pepper 10% + *A. alternata*	160	127	33	59	26	9
17- *Trichoderma* culture filtrate (CF)	228	66	11	33	6	16
18- *Trichoderma* filtrate + *P .expansum*	158	132	57	48	27	5
19- *Trichoderma* filtrate + *B. cinerea*	103	80	27	36	13	4
20- *Trichoderma* filtrate + *A. alternata*	141	106	36	38	30	2

TOT = Total number of spots
TOTD = Total number of deferential spots
ON = Number of spots present in the treatment compared to the unwounded control
OFF = Number of spots present in the unwounded control compared to the treatment
INCR = Number of spots whose intensity in the treatment increased more than two fold compared to the unwounded control
DECR = Number of spots whose intensity in the treatment decreased more than two fold compared to the unwounded control

We extracted a substantial number of proteins from all apple tissues analysed, and obtained a satisfactory 2D electrophoresis separation. PD-Quest analysis showed a consistent modification of all the interactive proteomes taken into account. In fact, the obtained differential spots (TOTD) varied from a minimum of 43 in tween 1% up to 166 in clove plus *P. expansum* treatment (Tab. 4). The data are summarized as follow:

A) Only pathogens treatment

The protein profile of the interaction between apple fruit and pathogens compared with the unwounded control indicated different responses. The apple proteome of the interaction with *P. expansum* showed 63 differential proteins (TOTD) of which 4 novel, 5 absent, 46 increased and 8 decreased. The proteome of the interaction with *B. cinerea* presented 107 differentially accumulated proteins of which 59 novel, 21 absent, 26 increased and 1 decreased. *A. alternata* treatment produced an apple proteome containing at least 74 differentially produced proteins of which 8 novel, 5 absent, 58 increased and 4 decreased.

B) Essential oils or *Trichoderma* culture filtrate treatment

The proteomes obtained from these interactions compared with the unwounded control were very different from each other. Proteome obtained from cinnamon oil treatment presented 55 differentially accumulated proteins of which 1 novel, 3 absent, 19 increased and 32 decreased. The apple proteome obtained after clove oil treatment contained 89 differentially accumulated proteins of which 18 novel, 45 absent, 24 increased and 2 decreased. Notably in the proteome of black pepper oil-treated apple 152 differentially accumulated proteins were detected of which 59 novel, 47 absent, 35 increased and 11 decreased. Similarly, the proteome obtained after *Trichoderma* CF treatment presented 66 differentially accumulated proteins of which 11 novel, 33 absent, 6 increased and 16 decreased.

C) Cinnamon oil plus pathogens treatment

Data from proteome analysis of the interaction between apple fruits, cinnamon oil and the three pathogens compared with unwounded control were interesting as well. The proteome profile of apple treated with cinnamon plus *P. expansum* showed 80 differentially accumulated proteins of which 9 novel, 3 absent, 46 increased and 2 decreased. Even the presence of *B. cinerea* and

cinnamon produced changes in the apple proteome; we identified 60 differentially accumulated proteins of which 9 novel, 3 absent, 46 increased and 2 decreased spots. Apple proteome from cinnamon plus *A. alternata* treatment showed 65 differentially accumulated proteins by which 20 novel, 23 absent, 0 increased and 22 decreased.

D) Clove oil plus pathogens treatment

Proteomes obtained from the interaction between apple fruits, clove oil and the three pathogens compared with unwounded control also indicated different responses by the fruit. Apple proteome after clove plus *P. expansum* treatment showed 166 differentially accumulated proteins of which 63 novel, 46 absent, 53 increased and 4 decreased. Otherwise, when clove plus *B. cinerea* were applied to apple fruit the obtained proteome showed 105 differentially accumulated proteins of which 34 novel, 52 absent, 9 increased and 10 decreased. Apple proteome obtained after clove plus *A. alternata* treatment presented 136 differentially accumulated proteins of which 47 novel, 45 absent, 18 increased and 26 decreased.

E) Black pepper oil plus pathogens treatment

The interaction between apple fruit, black pepper oil and pathogens also strongly changed the proteome of apple fruit tissue when compared with untreated control. Proteome obtained from black pepper oil plus *P. expansum* treatment showed 139 differentially accumulated proteins of which 41 novel, 56 absent, 37 increased and 5 decreased. In black pepper oil plus *B. cinerea* treated proteome we found 128 differentially accumulated proteins of which 29 novel, 54 absent, 20 increased and 25 decreased. While, black pepper oil plus *A. alternata* treated proteome showed 127 differentially accumulated proteins of which 33 novel, 59 absent, 26 increased and 9 decreased.

F) *Trichoderma* culture filtrate plus pathogens treatment

The apple protein profile obtained with *Trichoderma* filtrate plus *P. expansum* treatment showed 132 differentially accumulated proteins of which 57 novel, 48 absent, 27 increased and 5 decreased. When *Trichoderma* CF and *B. cinerea* were added the proteome showed 80 differentially accumulated proteins of which 27 novel, 36 absent, 13 increased and 4 decreased. While, *Trichoderma* filtrate plus *A. alternata* treatment caused changes in proteome of 106 differentially

accumulated proteins of which 36 novel, 38 absent, 30 increased and 2 decreased.

5.2.3 MALDI-TOF MS analysis of the most important protein spots

By comparing between all the apple proteome obtained, 29 of the most interesting differentially accumulated proteins were further identified by MALDI-TOF MS. To determine protein identity, these protein spots manually picked, digested with trypsin and subjected to a MALDI-TOF MS analysis. Because of the poorly solved apple genome a precise identification was very difficult. In fact, this analysis allowed us to classify with a high score three proteins from *Malus domestica,* one from *Fragaria ananassa*, one from *Arabidopsis thaliana*, one from *Allium cepa*, one from *Hordeum vulgare*, two from *Homo sapiens,* and twenty from different microorganisms (Tab. 5).

Table 5. Spots/proteins identified in *Malus domestica* cv Golden Delicious by peptide mass MALDI-TOF fingerprint (MF) and classified according to functional categories. Spot number, protein name, gene ID number, peptide sequence coverage, organism, peptide molecular weight, gene code, and protin function are listed.

Spot N	Prottein name	Gen Bank gi or Gene 3D	Peptide sequence coverage	Organism	Nominal Mass - Molecular weight (Da)	Gene	Function
7716	Mal d 1.0209	3.30.530.20	81	*Malus domestica*	17514	PR-10	Pathogenesis-related protein (PR-10) and defence response protein
5321	Mal d 1 (isoform 1)	3.30.530.20	45	*Malus domestica*	17528	PR-10	Pathogenesis-related protein (PR-10) and defence response protein
6707	Mal d 1 (isoform 2)	3.30.530.20	49	*Malus domestica*	17528	PR-10	Pathogenesis-related protein (PR-10) and defence response protein
6609	Allergen Fra a 1	3.30.530.20	58	*Fragaria ananassa*	11113	PR-10	Pathogenesis-related protein (PR-10) and defence response protein
2407	Brevinin-2Dye	-	75	*Rana dybowskii*	3673	-	Antimicrobial protein
7309	Chalcone-flavonone isomerase	3.50.70.10	55	*Allium cepa*	24156	-	It is one of the key enzymes in the flavonoid (antimicrobial) biosynthesis.
3317	Ribosomal RNA small subunit methyltransferase H	4784173	44	*Methylibium petroleiphilum*	34315	rsmH	Specifically methylates the N4 position of cytidine in 16S rRNA
4314	Ribosomal protein L10	5967423	100	*Mycobacterium abscessus*	18157	rplJ	helps coordinate tRNA movement through the large subunit
2318	Ribosomal protein S20	281416192	97	*Rhodoferax ferrireducens*	10783	rpsT	Binds directly to 16S ribosomal RNA
4318	50S ribosomal protein (isoform 1)	3.30.1390.20	39	*Chlorobium sp.*	6948	rpmD	Translational repressor protein
4211	30S ribosomal protein (isoform 1)	2736539	49	*Bdellovibrio sp.*	10172	rpsQ	Binds together with S18 to 16S ribosomal RNA
2308	50S ribosomal protein (isoform 2)	3.30.190.20	37	*Liberibacter asiaticus*	24700	rplA	Translational repressor protein
6607	30S ribosomal protein (isoform 2)	7379047	29	*Thermotoga neapolitana*	18013	rpsG	Binds together with S18 to 16S ribosomal RNA
4417	Bifunctional cystathionine gamma-lyase	-	63	*Arabidopsis thaliana*	34304	DES1	Involved in maintaining cysteine homeostasis through desulfuration of L-cysteine.

6308	Alcohol dehydrogenase	269929286	74	*Thermoplasma acidophilum*	35885	-	Alcohol dehydrogenase is our primary defense against alcohol.
6307	Protein translocase subunit SecA	5424713	24	*Xanthobacter autotrophicus*	10180	secA	ATP-driven molecular motor driving
6408	Tubulin polymerization-promoting protein family	395841984	80	*Homo sapiens*	25000	TPPP	This family encodes a 25 kDa protein that is phosphorylated by a Ser/Thr-Pro kinase.
6509	Junctional adhesion molecule A	338723351	64	*Homo sapiens*	32562	F11R	Interacts with the orthoreovirus sigma-1 capsid protein
4506	Virion infectivity factor	2.60.40.10	44	Simian immunodeficiency virus	26070	vif	Counteracts the innate antiviral activity of APOBEC3G
4520	Cysteine-tRNA ligase	1480253	25	*Methanosarcina mazei*	53827	cysS	Has a role in aminoacyl-tRNA synthetase and ATP binding.
5305	dual-specificity RNA methyltransferase	6319006	33	*Clostridium botulinum*	39746	rlmN	Intermediate methylation of a conserved cysteine residue
5520	Dihydroorotate dehydrogenase (quinone)	7062774	26	*E. coli*	36794	pyrD	It catalyzes the conversion of dihydroorotate to orotate with quinone.
5524	Thiazole synthase	6332377	31	*Sulfurihydrogenibium sp.*	28689	thiG	Has a role in thiamine biosynthesis.
4408	Thiazole synthase	3.20.20.70	33	*Salmonella choleraesius*	26808	thiG	Has a role in thiamine biosynthesis
3210	Mycothiol acetyltransferase	9166933	21	*Thermobispora bispora*	32168	mshD	The biosynthetic pathway for mycothiol is catalyzed by mycothiol synthase (MshD), which acetylates the cysteinyl amine of cysteine–glucosamine–inositol
2415	Ornithine carbamoyltransferase	-	85	*Pseudomonas putida*	4144	arcB	It catalyzes the formation of the amino acid citrulline
2409	Subtilisin-chymotrypsin inhibitor	3.30.10.10	80	*Hordeum vulgare*	8958	-	Protease inhibitor and serine protease inhibitor
2403	Ferrous iron transport protein	1.10.10.10	41	*Klebsiella pneumoniae*	8795	feoC	It works as a transcriptional regulator that controls feoABC expression.
7505	Flagellar transcriptional regulator	5816233	34	*Bordetella petrii*	11754	flhD	Activates expression of class 2 flagellar genes

5.2.4 *In silico* protein identification

As expected, all the treatments modified the proteome of apple fruits by activating the accumulation of a variety of different proteins compared with the control. Between all the differential expressed proteins, 29 proteins were further analysed by using MALDI-TOF and identified by *in silico* analysis. They were subdivided in 4 groups: pathogenesis-related proteins belonging to PR-10 sub-family, antimicrobial proteins, enzymes involved in the biosynthesis of antimicrobial compounds pathway and ribosomal proteins (Tab. 6).

Table 6. Proteome spots of 29 deferentially accumulated proteins in apple fruits treated as indicated below.

Spot N	Protein name	Treatments																				
		Wou.	Twe.	Pen.	Bot.	Alt.	Cin.	Cin.+ Pen.	Cin.+ Bot..	Cin.+ Alt.	Clo.	Clo.+ Pen.	Clo.+ Bot.	Clo.+ Alt.	Pep.	Pep.+ Pen.	Pep.+ Bot.	Pep.+ Alt.	Fil.	Filt.+ Pen.	Filt.+ Bot.	Filt.+ Alt.
7716	Mal d 1.0209	17.5	14.5	35.8	35	3.7	29	4.3	39.9	12.4	12.5	40	5	39	9.4	21.2	7.9	26.1	0	0	0	0
5321	Mal d 1 (isoform 1)	0	0	0	0	0	17.2	2.3	0	0	0	0	29.7	0	7.9	0	0	0	1.5	0	0	0
6707	Mal d 1 (isoform 2)	0	0	0	0	2.2	0	0.5	0	0	0	0	0	0	2.6	1.2	0	0	3.4	0	0	0
6609	Allergen Fra a 1	0.4	0	3.4	22.3	1.3	1.6	0	0	0	0	6.4	0	0	0	0	0	0	0	0	0	0
2407	Brevinin-2Dye	0	0	0	0	0	0	0	0	2.6	0	0	0	22.2	67	0	0	34.6	2.2	3.8	0	0
7309	Chalcone-flavonone isomerase	4.5	0	0	0	33	2	1	5.2	0	0	0	0	0	12.7	0	0	0	0.5	0	0	0
3317	Ribosomal RNA small subunit methyltransferase	0	0	0	0	0	2	4.9	0	8.1	0	0	0	0	9.7	0	0	0	3.5	0	0	0
4314	Ribosomal protein L10	5	0	0	8.4	0	10	0	40.9	0	0	0	0	0	46.7	0	36.5	0	0.2	18.1	0	0
2318	Ribosomal protein S20	33.5	76.5	0	0	0	0.6	0	0	0		0	0	0	0	0	0	0	0	0	0	0
4318	50S ribosomal protein	0	0	5	0	0	1.1	0	7.2	0	0	0	0	0	9.6	0	0	0	0	0.7	0	0
4211	30S ribosomal protein	0	0	0	0	0	0	0	0	0	0	0	0	0	5.7	0	0	0	0	0	0	0
2308	50S ribosomal protein	0	0	0	0	0	0.6	0	0	0	0	0	0	0	9.9	0	0	0	0.4	0	0	0
6607	30S ribosomal protein	8.1	3.2	2	13	6.7	1.8	1.5	3.7	0	6.4	5.1	7.5	2.4	1.6	5.1	2.7	2	0.4	12.7	8.8	33.2
4417	Bifunctional cystathionine gamma-lyase	0	9.9	0	0	0	1.5	1.2	11.3	5.7	0	0	6.1	0	9.4	0	0	0	1.2	0	0	0
6308	Alcohol dehydrogenase	15	18.4	39	30	55	7.5	7.3	43	3.2	8.2	3	16.1	3.1	8.9	4.2	6.2	1.9	0.5	13.5	9.1	29.2
6307	Protein translocase subunit SecA	0	55.9	0	71	40.2	23.5	5.3	0	0	55	28	45	20	0	0	14.3	30.1	4	55.5	36	48
6408	Tubulin polymerization-promoting	0.9	0	0	0	15.2	1.1	0.5	20.8	0	0	0	0	0	0	0	0	0	0.4	0	0	0
6509	Junctional adhesion molecule A	14.2	7.6	18	10.9	42	4.5	2.6	45	0	12.4	23	18.3	21	23.7	13.5	4.3	17.2	1.8	24.9	12.3	42.3
4506	Virion infectivity factor	14.2	0	0	0	15.3	1.4	0	0	0	0	43	0	8.4	3.3	4.7	0	0	0.2	6.1	0	15.2
4520	Cysteine-tRNA ligase	0	0	0	15.3	25.8	1.4	0.4	0	0	0	0	0	0	0	2.4	0	0	0	7.5	0	0
5305	Dual-specificity RNA methyltransferas	18	13.8	34	5.1	16.5	5.1	0	45	11	41.4	34.7	17.2	20.6	31.1	39.1	8.4	5.6	3.7	32	8.1	38.2
5520	Dihydroorotate dehydrogenase	5.1	0	0	0	0	2.1	0	10.7	0	6.7	0	0	0	13.1	5.6	0.6	0	1.2	1	0.3	0
5524	Thiazole synthase	0	0	7.2	0	23.8	0	0	0	0	0	0	15.2	0	0	0	0	2.5	1.5	0	0	0
4408	Thiazole synthase	27	0	56	76	0	50	0	80	0	12.3	15.4	23.3	0	45.2	0	0	0	40.5	50.5	0	0
3210	Mycothiol acetyltransferase	0	0	0	0	0	1.3	1.2	0	0	0	0	0	0	0	0	2.5	0	1.5	0.5	0	0
2415	Ornithine carbamoyltransferase	0	0	0	0	0	0	0	27.5	0	0	0	0	0	0	0	0	0.4	0.7	58.3	0	0
2409	Subtilisin-chymotrypsin inhibitor	0.7	0	0	0	0	1	1	11.9	0	0	0	0	0	0	0	0	0	7.1	0	0	0
2403	Ferrous iron transport protein	1.9	0	0	0	0	0	0	14.1	7.8	0	0	0	0	0	0	0	0	4.5	0	24.9	21.1
7505	Flagellar transcriptional regulator	1.8	0	2.1	0	12.1	0	0.6	0	0.8	0	0	0	0	1.8	3.6	1.2	1.2	0.4	0.1	0	8.2

The used treatments as following:
(**Unwou.** = Unwounded fruit), (**Twe.** = Tween 1%), (**Pen.** = *P. expansum*), (**Bot.** = *B. cinerea*), (**Alt.** = *A. alternata*), (**Cin.** = Cinnamon 10%), (**Cin. + Pen.** = Cinnamon 10% + *P. expansum*), (**Cin. + Bot.** = Cinnamon 10% + *B. Cinerea*), (**Cin. + Alt.** = Cinnamon 10% + *A. Alternata*), (**Clo**. = Clove 10%), (**Clo. + Pen.** = Clove 10% + *P. expansum*), (**Clo. + Bot.** = Clove 10% + *B. Cinerea*), (**Clo. + Alt.** = Clove 10% + *A. Alternata*), (**Pep.** = Black pepper 10%), (**Pep. + Pen.** = Black pepper 10% + *P. expansum*, (**Pep. + Bot.** = Black pepper 10% + *B. Cinerea*), (**Pep. + Alt.** = Black pepper 10% + *A. alternata*, (**Filt.** = *Trichoderma* filtrate, (**Filt.+ Pen.** *Trichoderma* filtrate + *P .expansum*), (**Filt. + Bot.** = *Trichoderma* filtrate + *B. cinerea*), and (**Filt. + Alt.** = *Trichoderma* filtrate + *A. Alternata*).

Pathogenesis-related protein (PR-10)

We detected four proteins belonging to PR-10 sub-family. Protein spots n° 7716, 5321, 6707, and 6609 were identified as Mal d 1.0209, Mal d 1 (isoform 1), Mal d 1 (isoform 2), and Allergen Fra a 1 respectively (Tab. 6). These PR proteins were strongly overexpressed in the proteome of apple interacting with pathogens alone or in combination with the essential oils, while application of *Trichoderma* CF slightly overexpressed Mal d 1 isoform 1 and isoform 2, but this accumulation was lost when pathogens were applied. Interestingly, Mal d 1 isoform 1 accumulation was not existed in unwounded fruits but seemed to be specifically accumulated when cinnamon, black pepper or clove plus *B. cinerea* were applied to the wounded fruits. Also, Mal d 1 isoform 2 was not existed in unwounded fruits but specifically accumulated when cinnamon, cinnamon plus *P. expansum*, clove plus *B. cinerea* or black pepper were applied. In the same way the protein corresponding to Allergen fra 1 seemed to be exclusively induced by the three pathogens, cinnamon oil or clove plus *P. expansum* application (Tab. 6).

Antimicrobial proteins

The spot n° 2407 corresponding to the antimicrobial protein Brevinin-2Dye was found to be specifically accumulated in the proteomes obtained when *A. alternata* was treated with the 3 essential oils (cinnamon, clove or pepper) (Tab. 5). Spot n° 7309 corresponding to chalcone-flavonone isomerase, an enzyme involved in the biosynthesis of antimicrobial compounds pathway, was found overexpressed only when *A. alternata* or pepper essential oil were applied to the wounded fruits (Tab. 6).

Ribosomal proteins

Between the 29 differential protein spots analysed, seven spots (3317, 4314, 2318, 4318, 4211, 4211, 2308, 6607) corresponded to ribosomal protein were detected (Tab. 6). Ribosome is an intracellular organelle consisting of RNA and protein. It is the site of protein biosynthesis resulting from translation of messenger RNA (mRNA). It consists of two subunits, one large and one small, each containing only protein and RNA. Both the ribosome and its subunits are characterized by their sedimentation coefficients, expressed in Svedberg units (symbol: S). Hence, the prokaryotic

ribosome (70S) comprises a large (50S) subunit and a small (30S) subunit, while the eukaryotic ribosome (80S) comprises a large (60S) subunit and a small (40S) subunit. Two sites on the ribosomal large subunit are involved in translation, namely the aminoacyl site (A site) and peptidyl site (P site). Ribosomes from prokaryotes, eukaryotes, mitochondria, and chloroplasts have characteristically distinct ribosomal proteins. The ribosomal proteins are generally involved in the biosynthesis of constituent macromolecules, assembly, and arrangement of constituent parts of a large ribosomal subunit; includes transport to the sites of protein synthesis. Treatments with pepper essential oil are stronger inducer of this class of proteins with the exeption of the ribosomal protein S20 that was strongly activated only by the wounding and tween treatments. Cinnamon and cinnamon plus *B. cinerea* treatments strongly induced only the ribosomal protein L10 and 50S, while clove treatments were not inducer for this class of protein except for a 30S ribosomal protein.

Other proteins

Spot n° 6308 corresponded to an alcohol dehydrogenase. These enzymes convert alcohol to acetaldehyde, which is then quickly transformed into acetate and other molecules that are easily utilized by the cells. Alcohol dehydrogenase was strongly overexpressed in the proteome of apple interacting with pathogens, cinnamon plus *B. cinerea* or *Trichoderma* CF plus *A. alternata*.

Spot n° 6307 corresponded to SecA protein which functions as an ATP-driven motor. Protein translocation and membrane protein insertion are essential processes for the biogenesis of any living cell. In bacteria, the main route for protein secretion and membrane protein insertion is formed by the Sec-pathway. We found that the protein translocase subunit SecA was strongly expressed when pathogens, clove plus pathogens or *Trichoderma* CF plus pathogens were applied to apple fruits.

Spot n° 6408 corresponded to a tubulin polymerization-promoting protein was overexpressed in *A. alternata* and cinnamon plus *P. expansum*, while it disappeared in the majority of other treatments.

Spot 6509 is a junctional adhesion molecule these are a family of glycoproteins, which bind to several ligands, in both a homophilic and heterophilic manner, and are associated with several cytoplasmic partners. Junctional adhesion molecule A protein was found overexpressed in *A. alternata,* cinnamon plus *B. cinerea*, clove plus *P. expansum,* black pepper, *Trichoderma* CF plus *P. expansum* or *Trichoderma* CF plus *A. alternata* treatment (Tab. 6).

Spot n° 4506 was found to be similar to a virion infectivity factor protein. These kinds of proteins are viral virulence factors. We found a similar protein overexpressed in apple fruits when clove

plus *P. expansum* were applied to the fruit.

Spot 4520 corrsponded to a cysteine-tRNA ligase. This enzyme participates in cysteine metabolism and aminoacyl-tRNA biosynthesis. The accumulation of this protein was up-regulated when *B. cinerea* or *A. alternata* were applied to apple fruits.

Spot n° 5305 is a RNA methyltransferase. It has the ability to intermediate methylation of a conserved cysteine residue. In apple fruits it was strongly accumulated especially when *P. expansum*, *P. expansum* plus clove, black pepper or *Trichoderma* CF were applied to apple fruit.

Spot n° 5520 corresponded to dihydroorotate dehydrogenase. This enzyme catalyzes the conversion of dihydroorotate to orotate with quinone. This protein spot showed over expression only in cinnamon plus *B. cinerea* and black pepper treatments, while it less accumulated or disappeared in the other treatments.

Spot n° 4408 is a thiazole synthase; it has a role in thiamine biosynthesis. This protein spot exhibited over expression in *P. expansum*, *B. cinerea*, cinnamon, cinnamon plus *B. cinerea*, black pepper and *Trichoderma* CF plus *P. expansum,* while it less accumulated or disappeared in the other treatments.

Spot n° 3210 is a mycothiol acetyltransferase. The biosynthetic pathway for mycothiol is catalyzed by mycothiol synthase, which acetylates the cysteinyl amine of cysteine–glucosamine–inositol. Mycothiol acetyltransferase protein was not existed in unwounded fruits, but accumulated in cinnamon, cinnamon plus *P. expansum,* black pepper plus *B. cinerea*, *Trichoderma* CF or *Trichoderma* CF plus *P. expansum* treatments. While it less accumulated or disappeared in the other treatments.

Spot n° 2415 was found to be similar to ornithine carbamoyltransferase. It catalyzes the formation of the amino acid citrulline. This protein was not existed in unwounded fruits, but accumulated in cinnamon plus *B. cinerea* or *Trichoderma* CF treatments.

Spot n° 2409 is a subtilisin-chymotrypsin inhibitor; it is a protease inhibitor enzyme. This protein overexpressed in cinnamon plus *B. cinerea* or *Trichoderma* CF treatments, while it disappeared in the majority of the other treatments.

Spot n° 2403 corresponded to ferrous iron transport protein. It works as a transcriptional regulator that controls feoABC expression. It was strongly overexpressed in the proteome of apple interacting with cinnamon plus (*B. cinerea* or *A. alternata*), also it was highly overexpressed in the proteome of apple interacting with *Trichoderma* CF alone or plus (*B. cinerea* or *A. alternata*).

Spot n° 7505 is a flagellar transcriptional regulator protein; it activates the expression of class 2

flagellar genes. It was overexpressed in the proteome of apple interacting with *A. alternata*, black pepper plus *P. expansum* and *Trichoderma* CF plus *A. alternata*.

Discussion

Plant oil extracts have been used for a wide variety of purposes for many thousand years (Jones 1996). Recently, essential oils and other extracts from plants have attracted interest as novel sources of natural products for agriculture use. They have also been screened for potential applications as alternative remedies to treat human infectious diseases and preserve commodities. Particularly, the antimicrobial activity of plant oils is considered for industrial applications. Plant essential oils can be extracted by using several different methods: in this study we used steam distillation because it is practical, inexpensive and generally regarded as a safe method. The yield of essential oil obtained by using the steam distillation may be variable because it depends on the distillation technique, the temperature, the pressure and the time of treatment, as well as the plant part used for the extraction (Stanojevic *et al.,* 2011; Yazdani *et al.,* 2005). For example, the amount of cinnamon oil that can be extracted from leaves is different from that extractable by using the bark. In this study, we managed to obtain an average yield of 0.9 % of essential oil from cinnamon bark, 8.35 % from clove buds and 1.63 % from black pepper fruits. These results are in agreement with those reported in the most recent literature (Li *et al.,* 2013; Makhaik *et al,* 2005; Rouatbi *et al,* 2007).

We used gas chromatography to identify the major components of essential oil. In the case of cinnamon oil, our finding was in agreement with previous reports (Li *et al.,* 2013). GC-MS analysis showed that cinnamaldehyde (73.2 %) is the most abundant component of cinnamon oil, followed by 2-Propen-1-ol,3-phenyl-,acetate (6.95 %), caryophyllene (6.28 %) and eugenol (3.62%). GC-MS analysis of clove oil, also in accordance with the literature (Makhaik *et al.*, 2004) indicated eugenol (55.1%) followed by caryophyllene (33,12 %) a the main components. In black pepper oil, we principally found caryophyllene (21.4 %), followed by limonene (15.53%) and α-pinene (7.19%). This result is different from the report of Fan *et al.* (2011), where they found 35.06% of limonene, 4.31% of α-pinene and 3.98% of caryophyllene. These differences are probably due to the diverse plant material use, while many papers indicated that chemical composition of essential oils is affected by the origin or the vegetative stage of plant material used (Basu *et al.,* 2009; Garcia *et al.,* 2002; Sangwan *et al.,* 2001). In fact, Fan *et al.,* (2011) prepared the essential oil from fresh fruits while we used dried fruits.

Meyer and collaborator (2008) conducted studies on cucumber (*Cucumis sativus*), muskmelon (*Cucumis melo*), pepper (*Capsicum annuum*) and tomato (*Solanum*

lycopersicum) seedlings by applying to soil 0, 2, 5, and 7 days before transplant 0.1%, 0.2%, and 0.3% of clove oil, and found that tomato seedlings were the most sensitive. The 0.2% and 0.3% clove oil concentrations applied as a drench at transplant (0 day) were particularly toxic to seedlings on different vegetable species. Concerning phytotoxicity of cinnamon and black pepper oil, no data are available in the literature. In our study, cinnamon and clove oil, but not black pepper oil, showed a strong phytotoxicity on tomato seedlings.

Plant essential oils have shown antimicrobial activity against a variety of plant pathogens and pests (Cowan 1999). Several studies have explored the potential usefulness of compounds contained in essential oils as antifungal agents (Abdolahi *et al,* 2010). Many of them are monoterpenes (d-limonene, cineole, b-myrcene, anethole, anisaldehyde, carvacrol, carvone, α-felandreno, α-pinene), reported to be able to inhibit spore germination of several plant pathogens (Pereira *et al.,* 2011).

In general, we found the cinnamon oil to represent the most effective mixture in controlling pathogen development, both *in vitro* and *in vivo*. The antifungal activity of cinnamon oil is well known. Kishore *et al,* (2007) reported that cinnamon oil inhibits by 43.6% *A. alternata* growing *in vitro*. It is known that the main component (73.2 %) of cinnamon oil is cinnamaldehyde, an unsaturated aldehyde with a phenyl group attached to it. The most obvious application for cinnamaldehyde is as flavouring agent in chewing gum, ice cream, candy, and beverages, but it is also used as fungicide (Abd Alla *et al.,* 2008) and insecticide. Previous studies showed that treatments with essential oils (1% and 10%) from oregano and thyme controlled apple fruits decay caused by *B. cinerea* and *P. expansum* (Lopez-Reyes *et al,* 2010). Cinnamon oil 0.16% (v/v) or clove oil 0.2% (v/v) were effective in controlling post-harvest fungal pathogens of banana (Ranasinghe *et al.,* 2005). Similarly, our results demonstrated that the *B. cinerea* development on apple fruits can be completely blocked by using cinnamon oil at 10% (v/v) concentration. Furthermore, pathogen development was also strongly impaired by cinnamon oil at 5%.

A good antifungal activity against the three tested pathogens was shown by clove oil applied *in vitro* and *in vivo*. The efficacy of clove oil as an antifungal agent has been previously reported for the control of *P. citrinum* (Xing *et al.,* 2011) and *A. alternata* (Kishore *et al.,* 2007). The main components of clove oil are eugenol (55.11%) and caryophyllene (33.12%); the first is well known for its antiseptic activity and the latter for its antinflammatory effect on mammal cells. Sukatta *et al.,* (2008) suggested that the antimicrobial activity of pure eugenol oil is due to the presence of an aromatic nucleus and a phenolic OH group, which is known to be highly reactive and able to

deactivate enzymes in fungi by forming hydrogen bonds with –SH groups found in the active sites. Our results show that clove oil is effective in controlling *B. cinerea* development *in vivo*. In fact, clove oil at 10% inhibited decay of apple fruits up to 46 %. Also *B. cinerea* development was strongly impaired by treatment with clove oil at 5%. In *in vitro* antifungal assays clove oil applications were the less effective in controlling the three pathogens, especially in the direct application experiments.

Also black pepper essential oil was active both *in vitro* and *in vivo*. In *in vitro* assays, black pepper oil was more active in controlling *A. alternata* and *B. cinerea* than *P. expansum* radial growth. In fact, 80% of RGI of *A. alternata* and *B. cinerea* was obtained when black pepper essential oil was applied at 5%, while *P. expansum* was only inhibited by 20% in the same condition. Singh *et al.,* (2004) reported that black pepper oil was effective in controlling the mycelial growth of *Fusarium graminearum in vitro*. In this work, black pepper oil inhibited the decay development on apple fruits by more than 11%. We suggest that the weak antifungal activity of black pepper oil, as compared to cinnamon and clove oils, was due to the high quantity of volatile components present in the mixture. In fact, black pepper oil was the strongest inhibitor of spore germination on the three pathogens, as tested in the volatile antifungal assay (*in vitro*), compared to cinnamon and clove extracts.

Trichoderma CF was active in controlling the decay of apple fruits; its direct applications produced a 9% of decay reduction against the three pathogens. Application time of the antagonist is an important factor to consider for efficient biocontrol activity. *Trichoderma* CF exhibited its biocontrol activity strongly when applied prior to the pathogens. Chalutz and Wilson (1990) also reported that the efficacy of *Debaryomyces hansenii* in controlling rot in citrus fruit was maintained when applied simultaneously or prior to inoculation with the postharvest pathogen *P. digitatum.* Several papers have demonstrated the effectiveness of CF of *Trichoderma spp.* in inhibiting spore germination and germ-tube elongation of pathogenic fungi (Lorito *et al.,* 1993a and 1993b; Lorito *et al.,* 1994c; Ghisalberti and Rowland, 1993; Schirmbock 1994). Furthermore, they inhibit or degrade pectinases and other enzymes that are essential for plant-pathogenic fungi, such as *B. cinerea*, to penetrate leaf surfaces (Harman *et al.,* 2004). Moreover, Palmieri *et al.,* (2012) mentioned that the severity of downy mildew was significantly reduced in grapevines treated with *T. harzianum* strain T39. Moreover, application of *T. harzianum* CF on rambutans fruits give a good control of major fungal pathogens during post-harvest (Sivakumar *et al.,* 2001). Also, it was effective against *Pythium aphanidermatum* in the production of baby beets (Pill *et al.,* 2011). Few more paper reported results

of application of enzymatic preparations, obtained from BCAs, for post-harvest disease control of pome fruits (Jijakli and Lepoivre 1998). To our best knowledge there are no commercial products based on *Trichoderma* CF for post-harvest apple disease biocontrol.

Induction of natural disease resistance (NDR) in harvested horticultural crops by using biological and/or chemical elicitors has received increasing attention in recent years, and it is considered a preferred strategy for disease management (Terry and Joyce 2004). The ability of *Trichoderma spp.* to induce both localized and systemic resistance in plants against a variety of pathogens was confirmed (Harman *et al.,* 2004, 2006; Walters *et al.*, 2005; Harman 2006). For instance, the yeast *Candida oleophila* applied to wounded lemon fruits was able to increase resistance to *P. digitatum* (Droby *et al.,* 2001)**.** Moreover, application of jasmonic acid can suppress *P. digitatum* on grapefruit through the enhancement of phytoalexin accumulation (Droby and Chalutz 1999).

The ability of essential oils to increase resistance in apple fruits has not been studied so far. Our results provide evidence that cinnamon, clove and black pepper essential oils or *Trichoderma* CF applied to wounded apples are able to increase resistance to *P. expansum, B. cinerea* and *A. alternata* on apple fruits. A good level of induced resistance (up to 29 % disease development reduction) was obtained by using cinnamon and clove oils (at 10% and 5%) against *P. expansum* and *B. cinerea*. *Trichoderma* CF and black pepper oil also induced systemic resistance (up to 8% of disease reduction) against the three tested pathogens. Macarisin *et al.,* (2010) observed that the application of the yeasts *Metschnikowia fructicola* (strain 277) and *Candida oleophila* (strain 182) generate greater levels of super oxide anion (O_2^-) on intact citrus and apple fruit. A recent study by Jin *et al.,* (2009) showed that the increased resistance in peach to *B. cinerea*, *P. expansum* and *Rhizopus stolonifer* after application of methyl jasmonate was associated with a significant increase in H_2O_2 levels in the fruit exocarp. Our results demonstrate for the first time that cinnamon, clove or black pepper essential oils together with *Trichoderma* CF are able to induce systemic resistance in apple fruits during post harvest. This has been demonstrated by using *in vivo* antifungal tests and phytoalexins analysis. The HPLC analysis of apple fruits peel extracts showed that cinnamon and clove oil produce a significant increase in the accumulation of the three phytoalexins scopoletin, umbelliferone and scoparone 24 and 72 hours after treatment. Black pepper and *Trichoderma* CF induced, instead, umbelliferone and scoparone 24 hours after treatment, and scopoletin and scoparone 72 hours after treatment. Phytoalexins are produced by the plants as a defence mechanism in response to biotic and abiotic stresses (Afek and Sztejnberg, 1988). Both scoparone

and scopoletin have been reported to be induced in citrus fruits by treatments with $NaCo_3$ and Na_2Co_3 (Ahmed 2011). To the best of our knowledge no one reported before the phytoalexins induction by essential oils in apple fruits.

The detected activation of systemic resistance in apple fruits treated with the three essential oils and *Trichoderma* CF was further investigated by proteomic analysis. Proteomics is a leading field of science with huge potential. Wilkins and co-workers conceptualised the term 'proteome' to define the expressed complement of a genome (Wasinger *et al.,* 1995; Wilkins *et al.,* 1995). In recent years, research on *A. thaliana* and other plant species demonstrated that proteomic is a very powerful tool in the study of molecular mechanisms in plants (Song *et al.,* 2006). Apple fruit tissues are notoriously recalcitrant to proteomic analysis, due to a low protein content and high concentration of interfering substances such as pigments, polyphenols and carbohydrates, including polysaccharides and starch (Guarino *et al.,* 2007). To overcome this problem, we used phenol extraction followed by ammonium acetate/methanol precipitation. The protein yield obtained (1 mg of total protein from 1 gr of fresh tissue) was higher than that obtained by others researchers (Song *et al*, 2006). Moreover, the quality of the extracted proteins allowed the separation of hundreds of proteins by 2D-electrophoresis, which provided a representative picture of the proteins expressed during the interactions studied. All the treatments applied to the apples strongly modified the protein pattern as compared to the untreated controls. An interesting result concerned the PR-proteins accumulation in apple fruits treated with pathogens as well as with essential oils and *Trichoderma* CF. The over-accumulated proteins, such as Mal d 1.0209, Mal d 1 (isoform 2) and Allergen Fra 1, belong to the PR-10 subfamily. Physical, chemical and biological stress factors, such as microbial infection, up-regulate the transcription levels of a number of plant genes, including those coding for the so-called pathogenesis-related (PR) proteins. PR-10 proteins have a wide distribution throughout the plant kingdom and the class members share size and secondary structure organization. PR-10 proteins are rather small (~ 160 amino acids) with a fold consisting of three α helices and seven antiparallel ß strands. These structural elements enclose a large hydrophobic cavity that is most probably the key to their functional relevance. Also, the outer surface of these proteins is of extreme interest, as epitopes from a PR-10 subclass cause allergic reactions in humans. Evidence that PR-10 proteins might be involved in general defence mechanisms comes from the observation that some of them are induced and accumulate around sites of invasion by viruses (Xu *et al,* 2003), bacteria (Bahramnejad *et al.,* 2010) and fungi (Puhringer *et al.,* 2000). However, the biological function of class-10 PR proteins remains unclear,

despite two decades of scientific research (Fernandez *et al*., 2013). Our results demonstrate for the first time that essential oils and *Trichoderma* CF, similarly to pathogens, can strongly induce PR-10 proteins. This finding is in according with the induction of resistance mechanisms found in apple fruits. Many others proteins were found over-accumulated in the proteomes of apple fruits interacting with pathogens, essential oils and *Trichoderma* CF. They include the antimicrobial proteins Brevinin (Xu *et al.,* 2012) and Chalcone-flavonone isomerase, also correlated with the systemic resistance induction. Wurms and co-workers (2011) found that a significant decrease (80% reduction) of chalcone-flavonone isomerase (CHI) gene expression corresponded to a significant reduction of rot disease incidence in kiwi fruits.

Recently, the use of bioactive carbohydrates, such as chitosan, to control post-harvest microbial diseases has attracted some attention (Badawy and Rabea 2011). Chitosan based commercial products are available and they have been shown the same effectiveness if formulated as biopolymers dissolved in acid solution. Chitosan is known to be able to trigger a defence response in the plant, leading to the formation of physical and chemical barriers against invading pathogens (Walker *et al.,* 2004). Furthermore, chitosan has a double mechanism of action on postharvest diseases: it reduces the decay caused by fungi by direct inhibition and inducing resistance response in fruit tissues (Romanazzi 2010). In fact, Romanazzi *et al.,* (2006) reported that incidence and disease severity of inoculated table grapes with *B. cinerea* were significantly reduced by pre-harvest treatment by chitosan (1%). Its antimicrobial activity depends on the type of chitosan (native or modified), its degree of polymerization, type of plant tissue treated, and environmental conditions. In some studies, the antimicrobial activity increased with the increase of molecular weight, with the effect being stronger on fungi than bacteria (El Hadrami *et al.,* 2010). A new effective bio-formulation has been designed by combining chitosan as emulsifier for black pepper oil and *Trichoderma* CF. The obtained mixture was particularly active against the most important post-harvest pathogen of apple (*P. expansum*) by using very low, homeopathic-level doses. A synergistic interaction in controlling the disease development was found when black pepper oil or *Trichoderma* CF were mixed with chitosan, while the stronger activity was obtained when the three components were applied together. In this case the inhibition of apple decay caused by *P. expansum* was reduced up to 50%. Similarly, Perdones *et al.,* (2012) enhanced the chitosan antifungal activity during cold storage in strawberries inoculated with a spore suspension of *B. cinerea* by adding lemon essential oil.

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