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NEW SUSTAINABLE APPROACHES FOR CONTROL OF POSTHARVEST DISEASES OF CITRUS FRUIT

This thesis is presented for the degree of
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ABSTRACT

Injuries sustained by citrus fruit during harvest and postharvest handling and storage allow the entry of wound pathogens, including *Penicillium digitatum* Sacc (green mold) and *P. italicum* Wehmer (blue mold), the two major pathogens of citrus fruit being the causal agents of green and blue mold diseases, respectively.

Control of these pathogens has classically been conducted with chemical fungicides, but a growing international concern over the often indiscriminate use of synthetic fungicides, the presence of residues in the food and in the environment, as well as the development of fungal resistance and the possible harmful effects on human health, make it necessary to undertake new approaches for disease control that minimize health and environmental risks.

In this work we evaluated the effectiveness of inducers of disease resistance for postharvest control of green mold of citrus. The chemical inducers (acibenzolar-s-methyl-ASM, chitosan, β-aminobutyric acid-BABA) exerted direct antimicrobial activity against of *P. digitatum*, *P. italicum* and *Geotrichum candidum* when tested *in vitro* on PDA and on orange peel extract agar media, with differences related to their concentrations and challenged pathogens. The antimicrobial efficacy of BABA and chitosan was also confirmed by their ability to inhibit *in vitro P. digitatum* conidial germination. Moreover, BABA and chitosan significantly reduced disease incidence and severity when applied on citrus fruit (oranges cvs. Tarocco, Valencia and Washington navel, lemons cv. Femminello and grapefruits cv. Marsh Seedless). On the opposite, none of ASM concentrations conferred protection against *P. digitatum*. The induction of resistance of BABA compound was detected on lemon and grapefruit when *P. digitatum* was inoculated into wounds made at 1 cm from the original BABA-treated sites.

Curative and preventive treatments with *Pseudomonas syringae*, *Trichoderma atroviride* and *Wickerhamomyces anomalus* biocontrol strains were evaluated alone and in different combined mixtures with hot water, sodium carbonate, sodium bicarbonate, ASM and chitosan. Combined applications of sodium bicarbonate, hot water and biocontrol agents efficiently controlled green mold

when treatments were applied 24 and 48 hours before the pathogen inoculation. When sodium carbonate, hot water and biocontrol agents were co-inoculated with *P. digitatum* green mold was significantly controlled as well even if less effectively, whereas fruit inoculated 24h before treatments developed green mold, and no treatment managed to control significantly *P. digitatum.*

We have analysed gene expression levels of chitinase, β-1,3-glucanase, phenylalanine ammonia lyase, gluthatione peroxidase in responses to curative and preventive biocontrol treatments. On the whole, CHI1, PAL and GPX1 over-expressions were observed within 2h after treatments, but only CHI1 over-expresion was observed for each treatment (curative and protectives). Quantitative RT-PCR technique showed that treatment with sodium bicarbonate, hot water and *Pseudomonas syringae* pv. *syringae* 48SR2 induced the highest increases in the levels of chitinase gene transcripts 48h after treatments and 2h after pathogen inoculation. Despite gene expression, only a partial correlation was found with the ability of other treatments to control green mold *in vivo*.

P. syringae pv. *syringae* strains are able produce cyclic lipodepsipeptides correlated with the ability to control postharvest fungal pathogens. In this work treatment with sodium bicarbonate, hot water and *P. syringae* pv. *syringae* 48SR2 generated weak over-expression of *syr*B1 gene and high over-expression of *syp*A in citrus peel tissue 48h after teatment and in absence of *P. digitatum.* However, *P. digitatum* inoculation strongly induced *syr*B1 gene expression and, on the opposite, weakly induced *syp*A genes expression. Pathogen inoculation resulted to be strongly stimulatory only to *syr*B1 expression, suggesting that at least *syr*B1 gene is involved in biocontrol activity.

These data indicate type of treatments that could be successfully used to control post-harvest fruit diseases and suggest that a signal produced by the pathogen is essential for triggering enhanced transcription levels of both defence-related and lipodepsipeptides synthetase genes.

Chapter 1.

INTRODUCTION

Citrus is one of the most important crop in the world, not only for the production, which exceeds by far that of other fruit species, but also, and mainly, because citrus is grown in over 100 countries on six continents, with a worldwide crop production of 122.368.732 tonnes (FAO 2009) exceeding that of watermelons, bananas and grapes. Citrus fruit is enjoyed around the world for its taste, nutritional value and relatively cheap price.

Oranges constitute approximately 57% of the world's citrus production followed by tangerines, mandarins and clem at 18%, lemons and limes at 11%, citrus fruit nes (9%) and grapefruit at 5%. The most popular orange varieties are the Navel and 'Valencia'. Clementine mandarins are highly favoured by consumers for their seedlessness, portability and ease of peeling. Pigmented oranges Tarocco, Valencia and Washington navel, Pigmented grapefruit is gaining popularity over white varieties, with the 'Ruby Red', 'Rio Red', 'Ray Ruby' and 'Star Ruby' cultivars most common.

The Mediterranean basin is the first citrus producing region with a production estimated to be about 20 million tons which is concentrate on fresh market, and the leader producing countries in this area are Spain, Italy, Egypt, Turkey and Morocco.

The majority of the world's citrus crop is harvested manually from October to June in the Northern Hemisphere and from April through November in the Southern Hemisphere. Consequently, citrus is available year-round.

Fruit passing maturity standards is transported in bulk bins to the packinghouse where it is usually drenched with a mixture of fungicide and chlorine to guard against decay prior to packing. Early season citrus is degreened with 1–5 ppm ethylene in rooms maintained at 21–30°C and 92–96% relative humidity for 24–72 hrs to stimulate chlorophyll breakdown. It is then washed, sorted by size, graded for colour and blemishes and treated with fungicides, either separately or in the wax.

1.1 Major Post-harvest Citrus Diseases.

The major post-harvest diseases of citrus can be separated into two categories based on their initial infections. Pre-harvest infections include *Diplodia natalensis* (Pole-Evans), *Phomopsis citri* (Fawcett), *Colletotrichum gloeosporioides* (Penz.) Sacc., *Phytophthora* species and *Alternaria citri* (Ellis & Pierce), and post-harvest infections include *Penicillium digitatum* (Pers.:Fr.) Sacc., *P. italicum* (Wehmer) and *Geotrichum citriaurantii* (Ferraris R. Cif. & F. Cif.) (Whiteside *et al*., 1988; Brown and Miller, 1999; Ohr and Eckert, 1985).

The types of decay and severity are dependent on the climate, varieties, agricultural practices and post-harvest handling practices.

Infection by fungi may occur during the growing seasons, at harvest time, during handling, storage, transport, and marketing (Whiteside *et al*., 1988). Furthermore, handling procedures in the field and during harvest and marketing processes cause various kinds of mechanical injuries (wound, bruising), which are the main sites of entry of pathogens, particularly in environments which are highly contaminated by conidia of *P. digitatum* and *Penicillium italicum* (packing

lines, storage rooms, means for transportation, etc) (Jeffries and Jeger, 1990), responsible for severe economic losses worldwide (Eckert and Eaks, 1989).

1.1.1 Green and blue molds of citrus

The genus *Penicillium* includes 150 species. Relatively few species are economically important plant pathogens (Pitt, 1979). Among the most notable species are *P. digitatum* (Pers.: Fr.) Sacc. and *P. italicum* Wehmer, which cause green and blue mold of citrus, respectively. Postharvest losses of citrus fruits caused by *P. digitatum* and *P. italicum* can account for more than 90% of all postharvest losses in semi-arid production areas of the world.

Green mould caused by *P. digitatum* is a universal postharvest disease of citrus. It is identified by the mass of olive green spores produced on infected fruit. Infection takes place only through wounds where nutrients are available to stimulate spore germination and fruit decay begins at these infected injury sites.

The early infection area appears as a soft watery spot and sometimes it is referred to as clear rot. As the lesion progresses, white mycelia develop and these produce the green spores. The white mycelium develops into a broad zone surrounding this sporulating area and the mycelium produces cell wall-degrading enzymes that cause a break-down of the fruit cell walls.

Within a few days the entire fruit can be covered with green spores. Spoilage of fruit, caused by the spread of spores from diseased fruit onto adjacent fruit, can occur within the shipping container, but green mould spores will only infect damaged fruit in packed cartons.

Blue mould is caused by *P. italicum*, which develops on citrus fruit via injuries like green mould. It occurs in all citrus-producing regions of the world, although it is not as prevalent as green mould. Blue mould is recognised by the mass of blue spores produced in decayed fruit.

Initial lesions are similar to the lesions of green mould, but the spores are blue in colour and are surrounded by a narrow band of white mycelium encompassed by water-soaked rind. Blue mould develops less rapidly than green mould under ambient conditions so that the green mould is often observed in mixed infections. Blue mould is more common in fruit held in cold storage during the summer and

it can spread in packed cartons more readily than green mould, causing a "nest" of decayed fruit. At low relative humidity, the rotted fruit becomes a dry mummy.

Due to that ability to produce large masses of spores, fungi have the potential to develop resistance to postharvest fungicides (Brown *et al*., 1995). Millions of spores of the causal fungi are produced on the surface of infected fruit and these spores are present practically everywhere in the field, packing area, storage room, transit containers, and market place.

In packed containers, fruits infected by *P. italicum* may contaminate healthy ones, producing nests of rotting fruits, whereas *P. digitatum* can only cause infection at injuries to the rind that occur during harvesting and packing, when moisture and particular peel compounds are present to stimulate spore germination (Pelser and Eckert, 1977).

Volatiles emitted from wounded peel tissue of various citrus cultivars had a pronounced stimulatory effect on germination and germ tube elongation of both *Penicillium digitatum* and *P. italicum*; however, *P. digitatum* appeared to be more sensitive to the stimulatory action of citrus peel volatiles (Droby *et al*., 2009). Enzymes such as pectin methylesterase, polygalacturonase, pectin lyase facilitate hyphal penetration by degrading the middle lamella between cells of the rind (El Shaieb and Malibari, 1995).

Other species of *Penicillium,* like *P. ulaiense* causing whisker mould (Hsieh *et al*., 1987), has been considered important on citrus, especially in packing-houses. (Frisvad *et al*., 2000).

1.1.2 Sour rot of citrus

Sour rot, caused by *Geotrichum citri-aurantii*, is second in importance only to *Penicillium* decays as a wound-mediated disease of citrus (Eckert and Brown, 1986). It has been reported in most areas where citrus is grown, occurring on all cultivars. It is particularly troublesome on fruit held in long- term cold storage.

The fungus only infects fruit through injuries and in particular deep injuries that involve the albedo tissue. Sour rot develops more frequently on mature to over-mature fruit with high peel moisture.

The initial symptoms are water-soaked lesions, light to dark yellow and slightly raised, with the cuticle being more easily removed from the epidermis than lesions caused by green or blue mould.

Decayed fruit tissue has a sour odour that attracts fruit flies and these can spread the fungus to other injured fruit during storage. The fungus is present in soil and can reach the fruit surface from windblown or splash dispersed soil and by fruit-soil contact.

Contaminated fruit can spread the pathogen through drenching equipment, soak tanks, pallet bins, washer brushes, belts and conveyors. Infected fruit spreads the disease to sound fruit within the container. The disease develops rapidly at warm temperatures, with an optimum of about 27 °C.

1.2 Post-harvest decay control strategies.

Occurrence and severity of post-harvest decays depend on many factors including the growing region, fruit variety, tree condition, cultural practices, pre-harvest treatments, harvesting method and post-harvest handling procedures.

An effective control can be accomplished using an integrated approach to prevent, reduce, and/or eradicate pathogen infections and disease development during pre- and postharvest stages.

Good cultural practices, including adequate fertilization and pest control, pruning low-hanging branches, use of herbicides to reduce wetness, which results in lowering pathogen populations and disease pressure of *C. gloeosporioides, D. natalensis, P. citri* and *Phytophthora* spp. Post-harvest green mold can be reduced

by removing fallen fruit under citrus trees since fruit infected with *P. digitatum* produce air-borne spores that contaminate fruit surfaces.

Pre-harvest application of chemicals is a common and effective practice for control of certain diseases that occur after harvest. Field application of fludioxonil and azoxystrobin have been effective in post-harvest decay control (Zhang, 2002).

Harvesting is a major source of fruit injuries, providing infection channels and nutrients for *Penicillium* species and *G. citri-aurantii*. Care must be taken to minimize fruit damage during harvesting. Fruit should not be allowed to come in contact with the soil since abrasive soil particles cause injuries during fruit handling and harbor inoculum of *G. citri-aurantii* (Brown and Miller, 1999). Harvest time should be delayed for several days after irrigation or heavy rains to reduce decay and peel injuries.

1.2.1 Physical treatments

Drenching

Drencing fruit with fungicides is a common and effective practice to control post-harvest green mould. Thiabendazole and imazalil are commonly used in the drench treatment for the control of *Penicillium* decays, but have no activity against sour rot. Chlorine is added to thiabendazole drench suspension to control *G. citri-aurantii*, *Phytophthora* species and thiabendazole resistant strains of *Penicillium* species. Recommended concentrations of thiabendazole and free chlorine are 1,000 ppm and 50 ppm, respectively. The optimum pH range of the drench with chlorine is 6.5–7.5. Chlorine cannot be added to imazalil drench since they are not compatible.

However, a heated imazalil drench is used in some packinghouses; it works well since heat can enhance the efficacy of imazalil and it has some sanitizing activity against sour rot and moulds. Three new fungicides, azoxystrobin, fludioxonil, and pyrimethanil, that belong to different chemical classes, are highly effective in managing post-harvest citrus green mold and are being registered for postharvest use in the United States.

Hot water treatments

The use of hot water treatments was one of the earliest nonchemical methods of control investigated to reduce postharvest decay. Hot water treatment are of short duration, easily monitored, leave no chemical residue on the fruit surface, and pathogens may be eradicated even after they have entered the fruit. Curing fruit at 32–36 °C for 36 to 72 hrs is an effective method for the control of green and blue moulds (Ben-Yehoshua *et al.*, 1992; Stange and Eckert, 1994; Plaza *et al.*, 2003). Curing stimulates the formation of phenolic-type compounds and lignin resulting in healing of injuries and increased resistance to decay (Ismail and Brown, 1975). Hot water (50–56 °C) treatments including dipping, drenching, brushing and rinsing reduce post-harvest decay caused by green mould (Ben-Yehoshua *et al.*, 1992; Porat *et al.*, 2002; Smilanick, 2003).

Immersion of lemons in 3% sodium carbonate at 35 °C for 30 seconds is a common commercial practice for control of moulds and sour rot (Smilanick *et al.*, 2002). Ozone is also used in dump tanks to disinfect fruit in some packinghouses. The fungicide sodium o-phenylphenate (SOPP), the only registered post-harvest fungicide having some activity against sour rot, is often applied for decay control. Care must be taken to avoid injury to fruit, often caused by prolonged brushing and excessive brush speed (Miller *et al.* 2001). Washing is followed by a potable water rinse to remove surface contaminants. Fruit is then dried with heated air and graded to remove defective fruit.

Ultraviolet light (UV)

Low doses of short-wave ultraviolet light (UV-C, 240-280 nm wavelength) can be used to reduce fungal decay in harvested fruits and vegetables. At low doses UV-C irradiation target the DNA of microorganisms, and for this reason UV-C treatment has been used as a germicidal or mutagenic agent. The efficacy of the UV-C treatment is affected by a variety of factors including, positioning of the fruit in relation to the irradiation source, type of fruit, cultivar or fruit maturity, which can cause significant variations in efficacy (Janisiewicz *et al.*, 2010).

The detrimental effect of UV-C includes tissue structural damage, changes in cytomorphology and changes in water permeability of inner epidermal cells (Lichtscheidl-Schultz, 1985). Nevertheless, low doses of UV-C irradiation stimulated beneficial reactions in biological organs, a phenomenon known as hormesis (Sharma, 2009). It has been reported that hormetic doses of UV-C can prolong the postharvest life and maintain the quality of tropical fruits. These effects include delay of senescence process and fruit ripening (Gonzalez-Aguilar *et al*., 2007), induction of natural defence and elicitors against fungi and bacteria (Alothman *et al*., 2009).

In addition to the direct germicidal activity, UV-C irradiation can modulate induced defence in plants. UV-C can stimulate accumulation of the stress-induced phenylpropanoids and PR proteins associated with induced resistance and reduction of lemon and grapefruit susceptibility to *P. digitatum* (Ben-Yehoshua *et al*., 1992; Porat *et al*., 2003). Increased UV-C dose lead to higher concentrations of the phytoalexin scoparone induced in flavedo tissue (Ben-Yehoshua *et al*., 1992).

In fruits such as apple, peach, citrus fruits, grapes or tomato, significant reductions in postharvest decay have been observed after UV treatment (Wilson *et al*., 1994; Wilson *et al*., 1997; Chalutz *et al*., 1992; Liu *et al*., 1993; Stevens *et al*., 2005).

Ozone

Ozone has traditionally been used as a water disinfectant throughout the world (Nickils and Varas, 1992). It can also be used to reduce fungal spore contamination in water used for handling fruits and vegetables in packinghouses, and therefore reduce new infections (Smilanick *et al*., 2002).

Gaseous ozone continuously released at low doses (0.3 or 1 ppm, v/v) is able to inhibit the sporulation of several important postharvest pathogens of table grapes, stone fruit, and citrus fruit (Palou *et al*., 2001). Sporulation of *Penicillium digitatum* and *P. italicum* on cold-stored oranges or lemons was suppressed without injuring the fruit.

Ozonated water, however, has been reported to be not effective in controlling infections from wounds on pears or citrus fruits inoculated before ozone treatment (Smilanick *et al*., 2003; Spots and Cervantes, 1992).

1.3 Fungicide application.

In the packinghouse is one of the most important steps for successful control of post-harvest decay. Currently, the blue and green moulds are controlled with pre and postharvest applications of fungicides such as sodium ortho1phenylphenate (SOPP), thiabendazole (TBZ) and imazalil (IMZ) (Ismail and Zhang, 2004; Kinay *et al*. 2007). These fungicide treatments are effective in controlling preexisting, established or new infections occurring in pack houses.

Imazalil and thiabendazole are used in an aqueous suspension before waxing or in the water emulsion wax. Recommended rates are 1,000 ppm for aqueous application and 2,000 ppm when used in the wax. The higher rates of the fungicides used in wax are due to the reduced efficacy of fungicide-wax combinations. Applying heat to imazalil greatly enhances its efficacy in decay control. Imazalil also has good activity for control of green and blue mould sporulation and it provides some activity for control of *Alternaria* stem-end rot.

Refrigerated storage and shipping of citrus fruit is an effective measure for decay control, maintenance of fruit quality and extension of shelf life. Appropriate temperatures of storage for citrus fruit depend on many factors including variety, susceptibility to chilling injury and production region. Recommended storage temperatures vary with the growing region. In Florida, grapefruit is stored at 10 to 15 °C; tangerines at 4.4 °C; and oranges at 0 to 1.1 °C. Oranges grown in other regions such as California, Egypt or Spain are usually shipped and stored at higher temperatures than those recommended for Florida oranges.

Packinghouse sanitation is an important component of the overall strategy to minimize decay, reduce losses and increase grower returns. Numerous sanitizers are approved for use on fruit and/or packinghouse equipment and chlorine is by far the most commonly used. At a concentration of 200 ppm, chlorine is effective against most microbial agents, including moulds and bacteria (Brown and Miller, 1999; Pao and Kelsey, 1999) in soak tanks and on the surface

of fruit. To achieve maximum sanitizing effect from chlorine, the pH must be maintained at 6.8 to 7.0. Quaternary ammonium compounds are commonly used to sanitize equipment and harvesting containers. Although these compounds are not approved for direct use on fruit, their regular application in the packinghouse contribute to lowering the inoculum levels and reducing post-harvest decay. Formaldehyde at 1 to 3% is used to fumigate packinghouse storage rooms (Brown and Miller, 1999) and is effective in eradicating spores of green and blue moulds as well as mycelia of the sour rot causing fungus.

Fungicide resistance management

Penicillium resistance to thiabendazole, imazalil and SOPP is a severe problem since resistant strains develop readily in large populations of spores. A number of strategies are used to reduce fungicide resistance problems: 1) sanitation of packinghouses, of storage rooms and use of sanitizers such as chlorine and quaternary ammonium; 2) rotation and/or mixing of fungicides with different modes of action; 3) application of broad spectrum biocides such as sodium bicarbonate and biological control agents to fruit before storage; 4) application of fungicides with specific modes of action immediately before shipment (Brown and Miller, 1999). Pyrimethanil, fludioxonil and azoxystrobin have different modes of action to thiabendazole, imazalil and SOPP and these new chemicals could provide more chemical tools to manage fungicide resistance.

Non-fungicidal measures to reduce postharvest decay

Some markets require fruit that has not been treated with any post-harvest fungicides. To provide such fruit, care must be exercised in the grove, implementing proper cultural practices, and during packing, shipping and storage to minimize decay (proper sanitation of the packinghouse, minimizing injuries to fruit during harvesting, packing, storage and shipping) thus reducing infections by mold and sour rot pathogens.

1.4 Biological control.

Postharvest losses of fruits and vegetables are caused to a large degree by pathogenic microorganisms and affect the cost and acceptability of the product, reduce storage time, and represent a source of risk for human and animal health because of the accumulation of cancerogenic mycotoxins in contaminated food.
Losses from postharvest spoilage of fruit and vegetables have been managed primarily by applying fungicides (Holmes and Eckert, 1999) but often pre- and postharvest treatments are inefficient and significant levels of rots occur during storage, transport and marketing. However, the use of chemicals is becoming increasingly restricted because of the development of pathogen resistance and consumer concerns about fungicide residues in the food. These issues have resulted in an intensive search for safer control options that pose minimal risk to human health and to the environment (Wilson *et al*., 1994).

Biological control holds great promise of providing safer alternatives to present postharvest synthetic fungicides and reducing the health and environmental hazards that they pose. Use of fungi, yeast and bacteria to control postharvest decay of several agricultural commodities has been studied and examples of successful disease control exit (Janisiewicz and Korsten, 2002; Fravel, 2005; Cirvilleri, 2008; Sharma *et al*., 2009).

1.4.1 Bacteria in post-harvest disease control of citrus

During the last 25 years, the use of bacterial strains to control postharvest decay of fruits has been extensively studied and several strains of *Bacillus, Burkholderia* and *Pseudomonas* spp. able to reduce disease caused by a variety of postharvest pathogens were found and some of these have been developed into commercial products.

Bacillus spp.

Several studies focused on *Bacillus* spp. strains as biological control agents because of their production of broad-spectrum antibiotics and their ability to form resistant endospores (Emmert and Handelsman, 1999). *B. subtilis* strains have been commercialized as a biocontrol agent for field applications, for control

of root diseases and for control of various seedling pathogens. The efficacy of *B. subtilis* against fruit diseases was subsequently reported, confirming the ability to control green mold, sour rot, stem end rot on citrus and brown rot on stone fruits
One of the first studies was reported by Gutter and Littauer (1953), who isolated an antagonistic *B. subtilis* strain from the surface of citrus fruit that effectively inhibited the development of citrus fruits pathogen. The efficacy of *B. subtilis* against fruit diseases was subsequently reported, confirming their ability to control green mold, sour rot, stem end rot on citrus (Singh and Deverall, 1984) and brown rot on stone fruits (Utkhede and Sholberg, 1986). *B. subtilis* strains F1, L2 and L2-5, isolated from citrus fruit surfaces, reduced significantly the incidence of *P. digitatum* and *P. italicum* molds on artificially inoculated "Valencia" and "Shamouti" orange fruit, but were not effective as the fungicide treatment (quazatine plus imazalil) which gave complete control of both diseases (Obagwu and Korsten, 2003). Huang *et al*, (1992) reported the use of a *B. pumilus* strain that showed strong antagonistic activity against *P. digitatum* in Valencia oranges, which was as effective as imazalil and was significantly better than benomyl treatment.

Intensive screening and field testing have led to the commercial development of diverse *Bacillus* strains as biological control agents of interest in postharvest. Serenade (Agraquest Inc., Davis, CA) based on *B. subtilis* strain QST713, is labeled for the management of a variety of plant diseases (early blight, fire blight, downy mildew, tomato leaf spots) and for the use against *B. cinerea* in grapes, strawberry and stone fruit in pre- and post-harvest control. Part of the biocontrol activity of Serenade appeared to be due to preformed antibiotics contained within the commercially formulated product, in addition to the production of antibiotics by *B. subtilis in vivo*. Serenade was also evaluated on rough lemon for the control of citrus scab (*Elsinoe fawcettii*), on grapefruit for the control of melanose (*Diaporthe citri*), and on tangerine for the control of alternaria brown spot (*Alternaria alternata*), and its effectiveness was probably due to its protective activity based on antagonistic effects of *B. subtilis* on germinating fungal spores (Agostini *et al*., 2003).

***Burkholderia* spp.**

Burkholderia species are widely distributed in the natural environment and can be found in soil, water, rhizosphere, in humans, and in the hospital environment (Parke and Gurian-Sherman, 2001). Traditionally, *Burkholderia* species are known as plant pathogens, but several of them have been also studied as biocontrol agents (Fridlender *et al*., 1993; Hebbar *et al*., 1998; Parke and Gurian-Sherman, 2001; Parke *et al*., 1991). Moreover, commercial products containing *B. cepacia* strains are available in the market for control of soil borne pathogens (Fravel *et al*., 1998).

Smilanick and Denis-Arrue (1992) reported that a *B. cepacia* strain was capable of suppressing green mold of lemons. An antibiotic producing strain of *B. cepacia* (ID 2131), tested on a semi-commercial scale, gave good biocontrol of *P. digitatum* on postharevest oranges (Huang *et al*., 1993a; Huang *et al*., 1993b).
Some studies showed that strains of *B. gladioli* have antagonistic activity against plant pathogenic bacteria and fungi (Attafuah and Bradbury, 1989; Mao and Cappellina, 1989; Walker *et al*., 1996). Recent studies showed that strains of *B. gladioli* have antagonistic activity against plant pathogenic bacteria and fungi including *P. digitatum in vitro* and on orange and lemon fruit (Scuderi *et al*., 2008).

***Pantoea* spp.**

Among bacteria used as biological control agents, *Pantoea agglomerans* strains, formerly known as *Erwinia herbicola* or *Enterobacter agglomerans* (Gavini *et al*., 1989), have been found as effective biocontrol agents of postharvest diseases. Among them, *Pantoea agglomerans* strain CPA-2 isolated from apple surface has been reported to be an effective antagonist to the major post-harvest pathogens of citrus and pome fruit (Usall *et al*., 2001; Teixidò *et al*., 2001; Nunes *et al*., 2002).

***Pseudomonas* spp.**

P. syringae is one of the bacteria which is actually studied as a biological control agent on numerous cultivated plants in pre and post-harvest. *P. syringae* is

a common epiphytic bacterium on fruit and vegetable crops, capable of producing several antibiotics (Iacobellis *et al*., 1992), to induce resistant responses in plants, (Smith and Metraux, 1991), and to produce biosurfactants that may have antimicrobial effects facilitating dispersal of the bacterium on plant surfaces (Lindow and Brandl, 2003).

Strains of *P. syringae* have been reported to be effective as biocontrol agents against *P. digitatum* on citrus (Bull *et al*., 1997; Cirvilleri *et al*., 2005) *P. expansum* and *B. cinerea* on pears and apples (Janisiewicz and Jeffers, 1997; Janisiewicz and Marchi, 1992; Sugar and Spotts, 1999; Zhou *et al*., 2001; Zhou *et al*., 2002), *M. fructicola* and *R. stolonifer* on peaches (Zhou *et al*., 1999), *V. inequalis* on apples (Zhou and DeYoung, 1996), *E.coli* 0157:H7 on apple (Janisiewicz *et al*., 1999), and as agent of induced resistance against *Plasmopara viticola* and *Uncinala necator* on grape (Kassemeyer *et al*., 1998).

In 1995, the US Environmental Protection Agency (EPA) registered the first biological control products against postharvest diseases of fruits, BioSave 10 and BioSave 11, based on *P. syringae* strains ESC-10 and ESC-11 (Janisiewicz and Marchi, 1992). The commercial products were approved for postharvest use on citrus, apple, and pear fruits against the genera *Botrytis, Penicillium, Mucor and Geotrichum* (Janisiewicz and Jeffers, 1997; Jeffers and Wright, 1994; Smilanick *et al*., 1995).

Strains of *P. syringae* produce two classes of pore-forming lipodepsipeptide phytotoxins that target host plasma membranes, syringomycin and syringopeptin. that are synthesised by modular nonribosomal peptide synthethase.

The production of lipodepsipeptides by *P. syringae* strains has been correlated with their ability to control postharvest diseases of citrus, apple and pear fruit Bull *et al*., (1998) demonstrated that *in vivo* production of syringomycin is likely depend on the physiological state of the bacteria and on nutrient availability and presence of plant signal molecules . Mo and Gross (1991) suggested that *syr* genes are activated shortly after bacterial penetration of host tissues, showing that plant signals like phenolic compounds and sugars, promote the expression of *syr* genes required for syringomycin production.

Lipodepsipeptides can be detected by PCR amplification using specific primers to amplify the *syr*B and *syr*D genes (Sorensen *et al*., 1998). This analysis has been applied for the identification and characterization of *P. syringae* strains that produce lipodepsipeptides (Bultreys and Gheysen, 1999), for identification of putative antagonistic *P. syringae* strains (Cirvilleri *et al*., 2005) and seems also useful for marker-assisted selection of bacterial BCAs and for tracking the behaviour of the biocontrol agents in the environment.

Mode of action of biological control bacteria

Although the mode of action of bacterial biological control agents of postharvest diseases are still incomplete, competition for nutrients and space, production of siderophores, antibiotics and hydrogen cyanide or induction of plant defense mechanisms appear to be significant for many of them (Cirvilleri, 2008; Anderson *et al*., 1988; Bull *et al*., 1991; Bakker *et al*., 1991; Weller, 1988; Berg *et al*., 2002; Pieterse *et al*., 2001).

The ability of biological control agents to survive and multiply at a sufficient population level in specific niches of fruit surfaces or fruit wounds after application increases their capacity to colonize larger areas preventing pathogen colonization. Competition for space and nutrients seems to be an important mode of action of bacterial antagonists in postharvest biocontrol such as *P. agglomerans* CPA-2 (Poppe *et al*., 2003), *P. syringae* ESC-10 and ESC-11 (Bull *et al*., 1997), *P. cepacia* LT-4-12W (Smilanick and Denis-Arrue, 1992). The mode of action involved in the control of green mold on citrus fruits by *P. syringae* ESC-10 and ESC-11 is mainly due to space and nutrient competitions, as well as to syringomycin E production (Bull *et al*., 1997; Bull *et al*., 1998). Antagonistic *P. syringae* strains occupy wounds on citrus fruits and reduce the disease incidence (Smilanick and Denis-Arrue, 1992; Smilanick *et al*., 1995).

Biological control based on competition for limited nutrients can be nullified by increasing the concentrations of nutrients. Poppe *et al*, (2003) observed this phenomenon for *P. agglomerans* CPA-2 which clearly reduced the germination of *Penicillium* conidia when diluted orange peel extract was the nutrient source, but not in undiluted orange peel extract thus indicating that competition for nutrients

may play a role but not at high nutrient concentrations. *P agglomerans* CPA-2 was unable to produce antibiotics or chitinolitic enzymes, and no significant increase in phenyalanin ammonia lyase and peroxidase activities were observed after orange inoculation with CPA-2 in presence or absence of the pathogen (Poppe *et al*., 2003).

Antibiosis is an important mechanism that has been suggested to operate also on postharvest biocontrol. Some of the most active antagonistic bacteria produce antibiotics, whose action, at least partially, determines their effectiveness. However antibiotic production, as demonstrated through in vitro assay, is not necessarily correlated with the inhibition ability of the pathogens in vivo (Fravel, 1988).

Pyrrolnitrin, an antibiotic with broad-spectrum antifungal activity firstly isolated from *Pseudomonas pyrrocina* (Arima *et al*., 1964) and subsequently from several strains of *Pseudomonas* and *Burkholderia* spp., has been suggested to play an important role in postharvest biological control (Janisiewicz *et al*., 1991; Roitman *et al*., 1990). This antibiotic was isolated, purified and identified in the *B. cepacia* strain LT-4-12W (Janisiewicz and Roitman, 1988), a biological control agent for apple and pear (Janisiewicz *et al*., 1991), citrus (Smilanick and Denis-Arrue, 1992), and stone fruits (Smilanick *et al*., 1993). However, the role of pyrrolnitrin in biocontrol is not clear, since *B. cepacia* strain LT-4-12W still provided substantial control of blue mold decay on oranges inoculated with pyrrolnitrin resistant mutants of *P. italicum* (Janisiewicz and Korsten, 2002). Smilanick and Denis-Arrue (1992) likewise showed that pyrrolnitrin-resistant isolates of *P. digitatum* were controlled by pyrrolnitrin producing strain of *P. cepacia.* Studies on the mechanism of action indicated that pyrrolnitrin may have systemic activity in mammals (Cordee and Thomas, 1969) and may inhibit fungal growth by inhibiting the respiratory electron transport (Tripathi and Gotthieb, 1969). Moreover, pyrrolnitrin deficient mutant still protected orange fruits against green and blue molds (Meziane *et al*., 2006).

Production of cyclic lipodepsipeptides (LDPs) by *P. syringae* strains has been correlated with the ability of bacteria to control plant diseases caused by postharvest fungal pathogens (Bull *et al*., 1998). Pseudomonas LDPs proved to be

quite effective in the growth inhibition of a wide array of fungi and bacteria (Lavermicocca *et al*., 1997) and clinically important fungal pathogens (Sorensen *et al*., 1996). In addition to their antifungal properties, the cyclic lipodepsinonapeptides syringomycin E, syringotoxin B, and syringostatin A caused erythrocyte lysis (Sorensen *et al*., 1996). Syringomycins (SRs) are significantly more toxic to fungi than to plant tissues and bacteria (Iacobellis *et al*., 1992), while syringopeptins (SPs) are more phytotoxic. It has been hypothesized that the low activity of SP on fungi found *in vitro* is due to the barrier effect of the fungal wall, and that the efficacy of this toxins *in vivo* is mediated by the action of enzymes capable of increasing cell permeability (Lorito *et al*., 1996). Recently, a marked synergism between antimicrobial syringomycin SRE and syringopeptin SP25 produced by antagonistic *P. syringae* strains and chitinases and glucanases from antagonistic *T. harzianum* strains in the inhibition of several pathogenic and non-pathogenic fungi has been shown (Fogliano *et al*., 2002).

The role of syringomycin E in biocontrol of *P. syringae* ESC-10 and ESC-11 (BioSave) is in doubt because efforts to isolate this compound from fruit wounds treated with the antagonist have been unsuccessful, and rapid growth and colonization of the wounds suggests that competition for nutrients and space may play a major role (Bull *et al*., 1997; Bull *et al*., 1998).

Syringomycin and syringopeptin are synthesized by nonribosomal peptide synthetases which are encoded by the syringomycin (*syr*) and syringopeptin (*syp*) genomic island of *Pseudomonas syringae* pv. *syringae*. Previous studies demonstrated that expression of the *syr*-*syp* genes was controlled by the *sal*A-*syr*F regulatory pathway, which in turn was induced by plant signal molecules (Whang *et al*., 2006). Differential regulation of bacterial genes critical to biological control by plant signal molecules may alter biological control. Using genetic tools, it will be possible to investigate the requirement of syringomycin E production for biological control and to determine if this antibiotic is produced in wounds.

1.4.2 Yeasts in post-harvest disease control of citrus

Several type of yeasts isolated from the surface of fruits and vegetables are reported as alternative methods to control post-harvest diseases for their capability to grow rapidly, colonize the fruit surface, use nutrients of the surface and proliferate very fast limiting nutrient availability to pathogens (Richard and Prusky, 2002), produce extra-cellular proteins that block the growth of the pathogens propagules (Wisniewski and Wilson, 1992; Sharma *et al*., 2009).

Among the mechanisms that have been reported to play a significant role in the biocontrol activity of antagonistic yeasts, interaction between yeast and post-harvest pathogens is involved. It has been suggested that attachment of the yeast to fungal hyphae and production of extracellular saccharides, and extensive production of extracellular enzymes such as gluconase, chitinase, proteinases may play a key role by either enhancing nutrient competition or by giving the ability to degrade the pathogen cell wall, or by some other undetermined mechanisms (Jiakli and Lepoivre, 1998; Chan and Tian, 2005).

Several yeasts have been reported to be effective as biocontrol agents on citrus. *Pichia anomala* and *Pichia gulliermondii* are two yeasts commonly used for controlling post-harvest green mold of citrus fruit (Lahlali *et al*., 2004; 20011a; 2011b; Wilson and Chalutz, 1990). *Debayromyces hansenii* has been reported to control green and blue mold of citrus fruit (Singh, 2002). *Metschnikowia fructicola* and *M. pulcherrima* have been described as effective biocontrol agents against *P. digitatum* (Kinay and Yildz, 2008) and *P. italicum* (Droby, 2006) on citrus. *Kloeckera apiculata* strain 34-9 provided a good application of controlling *P. italicum* on citrus (Long *et al*., 2006; 2007).

Some yeast strains produce extracellular protein toxins designated as “killer” proteins or killer toxins which are lethal to sensitive microbial cells (Hemandez *et al*., 2008; Schmitt and Breinig, 2002). Killer protein production appears to be a widespread characteristic among yeast species of different genera including *Saccharomyces, Hansenula, Kluyveromyces, Pichia* conferring an ecological advantage over their competitors (Comitini *et al*., 2009; Magliani *et al*., 2008). Among the species with a killer phenotype *Wickerhamomyces anomalus* (formerly *Pichia anomala*) NCYC 434 has been extensively studied and its killer

protein, Panomycocin, has been suggested as a potential antifungal agent (Izgu and Altinbay, 2004; Izgu *et al*., 2011). Furthermore, *W. anomalus* currently has QPS (Qualified Presumption of Safety) status from EFSA (European Food Safety Authority) and this has benefits in terms of public perspectives of food biotechnology and acceptability of novel microorganisms in food (Sundh and Melin, 2010).

1.4.3 Fungi in post-harvest disease control of citrus fruit

Other promising microorganisms that have potential to protect citrus fruit from disease are the imperfect fungi in the genus *Trichoderma*. They are worldwide in occurrence and easily isolated from soil, decaying wood, and other forms of plant organic matter. Rapid growth rate in culture and the production of numerous spores that are varying shades of green characterize fungi in this genus.

Several mechanisms are employed by *Trichoderma* spp. as biocontrol tools of plant diseases pathogens. *Trichoderma* spp. have been reported to compete for nutrients, secrete antifungal compounds, parasitize fungal pathogens, and induce systemic resistance in the host plant (Harman *et al*., 2004 2001; Whipps and Lumsden, 2001). The parasitic activity of *Trichoderma* is mediated by the production of cell wall-degrading enzymes (CWDEs) and of antibiotics such as gliotoxin (Haran *et al*., 1996; Lorito, 1998; Zhihe *et al*., 1998; Chet, 1987).
In addition, the treatment with *Trichoderma* spp. can have indirect effect on controlling plant pathogens by increasing plant vigour's and resistance (Howell, 2003; Harman, 2000).

Trichoderma spp. have been used also to control postharvest pathogens, including *Botrytis cinerea* on grapes, apples, strawberries and cucumbers (Dubois, 1984; Harman *et al*., 1996; Tronsmo and Raa, 1977; Batta, 1999; Elad *et al.*, 1993) *Colletotrichum gloeosporioides*, *Botryodiplodia theobromae* and *Gliocephalotrichum microchlamydosporum* on rambutan fruits (Sivakumar *et al*., 2000) and *P. digitatum* on orange fruits (Dìaz and Vila, 1990).

1.4.4 Commercial biological products

Some antagonistic microorganisms are constituents of commercial products for postharvest use: Bio-Save^{TM} 10LP and Bio-SaveTM 11LP (JET Harvest Solution, Longwood, FL, USA) based on the bacterial strains ESC-10 and ESC-11 of *P. syringae*, respectively; Aspire (Ecogen, US) based on the yeast *Candida* oleophila; Serenade^{TM} (AgraQuest, Davis, CA, USA) based on the strain QST 713 of *Bacillus subtilis* (Ehrenberg) Cohn; YeldPlus^{TM} (Anchor Yeast, Cape Town, South Africa) based on the yeast *Cryptococcus albidus* (Saito); Shemer^{TM} (Bayer CropScience, AG) registered in Israel and based on the yeast *Metschnikowia fructicola* Kurtzman and Droby.

The two biocontrol products, *Candida oleophila* (Aspire) and *Pseudomonas syringae* (BioSave), were registered by the US-EPA for commercial post-harvest treatment of citrus fruit for control of green and blue moulds (Brown and Miller, 1999). The biocontrol agents show preventive activity against *Penicillium* species with limited or no eradication activity when compared to imazalil and thiabendazole. They are not compatible with wax, soaps or sanitizers such as chlorine. These bio-fungicides might have potential for the control of fungicide-resistant strains of *Penicillium* species. They are acceptable for use on citrus when customers require non-use of post-harvest chemicals, chemical-free citrus.

At present, Biosave is still available on the market for postharvest use, whereas Aspire and YieldPlus are no longer available.

1.5 Chemical inducers of resistance.

Induction of resistance appears to be another approach for the control of post-harvest disease of fruit. Some chemical inducers of resistance, such as acibenzolar-s-methyl (ASM), β-aminobutyric acid (BABA), and chitosan, have been shown to enhance defence responses in fruit and to reduce disease incidence.

1.5.1 Acibenzolar S-methyl (ASM) in post-harvest disease control of citrus

Acibenzolar S-methyl (ASM), a functional analogue of salycilic acid (SA) (Tally *et al*., 2000) is perhaps the most potent synthetic activator of SAR, the inducible defence mechanism that play an important role in disease resistance. It

is commercialized in several countries, including Italy (commercial name BionTM), where it can be used on tomatoes, tobacco, cucurbits, pear and hazelnut trees. It could be consider as crop protection agent because it is not phytotoxic and increase the resistance.

ASM do not show any antimicrobial activity in vitro and activate resistance against the same spectra of pathogens as the biological inducers of SAR (Oostendorp *et al*., 2001).

Acibenzolar has effective role of elicitor in both monocotyledons (Gorlach *et al*., 1996) and dicotyledons (Tally *et al*., 2000) when applied as a foliar spray.

Huang *et al*. (2000) have demonstrated that pre-flowering spray-treatment with acibenzolar on melon leaves significantly reduced incidence and severity of postharvest fruit diseases caused by *Fusarium, Alternaria, Rhizopus* after low temperature storage of the fruit. The same research also reported no ASM activity against mycelial growth of the fungi tested *in vitro*. Authors thus hypotethesised that ASM was able to decrease postharvest disease in melon after foliar application by activating long-lasting systemic resistance in the fruit. They suggested a strong possibility that ASM or a second messenger affects fruit-generating cells in the flower. Rapid uptake from foliar sprays is followed by metabolic conversion in plants to a closely related products that moves up and down through the floem. Binding to receptor sites throughout plants is presumed to precede the known activation of genes and the accumulation of proteins which different consequences, including impediments to pathogen penetration, hydrolysis of components of fungal walls, more rapid cell reaction to infection.

In mango (*Mangifera indica* L.) fruit, salycilic acid treatment induced significant increases in the activities of several pathogenesis-related (PR) proteins and other defense-related enzymes, such as phenylalanine ammonia-lyase and peroxidase, and in the generation rate of reactive oxygen species, and inhibited anthracnose rot caused by *Colletotrichum gloeosporioides* (Zeng *et al*., 2006).

In the Yali pear (*Pyrus bretschneideri* Rehd.), ASM treatment significantly increases the activities of phenylalanine ammonia-lyase, peroxidase, chitinase and superoxide dismutase, and reduced the incidence and the area of lesions caused by

P. expansum (Cao *et al*., 2005), with similar results obtained for ASM-treated peach (*Prunus persica* L.) fruit (Liu *et al*., 2005).

The activation of defense responses has also been reported as one of the mode of action (and sometimes the main or unique mode) of ASM (Cao *et al*., 2005; Liu *et al*., 2005; Zhu *et al*., 2008) and BABA (Porat *et al*., 2003) in several fruit-pathogen interaction.

1.5.2 β-aminobutyric acid (BABA) in post-harvest disease control of citrus

Aminobutyric acid (BABA) is a non-protein aminoacid, which induces resistance against a broad range of disease-causing organisms, including fungi, bacteria, viruses and nematodes (Cohen, 2002; Cohen, 2001; Jakab *et al*., 2001).

Most of the studies describing the phenomenon of BABA-induced disease resistance were done in annual weedy plant species, especially those belonging to the Solanaceae family, such as tomato and potato, and to the Cucurbitaceae family, such as tobacco, pepper, cucumber and melon (Cohen, 1994; Cohen and Gisi, 1994; Hong *et al*., 1999; Siegrist *et al*., 2000). In addition, it was reported that BABA induced disease resistance in other weedy species such as *Arabidopsis*, cotton, cauliflower and sunflower (Tosi *et al*., 1998; Zimmerli *et al*., 2000; 2001; Silue *et al*., 2002).

More recently, it was demonstrated that BABA may also induce disease resistance in woody plants, such as grapevines (Cohen *et al*., 1999; Reuveni *et al*., 2001).

However, as far as we know, no report is yet available regarding the effects of BABA on the induction of disease resistance in fruit tissues.

The reduction in disease incidence that follows the application of BABA must result from its effects on the induction of pathogen defense responses in the host, since the compound itself did not seem to have any direct antifungal activity and did not affect the growth of various pathogens *in vitro* (Cohen, 1994; 2001; Cohen *et al*., 1994; Sunwoo *et al*., 1996; Tosi *et al*., 1998).

Moreover, in many cases, BABA induced systemic resistance: application to the root system or to the lower leaves of the plant induced pathogen resistance

in other distant non-treated portions of the plant (Cohen, 1994; Hong *et al*., 1999; Oka *et al*., 1999; Silue *et al*., 2002).

It was concluded that BABA protects plants from infection by potentiating pathogen-specific resistance mechanisms (Zimmerli *et al*., 2000; 2001).

The mode of action of BABA in inducing plant pathogen resistance is not yet fully understood. However, several reports indicated that BABA activated pathogenesis-related (PR) protein accumulation. For example, BABA induced PR-1a, chitinase, and β-1,3-glucanase protein accumulation in pepper, tomato and tobacco (Cohen, 1994; Cohen *et al*., 1994; Hwang *et al*., 1997; Siegrist *et al*., 2000).

On the other hand, although BABA increased disease resistance it did not induce PR-protein accumulation in cauliflower, *Arabidopsis* or tobacco, suggesting that activation of PR-proteins cannot be its only mode of action (Cohen, 1994; Jakab *et al*., 2001; Silue *et al*., 2002).

Other host pathogen defense responses that were reported to be induced by BABA include induction of the hypersensitivity response, callose deposition and lignin accumulation (Cohen *et al*., 1999; Siegrist *et al*., 2000; Zimmerli *et al*., 2000).

In any case, BABA may induce pathogen resistance in plants either through the activation of a signaling pathway that is dependent on salicylic acid (SA) or through the activation of a novel signaling cascade that is not dependent on the SA, jasmonic acid (JA) or ethylene signaling pathways (Zimmerli *et al*., 2000; 2001).

In grapefruit (*Citrus paradise* Macfad.), BABA treatment induced resistance against *Penicillium digitatum* (Pers.) through enhancement of chitinase gene expression and phenylalanine ammonia-lyase activity (Porat *et al*., 2003).

1.5.3 Chitosan in post-harvest disease control of citrus

Chitosan is obtained from the deacetylation of chitin, and consists of polymers of β-1,4-glucosamine subunits (Palma-Guerrero *et al*., 2008).

The antimicrobial activity of chitosan and its derivatives (eg, glycolchitosan or carboxy-methyl chitosan) depends mainly on the degree of

deacetylation, pH of the medium, temperature and the presence of food components (Janisiewicz, 2010). Chitosan has numerous physiological and biological properties in industries such as agriculture, food, biotechnology, pharmacology, medicine, cosmetology (Bautista-Banos *et al*., 2006).

Chitosan has dual function: working as fungistatic and as a biostimulant.

Numerous previous studies have shown that chitosan could directly inhibit spore germination, germ tube elongation and mycelial growth of many phytopathogens, such as *Botrytis cinerea* (Chien *et al*., 2006), *Fusarium solani* (Eweis *et al*., 2006), *Rhizopus stolonifer* (Hernandez-Lauzardo *et al*., 2008), *Penicillium* (Chien *et al*., 2006) and *Sclerotium rolfsii* (Eweis *et al*., 2006).

Chitosan is able to create a semi-permeable film, used as an ideal preservative coating for fresh fruit and vegetables because produce a coating on the surface of treated fruit, also inhibits the growth of fungi because stimulate defense responses (Romanazzi *et al*., 2006).

Chitosan is an exogenous elicitor, and chitin oligomers have been reported to stimulate systems involved in resistance, such as lipoxygenase, phenylalanine ammonia lyase activities, chitinase, β-1,3-glucanase and phenolic compound, synthesis of phytoalexins and induction of lignification (Bautista-Banos *et al*., 2006).

Chitosan at low molecular weight has been reported to control post-harvest diseases of citrus fruit (Chien *et al*., 2007). Low molecular weight chitosan coating beneficially influenced firmness, total soluble solid content, titratable acidity, ascorbic acid content and water content of citrus fruit after 56 days of storage at 15 °C.

The combination of *Candida saitoana* with glycolchitosan was more effecting in controlling decay of apple and citrus fruit than *C. saitoana* or glycolchitosan treatment alone (El-ghaouth and Wilson, 1997).

Verticillium lecanii as well as chitosan are equally capable of inducing a striking response in *P. digitatum* infected citrus fruits when exogenously applied prior to fungal infection. Although it is clear that reduction of disease incidence results from the synergistic action of several mechanisms, the induction of a

specific host response appears to play a key role in the control of citrus green mold by *V. lecanii* (Benhamou, 2004).

1.6 Bicarbonate and carbonate salts in post-harvest disease control of citrus.

Sodium carbonate, sodium bicarbonate, calcium chloride, and ethyl alcohol are GRAS substances frequently utilized in integrated biological control programs (Janisiewicz and Korsten, 2002).

Sodium bicarbonate (SBC) ($NaHCO_3$, baking soda) and sodium carbonate (SC) (Na_2CO_3, soda ash) are common food additives permitted by the United States Food and Drug Administration with no restrictions.

The effect of the SBC and SC treatments are to delay spore germination since they have only fungistatic effect. They can be useful tools to manage citrus postharvest decays because in addition to their considerable antimicrobial activity, they are inexpensive, readily available, and can be used with a minimal risk of injury to the fruit (Usall *et al*., 2008). Several reports suggest that alkalinization of the fruit surface with alkalyzing agents, e.g., $NaHCO_3$, would provide an attractive approach for control of acidifying pathogens such as *P. expansum, P. digitatum and P. italicum* (Porat *et al*., 2002; Smilanick *et al*., 1999; 2005).

For the future prospective exists the possibility that through genetic engineering and conventional background it's could be possible to produce fruits and vegetables that have epiphytic populations that make them more resistant to postharvest.

1.7 Combined treatments.

Several studies have demonstrated that the efficacy in biocontrol of postharvest pathogens can be increased by the use of combinations of microorganisms or their metabolites.

Mixtures of complementary and non-competitive antagonists have a wider spectrum of activity (different fruits and cultivars) and can control more than one disease simultaneously, thus providing a larger protection than when used alone. In this case, the antagonistic action will result not from the activity of one species

but rather from the action of a community of microorganisms that suppress a target pathogen through different mechanisms of action.

Furthermore, in a mixture, the presence of an antagonist could improve the efficacy of the other biocontrol agent. For example, bacterial biocontrol agents could utilize the nutrients released by chitinolytic enzymes from hyphae of target fungi for proliferation, and the subsequent increase in bacterial populations should enhance their ability to act as biocontrol agents (Lorito *et al*., 1993). Combination of cyclic lipodepsipeptides produced by *P. syringae* and cell wall degrading enzymes from *Trichoderma* strains synergistically enhances the antifungal activity of *Pseudomonas* LPDs, by facilitating their access to the cell membranes.

The application of antagonist mixtures has reduced variability and improved efficacy of biocontrol in many systems, some of which include pathogens that infected fruits.

Janisiewicz (1988) showed that *B. cinerea* and *P. expansum* rots of apple were simultaneously controlled with mixtures of antagonistic bacteria (*Pseudomonas* spp.) and yeast (*Acremonium breve*), and that the antagonistic strains were less effective when tested separately then when applied in mixture.

Leibinger *et al*, (1997) demonstrated that mixtures of *Aureobasidium pullulans*, *Rhodotorula glutinis* and *B. subtilis,* applied to apple trees, reduced the size and the number of lesions caused by *P. expansum, B. cinerea* and *Pezicula malicorticis* to a greater extent as compared with the same strains applied individually. These results were in agreement with those of Janisiewicz and Bors (1995), who demonstrated that *P. syringae* and *Sporobolomyces roseum* strains controlled blue mold on apple more effectively when mixed than when applied separately. The broad nitrogen-utilizing capacity of *P. syringae* and the broad carbon-utilizing capacity of *S. roseum* allowed both antagonists to form a stable community dominated by *P. syringae* in carbon rich apple and pear wounds. *S. plymutica* strains IC1270 and IC14 were applied separately and in combination on oranges, and green and blue mold control increased when both bacterial strains were combined (Meziane *et al*., 2006).

Compatibility between BCAs is also important. For example, when applied on apples in combination with *B. subtilis,* populations of *A. pullulans*

were reduced and the mixture was slightly less effective than mixture of *A. pullulans* and *R. glutinis*. Similar results were obtained when antagonistic *P. syringae* and *S. roseum* strains were applied in mixture on apples: populations of *S. roseum* were lower when applied with the bacteria than when applied alone (Janisiewicz and Bors, 1995), but the mixture enhanced biocontrol of *P. expansum* on Golden Delicious apples compared with antagonists applied separately.

However, the effectiveness of microbial antagonists depends upon the concentration of the antagonist, concentration of salt additive(s), their compatibility and duration and time at which they are applied. Usually, the cultures should be applied well before the initiation of infection process (Barkai-Golan, 2001).

For the most effective control of decays, biological control can be integrated with other alternative methods. This approach includes the utilization of a biological control agent in combination with substances Generally Regarded as Safe (GRAS) or other methods. Sodium carbonate, sodium bicarbonate, calcium chloride, and ethyl alcohol are GRAS substances frequently utilized in integrated biological control programs (El-Ghaouth *et al*., 2004; Janisiewicz and Korsten, 2002; Janisiewicz *et al*., 2010).

In combining SBC or SC with any antagonist, it is important that the antagonist is compatible with the carbonate solution and able to proliferate in fruit wounds in their presence (Janisiewicz *et al*., 2010).

Sodium carbonate and sodium bicarbonate have been reported to reduce conidial germination of *P. digitatum* and *P. expansum* (Janisiewicz *et al*., 1998; Smilanick *et al*., 1997; Smilanick *et al*., 1999), and development of *P. digitatum* and *P. italicum,* with reduction of up to 90% in the incidence of both molds after fruits treatments (Palou *et al*., 2001). Treatments of 3% sodium carbonate with *P. syringae* ESC-10 were superior to treatments alone in controlling green mold on citrus (Smilanick *et al*., 1999).

Several isolates of *Bacillus subtilis* were evaluated for control of green mould and blue mold on citrus fruits. While one of the antagonist isolates or SBC alone reduced decay to 10–20%, the combination resulted in complete control of both diseases (Obagwu and Korsten, 2002). A significant increase in biocontrol

activity of *B. subtilis* strains F1, L2, and L2-5, antagonists of citrus green and blue molds, was observed when the antagonists were combined with sodium bicarbonate (1-3% for 2 min) or when were applied following hot water treatment (45°C for 2 min) (Pusey *et al*., 1986).

The antagonist *Pantoea agglomerans* CPA-2 combined with SC and SBC reduced incidence of green mold from approximately 90% among control oranges to 55% and 25%, respectively after treatment with SC and SC + the antagonist, and 60% and 40%, respectively after treatment SBC and SBC + the antagonist (Usall *et al*., 2008). The efficacy of *P. agglomerans* for the control of green mold of oranges was improved when combined with bicarbonate, or baking soda (Teixidó *et al*., 2001).

The control of postharvest fruit rots achieved with antagonistic bacteria might be improved when combined with heat treatments (Lurie, 1998). Biocontrol activity of *Pseudomonas glathei* strain ID 2859 was significantly enhanced when citrus fruits were incubated at 30°C for 24 h after *P. digitatum* inoculation (Huang *et al*., 1995). The heat treatment retarded the fungal spores germination, and the antagonistic bacterial cells multiplied quickly before infection occurred. While heat treatment acts as an eradicant significantly reducing the pathogen population on the fruit surface, it provides little residual protection. The residual (protectant) protection from the antagonist adds to the control provided by the heat treatment to provide a complete decay control strategy. Since biocontrol of postharvest diseases has little eradicative activity, a combination of hot water treatment followed by biocontrol would be effective in providing both eradicant and protectant activities.

1.8Induction of disease resistance.

When a plant detects pathogen attack, several mechanisms of resistance, such as systemic acquired resistance (SAR) and induced systemic resistance (ISR) are activated. Several pathogenesis-related (PR) proteins, included in SAR group, have anti-fungal and anti-bacterial action, so that over expression of genes codifying products with antifungal activity is one of the strategies to improve plant defense against fungal attack. It should be mentioned that these processes

have mostly been studied in vegetative organs of plants, while mature fruits have received less attention.

1.8.1 Systemic acquired resistance (SAR)

SAR is major mechanism of induced defence that may confer long-lasting protection against a broad spectrum of microorganisms (van Loon *et al*., 2006), and it is characterised by the accumulation of SA and by the expression of PR genes (Hammerschmidt *et al*., 2001), such as chitinase (CHI1) and glucanase (GNS1). SAR refers to a distinct signal transduction pathway that plays an important role in stimulation of plant resistance system against pathogens after initial contact with the pathogen (Ryals *et al*., 1996). It is activated systemically in induced plants following localized inoculation with pathogens which can be viruses, bacteria or fungi (Kùc, 1982; Kessmann *et al*., 1984). SAR is mediated by salicylic acid (SA), a compound which is frequently produced following pathogen infection like a signaling molecules, environmental stresses and active defense reactions against pathogen invasion (Durner *et al*., 1997; Terry and Joyce, 2004). Plants, when appropriately stimulated, are able to substantially enhance their defensive capacity in either SA-dependent or SA independent manner, both leading to an increased protection against various types of pathogens (Pieterse and Van Loon, 1999). The development of SAR is associated with various cellular defence responses. These include synthesis of pathogenesis-related (PR) proteins, phytoalexins, accumulation of active oxygen species (AOS), rapid alterations in cell walls, and enhanced activity of various defence-related enzymes. The association SAR of with PRs has led to the hypothesis that accumulation of PRs is not a prerequisite for the induction of resistance, but that PRs contribute to the protective state (Van Loon, 1997).

1.8.2 Induced systemic resistance (ISR)

ISR depends on responsiveness to the plant hormones jasmonic acid (JA) and ethylene (Beckers and Conrath, 2007) which are produced following applications of some nonpathogenic rhizobacteria. Jasmonates play an important role as signal molecules in plant defence responses against pathogen attack,

induce the synthesis of antioxidants such as vitamin C, phenolic compounds, and increase the activity of enzymatic antioxidant system (Chanjirakul *et al*., 2006). Enhancement of ethylene production commonly occurs in most diseased or injured plants (Williamson, 1950). Ethylene is an important plant hormone in many developmental and stress processes, and exerts its signaling role through regulation of its biosynthesis, perception, and signal transduction, to modulate a wide array of responses that include gene expression changes (Wang *et al*., 2002).

As applications of SA prime cells for rapid expression of defense genes upon invasion by pathogens, the activity of applied JA and MeJA depends on the subsequent action of ethylene (Walling, 2001). There can be signal cross-talk between SA- and JA-induced pathways resulting in additive or synergistic effects (Bostock, 2005). Application of SA and MeJA induces resistance to various pathogens in different fruits, including resistance to brown rot (caused by *M. fructicola*) and blue mould (caused by *P. expansum*) in sweet cherry (Yao and Tian, 2005; Chan *et al*., 2008); fungal decay in papaya (Gonzalez-Aguilar *et al*., 2003), anthracnose (caused by *Colletotrichum cocodes*) in tomatoes (Tzotzakis, 2007), anthracnose (caused by *C. acutatum*) in loquat fruits (Cao *et al*., 2008), green mould (caused by *P. digitatum*) in grapefruits (Droby *et al*., 1999) and apple decay (Saftner *et al*., 1999).

1.8.3 Reactive oxygen species (ROS)

Another mechanism involved in plant disease resistance for protection against attack of pathogens such as fungi, bacteria and viruses, and also against adverse environmental conditions, is the production of reactive oxygen species (ROS). The transient and rapid production of ROS, including superoxide, hydroxyl radical, and H_2O_2, is the first signal of HR. H_2O_2 increases biosynthesis of gluthatione-S-transferase (GST) and gluthatione peroxidase (GPX), involved in resistance to pathogen infection. Since jasmonates (JA) are important signal molecules involved in ISR, phenylalanine ammonia-lyase (PAL) is responsive to stress conditions in plants and it results activated by the JA/ethylene pathway. The involvement of PAL gene expression and the synthesis of phenylpropanoid-derived compounds have been reported previously in the citrus response to

biocontrol agents and to the citrus postharvest pathogen, *Penicillium digitatum* (Ballester *et al*., 2006; González-Candelas *et al*., 2010; Mittler *et al*., 1999).

1.8.4 Pathogenesis related proteins (PRs)

Plant PR proteins are defined as proteins encoded by host plants that are induced in pathological or related situations, and represent major quantitative changes in soluble protein during the defense response (van Loon *et al*., 1994; Stintzi *et al*., 1993). In incompatible host-pathogen interactions, damage caused by the pathogen remains defined as result of the plant defensive response. This response is associated with a coordinated and integrated set of metabolic alterations that are active in impeding further pathogen ingress, as well as in enhancing the capacity of the host to limit subsequent infection by different types of pathogens (Van Loon, 1999). Pathogenesis-related proteins (PRs) have two subclasses: an acidic subclass, secreted to cellular space, and a vacuolar basic subclass (Kitajima and Sato, 1999). Signaling molecules mediate induction of PR proteins in plants during pathogen infection including SA (salicylic acid) for acidic PR genes as well as ethylene and MeJ for basic PR genes (Kitajima and Sato, 1999). In addition, PR genes (basic in general) also are present constitutively in some plant organs or tissues, including roots, leaves and floral tissues.

PR proteins were first classified into PR-1 to PR-5 families, based on serological properties and later on sequence data; more recently PR proteins comprise a large group of 17 protein families, even though PR-15 to PR-17 families have been recognized (van Loon *et al.*, 2006).

Antimicrobial activities have been demonstrated *in vitro* for members of PR protein families through direct hydrolytic activities on cell walls and contact toxicity; whereas indirect activities perhaps avoid an envelopment in defense signaling (van Loon *et al.*, 2006). Ten PR families are recognize as direct activities against fungi pathogens (PR-1, PR-2, PR-3, PR-4, PR-5, PR-8, PR-11, PR-12, PR-13, PR-14 families) (Tab. 1).

Development of induced resistance in plants is also associated with the coordinate expression of a complex set of PR proteins, so-called ‘SAR genes’

(Conrath *et al*., 2001). The enzymatic activities of several PR proteins have been identified and include 1,3-glucanases (PR-2) and chitinases (PR-3), which possess direct antimicrobial activity by degrading microbial cell wall components (Van Loon, 1997). Some plant chitinases also have lysozyme activity and can therefore hydrolyse bacterial cell walls (Boller *et al*., 1983; Heitz *et al*., 1994). Furthermore, breakdown products of pathogen and/or plant cell wall components released by the activity of these enzymes have been shown to act as elicitors of plant defence responses (Van Loon, 1997). The expression of PR genes and the associated accumulation of the encoded PR proteins have often been considered as the molecular basis of induced resistance. A correlation was also found between induced resistance and accumulation of chitinase.

β-1,3-glucanases (GNS1) are PR proteins involved in plant defence against pathogen attack; they are able to hydrolyze 1,3-β-D-glucoside a compound present in the constituent of fungus cell wall. This ability to hydrolyze 1,3- β-D-glucoside inhibites directly fungal growth (Porat *et al*., 2002). These families proteins are also able to synthesis lignin and phytoalexins (You *et al*., 2002). The activation of β-1,3-glucanases may stimulate the secondary plant pathogen defence responses and the over expression of this gene may determine in the tissue more resistance to pathogen attack (Zhu *et al*., 1994).

Chitinases (CHI) catalyse the hydrolysis of chitin, a major component of cell walls of phytopathogenic fungi, and serve as potent inhibitors of fungal growth (Lorito and Scala, 1999). This enzyme is involved in the synthesis of lignin and phytoalexins. The activity of CHI can be stimulated by wound in the tissue of fruit, infection of tissue with pathogens, exposition to ethylene.

Family	Type member	Properties	Gene symbols
PR-1	Tobacco PR-1°	Unknown	*Ypr1*
PR-2	Tobacco PR-2	B-1,3-glucanase	*Ypr2, [Gns2 ('Glb')]*
PR-3	Tobacco P,Q	Chitinase type I,II,IV,V,VI,VII	*Ypr3, Chia*
PR-4	Tobacco 'R'	Chitinase type I,II	*Ypr4, Chid*
PR-5	Tobacco S	Thaumatin-like	*Ypr5*
PR-6	Tomato Inhibitor I	Proteinase-inhibitor	*Ypr6, Pis ('Pin')*
PR-7	Tomato P_{69}	Endoproteinase	*Ypr7*
PR-8	Cucumber chitinase	Chitinase type III	*Ypr8, Chib*
PR-9	Tobacco "lignin-forming peroxidase"	Peroxidase	*Ypr9, Prx*
PR-10	Parsley "PR1"	Ribonuclease-like	*Ypr10*
PR-11	Tobacco "class V" chitinase	Chitinase, type I	*Ypr11, Chic*
PR-12	Radish Rs-AFP3	Defensin	*Ypr12*
PR-13	Arabidopsis THI2.1	Thionin	*Ypr13, Thi*
PR-14	Barley LTP4	Lipid-transfer protein	*Ypr14, Ltp*
PR-15	Barley OxO2 (germin)	Oxalate oxidase	*Ypr15*
PR-16	Barley OxOLP	Okalate-oxidase-like	*Ypr16*
PR-17	Tobacco PRp27	Unknown	*Ypr17*

Table 1. Families of pathogenesis related proteins (van Loon *et al*., 2006).

1.8.5 Induction of resistance against *Penicillium digitatum* in citrus fruit

The increase of the fruit's natural defense capabilities through induction of resistance has attracted great interest as a means to reduce the use of chemical fungicides during postharvest handling and storage of fruit and has been proven to be effective in laboratory and field trials cases (Tian and Chan, 2004).

In fully mature citrus fruit, increased resistance against *P. digitatum* infection can be achieved by application of physical (Kim *et al*., 1991; Ben Yehoshua *et al*., 1992; Rodov *et al*., 1992; Droby *et al*., 1993; Arcas *et al*., 2000), chemical (Porat *et al*., 2001; 2002; Venditti *et al*., 2005), or antagonistic microorganism treatments (Arras, 1996; Fajardo *et al*., 1998; Droby *et al*., 2002). The efficacy of these treatments in eliciting induced resistance is variable and, in many instances, depends on the maturity of the fruit.

Treatments include UV irradiation (Droby *et al*., 1993; Porat *et al*., 1999a) and hot water rinsing and brushing (Porat *et al*., 2000); the application of natural compounds such as the plant growth regulator jasmonic acid (JA) (Droby *et al*., 1999), chitosan (Benhamou, 2004) and the amino acid derivative β-aminobutyric

acid (BABA) (Porat *et al*., 1999b); and use of the biocontrol yeast antagonists *Candida oleophila* (Droby *et al*., 2002).

Induction of plant pathogen resistance is a complex mechanism involving the activation of various processes, including the induction of expression of defence-related genes, accumulation of pathogenesis-related (PR) proteins, increased synthesis of phytoalexins and other antifungal secondary metabolites, either after fungal infection or after treatments that induce protection against fungal infection (Afek *et al*., 1999; McCollum, 2000; Porat *et al*., 2001; 2002; Del Rìo *et al*., 2004). Nevertheless, little is known about the molecular mechanisms involved in the defence response of citrus fruits against fungal infection.

UV irradiation, hot water and biocontrol yeast elicitation of fruit resistance are accompanied by induction of the PR proteins chitinase and β-1,3-glucanase (Porat *et al*., 1999, 2001). Lignification and accumulation of phenolic compounds have also been associated with the resistance of citrus fruit to *P. digitatum* infection (Angioni *et al*., 1998; Ortuno *et al*., 2006).

Hot water dips and curing reduce postharvest citrus decay development after storage (Schirra and D'hallewin, 1997) and enhance resistance against the green mold pathogen *P. digitatum* (Kim *et al*., 1991). Two major groups of proteins may be activated by the hot water treatments that induce fruit resistance: heat shock proteins (HSPs) and pathogenesis-related (PR) proteins. PR proteins coded by host plant genes are induced by pathogen infection or related situations, and are thought to play a major role in plant defence responses against a wide variety of pathogens (Van Loon and Van Strien, 1999).

Phenylalanine ammonia lyase (PAL) and peroxidase activities were induced in grapefruit after elicitation of resistance by UV irradiation (Droby *et al*., 1993) or by the biocontrol yeast *Candida oleophila* (Droby *et al*., 2002).

PAL is a key enzyme in the JA/ethylene biosynthetic pathway, and is the first enzyme in the phenylpropanoid pathway, from which phytoalexins scoparone and scopoletin are synthesized.

Induction of this citrus phytoalexin has been observed in UV-irradiated fruit (Rodov *et al*., 1992) or after elicitation of resistance by antagonistic yeasts (Arras, 1996; Droby *et al*., 2002). The levels of the antifungal scoparone in the

flavedo of citrus fruit increased in pathogen-inoculated fruit that were subsequently subjected to a heat treatment, whereas pathogen infection did not promote such an increase (Kim *et al*., 1991). Induction of this citrus phytoalexin has also been observed in UV-irradiated fruit (Rodov *et al*., 1992) or after elicitation of resistance by antagonistic yeasts (Arras, 1996; Droby *et al*., 2002). Therefore, scoparone has been considered a good marker of induced resistance in citrus fruit.

The production of H_2O_2 increases the byosinthesis of glutathione peroxidase (GPX), involved in the resistance to pathogen. Peroxidases play a key role at a later stage in the pathway during the synthesis of lignin, which acts as a cell wall reinforcement enhancing resistance against multiple pathogens, and may alter the antioxidant ability of citrus fruit to cope with *Penicillium* infection (Ballester *et al*., 2006). In this regard, it is noteworthy that both enzymes have been suggested to play a role in the defense response of citrus fruit against *P. digitatum* (Ballester *et al*., 2006), although their transcriptional regulation during development of induced resistance in citrus fruit remains unknown.

The most thoroughly carefully investigated chemical elicitors are those interfering with salicylic acid (SA) pathway, such as INA (2,6-dichloroisonicotinic acid) or ASM (benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester; BTH). ASM is commercialized in Italy and in some other countries under the tradename of BION and it is used on tomatoes, tobacco, cucurbits, pear and hazelnut trees (Friedrich *et al*., 1996; Benhamou and Belanger, 1998). It has proved to be effective on the control of diseases caused by viral, bacterial and fungal pathogens in different plant species (Görlach *et al*., 1996; Benhamou and Belanger, 1998; Cole, 1999). It has been used as potential elicitor of fruit resistance to postharvest diseases in melon (Huang *et al*., 2000), strawberry (Terry and Joyce, 2000), passionfruit (Willingham *et al*., 2002), Yali pear (Cao *et al*., 2005), peach (Liu *et al*., 2005) and mango fruits (Zhu *et al*., 2008).

A new class of resistance-inducing compounds belongs to aminobutyric acid. DL-β-aminobutyric acid (BABA) induces resistance against a broad range of disease-causing organisms, including fungi, bacteria, viruses and nematodes

(Cohen, 2001; 2002; Jakab *et al*., 2001; Oka *et al*., 1999). The mode of action of BABA in inducing resistance in plants is still poorly understood. It is not clear if BABA-induced resistance involves the salicylic acid pathway or another pathway. BABA treatments promote callose deposition and lignin accumulation, enhance the production of phenolics and peroxides and induce accumulation of PR-proteins in some host-pathogen systems (Cohen *et al.*, 2002).

Few reports have shown that BABA activated pathogenesis-related (PR) protein accumulation in tomato, pepper and tobacco (Cohen *et al*., 1994; Cohen, 1994; Hwang *et al*., 1997; Siegrist *et al*., 2000), whereas other authors reported that BABA did not induce PR-protein accumulation in cauliflower, *Arabidopsis* or tobacco, suggesting that activation of PR-proteins cannot be its only mode of action (Cohen, 1994; Jakab *et al*., 2001; Silué *et al*., 2002). Recently, application of BABA was capable of controlling *A. alternata* in apple fruits in the laboratory and in the field (Reuveni *et al.*, 2003), *P. digitatum* in grapefruit (Porat *et al.*, 2003) and *P. italicum* in sweet orange (Tavallali *et al*., 2008).

Until now, many authors have studied BABA without describing direct antifungal or antibacterial activities associated with BABA treatment (Cohen *et al*., 1994; Sunwoo *et al*., 1996; Jakab *et al*., 2001) and excluded a direct antimicrobial effect for efficient protection against biotic stress, except Porat *et al* (2003), who suggested a direct antifungal effect against *P. digitatum in vitro* and *in vivo* on grapefruits, and Fisher *et al*, (2009), who showed a direct antifungal effect against *B. cinerea in vitro* experiments.

Chitosan is a high molecular weight cationic polysaccharide β-1,4-glucosamine compound, derivated from shellfish exoskeletons or from cell walls of some microorganism after deacetylation of chitin (Hirano *et al.*, 1976). Chitosan is not toxic to plants and has been used as a surface coating on fruit and vegetables. The polymer exhibits antifungal activity against several fungi, forms a semi-permeable film that reduces transpiration losses and fruit ripening (Bautista-Baňos *et al.*, 2006; Romanazzi, 2010) and it can also induce defense responses in seeds (Benhamou *et al.*, 1994; Lafontaine and Benhamou, 1996), leaves (Trotel-Aziz *et al.*, 2006) or fruits (Fajardo *et al.*, 1998; Bautista-Baňos *et al.*, 2006) reducing disease caused by fungal pathogens. A chitosan coating is known

effective to prolong the storage life and control the decay of strawberries (El Ghaouth *et al.*, 1992; Park *et al*., 2005; Vargas *et al*., 2006), litchis (Zhang and Quantick, 1998), sweet cherries (Park and Zao, 2004), Murcott tangor fruits (Chien *et al*., 2007), apples (de Capdeville *et al*., 2002; Yu *et al*., 2007), peaches, Japanese pears, kiwi fruits (Du *et al*., 1997) and table grapes (Romanazzi *et al*., 2002, 2007; Munoz *et al.*, 2009).

Chapter 2.

OBJECTIVES

Green mold caused by *Penicillium digitatum* is one of the most important postharvest disease of citrus fruit that often causes extensive losses during storage and transportation. Synthetic fungicides such as imazalil (IMZ), thiabendazole (TBZ), and sodium o-phenylphenol (SOPP) are traditionally used to control green mold and they play an important role for the management of *P. digitatum* of citrus fruit.

There is increasing concern about the environmental contamination and human health problems caused by synthetic fungicides due to possible toxicological risks. Therefore, the need for alternative strategy to control postharvest disease of citrus fruit is urgent. However, chemicals' efficacy is frequently decreased by development of postharvest pathogen resistance.

Previous studies have demonstrated capabilities of increasing citrus resistance against green mold pathogen *Penicillium digitatum* by applying various treatments, such as UV (Droby *et al*., 1993), hot water (Porat *et al*., 2000), JA

(Droby *et al*., 1999), antagonistic microrganisms (Droby *et al*. 2001; Cirvilleri *et al*., 2005).

Thus, the activity of resistance inducers, alone or in combination with BCAs or carbonate salts, was evaluated in this study. In addition, preventive and curative activity, like possible alternatives to the use of synthetic fungicides in controlling post-harvest pathogens, was evaluated.

Objective 1. Direct antifungal activities of elicitors and BCAs in controlling post-harvest green mold *in vitro* and on citrus fruit.

Approaches:

- Direct antifungal activity of ASM, BABA and chitosan *in vitro*.
- Direct antifungal activity of ASM, BABA and chitosan on citrus fruit.
- Direct antifungal activity of yeasts *in vitro*.
- Direct antifungal activity of yeasts on oranges fruit.

Objective 2. Curative and preventive activity of biological control agents and elicitors in different combined treatments on oranges fruit.

Approaches:

- Combined treatment of *Pseudomonas syringae* pv. *syringae* strains (48SR2, 40SR4, 46P) hot water, sodium carbonate, ASM, chitosan.
- Combined treatment of *Pseudomonas syringae* pv. *syringae* 48SR2, *Trichoderma atroviride* P1 and chitosan.
- Combined treatment of *Pseudomonas syringae* pv. *syringae* 48SR2, *Wickerhamomyces anomalus* BS91, hot water, sodium bicarbonate.

Objective 3. Gene expression of defence-related genes and lipodepsipeptides synthetase genes in orange fruit in response to

antagonist/elicitors–pathogen interaction using quantitative RT-PCR technique.

Approaches:

- RNA citrus tissues and bacterial extraction from inoculated fruit.
- Selection of amplification primers.
- One step quantitative RT-PCR gene expression analysis (qRT-PCR) of chitinase, β-1,3-glucanase, phenylalanine ammonia-lyase and glutathione peroxides, syringomycin (*syr*B) and syringopeptin (*syp*A).
- Relative quantification of defence-related genes and of *syr*B1 and *syp*A genes.

Chapter 3.

MATHERIALS AND METHODS

3.1 Objective 1. Direct antifungal activities of elicitors and BCAs in controlling post-harvest green mold *in vitro* and on citrus fruit

3.1.1 Direct antifungal activity of ASM, chitosan and BABA *in vitro.*

Chemicals
Fruit
Fungal cultures
In vitro assays
Effect of chemicals on spore germination of P. digitatum in vitro

3.1.2 Direct antifungal activity of ASM, chitosan and BABA on citrus fruit.

Effects of ASM, chitosan and BABA on direct control of P. digitatum in vivo

Effect of BABA on the induction of resistance to P. digitatum
Statistical analysis

3.1.3 Direct antifungal activity of *yeasts in vitro.*
Biocontrol agent
In vitro assay 1
In vitro assay 2

3.1.4 Direct antifungal activity of yeasts on oranges fruit.
Effects of yeasts on direct control of P. digitatum *in vivo*
Statistical analysis

3.1.1 Direct antifungal activity of ASM, chitosan and BABA *in vitro*

Chemicals

ASM compound (Acibenzolar-S-Methyl-Bion WG 50; Novartis Ltd., Basel, Switzerland) was dissolved in sterile distilled water (SDW) at concentrations of 0.1, 0.2, 0.5, 1, 2, 5, and 10 mg/ml.

Chitosan oligosaccharide (Acros Organics, New Jersey, USA) was dissolved, under continuous stirring, in 0.5% (v/v) acetic acid plus 0.1% Tween 80 (Du *et al*., 1997; Romanazzi *et al*., 2002) to obtain a concentration of 10 mg/ml (stock solution) and pH adjusted to 5.6 by using 1N NaOH. Lower concentrations were obtained by appropriate dilutions (0.1, 0.2, 0.5, 1, 2, 5, and 10 mg/ml) with SDW (Romanazzi *et al*., 2007).

BABA (DL-3-amino-*n*-butanoic acid, DL-β-aminobutyric acid) was purchased from Sigma (Chemical Co., St. Louis, MO). A stock solution of BABA (1 M in sterile distilled water) was used. Lower concentrations were obtained by appropriate dilutions (0.1, 1, 10, 100 and 1000 mM) with SDW.

Fruit

Orange (*C. sinensis* (L) Osbeck) cv. Tarocco, Washington Navel and Valencia, yellow lemon (*C. lemon* Burm) cv. Femminello, and grapefruit (*Citrus paradisii* Macf) cv. Marsh Seedless fruit were obtained from local markets and used immediately or held at 4 ˚C for no longer than 1 week before use. Before each experiment, fruit showing no visible wound were carefully hand selected and were washed with tap water, surface-sterilized by dipping for 2 min in 2% of sodium hypochlorite, rinsed with sterile distilled water and then air-dried at the room temperature.

Fungal cultures

Culture of *Penicillium digitatum, P. italicum* and *Geotrichum candidum* were isolated from infected fruits in our mycological laboratory, stored on potato dextrose agar (PDA) slants at 4 °C, and grown on PDA plates for 1 week at 25 °C. Spore suspensions were prepared by flooding 7-day-old sporulating cultures of pathogens with SDW. Conidia were rubbed from the agar surface with a sterile glass rod. Conidia were counted with a haemocytometer and then were adjusted

with SDW to obtain an assorbance of 0.1 at 420 nm determined with a spectrophotometer. This density is approximately equivalent to $1x10^6$ spore/ml, and this inoculum densityb is recommended for evaluation of postharvest treatments to control citrus green mold (Eckert and Brown, 1986).

In vitro assays

The effects of ASM, chitosan and BABA compounds on mycelial growth of *P. digitatum P. italicum,* and *G. candidum* were tested *in vitro*. According to the method of Reuveni (2003) with some modifications, a 10 µl aliquot of the conidial suspension of each pathogen (1×10^6 conidia/ml) was spotted on potato dextrose agar (PDA, Oxoid, Basingstoke, UK) and on orange peel extract agar (100 g/l of orange peel extract; 20 g/l of agar Oxoid) amended to make final concentrations of:

a) 0.1, 0.2, 0.5, 1, 2, 5 and 10 mg/ml of ASM compound (aseptically added to the autoclaved media);

b) 0.1, 0.2, 0.5, 1, 2, 5 and 10 mg/ml (wt/vol) of chitosan (added to the media before being autoclaved);

c) 0.1, 1, 10 and 100 mM of BABA compound (aseptically added to the autoclaved media).

Unamended PDA and orange peel extract agar plates served as controls. Cultures were incubated at 25 °C for 7 days in the dark. At the end of the incubation period, radial growth reduction was calculated in relation to growth of the control as follows: %I = (C-T/C) × 100, where % I represented the inhibition of radial mycelial growth, C was radial growth measurement of the pathogen in the control plates and T was radial growth of the pathogen in the media amended with chemical compound. The mean mycelial growth reduction and the standard deviation were calculated. Three Petri dishes were used for each treatment concentration and experiments were conducted twice.

Effect of chemicals on spore germination of* P. digitatum *in vitro

The effects of different concentrations of ASM, chitosan and BABA on *P. digitatum* conidia germination were tested according to Porat *et al*. (2003). Spores of *P. digitatum* were suspended in potato dextrose broth (PDB, Difco) at final concentrations of $2x10^6$ spores ml^{-1}. Aliquots (450 µl) of spore suspensions were transferred to eppendorf tubes and aliquots of the different stock solutions were added to obtain:

a) ASM final concentrations of 0.1, 0.2, 0.5, 1, 2, 5 mg/ml;

b) Chitosan final concentration of 0.1, 0.2, 0.5, 1, 2, and 5 mg/ml (wt/vol);

c) BABA final concentration of 0.1, 1, 10, 100 and 1000 mM.

Conidial suspensions of *P. digitatum* in sterile distilled water were used as control.

Aliquots (30 µl) of the ASM/chitosan/BABA solutions were placed on ethanol-washed microscope slides (three drops per slide), kept in Petri dishes padded with moistened filter paper and incubated at 25 °C for 24 hours in darkness.

The number of germinating conidia was counted in three microscope fields, each contained 40-50 spores of the pathogen.

Three replicates per each treatment were used. The mean percent germination and the standard deviation were calculated.

3.1.2 Direct antifungal activity of ASM, chitosan and BABA on citrus fruit

Effects of ASM , chitosan and BABA on direct control of* P. digitatum *in vivo

Oranges, yellow lemons and grapefruits were wounded with a sterile needle to make four 2-mm deep and 5-mm wide wounds on their peel at the equatorial region (four wound for each fruit).

Thirty microliters of ASM solutions (concentrations of 0.2, 0.5, 1, 2, 5 mg/ml), chitosan solutions (concentrations of 0.2, 0.5, 1, 2 and 5 mg/ml), and BABA solutions (concentrations of 1, 10, 100, 1000 mM) were injected into each wound site. Wounds treated with the same amount of distilled sterilized water served as a control.

After 24 h incubation at 20 °C under humid conditions, the same wounds were inoculated with 20 µl of spore suspension (5×10^6 spores/ml) of *P. digitatum*.

Fruit were placed in plastic cavity packaging trays under humid conditions and incubated at 20 °C. Twelve fruits were used for each treatment (total of 48 wounds per treatment) and each experiment was repeated three times The number of wounds showing symptoms of infection was counted and incidence of disease was determined 3-4-5 days after the inoculation. Disease severity was evaluated with an empiric scale (1 = no visible symptoms; 2 = initial soft rot; 3 = presence of mycelium; 4 = sporulation). In all experiments, disease severity data were converted to percentage midpoint values (Campbell and Madden, 1990), where 1 = 0%, 2 = 35%, 3 = 65% and 4 = 90%.

***Effect of BABA on the induction of resistance to* P. digitatum**

For the evaluation of the protection conferred by the treatment with BABA (induction of resistance mediated by the compound), protocol described by Porat *et al*. (2003) with minor changes was used. The fruit were wounded with a sterile needle as previously described (four wounds for each fruit). Thirty microliters of BABA at concentrations of 1, 10, 100, 1000 mM were pipetted into each wound site.

After 24 h incubation at 20 °C under humid conditions, fresh wounds were made at 1cm from the original BABA-treated wounds, and the fresh wounds were inoculated with 20 µl of *P. digitatum* (5×10^6 spores ml^{-1}). The effects of BABA compounds mediated by induction of resistance were evaluated on oranges cv. Tarocco, lemon and grapefruits.

In a second set of trials, oranges cvs. Tarocco, Valencia and Washington navel were treated with 10 mM of BABA, and after 24 h incubation at 20 °C under humid conditions inoculated with *P. digitatum* into fresh wounds made at 1 cm from the original treated site. In all trials, control fruits were treated with sterile distilled water and pathogen suspension.

Fruit were placed in plastic cavity packaging trays under humid conditions and incubated at 20 °C. Twelve fruits were used for each treatment (total of 48 wounds per treatment) and each experiment was repeated at least three times with similar results. The number of wounds showing symptoms of infection was counted and incidence of disease was determined 3, 4, and 5 days after the inoculation. Disease severity was evaluated 3, 4, and 5 days after the inoculation with an empiric scale (1 = no visible symptoms; 2 = initial soft rot; 3 = presence of mycelium; 4 = sporulation). In all experiments, disease severity data were converted to percentage midpoint values (Campbell and Madden, 1990), where 1 = 0%, 2 = 35%, 3 = 65% and 4 = 90%.

Statistical analysis

The percentages of decayed tissues (incidence of disease) and disease severity ratings were subjected to an arcsine square root transformation before running the ANOVA. Subsequently, one-way ANOVA was performed. The mean values were separated using the Student–Newman–Keuls mean separation test, at $P \leq 0.05$.

3.1.3. Direct antifungal activity of yeasts *in vitro*

Biocontrol agents

Yeasts isolated from naturally fermented olives, belonging to DISPA collection and identified as *Saccharomyces cerevisiae* BS46, *S. cerevisiae* BCA61, *Wickerhamomyces anomalus* BCU24, *W. anomalus* BS91, *W. anomalus* BS92 and *W. anomalus* BCA15, were selected on the basis of the high killer capacity against sensitive strain of *Saccharomyces cerevisiae*, and the toxic principle was identified and characterized as a β-glucanase (Mucilli *et al*., 2010). Yeasts culture was maintained in 15% v/v glycerol at –80°C and subcultured on YPDA (g/l distilled water: yeast extract 10, peptone 10, dextrose 20, agar 20; Oxoid, Basingstoke, UK). Yeast suspensions were prepared from cells grown on YPDA for 24-48 h at 27°C. Yeast cultures were suspended in SDW and the concentrations were photometrically adjusted with sterile water to a cell density corresponding at $1–2\times10^9$ colony-forming units per milliliter (CFU/ml).

In vitro assay 1

Yests and fungi to be tested were grown, respectively, on YPDA for 24-48 h at 27°C and on PDA for 7-14 days at 25°C until sporulation.

Yeasts suspensions (20µl) obtained from cultures on YPDA (1×10^9 CFU/ml) were spotted on YPDA pH 7 on plates (two spots per plate). Plates were incubated for three days at 27°C; then, plates were sprayed with a suspension containing 1×10^6 conidia/ml (an absorbance of 0.1 at 420 nm determined with a spectrophotometer) of *P. digitatum*, *P. expansum*, *P. italicum* and *Geotrichum candidum*.

Non inoculated plates were used as control and were sprayed with conidia suspension. The plates were incubated at 27°C for 3-5 days. The presence and size of a clear zone around yeast colonies, indicating the inhibitory effect, was noted and measured. All test was repeated twice.

In vitro assay 2

Yeasts and mold strains to be tested were grown, respectively, on YPDA for 48-72 h at 25 °C and on PDA for 7-14 days at 25 °C until sporulation. To evaluate mold growth biocontrol by the yeast isolates, an assay in which both

fungi were grown side by side was done. Petri dishes with YPDA, at pH 7 and pH 4.5, buffered with citrate-phosphate, were inoculated with a 5-mm square plug of an actively growing fungal mycelium near the dish edge. A loop of the yeast cells was inoculated at the opposite edge. A control dish was prepared with only inoculation of the mold. The experiment was performed with 3 replicates, incubating the dishes at 25 °C. Mycelial growth was measured after 2, 4, 7 and 14 days and expressed as the distance (cm) from the plug to the edge side of the actively growing mould. The fungal mycelium was microscopically observed after 4 days to assess the possible hyphal damage caused by the killer toxin. Mycelium disks (5 mm in diameter), from the dish in which the yeast had inhibited the mold growth, were removed from the part located nearer to the yeast cells. The excess of culture medium was longitudinally cut to obtain thin mycelia layers which were studied by an upright optical microscope (Olympus, Hamburg, Germany). The hyphae were photographed and compared with the morphology of the control.

3.1.4. Direct antifungal activity of yeasts on oranges fruit

Effects of yeasts on direct control of* P. digitatum *in vivo

Oranges cv. Tarocco were wounded with a sterile needle to make four 2-mm deep and 5-mm wide wounds on their peel at the equatorial region (four wound for each fruit). Twenty microliters of yeast suspension (1×10^9 CFU/ml), prepared from cells grown on YPDA for 24 h at 25°C, were injected into each wound site. Wounds treated with the same amount of distilled sterilized water served as a control. After 72 h incubation at 20 °C under humid conditions, the wounds were inoculated with 20 µl of spore suspension (5×10^6 spores/ml) of *P. digitatum*.

Fruit were placed in plastic cavity packaging trays under humid conditions and incubated at 20°C. Four fruit were used for each treatment (total of 16 wounds per treatment) and each experiment was repeated three times.

The number of wounds showing symptoms of infection was counted and incidence of disease was determined 3-5-10 days after the inoculation. Disease severity was evaluated with an empiric scale (1 = no visible symptoms; 2 = initial soft rot; 3 = presence of mycelium; 4 = sporulation). In all experiments, disease severity data were converted to percentage midpoint values (Campbell and Madden, 1990), where 1 = 0%, 2 = 35%, 3 = 65% and 4 = 90%.

Statistical analysis

The percentages of decayed tissues (incidence of disease) and disease severity ratings were subjected to an arcsine square root transformation before running the ANOVA. Subsequently, one-way ANOVA was performed. The mean values were separated using the Student–Newman–Keuls mean separation test, at $P\leq 0.05$.

MATERIALS AND METHODS

3.2 Objective 2. Curative and preventive activity of biological control agents and elicitors in different combined treatments on oranges fruit.

3.2.1 Combined treatment of *Pseudomonas syringae* pv. *syringae* strains (48SR2, 40SR4, 46P), hot water, sodium carbonate, ASM, chitosan.

Reagents
Fruit
Fungal cultures
Biocontrol agents
Curative activity of combined treatments with P. s. pv. syringae 48SR2*, hot water, and sodium carbonate.*
Protective activity of combined treatments with P. s. pv. syringae *strains, acibenzolar-S-methyl, and chitosan.*
Statistical analysis

3.2.2 Combined treatment of *Pseudomonas syringae pv. syringae* 48SR2, *Trichoderma atroviride* P1 and chitosan

Reagents
Fruit
Fungal cultures
Biocontrol agents
Curative and preventive activity of combined application of P.s. pv. syringae 48SR2 T. atroviride P1 *and chitosan 0.5 mg/ml on the control of* P. digitatum *on cv. Tarocco stored at different temperatures after treatment.*
Statistical analysis

3.2.3 Combined treatment of *Pseudomonas syringae* pv. *syringae* 48SR2, *Wickerhamomyces anomalus* BS91, hot water, sodium bicarbonate.

Fruit
Fungal cultures
Biocontrol agents
Curative and preventive activity of combined treatments of hot water, sodium bicarbonates, P.s. pv. syringae 48SR2 *and* Wickerhamomyces anomalus BS91 *to control postharvest green mold of oranges cv. Tarocco.*

3.2.1 Combined treatment of *Pseudomonas syringae* pv. *syringae* strains (48SR2, 40SR4, 46P), hot water, sodium carbonate, ASM, chitosan

Reagents

Sodium carbonate (Na_2CO_3 99.5%, Sigma-Aldrich, Germany) was dissolved at 3% in hot water (45 °C); acibenzolar-S-methyl (ASM) (Bion WG 50; Novartis Ltd., Basel, Switzerland) was dissolved in distilled sterilized water to obtain final concentrations of 0.5 and 1.0 mg/ml; chitosan oligosaccharide (Chitosan, molecular weight: 100,000-300,000; Acros Organics, New Jersey, USA) was prepared at 0.5 mg/ml by dissolving, under continuous stirring, the chitosan in 0.5% (v/v) acetic acid (Du *et al*., 1997; Romanazzi *et al*., 2002). When dissolved, the pH of the chitosan solution was adjusted to 5.6 by using 1N NaOH.

Fruit

Oranges (*C. sinensis* (L) Osbeck) cv. Tarocco were obtained from local markets and used immediately or held at 4 ˚C for no longer than 1 week before use. Before each experiment, fruit showing no visible wound were carefully hand selected and were washed with tap water, surface-sterilized by dipping for 2 min in 2% of sodium hypochlorite, rinsed with sterile-distilled water (SDW) and then air-dried at the room temperature.

Fungal cultures

Penicillium digitatum was isolated from infected fruit in our mycological laboratory, stored on potato dextrose agar (PDA) slants at 4 °C, and grown on PDA plates for 1 week at 25 °C. Spore suspensions were prepared by flooding 7-day-old sporulating cultures of pathogens with SDW. Conidia were rubbed from the agar surface with a sterile glass rod. Conidia were counted with a haemocytometer and then were adjusted with SDW to obtain an assorbance of 0.1 at 420 nm determined with a spectrophotometer. This density is approximately equivalent to 1×10^6 spore/ml, and this inoculum density is recommended for evaluation of postharvest treatments to control citrus green mold (Eckert and Brown, 1986).

Biocontrol agents

The antagonistic *P. syringae* pv. *syringae* 40SR4, 48SR2 and 46P strains were originally isolated from the surface of orange fruit and had earlier been screened for their antagonistic properties (Cirvilleri *et al.,* 2005). Bacterial culture was maintained in 15% v/v glycerol at –80°C and subcultured on medium King'B (KB) as needed (King *et al.* 1954). Bacterial suspensions were prepared from cells grown on NA for 4 days at 27°C. The bacterial cultures were suspended in SDW and the concentrations were photometrically adjusted with sterile water to a cell density corresponding at 1×10^9 colony-forming units per milliliter (CFU/ml). The choice of this concentration was based on preliminary investigations (Cirvilleri *et al*., 2005)

Curative activity of combined treatments with* P. s. pv. syringae 48SR2*, hot water, and sodium carbonate.

The efficacy of combined treatments with hot water, sodium carbonate and *P. syringae* strains on oranges cv. Tarocco was evaluated. Orange fruit, sterilized as described above, were inoculated with *P. digitatum* by immersing a stainless steel rod with a probe tip 1 mm wide and 2 mm in length into the spore suspension (1×10^6 conidia/ml) and wounding each fruit four times.

After 3h, fruits were immersed in a water bath containing tap water at 45°C or in 3% SC at 45°C for 2 min. After air-drying, antagonistic *P. syringae* strain 48SR2 (1×10^9 CFU/ml) was spray-inoculated on fruits. Oranges inoculated with *P. digitatum* and immersed in sterile distilled water were used as a control.

Fruits were then incubated at 20°C in plastic trays under humid conditions. There were three replicates of 4 oranges per replicate and the experiment was conducted twice.The number of wounds showing symptoms of infection was counted and incidence of disease was calculated 5 days after the treatments. Disease severity was instead evaluated with an empiric scale (1 = no visible symptoms; 2 = initial soft rot; 3 = presence of mycelium; 4 = sporulation). For each experiment, disease severity data were converted to percentage midpoint values (Campbell and Madden, 1990), where 1 = 0%, 2 = 35%, 3 = 65% and 4 = 90%.

Protective activity of combined treatments with* P. s. pv. syringae *strains, acibenzolar-S-methyl, and chitosan

The protective activity of combined treatments with ASM and *P. syringae* strains on oranges cv. Tarocco was evaluated. The following treatments were applied: (A) non-wounded fruit were soaked in ASM solutions at concentrations of 0.5 mg/ml and 1.0 mg/ml for 5 minutes, incubated at 20 °C under humid conditions, and inoculated 72 h later with *P. digitatum;* (B) fruit were wounded, soaked in ASM solutions at concentrations of 0.5 mg/ml and 1.0 mg/ml for 5 minutes, incubated at 20 °C under humid conditions, and inoculated 72 h later with *P. digitatum;* (C) fruit were wounded, treated 2 h later in same wounds with 3 different antagonistic bacteria (*P.s.* pv. *syringae* strain 40SR4, 48SR2, 46P) (20 µl, 1×10^9 CFU/ml), incubated at 20°C under humid conditions, and inoculated 72 h later with *P. digitatum*; (D) fruit were wounded, soaked in ASM solution 0.5 mg/ml and 10 mg/ml, treated 2 h later with 3 different antagonistic bacteria (*P.s.* pv. *syringae* strain 40SR4, 48SR2, 46P), incubated at 20°C under humid conditions, and inoculated 72 h later with *P. digitatum*; (E) fruit were wound inoculated with *P. digitatum* (1×10^6 spore/ml) and untreated (control treatment).

The protective activity of combined applications of chitosan and *P. syringae* 48SR2 was evaluated. Oranges cv. Tarocco were wounded and dipped for 10 min in the following solutions: 1) chitosan at concentration of 0.5 mg/ml; 2) cell suspensions of *P. syringae* strain 48SR2 (1×10^9 CFU/ml); 3) cell suspensions of *P. syringae* strain 48SR2 (1×10^9 CFU/ml) plus chitosan (0.5 mg/ml). After 3 days, fruits were wound-inoculated with *P. digitatum* (1×10^6 conidia/ml) and incubated for 5 days at 20°C.

In both trials, there were three replicates of 4 oranges per replicate and the experiment was conducted twice. In all experiments, number of wounds showing symptoms of infection was counted and incidence of disease was determined 3, 4, 5 days after the inoculation. Disease severity was evaluated 3, 4, 5 days after the inoculation, with an empiric scale (1 = no visible symptoms; 2 = initial soft rot; 3 = presence of mycelium; 4 = sporulation). In all experiments, disease severity data were converted to percentage midpoint values (Campbell and Madden, 1990), where 1 = 0%, 2 = 35%, 3 = 65% and 4 = 90%.

Statistical analysis

The percentages of decayed tissues (incidence of disease) and disease severity ratings were subjected to an arcsine square root transformation before running the ANOVA. Subsequently, one-way ANOVA was performed. The mean values were separated using the Student-Newman-Keuls' mean separation test, at $P \leq 0.05$.

3.2.2. Combined treatment of *Pseudomonas syringae* pv. *syringae* 48SR2, *Trichoderma atroviride* P1 and chitosan

Reagents

Chitosan oligosaccharide (Acros Organics, New Jersey, USA) was dissolved, under continuous stirring, in 0.5% (v/v) acetic acid plus 0.1% Tween 80 (Du *et al*., 1997; Romanazzi *et al*., 2002) to obtain a concentration of 10 mg/ml (stock solution); when dissolved, the pH of the chitosan solution was adjusted to 5.6 by using 1N NaOH, and lower concentration of 0.5 mg/ml was obtained by dilution with sterile distilled water (Romanazzi *et al*., 2007).

Thiabendazole (2-4-thiazolylbenzimidazole, Tecto 20S (20% p.a.), Sygenta Crop Protection), was dissolved in sterile distilled water (SDW) and used at final concentration of 0.1%.

Fruit

Oranges (*C. sinensis* (L) Osbeck) cv. Tarocco were obtained from local markets and used immediately or held at 4 ˚C for no longer than 1 week before use. Before each experiment, fruit showing no visible wound were carefully hand selected and were washed with tap water, surface-sterilized by dipping for 2 min in 2% of sodium hypochlorite, rinsed with sterile-distilled water (SDW) and then air-dried at the room temperature.

Fungal cultures

Penicillium digitatum was isolated from infected fruit in our mycological laboratory, stored on potato dextrose agar (PDA) slants at 4°C, and grown on PDA plates for 1 week at 25° C. Spore suspensions were prepared by flooding 7-day-old sporulating cultures of pathogens with SDW. Conidia were rubbed from the agar surface with a sterile glass rod. Conidia were counted with a haemocytometer and then were adjusted with SDW to obtain an assorbance of 0.1 at 420 nm determined with a spectrophotometer. This density is approximately equivalent to 1×10^6 spore/ml, and this inoculum density is recommended for evaluation of postharvest treatments to control citrus green mold (Eckert and Brown, 1986).

Biocontrol agents

The antagonistic *P. syringae* pv. *syringae* 48SR2 (Pss48SR2) strain was originally isolated from the surface of orange fruit and had earlier been screened for its antagonistic properties (Cirvilleri *et al.,* 2005). Bacterial culture was maintained in 15% v/v glycerol at –80°C and subcultured on medium King'B (KB) as needed (King *et al.* 1954). Bacterial suspensions were prepared from cells grown on NA for 4 days at 27°C. The bacterial cultures were suspended in SDW and the concentrations were photometrically adjusted with sterile water to a cell density corresponding at 1×10^9 colony-forming units per milliliter (CFU/ml). The choice of this concentration was based on preliminary investigations (Cirvilleri *et al*., 2005).

The antagonistic *Trichoderma atroviride* P1 strain was obtained from the collection of Department of Arboricoltura, Botanica e Patologia Vegetale, University of Napoli. Fungal colture was grown on PDA plates for 1 week at 25°C before use. A spore suspension was obtained by flooding 2-week-old cultures with sterile distilled water containing 0.1% (v/v) Tween 80. Spores were counted with a haemocytometer and spore concentration was adjusted with sterile distilled water to obtain 1×10^6 spores per ml.

Curative and preventive activity of combined application of* Pseudomonas syringae *pv.* syringae 48SR2 T. atroviride *P1 and chitosan 0.5 mg/ml on the control of* P. digitatum *on cv. Tarocco stored at different temperatures after treatment.

Orange, wounded with a sterile needle to make four 2-mm deep and 5-mm wide wounds on their peel at the equatorial region (four wound for each fruit). Fruit were dipped in a solutions containing: bacterial suspension of *Pss* 48SR2 (1×10^9 CFU/ml); conidial suspension of *T. atroviride* P1 (1×10^8 spore/ml); chitosan at concentration of 0.5 mg/ml; thiabendazole at concentration of 0.1%; mixture of bacterial and conidial suspension 1:1 ratio, mixture of chitosan, 48SR2 and P1 in 1:1:1 ratio. Oranges dipped in distilled sterilized water served as a control.

Fruits were then placed in plastic cavity packaging trays under humid conditions and incubated at 20° and 6°C. Two temperatures of storage were chosen for the test: room temperature (20 °C) normally favours the growth of the pathogens (Spadaro *et al*., 2004), whereas 6 °C is one of the temperature of commercial fruit. After 2, 24 and 72 hours wounds made at the beginning were inoculated with 20 µl of spore suspension (1×10^6 spores/ml) of *P. digitatum*. Fruits were then re-placed in packaging trays and incubated at 20 °C and 6 °C for respectively 5 and 22 days. Four fruits were used for each treatment (total of 16 wounds per treatment) and each experiment was repeated three times.

The number of wounds showing symptoms of infection was periodically recorded during the incubation time at 20 °C and 6 °C, and incidence of disease was determined. Disease severity was evaluated with an empiric scale (1 = no visible symptoms; 2 = initial soft rot; 3 = presence of mycelium; 4 = sporulation). In all experiments, disease severity data were converted to percentage midpoint values (Campbell and Madden, 1990), where 1 = 0%, 2 = 35%, 3 = 65% and 4 = 90%.

Statistical analysis

The percentages of decayed tissues (incidence of disease) and disease severity ratings were subjected to an arcsine square root transformation before running the ANOVA. Subsequently, one-way ANOVA was performed. The mean values were separated using the Student–Newman–Keuls mean separation test, at $P\leq 0.05$.

3.2.3. Combined treatment of *Pseudomonas syringae* pv. *syringae* 48SR2, *Wickerhamomyces anomalus* BS91, hot water, sodium bicarbonate

Fruit

Oranges (*C. sinensis* (L) Osbeck) cv. Tarocco were obtained from local markets and used immediately or held at 4 ˚C for no longer than 1 week before use. Before each experiment, fruit showing no visible wound were carefully hand selected and were washed with tap water, surface-sterilized by dipping for 2 min in 2% of sodium hypochlorite, rinsed with sterile-distilled water (SDW) and then air-dried at the room temperature.

Fungal cultures

Penicillium digitatum was isolated from infected fruit in our mycological laboratory, stored on potato dextrose agar (PDA) slants at 4 °C, and grown on PDA plates for 1 week at 25 °C. Spore suspensions were prepared by flooding 7-day-old sporulating cultures of pathogens with SDW. Conidia were rubbed from the agar surface with a sterile glass rod. Conidia were counted with a haemocytometer and then were adjusted with SDW to obtain an assorbance of 0.1 at 420 nm determined with a spectrophotometer. This density is approximately equivalent to 1×10^6 spore/ml, and this inoculum density is recommended for evaluation of postharvest treatments to control citrus green mold (Eckert and Brown, 1986).

Biocontrol agents

The antagonistic *P. syringae* pv. *syringae* 48SR2 strain was originally isolated from the surface of orange fruit and had earlier been screened for its antagonistic properties (Cirvilleri *et al.,* 2005). Bacterial culture was maintained in 15% v/v glycerol at –80°C and subcultured on medium King'B (KB) as needed (King *et al.* 1954). Bacterial suspensions were prepared from cells grown on NA for 4 days at 27 °C. The bacterial cultures were suspended in SDW and the concentrations were photometrically adjusted with sterile water to a cell density corresponding at 1×10^9 colony-forming units per milliliter (CFU/ml). The choice

of this concentration was based on preliminary investigations (Cirvilleri *et al*., 2005).

Yeast *Wickerhamomyces anomalus* BS91, isolated from naturally fermented olives, was selected on the basis of the high killer capacity against sensitive strain of *Saccharomyces cerevisiae*, and the toxic principle was identified and characterized as a β-glucanase (Mucilli *et al*., 2010). Yeast culture was maintained in 15% v/v glycerol at –80°C and subcultured on YPDA (g/l distilled water: yeast extract 10, peptone 10, dextrose 20, agar 20; Oxoid, Basingstoke, UK). Yeast suspensions were prepared from cells grown on YPDA for 24-48 h at 27°C. Yeast cultures were suspended in SDW and the concentrations were photometrically adjusted with sterile water to a cell density corresponding at 1–2 · 10^9 colony-forming units per milliliter (CFU/ml).

Curative and preventive activity of combined treatments of hot water, sodium bicarbonates, Pseudomonas syringae 48SR2 and Wickerhamomyces anomalus BS91 to control postharvest green mold of oranges cv. Tarocco

Before each treatment Tarocco oranges were wounded with a sterile needle to make four 2-mm deep and 5-mm wide wounds on their peel at the equatorial region.

At different inoculation time, 20 µl of *P. digitatum* at 1×10^6 conidia/ml, 20 µl of antagonistic *P.s.* pv. *syringae* PVCT48SR2 cells (1×10^9 CFU/ml) and 20 µl of antagonistic *Wickerhamomyces anomalus* BS 91 (1×10^9 CFU/ml), prepared as described above, were applied to each wound on fruit and allowed to dry at room temperature.

The sodium bicarbonate (SBC) treatments (sodium bicarbonate-$NaHCO_3$ 99.5%, pH 8, Sigma-Aldrich, Germany, dissolved at 3% in hot water at 45°C) were done by placing fruit into plastic baskets and immersing them for 5 min in 3% (w/v) SBC solution at 45°C.

The following 12 treatments were applied on Tarocco oranges: (1) fruit were wound inoculated with *P. digitatum* and untreated (control treatment); (2) fruit were wound inoculated with *P. digitatum* then treated 2 h later in same wounds with the strain *Pss* 48SR2; (3) fruit were wound inoculated with *P. digitatum* and treated 24 h later with sodium bicarbonate (SBC); (4) fruit were

wound inoculated with *P. digitatum*, treated 24 h later with SBC, and treated 2 h later in same wound with *Pss*; (5) fruit were wounded, treated 24 h later with SBC, and inoculated 2 h later in same wound with *P. digitatum*; (6) fruit were wounded, treated 24 h later with SBC, inoculated 2 h later in same wound with *P. digitatum*, and treated 2 h later in same wound with *Pss* 48SR2; (7) fruit were treated with SBC, wounded and inoculated 2 h later with *P. digitatum*; (8) fruit were treated with SBC, wounded and inoculated 2 h later with *P. digitatum,* and then treated 2 h later in same wound with *Pss* 48SR2; (9) fruit were treated with SBC, wounded and treated 2 h later with *Pss* 48SR2, and inoculated 24 h later with *P. digitatum;* (10) fruit were treated with SBC, wounded and treated 2 h later with *W.a.* BS91, and inoculated 24 hours later with *P.d;* (11) fruit were treated with SBC, wounded and treated 2 h later with *Pss* 48SR2, and inoculated 48 hours later with *P.d.*; (12) fruit were treated with SBC, wounded and treated 2 h later with *W.a.* BS91, and inoculated 48 hours later with *P.d.*

Treated fruit were allowed to air-dry at room temperature and then placed on plastic packaging trays. The cavity trays isolate each fruit and prevent contact infections of adjacent fruits. To provide ample humidity for disease development, a wet paper towel was placed on empty cavity trays and the entire box was placed in a plastic bag. The bags were sealed and the fruits were incubated at 20°C for 6-8 days. Each treatment was applied to 2 replicates of four fruit each. Each treatment was repeated 3 times.

During incubation, the fruits were observed repeatedly and the number of wounds showing symptoms of infection was counted and incidence of disease was determined 1, 2, 3, 4, 6, 7 and 8 days after the inoculation. Disease severity was evaluated 4 days after the inoculation with an empiric scale (1 = no visible symptoms; 2 = initial soft rot; 3 = presence of mycelium; 4 = sporulation). In all experiments, disease severity data were converted to percentage midpoint values (Campbell and Madden, 1990), where 1 = 0%, 2 = 35%, 3 = 65% and 4 = 90%.

Statistical analysis

The percentages of decayed tissues (incidence of disease) and disease severity ratings were subjected to an arcsine square root transformation before running the ANOVA. Subsequently, one-way ANOVA was performed. The mean values were separated using the Student–Newman–Keuls mean separation test, at $P \leq 0.05$.

MATERIALS AND METHODS

3.3 Objective 3. Gene expression of defence-related genes and lipodepsipeptides synthetase genes in orange fruit in response to antagonist/elicitors–pathogen interaction using quantitative RT-PCR technique

3.3.1 RNA citrus tissues and bacterial extraction from inoculated fruit.

3.3.2 Selection of amplification primers.

3.3.3 One step quantitative RT-PCR gene expression analysis (qRT-PCR) of chitinase, β-1,3-glucanase, phenylalanine ammonia-lyase and glutathione peroxides, syringomycin *(syr*B*)* and syringopeptin *(syp*A*)*.

3.3.4 Relative quantification of defence-related genes and of s*yr*B1 and *syp*A genes.

3.3.1. RNA citrus tissues and bacterial extraction from inoculated fruit

Citrus peel RNAs were extracted and RT-PCR analysis carried out from citrus tissues treated according to the treatments 1, 4, 6, 11, 12 sampled 2h, 24h and 48h after *P. digitatum* inoculation. As mechanical injury can also induce resistance responses, the analysis was also performed on citrus peel from untreated and non-inoculated fruit and from citrus peel collected from intact fruit (non-wounded and non-inoculated) (control). Peel tissue samples (obtained using a sterile scalpel, 10 mm in diameter around the inoculation site; 100 mg) were taken for analysis and immediately utilized or stored at -80°C until required. Peel tissue samples were macerated for 1 h in 500 µl of RNA protect Bacteria Reagent (Qiagen) for RNA stabilization and protection. Citrus peel RNAs were extracted using the RNeasy® Mini Kit for plant tissue (Qiagen, MD, USA) in accordance with the manufacturer's instructions (Appendix 1).

P. syringae 48SR2 RNA was extracted from citrus tissues treated according to the treatment 11, at 2h, 24h and 48h after SBC+Pss treatment and 2h, 24h, and 48h after *P. digitatum* inoculation . Peel tissue samples (obtained using a sterile scalpel, 10 mm in diameter around the inoculation site; 100 mg) were taken for analysis and immediately utilized or stored at -80°C until required. Peel tissue samples were macerated for 1 h in 500 µl of RNA protect Bacteria Reagent (Qiagen) for RNA stabilization and protection. Bacterial RNAs from tissue samples were extracted using RNeasy® Mini Kit (Quiagen, Valencia, CA, USA) in accordance with manifacture's instructions (Appendix 2).

Concentrations of tissue and bacterial RNAs were determined by measuring the absorbance at 260 nm in a spectrophotometer (BioPhotometer, Eppendorf, UK).

RNA samples were treated with Turbo DNase-free kit (Ambion, Austin, TX, USA) (Appendix 3), adjusted to 5 ng/ µl, and aliquots of 5 µl of each sample were used for qRT-PCR.

PRIMER	SEQUENCE	AMPLICON	REFERENCE
EF 1 Elongation factor 1-alpha	**Forward** 5'-GGTCAGACTCGTGAGCATGC-3' **Reverse** 5'-CATCGTACCTAGCCTTTGAGTACTTG-3'	114	Distefano *et al*. (2008)
CHI 1 Chitinase	**Forward** 5'-AATGTTGCTAGCATTGTGACTCC-3' **Reverse** 5'-GCAGCATTCAGAAACGCATCT-3'	121	Scuderi *et al*. (2011)
GNS1 β-1,3-Glucanase	**Forward** 5'-TTCCGACGGATCGTTAAGTTACC-3' **Reverse** 5'-CCACCCACTCTCTGATATCACG-3'	148	Distefano *et al*. (2008)
PAL 1 Phenylalanine Ammonia-Lyase	**Forward** 5'-GTTGCATGAGATTGATCCTCTGC-3' **Reverse** 5'-TGTCATTCACCGAGTTGATCTCC-3'	143	Distefano *et al*. (2008)
GPX1 Phospholipid hydroperoxide gluthatione peroxidase	**Forward** 5'-GAATGTTGTTGAGCGTTATGCC-3' **Reverse** 5'-AGCTGATCATGCAAGTTGTAGCA-3'	231	Distefano *et al*. (2008)
16S rRNA	**Forward** 5'-ACACCGCCCGTCACACCA-3' **Reverse** 5'-GTTCCCCTACGGCTACCTT-3'	125	Kang and Gross (2005)
*Syr*B1 Syringomycin	**Forward** 5'-AATGTTGCTAGCATTGTGACTCC-3' **Reverse** 5'-GCTCAACGTCCGGGCTGCATCGCTCAC-3'	116	Scuderi *et al*. (2011)
*Syp*A Syringopeptin	**Forward** 5'-TGCGGGTCGAGGCGTTTTTG-3' **Reverse** 5'-TTCGCCGCGCCCTTGTCTGA-3'	248	Scuderi *et al*. (2011)

Table 2. Sense and antisense primers adopted in quantitative RT-PCR.

3.3.2. Selection of amplification primers

Primer pairs listed in Table 2 were derived from literature according to the sequences deposited in GeneBank: elongation factor 1-alpha (EF1), chitinase (CHI1), glucanase (GNS1), phenylalanine ammonia-lyase (PAL1), gluthatione peroxidase (GPX1) from *Citrus sinensis* published genome, and 16S housekeeping gene, syringomycin synthetase gene (*syr*B1), syringopeptin synthetase gene (*syp*A) from published genome sequences of *P.s.* pv. *syringae* B728a strain (Refseq: NC_007005).

These oligos were synthesized by Invitrogen-Life Technologies, Paisley, UK.

3.3.3 One step quantitative RT-PCR gene expression analysis (qRT-PCR) of chitinase, β-1,3-glucanase, phenylalanine ammonia-lyase, glutathione peroxides, syringomycin (syrB) and syringopeptin (sypA).

QRT-PCR one-step was performed on the iCycler Thermal Cycler using IQ SYBRGreen supermix PCR Reagents (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions.

RNA samples were added in triplicate to an iCycler iQ 96-well PCR plate (Bio-Rad) and the qRT-PCR was performed from two separate samples per treatment. A total of six data were obtained for each treatment and the mean of these values was used for further analyses.

To each well the following components were added to a total volume of 25 µl: 5 U MultiScribe-RT enzyme (Applied Biosystem, Courtaboeuf, France), 15 U RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen), 1× SyberGreen Master Mix (Bio-Rad), 0.2 µM of each forward and reverse primer and 5 µl of template (RNA 5 ng/µl). A negative control devoid of template (water sample) and one RT negative control (lacking of MultiScribe-RT enzyme) were included for each primer pair analysis to determine genomic contamination and to verify effectiveness of reverse transcriptase, too.

Real-time results were analyzed using iCycler iQ Optical System software, version 3.0a (Bio-Rad). The amplification profile for all primers consisted of an initial reverse transcription step of 30 min at 48°C, an activation step of 10 min at 95°C followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Specificity of the reaction was checked by analysis of the melting curve of the final amplified product.

3.3.4. Relative quantification of defence-related genes and of *syr*B1 and *syp*A genes.

The transcript levels were calculated by relative quantification of citrus target genes (CHI1, GNS1, PAL1, GPX1) normalized against citrus elongation factor 1-alpha (EF1) (Distefano *et al*. 2008), and by relative quantification of *P.s.* pv. *syringae* target genes (*syr*B1, *syp*A) normalized against 16S rRNA gene (Scuderi *et al*., 2011).

The relative quantification of target genes was performed by comparing ΔC_t (C_t target gene – C_t reference gene). The ΔC_t value of the control sample was used as the calibrator and fold-activation was calculated by the expression: $2^{-\Delta\Delta Ct}$, where $\Delta\Delta C_t$) = ΔC_t sample - ΔC_t control. The levels of the transcripts were expressed as relative units.

Statistical analysis

The paired Bonferroni comparison test was used for the statistical analysis of data (Sheskin, 2004). A value of $P < 0.05$ was considered as significant.

Appendix 1

Extraction protocol from orange tissue

Before to start, determine the amount of plant material, do not use more than 100 mg. Grind thoroughly with a pestle the tissue, add 450 µl Buffer RLT and vortex vigorously. Transfer the lysate to a QIAshredder spin column (liliac) placed in a 2 ml collection tube, and centrifuge for 2 minutes at full speed. Carefully transfer the supernatant of the flow-through to a new microcentrifuge tube. Add 0.5 volume of ethanol (96-100%) to the cleared lysate, and mix immediately by pipetting. Transfer the sample (usually 650 µl), including any precipitate that may have formed, to an RNeasy spin column (pink) placed in a 2 ml collection tube. Centrifuge for 15 seconds at 13,000g. Discard the flow-through. Add 700 µl Buffer RW1 to the RNeasy spin column. Centrifuge for 15 seconds at 13,000 g to wash the spin column membrane. Discard the flow-through. Add 500 µl Buffer RPE to the RNeasy spin column. Centrifuge for 15 seconds at 13,000 g. Discard the flow-through. Add 500 µl Buffer RPE to the RNeasy spin column. Centrifuge for 2 minutes at 13,000 g to wash the spin column membrane. Place the RNeasy spin column in a new 2 ml collection tube and discard the old collection tube with the flow-through, centrifuge at full speed for 1 minute. Place the RNeasy spin column in a new 1.5 ml collection tube. Add 50 µl of RNase-free water directly to the spin column membrane. Centrifuge for 1 minute at 13,000 g to elute the RNA. Repeat the last step using the eluate from the last step and centrifuge for 1 minute at 13,000 g. Discard the spin column and immediately transfer the 1,5 ml collection tube (containing the RNA) in -80°C.

Appendix 2

Extraction protocol from bacteria

Add 1 volume of bacterial culture to the tube. Mix immediately by vortexing for 5 seconds. Incubate for 5 minutes at room temperature (15-25°C). Centrifuge for 10 minutes at 5000g (12.000 rpm), (From RNeasy Mini Protocol for isolation of Total RNA from Bacteria).

Decant supernatant, and carefully remove all the supernatant. Re-suspended the bacteria in 100 µl of lysozyme-containing TE buffer (TE buffer exist ready to use

than add lysozyme: for Gram- bacteria 400 µg/ml; for Gram+ bacteria 3 mg/ml.) Vortexing. Incubate at room temperature for 3-5 minutes (Gram-bacteria) or 5-10 minutes (Gram+bacteria). Add 350 µl buffer RLT (before the use you have to add β-mercaptoethanol 10 µl β-Me per 1 ml Buffer RLT; it's stable for 1 month). Mix horougly by vortexing vigorously. If insoluble material is visible centrifuge for 2 minutes at maximum speed and use only the supernatant. Add 250 µl ethanol (96-100%) to the lysate. Mix by pipetting. Apply the sample (usually 700 µl) including any precipitate that may have formed to an RNeasy mini column placed in a 2 ml collection tube. Centrifuge at 10.000 rpm for 15 seconds. Discard the flow-through. Add 700 µl buffer RW1 to the RNeasy column. Centrifuge at 10.000 rpm for 15 seconds to wash the column. Discard the flow-through and the collection tube. Transfer the RNeasy spin column in a new 2 ml collection tube. Pipet 500 µl buffer RPE to the column. Centrifuge at 10.000 rpm for 15 seconds. Discard the flow-through. Add another 500 µl of buffer RPE to the column. Centrifuge for 2 minutes at 10.000 rpm to dry the RNeasy silica membrane gel. Discard the flow-through and the collection tube. Transfer the RNeasy column to a new 1,5 ml collection tube. Pipet 50 µl RNase free water directly onto the RNeasy silica-gel membrane. Centrifuge for 1 minute at 10.000 rpm. Using the eluate for another eluition, pipet 50 µl of the first eluate directly onto the RNeasy silica-gel membrane. Discard the column and store the RNA in -80°C.

Appendix 3

RNA purification treatment by TURBO DNase

2U of Turbo DNase and 0.1 volume of 10X Turbo DNase buffer were added to the RNAs in a 50 µl reaction volume. Tubes containing samples were incubated at 37°C for 30 min and treated with 0.1 volume of DNase inactivation reagent by flicking for 2 min. Tubes were centrifuged at 10.000 x g for 2 min and the supernatants (containing RNA) were carefully transferred into fresh tubes. RNA samples concentration was determined by spectrophotometer and adjusted to 5 ng μl^{-1}.

Chapter 4.

RESULTS

4.1 Direct antifungal activities of elicitors and BCAs in controlling post-harvest green mold *in vitro* and on citrus fruit

4.1.1 Direct antifungal activity of ASM, chitosan and BABA *in vitro*.

Chemicals
Fruit
Fungal cultures
In vitro assays
Effect of chemicals on spore germination of P. digitatum in vitro

4.1.2 Direct antifungal activity of ASM, chitosan and BABA on citrus fruit.

Effects of ASM, chitosan and BABA on direct control of P. digitatum in vivo
Effect of BABA on the induction of resistance to P. digitatum

Statistical analysis

4.1.3 Direct antifungal activity of *yeasts in vitro.*
Biocontrol agent
In vitro assay 1
In vitro assay 2

4.1.4 Direct antifungal activity of yeasts on oranges fruit.
Effects of yeasts on direct control of P. digitatum *in vivo*
Statistical analysis

4.1.1. Direct antifungal activity of ASM, chitosan and BABA in vitro

a) ASM *in vitro* assays

As shown in Table 1, ASM significantly reduced the growth rate of *P. digitatum* in both amended media at the concentrations of 1, 2, 5, and 10 mg/ml, whereas all the concentrations tested had very little or no effect on *P. italicum* and *G. candidum* mycelial growth (Tab. 3). For all three pathogens, the highest growth reduction was observed at the highest concentrations (5 and 10 mg/ml). However, the rate of reduction was low also in presence of these concentrations, except for *P. digitatum* (47-56% on PDA and 38% on orange peel extract agar).

Table 3. Effect of different ASM concentrations on the mycelial growth of *P. digitatum, P. italicum* and *G. candidum in vitro*

	Reduction of mycelial growth (%) ± SD[a]					
	P. digitatum		*P. italicum*		*G. candidum*	
ASM (mg/ml)	PDA	ORANGE	PDA	ORANGE	PDA	ORANGE
0	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
0.1	0.7±3.5 a	2.3±2.1 a	0.0 a	1.7±3.5 a	0.0 a	0.0 a
0.2	2.7±1.5 a	12.3±2.3 b	2.0±0.0 a	1.6±2.8 a	2.8±0.0 a	0.0 a
0.5	13.0±0.0 b	11.5±3.5 b	0.0 a	8.0±0.0 b	0.0 a	2.0±0.0 a
1.0	27.5±0.5 c	13.8±1.4 b	6.9±2.1 b	9.8±0.0 b	2.8±0.0a	0.0 a
2.0	38.4±3.5 d	15.4±0.0 b	8.0±0.0 b	12.3±2.1 b	0.7±0.7 a	0.0 a
5.0	47.1±2.1 e	38.5±0.0 c	10.0±0.0 c	18.0±0.0 c	14.3±0.0 b	22.0±0.0 b
10.0	56.5±0.0 f	38.5±0.0 c	10.0±0.0 c	24.0±0.0 d	14.3±0.0 b	22.0±0.0 b

[a] The values were expressed as mean ± standard deviation. Numbers within a column followed by the same letter are not significantly different according to the Student-Newman- Keuls'mean separation test, at P ≤0.05.

b) Chitosan *in vitro* assays

Chitosan always had strength to inhibit the pathogen growth *in vitro*, which was in a concentration-dependent manner: the higher the concentration used, the more inhibition emerged (Table 4).

At concentrations of 0.1, 0.2 and 0.5 mg/ml, chitosan weekly reduced colony diameter of all the pathogens with a different effectiveness depending on the substrate or the target fungus used. The growth reduction of *P. digitatum* and *G. candidum* was more evident on orange peel extract agar medium (17-24% and 11-12%, respectively) than on PDA medium (10-22% and 3-5%, respectively); on the contrary, *P. italicum* was suppressed stronger on amended PDA plates (22-33%) than on orange peel extract agar medium (15-28%). *G.candidum* was the less sensitive to fungicidal effects of chitosan *in vitro* assays.

At concentrations of 1.0, 2.0, 5.0, and 10.0 mg/ml, a complete inhibition of *P. digitatum*, *P. expansum* and *G. candidum* growth was observed on amended PDA and orange peel extract agar.

Table 4. Effect of different chitosan concentrations on the mycelial growth of *P. digitatum, P. italicum* and *G. candidum in vitro*

Chitosan (mg/ml)	Reduction of mycelial growth (%) ± SD[a]					
	P. digitatum		*P. italicum*		*G. candidum*	
	PDA	ORANGE	PDA	ORANGE	PDA	ORANGE
0	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
0.1	10.4±0.5 b	17.3±1.1 b	22.6±1.2 b	15.5±1.3 b	3.7±0.4 b	12.4±1.0 b
0.2	14.7±0.9 c	19.7±1.9 b	33.5±2.0 c	14.9±0.5 b	3.2±1.0 b	11.5±1.4 b
0.5	22.6±1.6 d	24.7±0.4 c	33.0±5.0 c	28.2±0.0 c	5.3±0.8 c	11.5±0.0 b
1.0	100 e	100 d	100 d	100 d	100 d	100 c
2.0	100 e	100 d	100 d	100 d	100 d	100 c
5.0	100 e	100 d	100 d	100 d	100 d	100 c
10.0	100 e	100 d	100 d	100 d	100 d	100 c

[a] The values were expressed as mean ± standard deviation. Numbers within a column followed by the same letter are not significantly different according to the Student-Newman-Keuls'mean separation test, at P ≤0.05.

c) BABA *in vitro* assays

As shown in Table 5, BABA had no or low significant effect on reduction of mycelial growth of the pathogens when tested at concentration of 0.1, 1 mM and 10 mM, whereas it significantly reduced the mycelial growth at 100 mM, both on PDA and orange peel extract agar.

The efficiency of the treatment was also dependent of the fungal pathogen: treatment with BABA at 100 mM induced a 78% inhibition of *P. digitatum*, 46% inhibition of *P. italicum* and 21% inhibition of *G. candidum*.

G. candidum was less sensitive to BABA in both culture media tested, but with some differences at 100 mM.

Treatment with BABA at 100 mM induced 21.8% inhibition of *G. candidum* on PDA, whereas the growth reduction was of only 1.2% on orange peel extract medium. The efficiency of the treatment was also dependent of the fungal pathogen: treatment with BABA at 100 mM induced a 78% inhibition of *P. digitatum*, 46% inhibition of *P. italicum* and 21% inhibition of *G. candidum*.

Table 5. Effect of different concentrations of BABA on the mycelial growth of *P. digitatum, P. italicum,* and *G. candidum in vitro.*

	Reduction of mycelial growth (%) ± SD[a]					
	P. digitatum		*P. italicum*		*G. candidum*	
BABA (mM)	PDA	ORANGE	PDA	ORANGE	PDA	ORANGE
0	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
0.1	0.0 a	1.5±2.1 a	21.9±2.1 b	5.0±0.0 a	0.0 a	0.0 a
1	3.1±1.4 a	7.4±3.5 a	28.9±1.4 b	12.1±2.1 a	8.0±0.0 b	0.0 a
10	23.8±1.4 b	38.5±4.9 b	40.5±1.4 c	46.4±3.5 b	15.0±0.0 c	1.2±1.4 a
100	78.5±2.1 c	70.3±7.1 c	46.3±1.4 c	46.0±0.0 b	21.8±0.7 d	1.2±1.4 a

[a] The values were expressed as mean ± standard deviation. Numbers within a column followed by the same letter are not significantly different according to the Student-Newman-Keuls'mean separation test at P ≤0.05

Effect of chemicals on spore germination of *P. digitatum in vitro*

a) ASM

Spore germination of *P. digitatum* was not affected by ASM (Table 6). Examination under a light microscope showed no significant differences in the number of germinating conidia between the control and the fungal suspensions treated with ASM. As showed figure 1.

Table 6. Effect of different concentrations of ASM on spore germination of *P. digitatum in vitro.*

Spore germination (%) ± SD [a]	
ASM (mg/ml)	2×10^6 spore ml^{-1}
0	100 b
0.1	98.8±2.6 b
0.2	99.3±1.7 b
0.5	99.4±0.7 b
1.0	99.7±0.7 b
2.0	95.3±2.6 a
5.0	94.8±1.1 a

[a.] Spores of *Penicillium digitatum* (2×10^6 spore ml^{-1}) were grown in Potato Dextrose Broth containing different concentrations of ASM. After 24 h, the spore germination percentage were measured in three light-microscope fields, each containing 40-50 spores. Data are mean values of three replication per experiment. Each experiment was repeated twice. The values were expressed as mean ± standard deviation. Columns marked with different letters are significantly different at $P \leq 0.05$ according to a Student-Newman-Keuls'one-way ANOVA test.

b) Chitosan

As shown in Table 7, chitosan was found to be able to totally inhibit the germination of *P. digitatum* spore at all the tested concentrations (0% spore germination). As showed figure 1.

Table 7. Effects of different chitosan concentrations on *P. digitatum* spore germination *in vitro*

	Spore germination (%)±SD[a]
Chitosan (mg/ml)	2×10^6 spores ml^{-1}
0	100 b
0.1	1.1±1.4 a
0.2	1.0±1.4 a
0.5	0.0 a
1.0	0.0 a
2.0	0.0 a
5.0	0.0 a

[a.] Spores of *Penicillium digitatum* (2×10^6 spore ml^{-1}) were grown in Potato Dextrose Broth containing different concentrations of chitosan. After 24 h, the spore germination percentage were measured in three light-microscope fields, each containing 40-50 spores. Data are mean values of three replication per experiment. Each experiment was repeated twice. The values were expressed as mean ± standard deviation. Columns marked with different letters are significantly different at $P \leq 0.05$ according to a Student-Newman-Keuls'one-way ANOVA test.

c) BABA

To examine the effects of BABA on *P. digitatum* spore germination *in vitro*, fungal spores were incubated in PDB containing different concentrations of BABA. The results (Tab. 8) show that at increasing concentrations of BABA from 0.1 to 1000 mM, a clear reduction in percentage of germination was observed. No evident reduction of spore germination was detected at concentration of 0.1 and 1 mM. BABA at concentrations of 10 and 100 mM significantly reduced the number of germinating conidia (67.9% and 28.7%, respectively). Finally, at a concentration of 1000 mM there was a complete inhibition of *P. digitatum* spore germination (Fig. 1). No differences were observed when different *P. digitatum* concentrations (2×10^4 and 2×10^6 spores ml^{-1}) were used.

Table 8. Effects of different BABA concentrations on *P. digitatum* spore germination *in vitro*

	Spore germination (%)±SD[a]	
BABA (mM)	2×10^4 spores ml^{-1}	2×10^6 spores ml^{-1}
0	95,3±5,3 d	100 d
0.1	95,3±5,9 d	100 d
1	95,7±7,8 d	100 d
10	67,9±7,5 c	61,6±10,9 c
100	28,7±6,3 b	28,2±6,3 b
1000	0,0 a	0,0 a

[a.] Spores of *Penicillium digitatum* (2×10^4; 2×10^6 spore ml^{-1}) were grown in Potato Dextrose Broth containing different concentrations of BABA. After 24 h, the spore germination percentage were measured in three light-microscope fields, each containing 40-50 spores. Data are mean values of three replication per experiment. Each experiment was repeated twice. The values were expressed as mean ± standard deviation. Columns marked with different letters are significantly different at $P \leq 0.05$ according to a Student-Newman-Keuls'one-way ANOVA test.

Spore germination

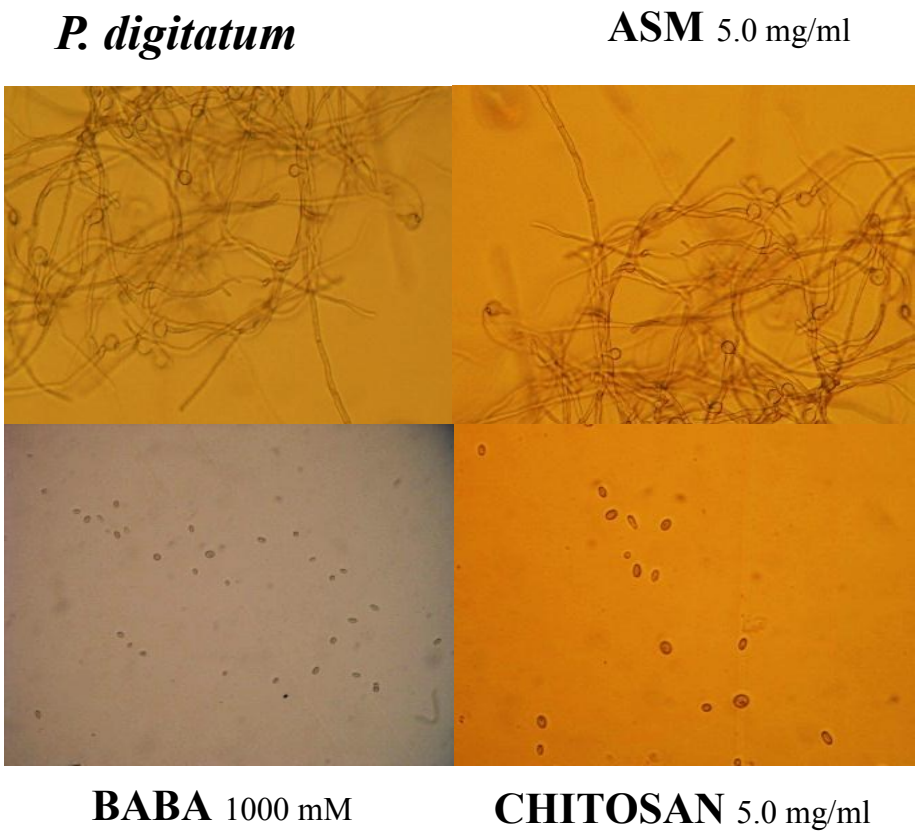


Figure 1. *P. digitatum* spore germination *in vitro*. Fungal spores were incubated in PDB at final concentration of 2×10^6 after 24 h and the spore germination percentage were measured in three light-microscope fields, each containing 40-50 spores containing different concentrations. *P. digitatum* conidia were used al control (in the top). Highest concentrations of ASM (5.0 mg/ml) BABA (1000 mM) and chitosan (5.0 mg/ml) were showed.

4.1.2. Direct antifungal activity of ASM, chitosan and BABA in citrus fruit

a) Effects of ASM on direct control of *P. digitatum in vivo*

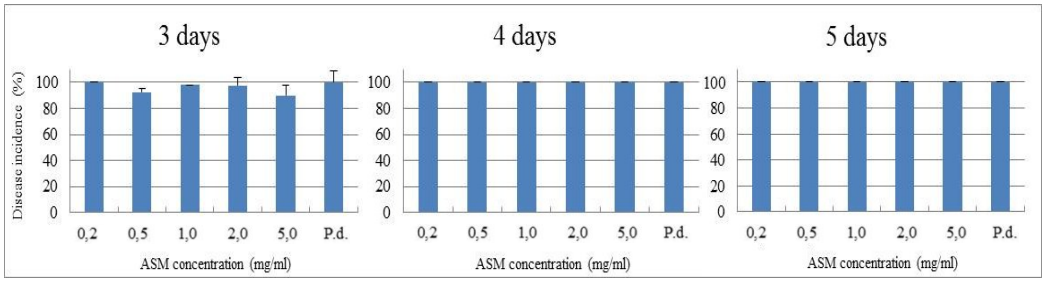
Early symptoms of green mold rot were characterized by water soaked lesions and initial soft rot after 1 to 2 days of incubation. After 2 to 3 days, on the surface of the maceration zone originating from the point of inoculation, white mycelia development and disease progressed in 3 to 4 days, followed the appearance of green spores (sporulation).

Three days post-inoculation with *P.digitatum,* ASM did not reduced the development of green mold in oranges cv. Tarocco and lemon cv. Femminello (100% disease incidence). Orange cvs. Valencia and Washington navel treated with ASM concentration of 0.2 up to 2 mg/ml showed a disease incidence of 60-70% as compared with the control (80-100%) after 3 days of incubation (Fig. 2). ASM reduced disease incidence on grapefruit at concentration from 0.2 to 5 mg/ml after 3 days of incubation (disease incidence of 20-40%, respectively) (Fig. 4).

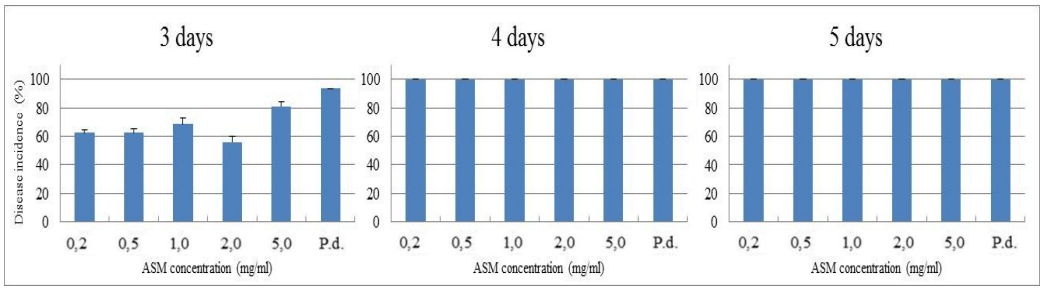
Application of ASM did not reduce the development of green mold in any treated fruit 5 days after *P. digitatum* inoculation; thus, both incidence and severity of disease in ASM-treated oranges (cv. Tarocco, Valencia and Washington Navel), lemons and grapefruits were similar to those observed in the untreated control (Fig. 2-3-4-5). Statistical analysis of data showed a low but significant decrease in disease severity only on grapefruits treated with ASM at concentrations of 2 and 5 mg/ml (50-56%, respectively) as compared with the control (70%).

Effects of ASM on direct control of *P. digitatum in vivo:* disease incidence

Orange cv. Tarocco



Orange cv. Valencia



Orange cv. Washington Navel

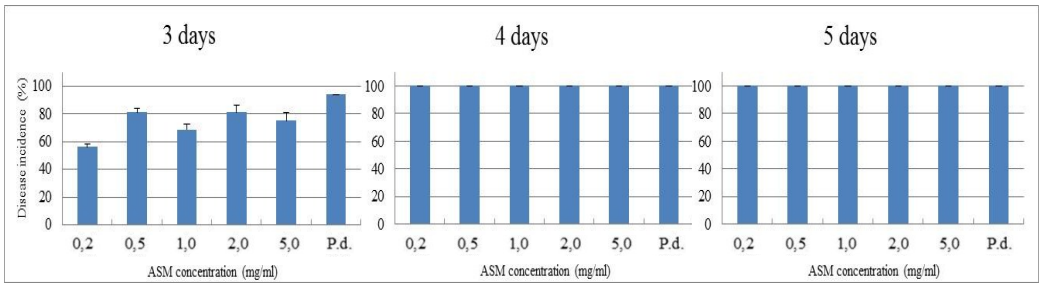
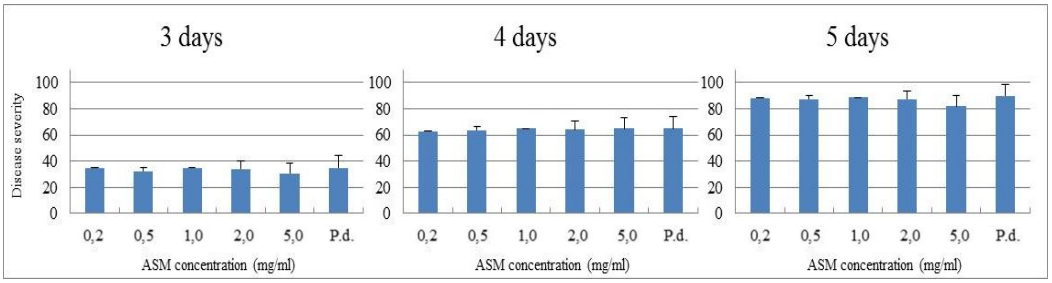


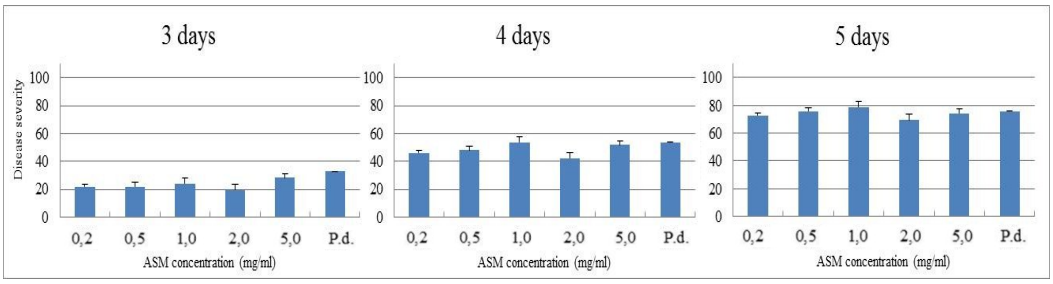
Figure 2. Disease incidence (percentage of infected wounds) on cvs."Tarocco", "Valencia" and "Washington Navel" oranges artificially inoculated with 30 µl of different concentrations of ASM (0,2; 0,5; 1,0; 2,0; 5,0 mg/ml) and after 24h inoculated with Penicillium digitatum (P.d.)10^6spore/ml. Disease incidence was evaluated after 3-4-5 days of incubation at 20°C. Data are the mean of 48 wounds per treatment. Columns without letters are not significantly different according to the Student-Newman-Keuls' one way ANOVA test at $P \leq 0.05$.

Effects of ASM on direct control of *P. digitatum in vivo:* disease severity

Orange cv. Tarocco



Orange cv. Valencia



Orange cv. Washington Navel

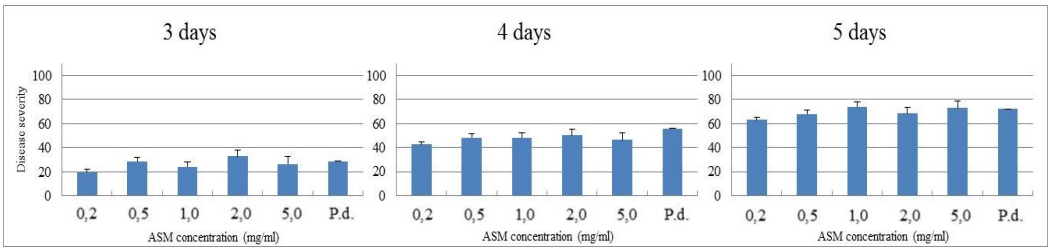
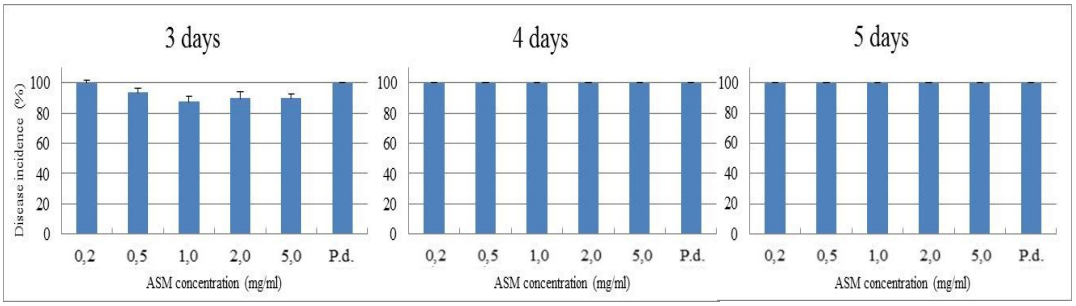


Figure 3. Disease severity on cvs."Tarocco", "Valencia" and "Washington Navel" oranges artificially inoculated with 30 µl of different concentrations of ASM (0,2; 0,5; 1,0; 2,0; 5,0 mg/ml) and after 24h inoculated with *Penicillium digitatum* (P.d.)10^6spore/ml. Disease severity was evaluated after 3-4-5 days of incubation at 20°C. Data are the mean of 48 wounds per treatment. Columns without letters are not significantly different according to the Student-Newman-Keuls' one way ANOVA test at P ≤0.05. Disease severity was evaluated with an empiric scale (1=no visible symptoms; 2=initial soft rot; 3=presence of mycelium; 4=sporulation) data were converted percentage midpoint values where 1=0%; 2=35%; 3=65%; 4=90%).

Effects of ASM on direct control of *P. digitatum in vivo:* disease incidence

Lemon cv. Femminello



Grapefruit cv. Marsh Seedless

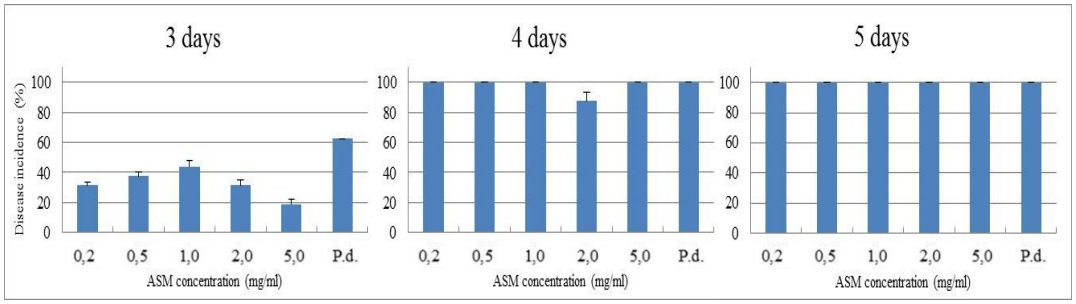
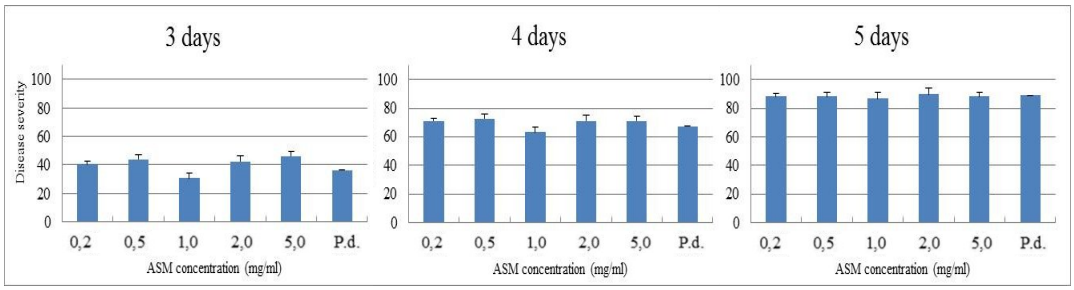


Figure 4. Disease incidence (percentage of infected wounds) on cv. "Femminello" lemon and cv. "Marsh Seedless" grapefruit treated with 30 µl of different concentrations of ASM (0,2; 0,5; 1,0; 2,0; 5,0 mg/ml) and after 24h inoculated with *Penicillium digitatum* (P.d.)10^6spore/ml. Disease incidence was evaluated after 3-4-5 days of incubation at 20°C. Data are the mean of 48 wounds per treatment. Columns without letters are not significantly different according to the Student-Newman-Keuls' one way ANOVA test at $P \leq 0.05$.

Effects of ASM on direct control of *P. digitatum in vivo:* disease severity

Lemon cv. Femminello



Grapefruit cv. Marsh Seedless

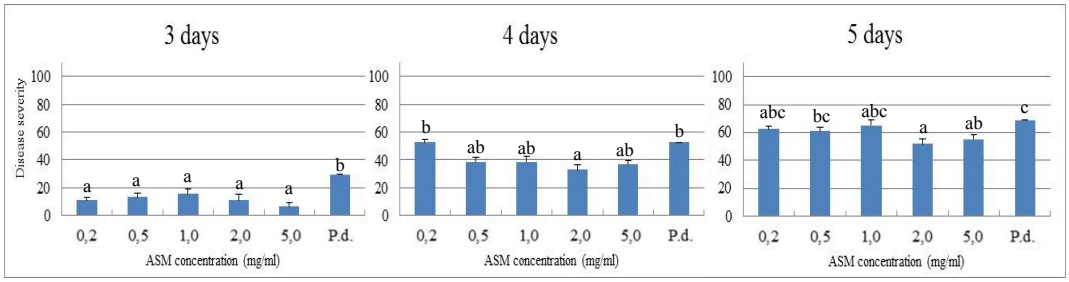


Figure 5. Disease severity on cv. "Femminello" lemon and cv. "Marsh Seedless" grapefruit treated with 30 µl of different concentrations of ASM (0,2; 0,5; 1,0; 2,0; 5,0 mg/ml) and after 24h inoculated with *Penicillium digitatum* (P.d.)10^6spore/ml. Disease severity was evaluated after 3-4-5 days of incubation at 20°C. Data are the mean of 48 wounds per treatment. Columns with the same letters (or without letters) are not significantly different according to the Student-Newman-Keuls' one way ANOVA test at P ≤0.05. Disease severity was evaluated with an empiric scale (1=no visible symptoms; 2=initial soft rot; 3=presence of mycelium; 4=sporulation) data were converted percentage midpoint values where 1=0%; 2=35%; 3=65%; 4=90%).

b) Effects of chitosan on direct control of *P. digitatum in vivo*

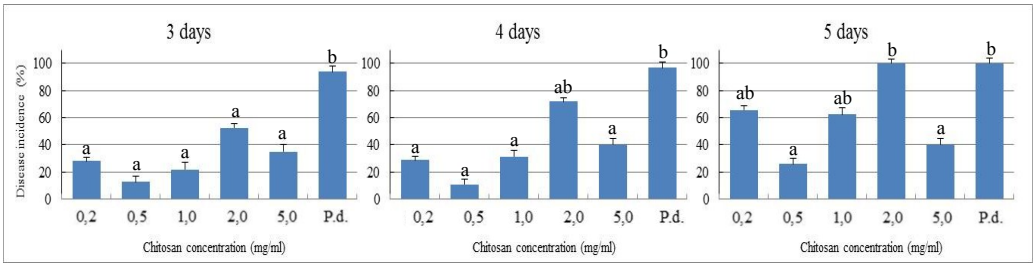
Chitosan significantly reduced green mold incidence and severity on oranges cv. Valencia and on grapefruit cv. Marsh Seedeless when applied at 0.2 - 0.5 mg/ml (incidence and severity < 10%), and on orange cv. Washington Navel and on lemon cv. Femminello when applied at 0.5 - 1 mg/ml (green mold incidence of 15% and green mold severity of 10%) compared with the control inoculated with *P. digitatum* only (Fig. 6-8).

On the contrary, disease incidence and disease severity were not significantly different among the various concentrations when chitosan was applied on orange cv. Tarocco (Fig. 6-7).

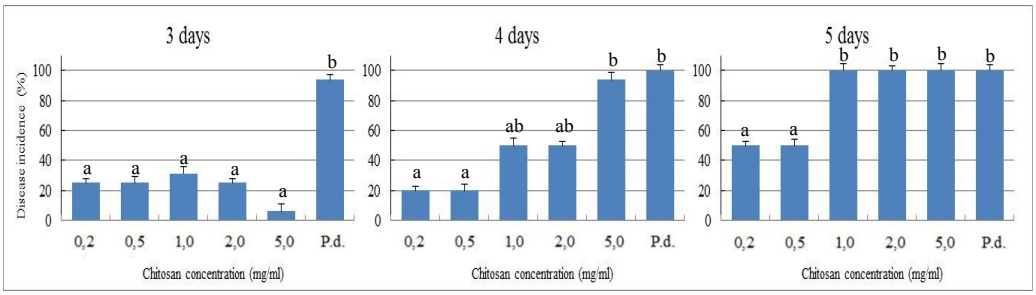
The results shown that the percentage of infected wounds, as well as disease severity, were significantly reduced on orange cv. Washington Navel on lemon and on grapefruit (Fig. 7-9).

Effects of chitosan on direct control of *P. digitatum in vivo:* disease incidence

Orange cv. Tarocco



Orange cv. Valencia



Orange cv. Washington Navel

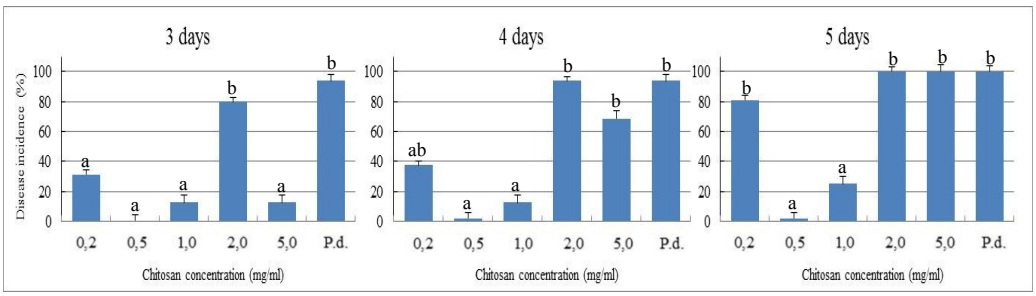
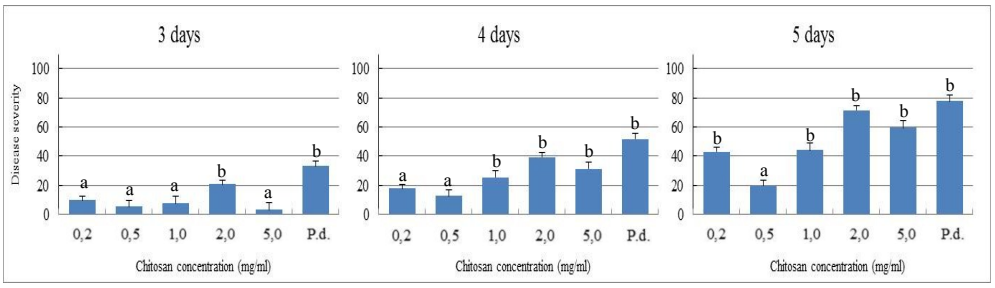


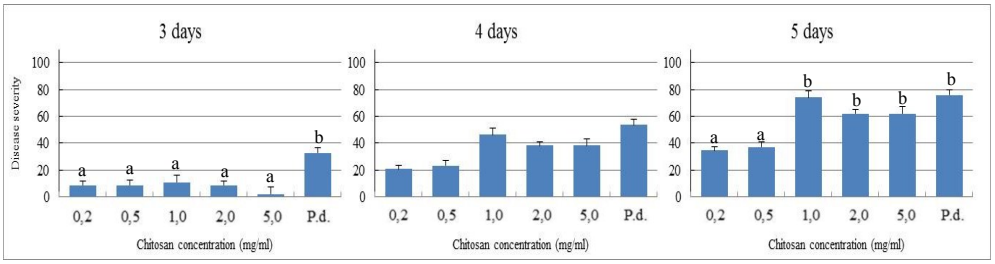
Figure 6. Disease incidence (percentage of infected wounds) on cvs."Tarocco", "Valencia" and "Washington Navel" oranges artificially inoculated with 30 µl of different concentrations of chitosan (0,2; 0,5; 1,0; 2,0; 5,0 mg/ml) and after 24h inoculated with *Penicillium digitatum* (P.d.)10^6spore/ml. Disease incidence was evaluated after 3-4-5 days of incubation at 20°C. Data are the mean of 48 wounds per treatment. Columns with the same letters are not significantly different according to the Student-Newman-Keuls' one way ANOVA test at $P \leq 0.05$.

Effects of chitosan on direct control of *P. digitatum in vivo:* disease severity

Orange cv. Tarocco



Orange cv. Valencia



Orange cv. Washington Navel

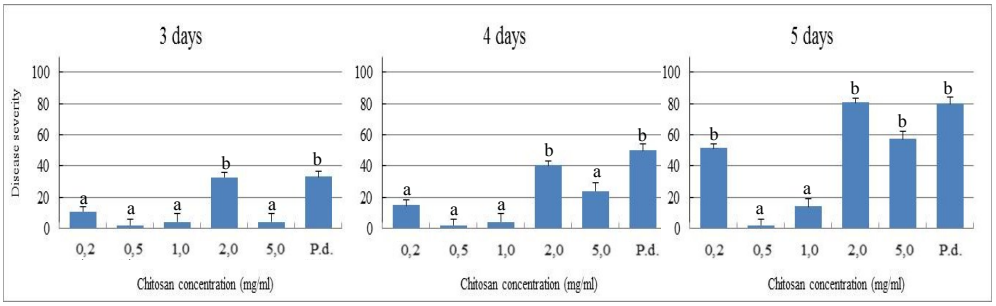
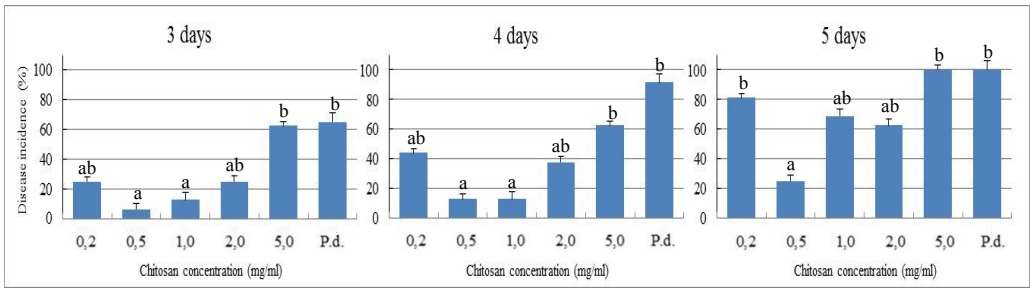


Figure 7. Disease severity on cvs."Tarocco", "Valencia" and "Washington Navel" oranges artificially inoculated with 30 µl of different concentrations of chitosan (0,2; 0,5; 1,0; 2,0; 5,0 mg/ml) and after 24h inoculated with *Penicillium digitatum* (P.d.)10^6spore/ml. Disease severity was evaluated after 3-4-5 days of incubation at 20°C. Data are the mean of 48 wounds per treatment. Columns the same letters (or without letters) are not significantly different according to the Student-Newman-Keuls' one way ANOVA test at $P \leq 0.05$. Disease severity was evaluated with an empiric scale (1=no visible symptoms; 2=initial soft rot; 3=presence of mycelium; 4=sporulation) data were converted percentage midpoint values where 1=0%; 2=35%; 3=65%; 4=90%).

Effects of chitosan on direct control of *P. digitatum in vivo:* disease incidence

Lemon cv. Femminello



Grapefruit cv. Marsh Seedless

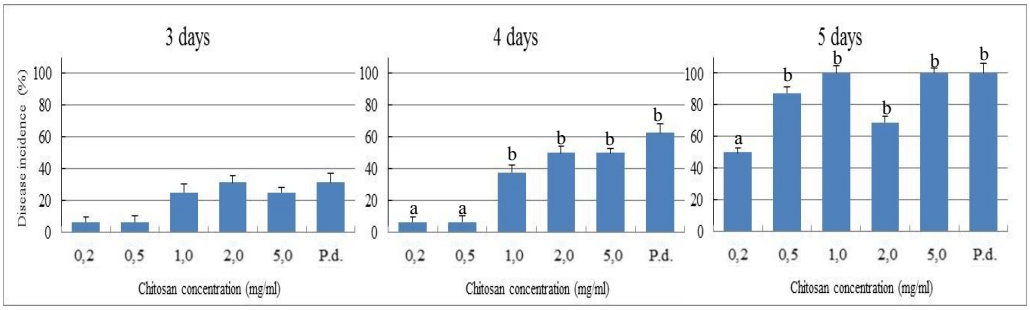
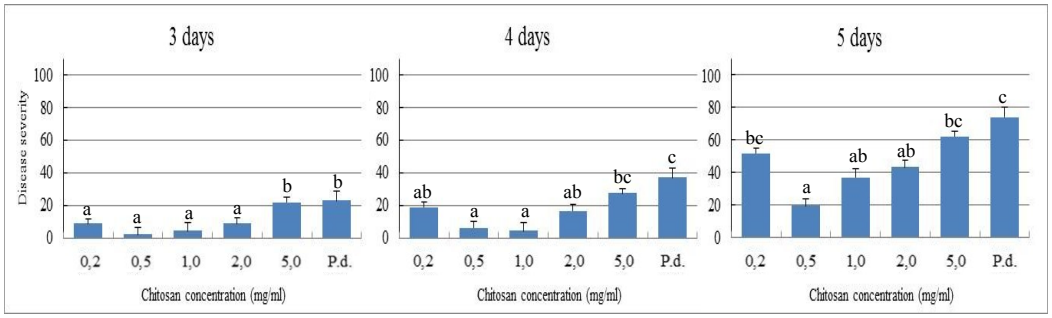


Figure 8. Disease incidence (percentage of infected wounds)) on cv. "Femminello" lemon and cv. "Marsh Seedless" grapefruit artificially inoculated with 30 µl of different concentrations of chitosan (0,2; 0,5; 1,0; 2,0; 5,0) and after 24h inoculated with *Penicillium digitatum* (P.d.)10^6spore/ml. Disease incidence was evaluated after 3-4-5 days of incubation at 20°C. Data are the mean of 48 wounds per treatment. Columns with the same letters (or without letters) are not significantly different according to the Student-Newman-Keuls' one way ANOVA test at P ≤0.05.

Effects of chitosan on direct control of *P. digitatum in vivo:* disease severity

Lemon cv. Femminello



Grapefruit cv. Marsh Seedless

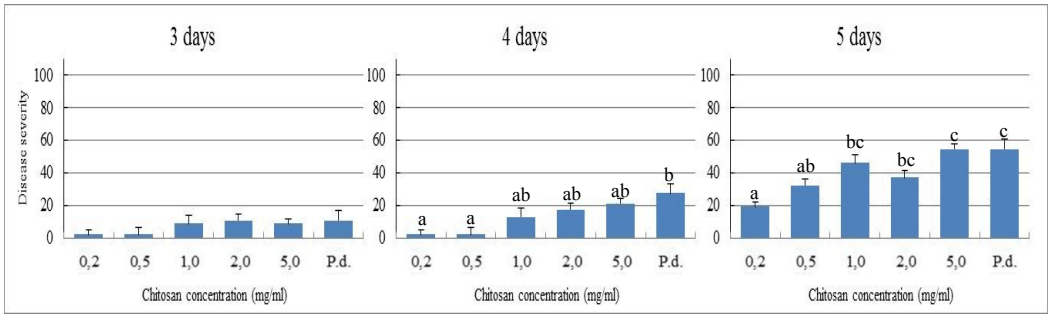


Figure 9. Disease severity on "Femminello" lemon and "Marsh Seedless" grapefruit artificially inoculated with 30 µl of different concentrations of chitosan (0,2; 0,5; 1,0; 2,0; 5,0) and after 24h inoculated with *Penicillium digitatum* (P.d.)10^6spore/ml. Disease severity was evaluated after 3-4-5 days of incubation at 20°C. Data are the mean of 48 wounds per treatment. Columns with the same letters (or without letters) are not significantly different according to the Student-Newman-Keuls' one way ANOVA test at P ≤0.05. Disease severity was evaluated with an empiric scale (1=no visible symptoms; 2=initial soft rot; 3=presence of mycelium; 4=sporulation) data were converted percentage midpoint values where 1=0%; 2=35%; 3=65%; 4=90%).

c) Effects of BABA on direct control and on the induction of resistance to *P. digitatum in vivo*

The effect of BABA treatments on decay of orange was influenced by concentration and cultivars tested **(**Fig. 10**)**. At the end of the storage period, the greatest reduction of green mold incidence and severity occurred on oranges cv. Tarocco and Valencia when this compound was applied at 10, 100 and 1000 mM. At these concentrations BABA totally inhibited or severely reduced the disease incidence on oranges cv. Tarocco (0%, 20% and 8% , respectively) and disease severity (≤ 10%), and strongly reduced green mold on oranges cv. Valencia (disease incidence of 10, 22 and -21%, respectively; disease severity ≤15%). BABA at 1 mM weakly reduced the percentage of infected wounds both in cv. Tarocco (60%) and in cv. Valencia (56%). All BABA concentrations tested were totally ineffective on oranges cv. Washington Navel, except when it was applied at the concentration of 1000 mM (50% of infected wounds; severity of 20%) (Fig. 10-11).

On lemon fruit BABA weakly reduced incidence and severity of disease, and the concentrations of 10, 100 and 1000 mM were more effective (disease incidence of 45, 50 and 60%, respectively) than 1 mM.

On the opposite, on grapefruit all treatments at concentration of 1, 10, 100, and 1000 mM significantly reduced disease incidence (20-30%) and severity (4.5 - 14%) compared with the control (80% of infected wounds, 58% of severity) (Fig. 12-13).

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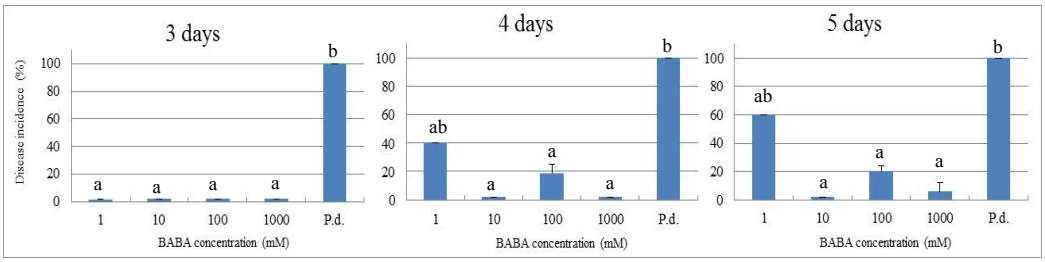
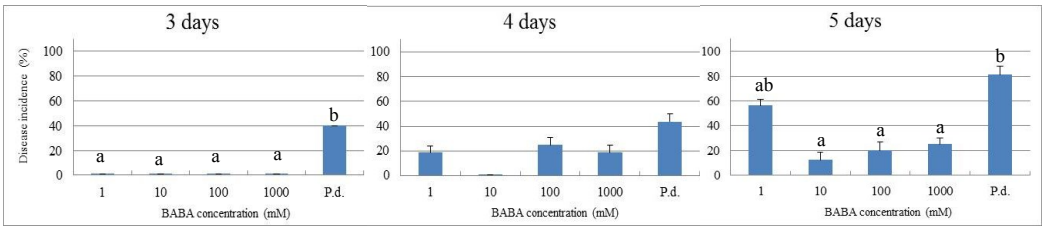
Effects of BABA on direct control of *P. digitatum in vivo:* disease incidence

Orange cv. Tarocco



Orange cv. Valencia



Orange cv. Washington Navel

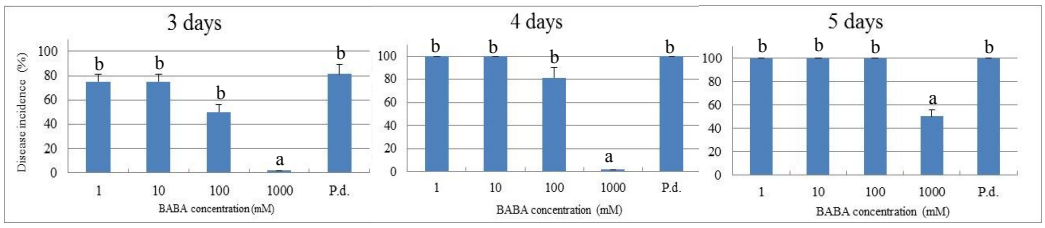
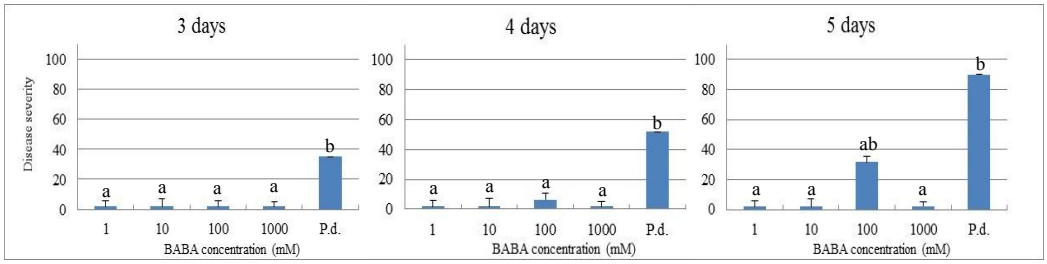


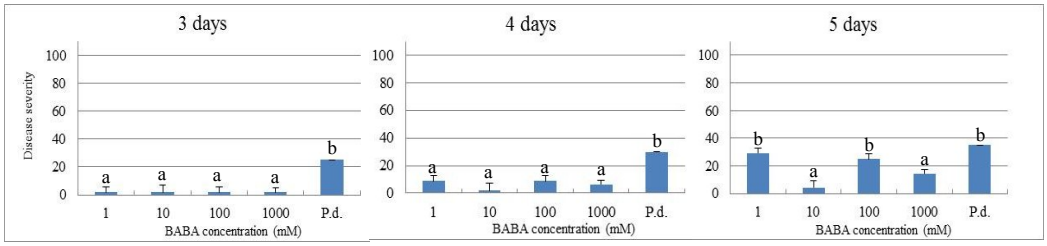
Figure 10. Disease incidence (percentage of infected wounds) on cvs."Tarocco", "Valencia" and "Washington Navel" oranges artificially inoculated with 30 µl of different concentrations of BABA (1; 10; 100; 1000 mM) and after 24h inoculated with *Penicillium digitatum* (P.d.)10^6spore/ml. Disease incidence was evaluated after 3-4-5 days of incubation at 20°C. Data are the mean of 48 wounds per treatment. Columns with the same letters (or without letters) are not significantly different according to the Student-Newman-Keuls' one way ANOVA test at P ≤0.05.

Effects of BABA on direct control of *P. digitatum in vivo:* disease severity

Orange cv. Tarocco



Orange cv. Valencia



Orange cv. Washington Navel

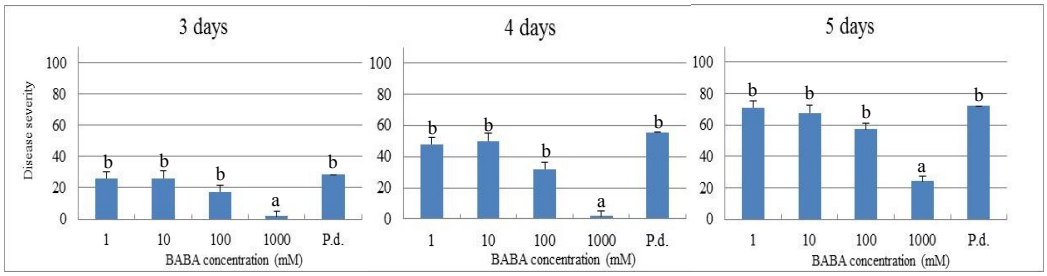
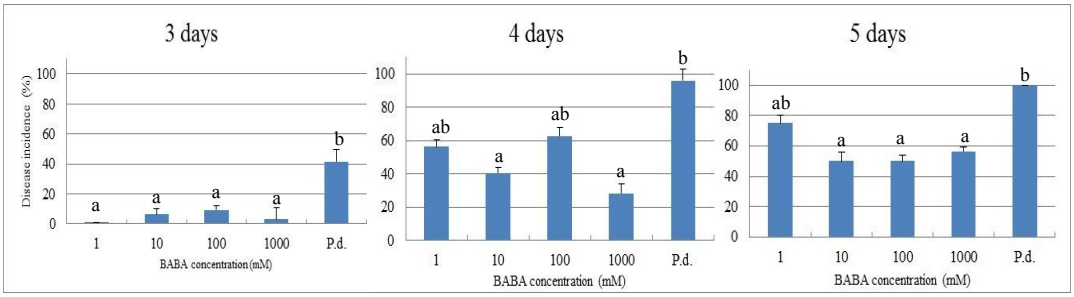


Figure 11. Disease severity on cvs."Tarocco", "Valencia" and "Washington Navel" oranges artificially inoculated with 30 µl of different concentrations of BABA (1; 10; 100; 1000 mM) and after 24h inoculated with *Penicillium digitatum* (P.d.)10^6spore/ml. Disease severity was evaluated after 3-4-5 days of incubation at 20°C. Data are the mean of 48 wounds per treatment. Columns with the same letters are not significantly different according to the Student-Newman-Keuls' one way ANOVA test at P ≤0.05. Disease severity was evaluated with an empiric scale (1=no visible symptoms; 2=initial soft rot; 3=presence of mycelium; 4=sporulation) data were converted percentage midpoint values where 1=0%; 2=35%; 3=65%; 4=90%).

Effects of BABA on direct control of *P. digitatum in vivo:* disease incidence

Lemon cv. Femminello



Grapefruit cv. Marsh Seedless

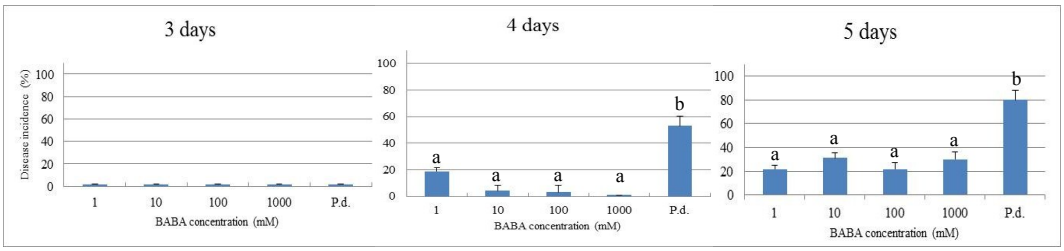
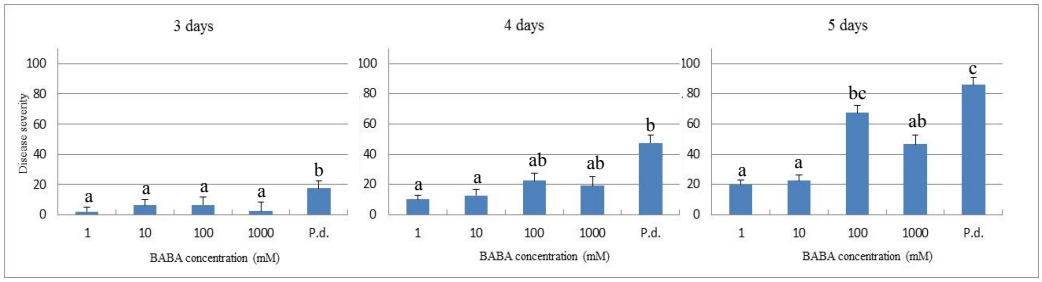


Figure 12. Disease incidence (percentage of infected wounds) on cv. "Femminello" lemon and cv. "Marsh Seedless" grapefruit treated with 30 µl of different concentrations of BABA (1; 10; 100; 1000 mM) and after 24h inoculated with *Penicillium digitatum* (P.d.)10^6spore/ml. Disease incidence was evaluated after 3-4-5 days of incubation at 20°C. Data are the mean of 48 wounds per treatment. Columns with the same letters (or without letters) are not significantly different according to the Student-Newman-Keuls' one way ANOVA test at P ≤0.05.

Effects of BABA on direct control of *P. digitatum in vivo:* disease severity

Lemon cv. Femminello



Grapefruit cv. Marsh Seedless

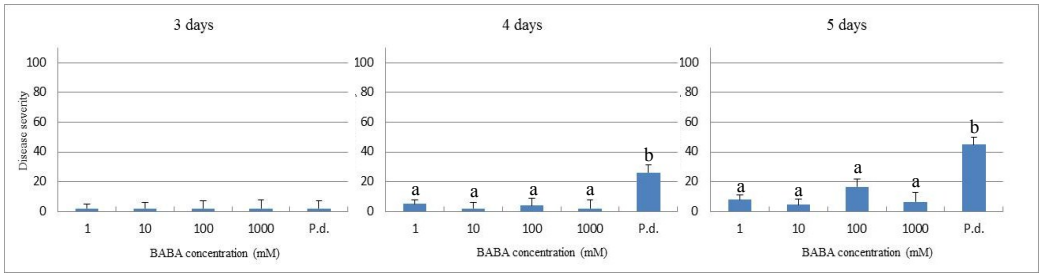


Figure 13. Disease severity on cv. "Femminello" lemon and cv. "Marsh Seedless" grapefruit treated with 30 µl of different concentrations of BABA (1; 10; 100; 1000 mM) and after 24h inoculated with *Penicillium digitatum* (P.d.)10^6spore/ml. Disease severity was evaluated after 3-4-5 days of incubation at 20°C. Data are the mean of 48 wounds per treatment. Columns with the same letters (or without letters) are not significantly different according to the Student-Newman-Keuls' one way ANOVA test at P ≤0.05. Disease severity was evaluated with an empiric scale (1=no visible symptoms; 2=initial soft rot; 3=presence of mycelium; 4=sporulation) data were converted percentage midpoint values where 1=0%; 2=35%; 3=65%; 4=90%).

To determine the effects of BABA on the induction of citrus fruit resistance to *P. digitatum*, specific wounds were pretreated with different concentrations of BABA (10, 100 and 1000 mM) and after 24 h fresh wounds were made at 1 cm from the BABA treated sites and inoculated with the pathogen. The results (Fig. 14-15) show that the percentage of infected wounds, as well as disease severity, were significantly reduced by different BABA concentrations on oranges, lemons and on grapefruits.

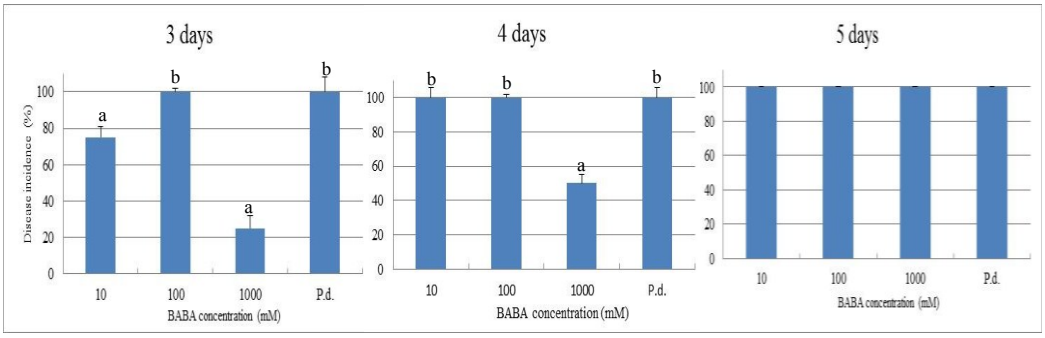
The largest reduction occurred when BABA was applied on orange cv. Tarocco at 1000 mM (disease incidence of 50%), on lemons at 10 mM (disease incidence of 46.9%) and on grapefruits at 10 mM (disease incidence of 15.6%).

To determine the effects of BABA on the induction of citrus fruit resistance to *P. digitatum* on oranges cvs. Tarocco, Washington Navel and Valencia, specific wounds were pretreated with BABA 10 mM and after 24 h fresh wounds were made at 1 cm from the BABA treated sites and inoculated with the pathogen.

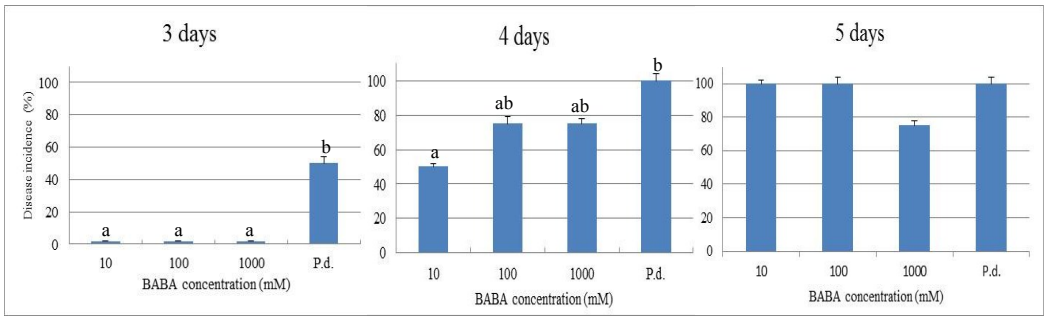
The results (Fig. 16-17) show that the percentage of infected wounds were significantly reduced only on orange cv. Valencia (disease incidence of 50%), whereas 10mM was totally ineffective on orange cvs. Washington Navel and Tarocco

Effect of BABA on induction of resistance: disease incidence

Orange cv. Tarocco



Lemon cv. Femminello



Grapefruit cv. Marsh Seedless

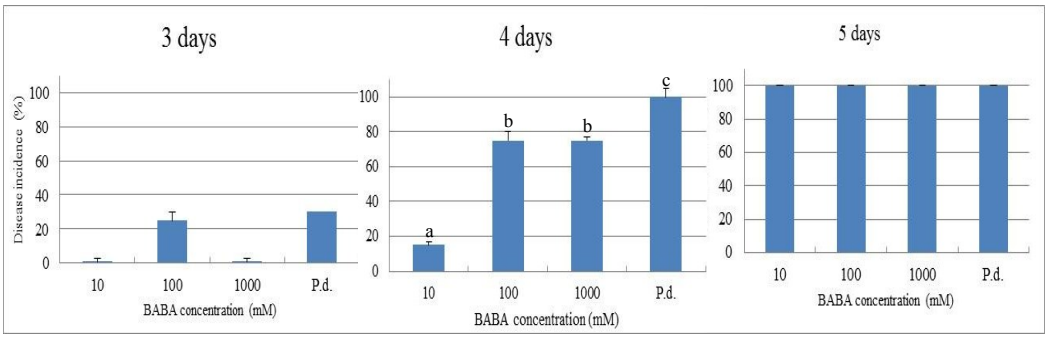
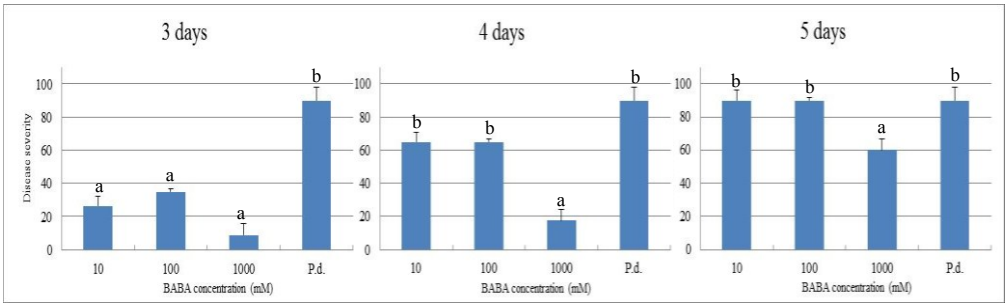


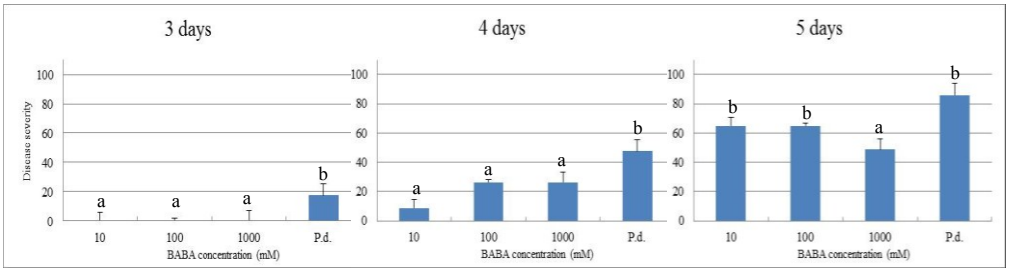
Figure 14. Effects of the distance from the BABA-treated wound sites on orange cv. Tarocco, lemon cv. Femminello and grapefruit cv. Marsh Seedless resistance to P. digitatum. Fruit were wounded and treated with 30 µl of sterile water or different concentrations of BABA (10; 100; 1000 mM) and after 24 h were wounded again at different distances 1 cm from the original BABA application site, and inoculated with *Penicillium digitatum* (P.d.)10^6spore/ml. Decay was evaluated after 3-4-5 days of incubation at 20 °C. The values are averages of three replications and four fruits per replication. Data are the mean of 48 wounds per treatment. Columns with the same letters (or without letters) are not significantly different according to the Student-Newman-Keuls' one way ANOVA test at $P \leq 0.05$.

Effect of BABA on induction of resistance: disease severity

Orange cv. Tarocco



Lemon cv. Femminello



Grapefruit cv. Marsh Seedless

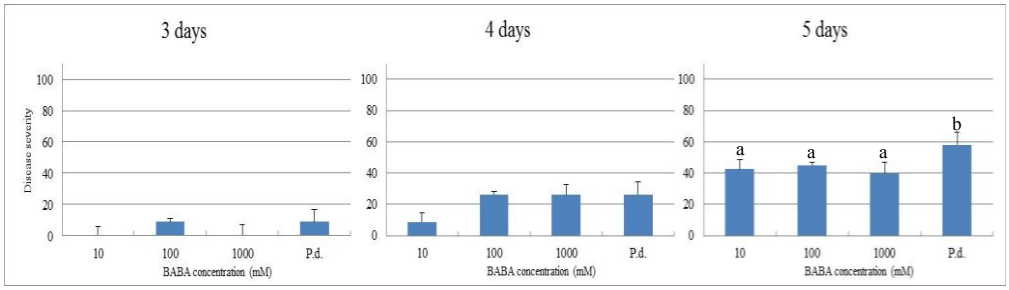
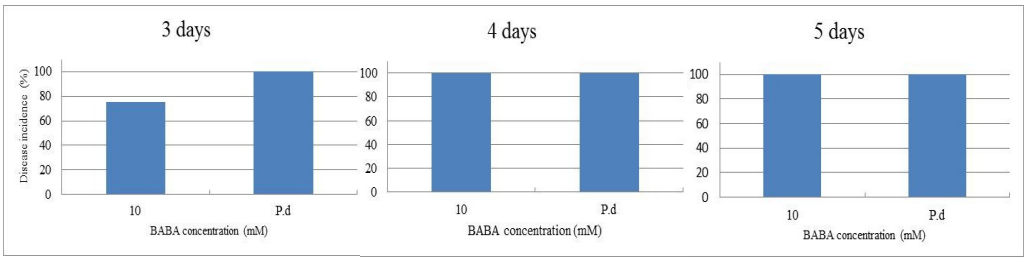


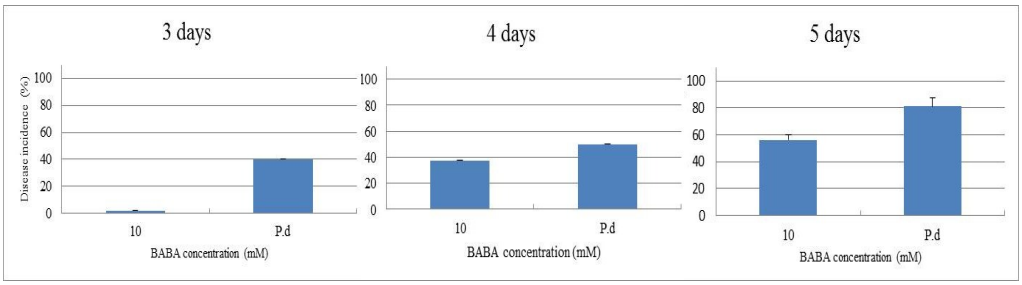
Figure 15. Effects of the distance from the BABA-treated wound sites on orange cv. Tarocco, lemon cv. Femminello and grapefruit cv. Marsh Seedless resistance to P. digitatum. Fruit were wounded and treated with 30 µl of sterile water or different concentrations of BABA (10; 100; 1000 mM) and after 24 h were wounded again at different distances 1 cm from the original BABA application site, and inoculated with *Penicillium digitatum* (P.d.)10^6spore/ml. Decay was evaluated after 3-4-5 days of incubation at 20 °C. The values are averages of three replications and four fruits per replication. Data are the mean of 48 wounds per treatment. Columns with the same letters (or without letters) are not significantly different according to the Student-Newman-Keuls' one way ANOVA test at P ≤0.05. Disease severity was evaluated with an empiric scale (1=no visible symptoms; 2=initial soft rot; 3=presence of mycelium; 4=sporulation) data were converted percentage midpoint values where 1=0%; 2=35%; 3=65%; 4=90%).

Effect of BABA on induction of resistance: disease incidence

Orange cv. Tarocco



Orange cv. Valencia



Orange cv. Washington Navel

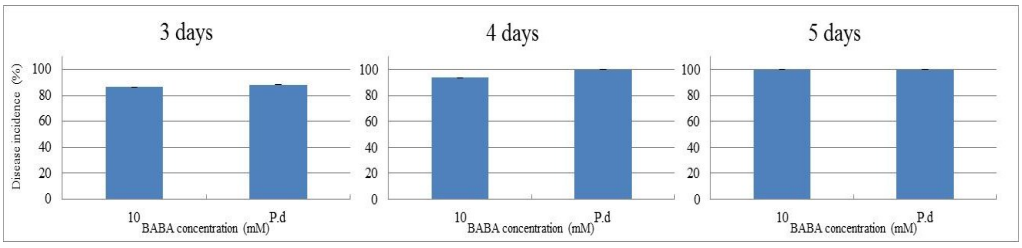
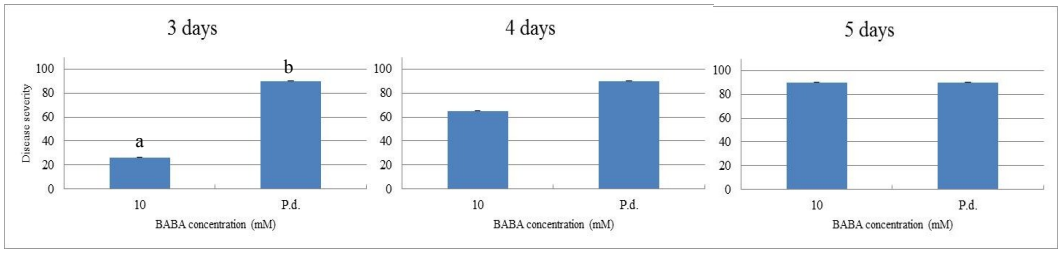


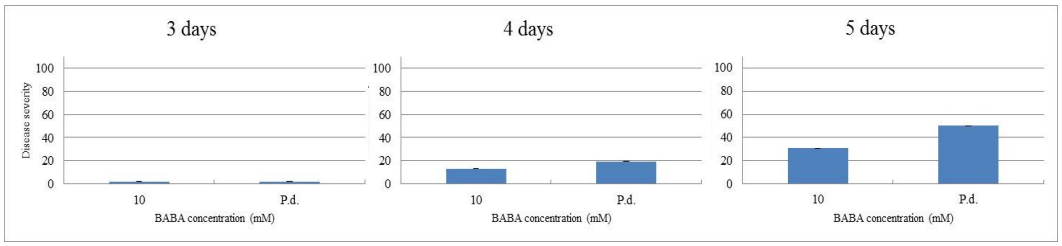
Figure 16. Effects of the distance from the BABA-treated wound sites on orange cvs. Tarocco, Valencia and Washington navel resistance to P. digitatum. Fruit were wounded and treated with 30 µl of sterile water or different concentrations of BABA (10 mM) and after 24 h were wounded again at different distances 1 cm from the original BABA application site, and inoculated with *Penicillium digitatum* (P.d.)10^6spore/ml. Decay was evaluated after 3-4-5 days of incubation at 20 °C. The values are averages of three replications and four fruits per replication. Data are the mean of 48 wounds per treatment. Columns without letters are not significantly different according to the Student-Newman-Keuls' one way ANOVA test at $P \leq 0.05$.

Effect of BABA on induction of resistance: disease severity

Orange cv. Tarocco



Orange cv. Valencia



Orange cv. Washington Navel

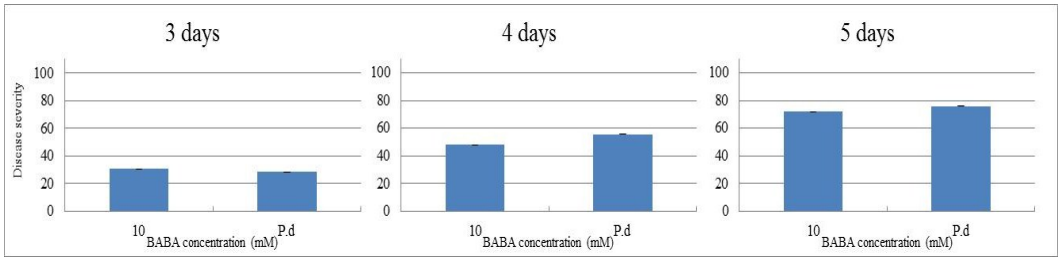


Figure 17. Effects of the distance from the BABA-treated wound sites on orange cvs. Tarocco, Valencia and Washington navel resistance to P. digitatum. Fruit were wounded and treated with 30 µl of sterile water or different concentrations of BABA (10 mM) and after 24 h were wounded again at different distances 1 cm from the original BABA application site, and inoculated with *Penicillium digitatum* (P.d.)10^6spore/ml. Decay was evaluated after 3-4-5 days of incubation at 20 °C. The values are averages of three replications and four fruits per replication. Data are the mean of 48 wounds per treatment. Columns with the same letters (or without letters) are not significantly different according to the Student-Newman-Keuls' one way ANOVA test at P ≤0.05. Disease severity was evaluated with an empiric scale (1=no visible symptoms; 2=initial soft rot; 3=presence of mycelium; 4=sporulation) data were converted percentage midpoint values where 1=0%; 2=35%; 3=65%; 4=90%).

4.1.3 Testing direct antifungal activity of *Wickerhamomyces anomalus* and *Saccharomyces cerevisiae* strains *in vitro*

In vitro assay 1

No reduction in the mycelial growth was observed. The presence of the yeasts before the inoculation of the pathogen didn't demonstrate any inhibitory effect on the mycelial growth, *in vitro*. All the yeasts tested showed no effect in reducing the diameter of the mycelium of *P. digitatum*, *P. expansum*, *P. italicum*, *G. candidum* both on PDA and on YPDA at 7 pH.

In vitro assay 2

The *in vitro* antifungal activity of *W. anomalus* and *S. cerevisiae*, able to produce killer toxins, was determined against *P. digitatum*, one of the major postharvest pathogen of citrus fruits, at two different pH values. As expected, no fungal growth inhibition was evidence at neutral pH. At pH 4.5, significant fungal growth inhibition, with a clear inhibition halo, was observed only for *W. anomalus* isolates (Fig. 19). The mycelial growth in the Petri dishes was significantly reduced, although different effects were observed among strains (Fig. 18). Slight reduction of *P. digitatum* growth were observed also for *S. cerevisiae* BS46 and BCA61 isolates but, in this case, pathogen grew in the proximity of the yeast streaks without overgrowing the yeast biomass with complete colonization of the plate (data not shown).

Thus, i*n vitro* fungal growth inhibition by *W. anomalus* was analyzed microscopically. Growth of the *P. digitatum* tested strain was compared with the pathogen growth control. Hyphal deformities were observed when mycelial samples were removed from the parts of the PDA medium located closer to the inhibition halo, appearing wilted, folded, coiled and with granulation along the hyphae (Fig. 20a), when compared with thin, elongated, well extended mycelial growth in the control (Fig. 20c). Moreover, microscopical analysis at 100x magnification evidenced collapsing of hyphae with loss of intracellular content (Fig. 20b).

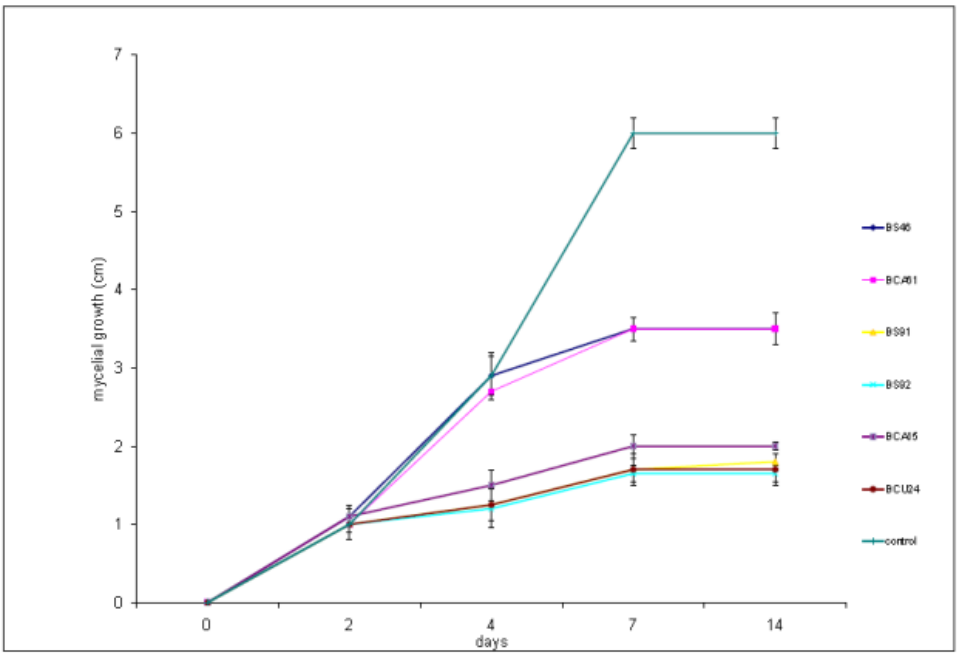


Figure 18**.** Mycelial growth (cm) of the mold growing in YPDA medium with the yeast strains compared with the control. Vertical bars represent the standard deviation of the mean of three replications for each strain.

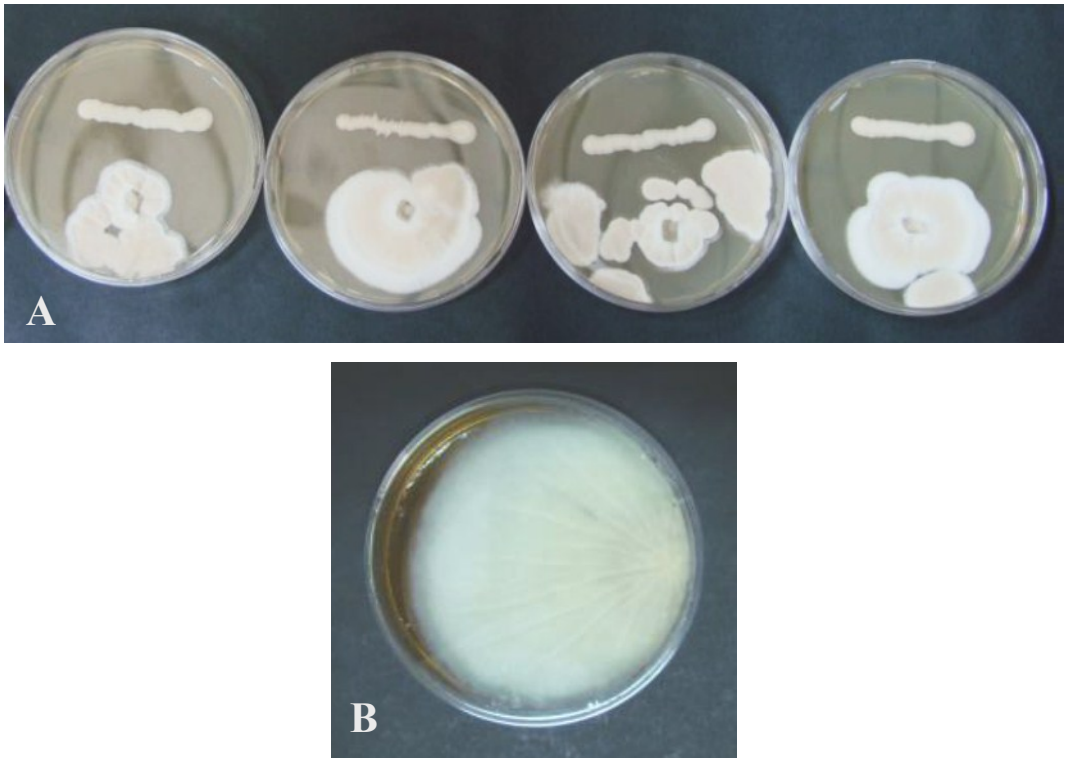


Figure 19. *In vitro* antagonism by the *Wickerhamomyces anomalus* isolates against the phytopathogenic mold *Penicillium digitatum*. (**A**) Petri dish with YPD medium inculated with BCU24, BS91, BS92 and BCA15; (**B**) Petri dish with medium inoculated solely with the mould.

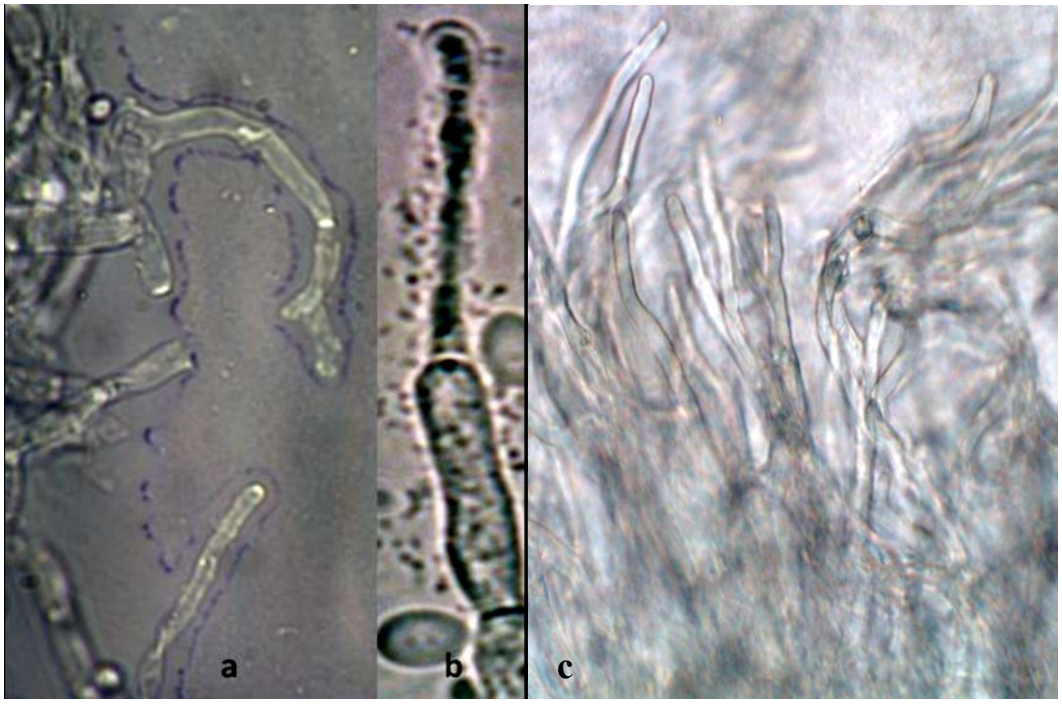


Figure 20. Hyphal morphology seen under optical microscopy. (**a**) Sample taken from YPD medium inoculated with the yeasts at 40×; (**b**) sample taken from YPD medium inoculated with the yeasts at 100×; (**c**) sample taken from YPD medium inoculated solely with the mold at 40×.

4.1.4. Testing direct antifungal activity of *Wickerhamomyces anomalus* and *Saccharomyces cerevisiae* strains on oranges fruit

After 3 days incubation, the strains BS91, BS92 and BCA15 belonging to *W. anomalus* species completely inhibited the growth of *P. digitatum* (0% of disease incidence) (Fig. 21A) compared with the control (100% of disease incidence). Similarly, the incidence of disease on oranges inoculated with strain BCU24 was significantly lower (13% infected wounds) than the control (100%) ($P < 0.05$), whereas strains BCA61 and BS46 reduced disease incidence to a lesser extent (50%).

After 5 days of incubation *W. anomalus* BCU24 and *S. cerevisiae* BCA61 and BS46 strains were totally ineffective (100% disease incidence).

On the opposite, *W. anomalus* BS91, BS92 significantly and strongly inhibited *P. digitatum* growth up to the 10th day (disease incidence of 13 and 17 %, respectively), whereas BCA15 reduced the incidence of disease to a less, even if significantly, extent (70%) compared to the control (100%).

BS91, BS92 and BCA15 strains belonging to *W. anomalus* significantly reduced disease severity (Fig. 21B), compared with the control (90% disease severity). In particular, BS91 and BS92 strains showed disease severity of 1% and 4%, respectively up to 10 days, while BCA15 reduced the severity of disease to a lesser extent (44%). On the opposite, BCU24 and BCA61 weakly reduced disease seerity (64% and 70%, respectively) after 5 days, while after 10 days were totally ineffective (100% disease severity) and sporulation occurred on infected wounds.

S. cerevisiae BS46 weakly reduced the severity of disease, only at the first stage of incubation (3 days) (disease severity of 18%), whereas failed to reduce the severity of disease since the 5th day of incubation and sporulation was observed on infected wound.

Effects of yeasts on direct control of green mold *in vivo*

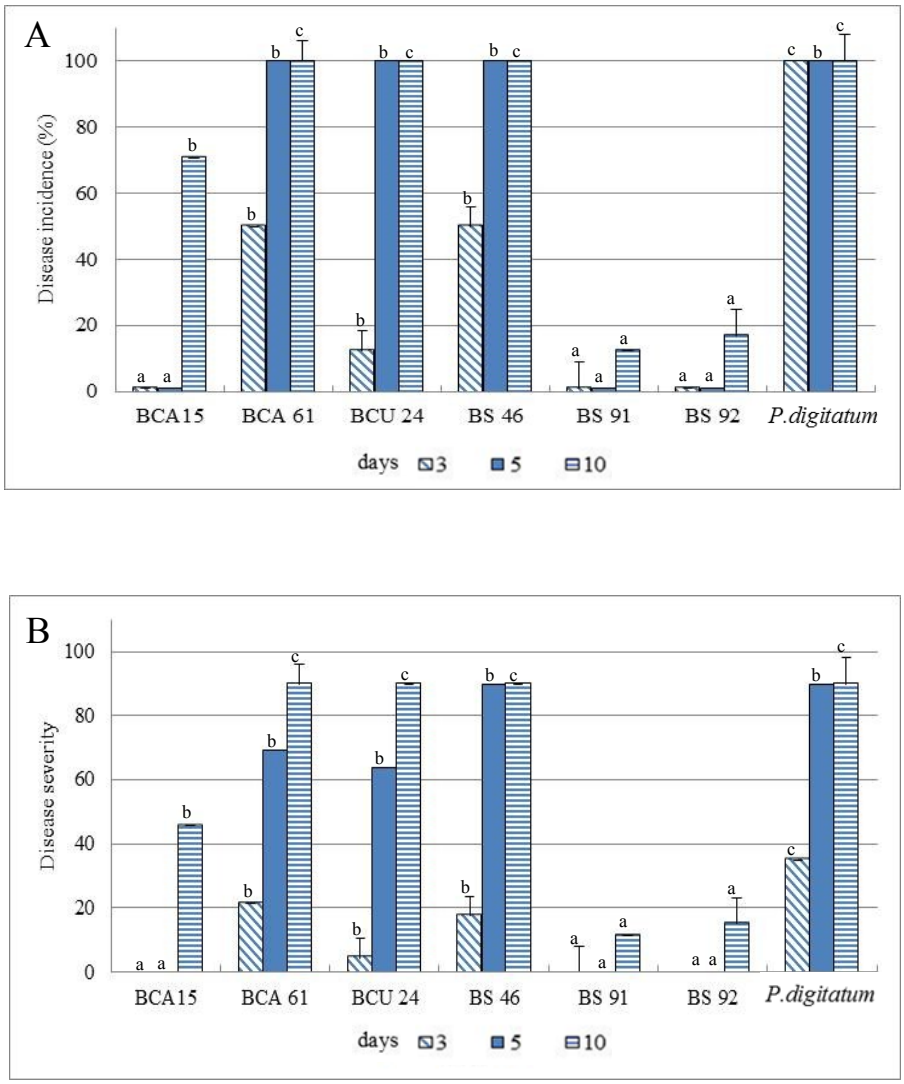


Fig 21. Disease incidence (A) and severity (B) on oranges cv. Tarocco, treated with yeasts W. anomalus BCA15, S. cerevisiae BCA61, W. anomalus BCU24, S. cerevisiae BS46, W. anomalus BS91, W. anomalus BS92 (109 CFU/ml) and 3 days later inoculated with P. digitatum (106spore/ml). Means ± standard deviations are shown. Bars with the same letter within each monitoring time (after 3, 5, 10 days) are not significantly different at P≤0.05 according to a Student-Newman-Keul's one-way ANOVA test. Disease severity was evaluated with an empiric scale (1=no visible symptoms; 2=initial soft rot; 3=presence of mycelium; 4=sporulation) data were converted percentage midpoint values where 1=0%; 2=35%; 3=65%; 4=90%).

RESULTS

4.2 Curative and preventive activity of biological control agents and elicitors in different combined treatments on oranges fruit.

4.2.1 Combined treatment of *Pseudomonas syringae* pv. *syringae* strains (48SR2, 40SR4, 46P), hot water, sodium carbonate, ASM, chitosan.

a) Curative activity of combined treatments with P. s. pv. syringae 48SR2*, hot water, and sodium carbonate.*

b) Protective activity of combined treatments with P. s. pv. syringae *strains, acibenzolar-S-methyl, and chitosan.*

4.2.2 Combined treatment of *Pseudomonas syringae pv. syringae* 48SR2, *Trichoderma atroviride* P1 and chitosan

Curative and preventive activity of combined application of P.s. pv. syringae 48SR2 T. atroviride P1 *and chitosan 0.5 mg/ml on the control of* P. digitatum *on cv. Tarocco stored at different temperatures after treatment.*

4.2.3 Combined treatment of *Pseudomonas syringae* pv. *syringae* 48SR2, *Wickerhamomyces anomalus* BS91, hot water, sodium bicarbonate.

Curative and preventive activity of combined treatments of hot water, sodium bicarbonates, P.s. pv. syringae 48SR2 *and* Wickerhamomyces anomalus BS91 *to control postharvest green mold of oranges cv. Tarocco.*

4.2.1. Combined treatment of *Pseudomonas syringae* pv. *syringae* strains (48SR2, 40SR4, 46P), hot water, sodium carbonate, ASM, chitosan

a) Curative activity of combined treatments with P. syringae 48SR2, hot water, and sodium carbonate

Fruit treatments with *P. syringae* pv. *syringae* PVCT48SR2 or hot water weakly reduced incidence and severity of decay (75-80% of infected wounds; 60-66.5% of severity) on fruit inoculated with *P. digitatum* 3 h before treatments (Fig. 22A-B), indicating lack of curative activity of spray-inoculated biocontrol agent and hot water treatments.

On the contrary, 3% SC treatment provided good control of the pathogen (37% of infected wounds; 24.8% of severity), thus suggesting a curative activity of sodium carbonate.

The curative activity was enhanced by biocontrol agent following hot water and sodium carbonate treatments. As shown in figure 22 (A-B), *P. syringae* PVCT48SR2 strain significantly improved the control of green mold when applied in combination with hot water treatments, and incidence and severity of disease (18.7 and 12.6%, respectively) were significantly lower than after treatments alone. Incidence and severity of disease were also consistently reduced when *P. syringae* PVCT48SR2 strain was combined with 3% SC and the level of control (20% of infected wounds; 12% of severity) was superior to *P. syringae* applied alone (Fig. 22A-B).

Curative activity of combined treatments with *P. syringae* pv. *syringae* 48SR2, hot water and sodium carbonate

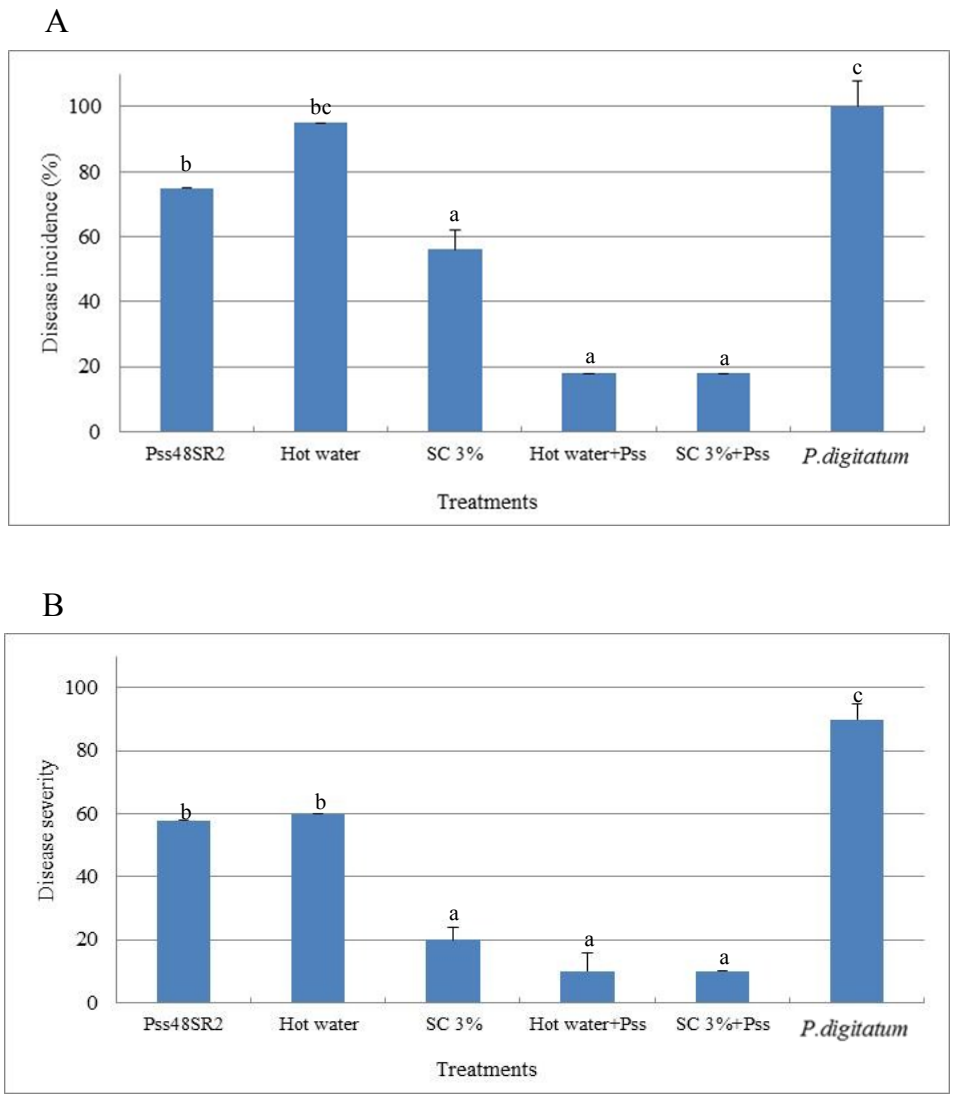


Figure 22. Incidence (A) and severity (B) of green mold on cv. Tarocco oranges 5 days after P. digitatum inoculation followed (3 h) by different treatments: antagonistic P. syringae PVCT48SR2 spray-inoculation; immersion in hot water (45°C); immersion in 3% SC (45°C), alone or in combination with P. syringae PVCT48SR2. Columns with the same letter are not significantly different using the Student-Newman–Keuls mean separation test at P □0.05

Protective activity of combined treatments with P. syringae strains* and *acibenzolar-S-methyl (ASM), and chitosan

The protective treatments with ASM were totally ineffective when applied on non-wounded oranges (100% disease incidence) (Fig. 23A-25A-27A). The ASM dip-treatments, when applied on wounded oranges, weakly reduced green mold decay at the two tested concentrations (80-85% of disease incidence afer 5 days of incubation), indicating in any case a modest protective effect (Fig.23B-25B-27B).

The protective activity was notable on wounded oranges treated with biocontrol bacteria and, 3 days later, inoculated with the pathogen. Treatment with Pss40SR4 Pss48SR2, and Pss46P reduced green mold incidence from 100% among the inoculated and untreated control fruit to 16, 18, and 37%, respectively (Fig. 23C-25C-27C), 5 days after *P. digitatum* inoculation.

A significant improving of green mold control was obtained when ASM treatments were followed by *P. syringae* PVCT40SR4 or PVCT48SR2 inoculation (Fig. 23D-25D). After these treatments, incidence and severity of green mold were completely reduced (0% disease incidence and severity) also 5 days after inoculation and the efficacy did not appear to increase by raising the concentration of ASM from 0.5 to 1.0 mg/ml (Fig. 23D-24D-25D-26D). On the contrary, ASM did not improve the efficacy of *P. syringae* strain PVCT46P and decay incidence and severity were significantly similar (using ASM 0.5 mg/ml) or higher (using ASM 1 mg/ml) to that observed following *P. syringae* treatment alone (Fig. 27C,D-28C,D).

Protective activity of combined treatments of ASM and *P. syringae* 40SR4 strain in controlling green mold *in vivo:* disease incidence

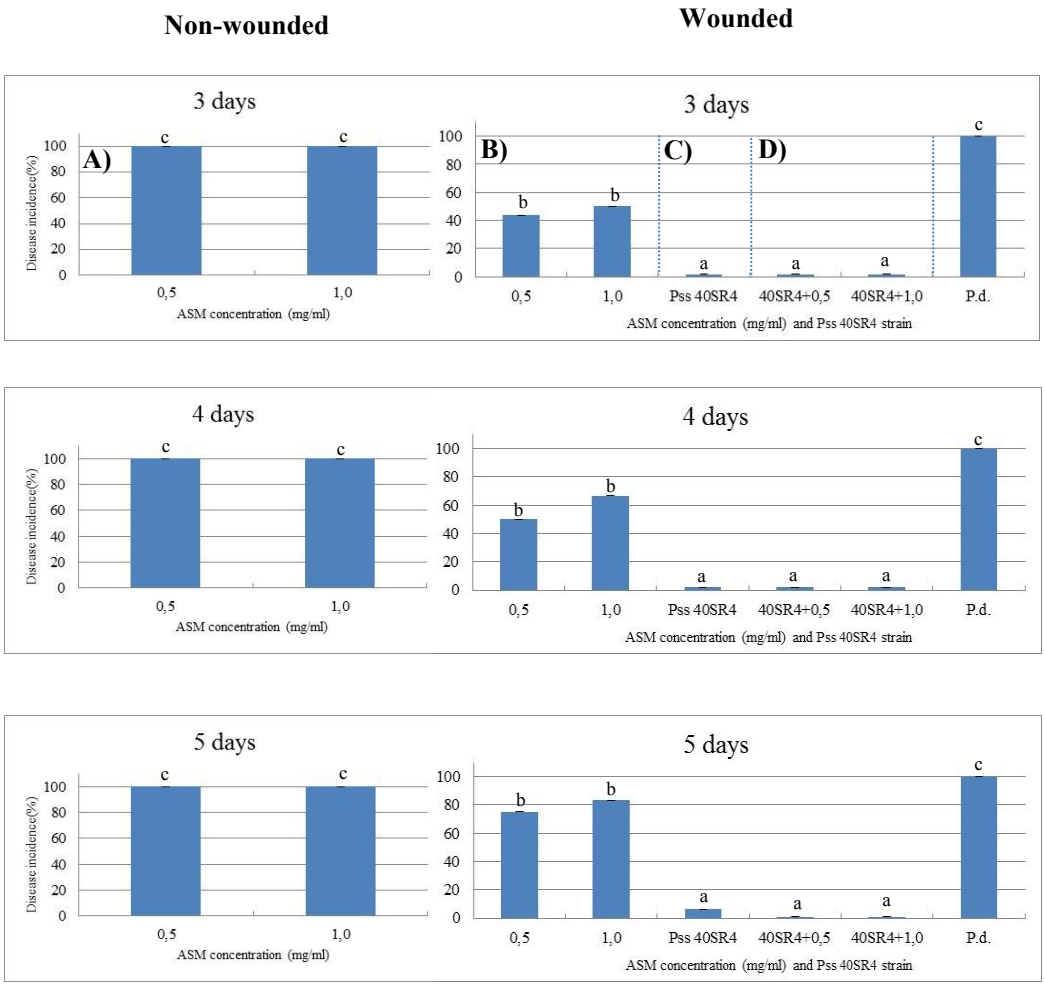


Figure 23. Disease incidence on oranges cv. "Tarocco": (A) non-wounded and soaked in ASM solutions (0,5 and 1,0 mg/ml); (B) wounded and soaked in ASM solutions (0,5 and 1,0 mg/ml); (C) wounded and treated with *P. syringae* pv. *syringae* PVCT40SR4 10^9ufc/ml (Pss40SR4); (D) wounded, soaked in ASM solutions (0,5 and 1,0 mg/ml) and treated with *P. syringae* pv. *syringae* PVCT40SR4 (40SR4+0,5; 40SR4+1,0) wounded and inoculated with *P. digitatum* (P.d.). After 72h all treatments were inoculated with P. digitatum (10^6spore/ml). Disease incidence was evaluated after 3-4-5 days of incubation at 20°C. Columns without letters within different combined treatments are not significantly different according to the Student-Newman-Keuls' one way ANOVA test at P ≤0.05.

Protective activity of combined treatments of ASM and *P. syringae* 40SR4 strain in controlling green mold *in vivo:* disease severity

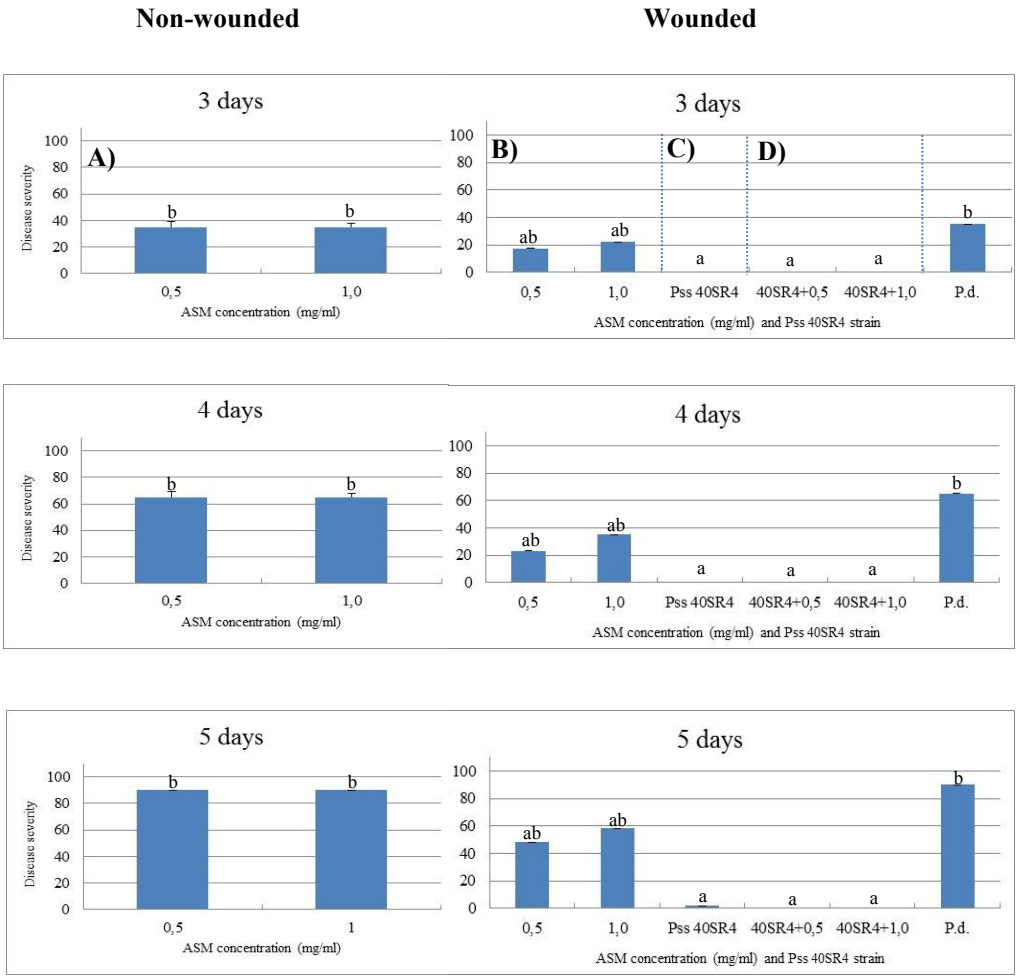


Figure 24. Disease severity on oranges cv. "Tarocco": (a) non-wounded and soaked in ASM solutions (0,5 and 1,0 mg/ml); (b) wounded and soaked in ASM solutions (0,5 and 1,0 mg/ml); (c) wounded and treated with *P. syringae* pv. *syringae* PVCT40SR4 10^9ufc/ml (Pss40SR4); (d) wounded, soaked in ASM solutions (0,5 and 1,0 mg/ml) and treated with *P. syringae* pv. *syringae* PVCT40SR4 (40SR4+0,5; 40SR4+1,0) wounded and inoculated with *P. digitatum* (P.d.). After 72h all treatments were inoculated with *P. digitatum* (10^6spore/ml). Disease severity evaluated after 3-4-5 days of incubation at 20°C. Columns without letters within different combined treatments are not significantly different according to the Student-Newman-Keuls'one way ANOVA test at P ≤0.05. Disease severity was evaluated with an empiric scale (1=no visible symptoms; 2=initial soft rot; 3=presence of mycelium; 4=sporulation) data were converted percentage midpoint values where 1=0%; 2=35%; 3=65%; 4=90%).

Protective activity of combined treatments of ASM and *P. syringae* 48SR2 strain in controlling green mold *in vivo:* disease incidence

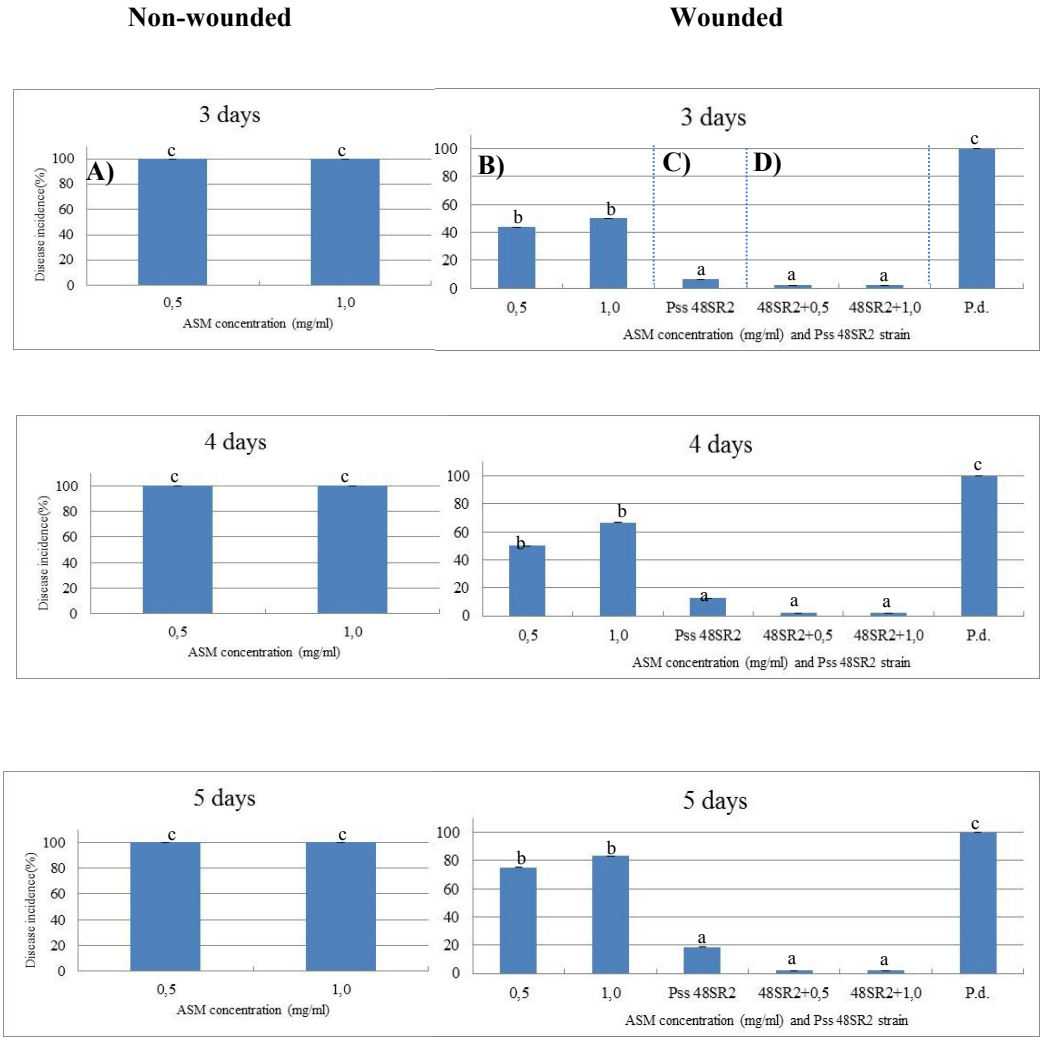


Figure 25. Disease incidence on oranges cv. "Tarocco": (a) non-wounded and soaked in ASM solutions (0,5 and 1,0 mg/ml); (b) wounded and soaked in ASM solutions (0,5 and 1,0 mg/ml); (c) wounded and treated with *P. syringae* pv. *syringae* PVCT48SR2 10^9ufc/ml (Pss48SR2); (d) wounded, soaked in ASM solutions (0,5 and 1,0 mg/ml) and treated with *P. syringae* pv. *syringae* PVCT48SR2 (48SR2+0,5; 48SR2+1,0) wounded and inoculated with *P. digitatum* (P.d.). After 72h all treatments were inoculated with *P. digitatum* (10^6spore/ml). Disease incidence was evaluated after 3-4-5 days of incubation at 20°C. Columns without letters within different combined treatments are not significantly different according to the Student-Newman-Keuls' one way ANOVA test at P ≤0.05.

Protective activity of combined treatments of ASM and *P. syringae* 48SR2 strain in controlling green mold *in vivo:* disease severity

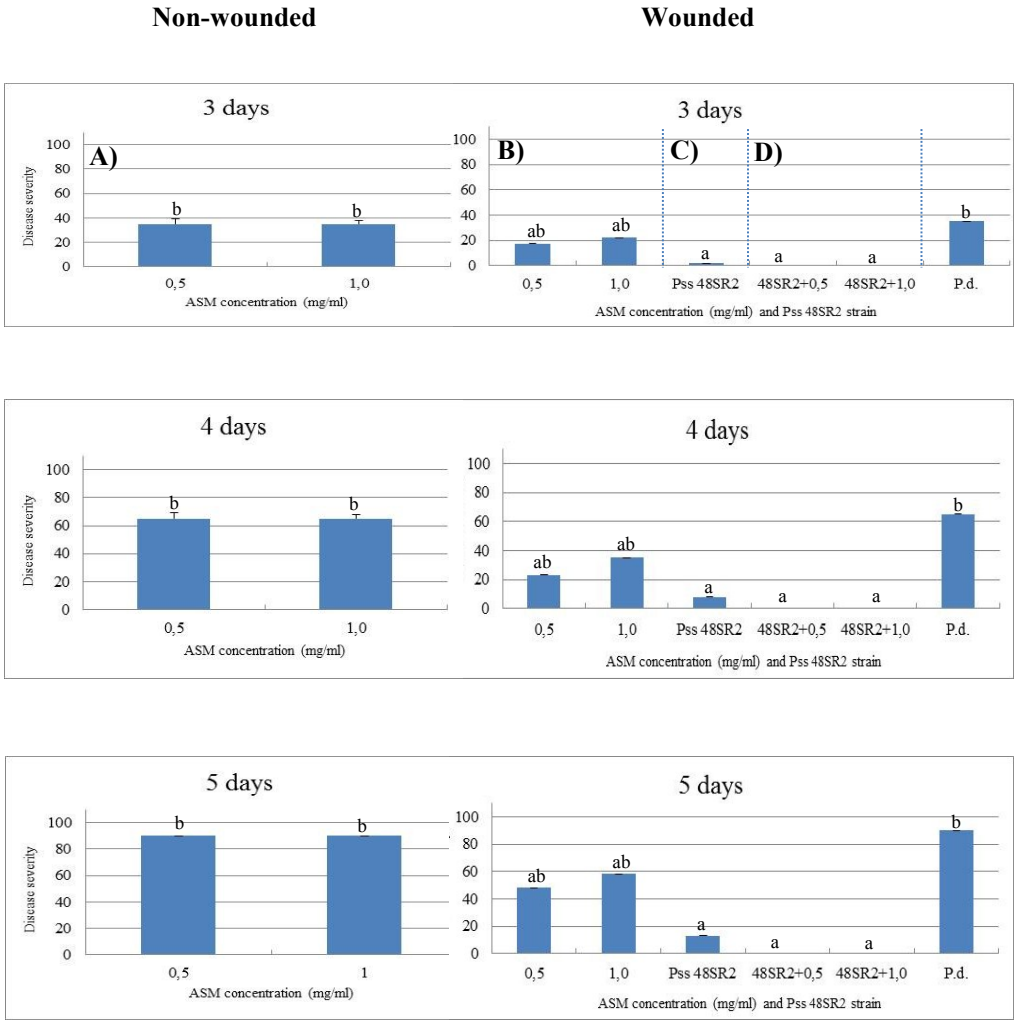


Figure 26. Disease severity on oranges cv. "Tarocco": (1) non-wounded and soaked in ASM solutions (0,5 and 1,0 mg/ml); (2) wounded and soaked in ASM solutions (0,5 and 1,0 mg/ml); (3) wounded and treated with *P. syringae* pv. *syringae* PVCT48SR2 10^9ufc/ml (Pss48SR2); (4) wounded, soaked in ASM solutions (0,5 and 1,0 mg/ml) and treated with *P. syringae* pv. *syringae* PVCT48SR2 (48SR2+0,5; 48SR2+1,0) wounded and inoculated with *P. digitatum* (P.d.). After 72h all treatments were inoculated with *P. digitatum* (10^6spore/ml). Disease severity evaluated after 3-4-5 days of incubation at 20°C. Columns without letters within different combined treatments are not significantly different according to the Student-Newman-Keuls'one way ANOVA test at P ≤0.05. Disease severity was evaluated with an empiric scale (1=no visible symptoms; 2=initial soft rot; 3=presence of mycelium; 4=sporulation) data were converted percentage midpoint values where 1=0%; 2=35%; 3=65%; 4=90%).

Protective activity of combined treatments of ASM and *P. syringae* 46P strain in controlling green mold *in vivo:* disease incidence

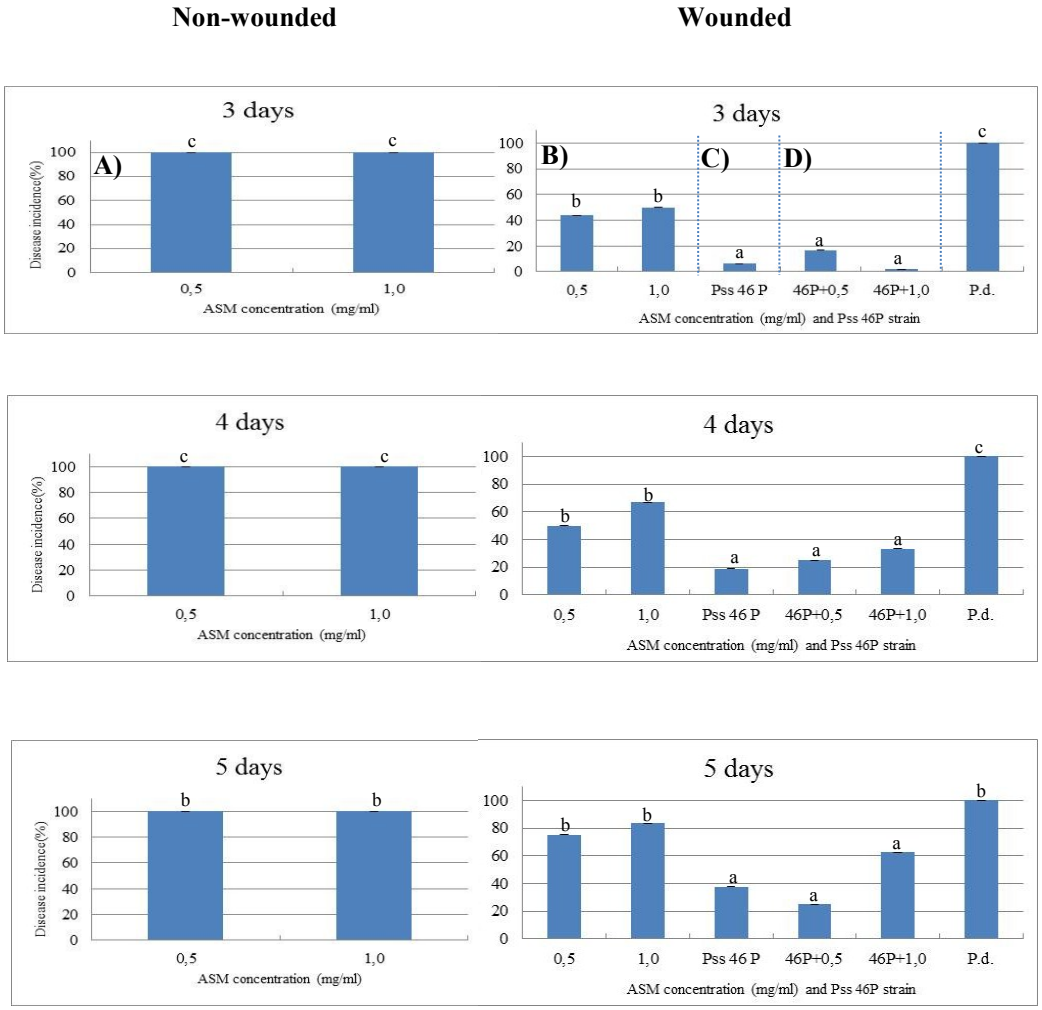


Figure 27. Disease incidence on oranges cv. "Tarocco": (1) non-wounded and soaked in ASM solutions (0,5 and 1,0 mg/ml); (2) wounded and soaked in ASM solutions (0,5 and 1,0 mg/ml); (3) wounded and treated with *P. syringae* pv. *syringae* PVCT46P 10^9ufc/ml (Pss46P); (4) wounded, soaked in ASM solutions (0,5 and 1,0 mg/ml) and treated with *P. syringae* pv. *syringae* PVCT46P (46P+0,5; 46P+1,0) wounded and inoculated with *P. digitatum* (P.d.). After 72h all treatments were inoculated with P. digitatum (10^6spore/ml). Disease incidence was evaluated after 3-4-5 days of incubation at 20°C. Columns without letters within different combined treatments are not significantly different according to the Student-Newman-Keuls' one way ANOVA test at $P \leq 0.05$.

Protective activity of combined treatments of ASM and *P. syringae* 46P strain in controlling green mold *in vivo:* disease severity

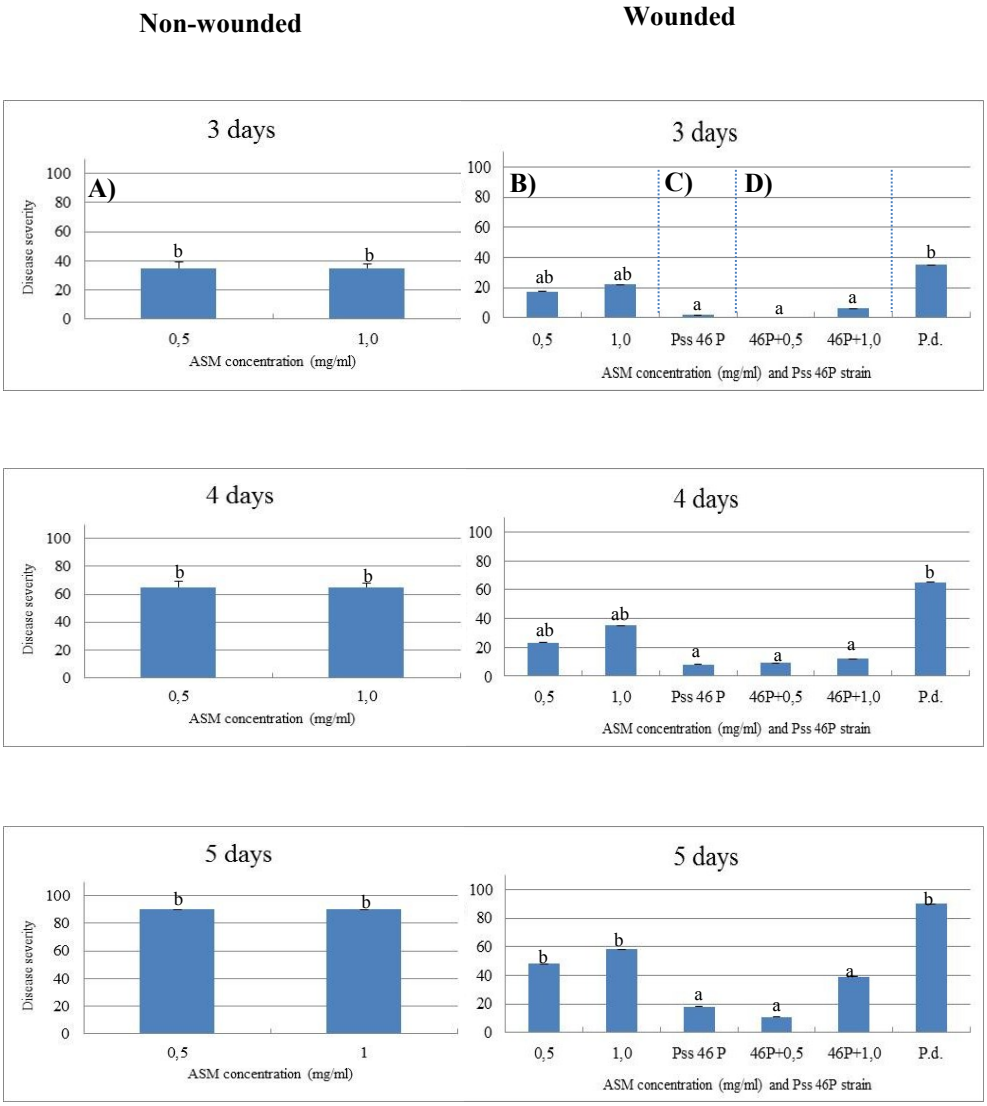


Figure 28. Disease severity on oranges cv. "Tarocco": (1) non-wounded and soaked in ASM solutions (0,5 and 1,0 mg/ml); (2) wounded and soaked in ASM solutions (0,5 and 1,0 mg/ml); (3) wounded and treated with *P. syringae* pv. *syringae* PVCT46P 10^9ufc/ml (Pss46P); (4) wounded, soaked in ASM solutions (0,5 and 1,0 mg/ml) and treated with *P. syringae* pv. *syringae* PVCT46P (46P+0,5; 46P+1,0) wounded and inoculated with *P. digitatum* (P.d.). After 72h all treatments were inoculated with *P. digitatum* (10^6spore/ml). Disease severity evaluated after 3-4-5 days of incubation at 20°C. Columns without letters within different combined treatments are not significantly different according to the Student-Newman-Keuls'one way ANOVA test at P ≤0.05. Disease severity was evaluated with an empiric scale (1=no visible symptoms; 2=initial soft rot; 3=presence of mycelium; 4=sporulation) data were converted percentage midpoint values where 1=0%; 2=35%; 3=65%; 4=90%).

Chitosan treatment at 0.5 mg/ml provided very low protection against infections in wounds made and inoculated with the pathogen 3 days after chitosan treatment (Fig. 29), whereas treatments with *P. syringae* strain PVCT48SR2 significantly reduced disease incidence and severity. The protective treatments of combined applications of chitosan and *P. syringae* 48SR2 showed a high antifungal activity (18.8-21.9% disease incidence; 13.4-11.4% disease severity) 5 days after *P. digitatum* inoculation (Fig. 29A-B). The addiction of biocontrol agent to chitosan-treated fruit significantly improved green mold control and compensated for the modest protective activity of chitosan treatment, whereas there were no synergistic results from the combination compared with *P. syringae* application alone.

Protective activity of combined treatments of *P. syringae* pv. *syringae* PVCT48SR2 and chitosan in controlling green mold *in vivo*

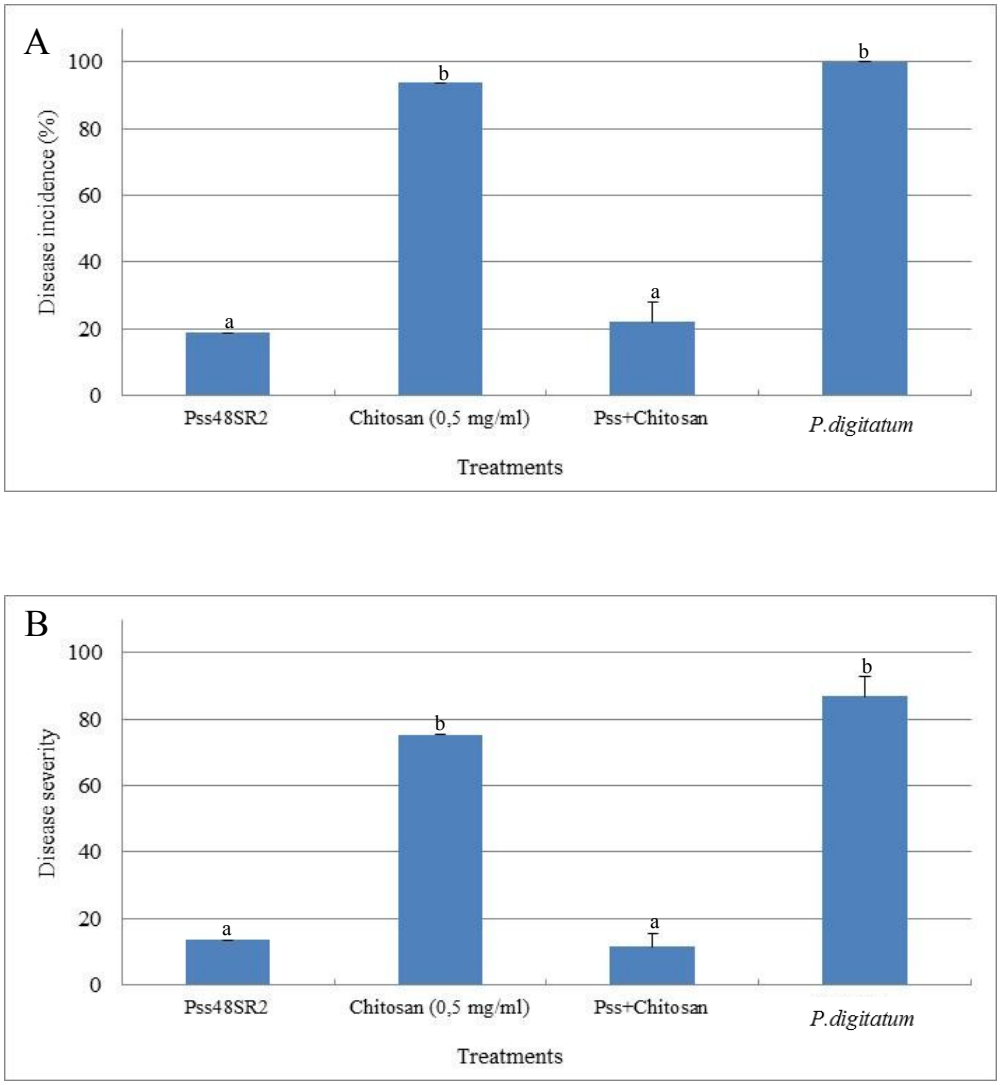


Figure 29. Protective activity of *P. syringae* pv. *syringae* strain PVCT48SR2 and chitosan, alone or in combination, on decay of "Tarocco" orange fruit caused by *P. digitatum*. *P. digitatum* inoculation was performed 72h after treatments. Disease incidence (A) and severity (B) were evaluated after 5 days of incubation at 20°C. Columns with the same letters within different combined treatments are not significantly different according to the Student-Newman-Keuls'one way ANOVA test at P ≤0.05. Disease severity was evaluated with an empiric scale (1=no visible symptoms; 2=initial soft rot; 3=presence of mycelium; 4=sporulation) data were converted percentage midpoint values where 1=0%; 2=35%; 3=65%; 4=90%).

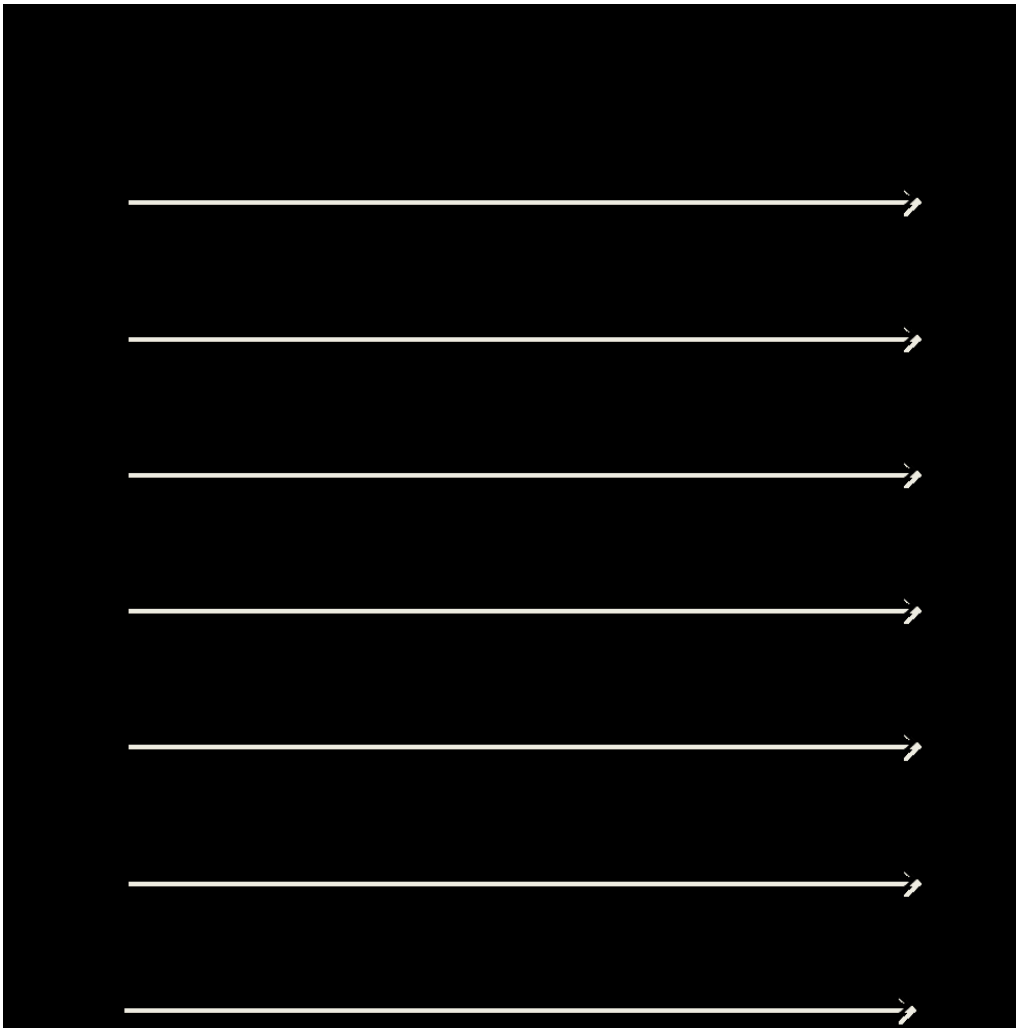
4.2.2. Combined treatment of *Pseudomonas syringae* pv. *syringae* 48SR2, *Trichoderma atroviride* P1 and chitosan

Curative and preventive activity of combined application of* P. syringae *PVCT48SR2* T. atroviride *P1 and chitosan 0.5 mg/ml on the control of* P. digitatum *on cv. Tarocco stored at different temperatures after treatment

Treatment of *P. digitatum*-inoculated oranges cv. Tarocco with *P. syringae* 48SR2, *T. atroviride* P1, and chitosan 0.5 mg/ml reduced green mold incidence from 100% among the inoculated and untreated fruit to 80% (Pss and chitosan treatments) and 60% (P1 treatment) after 4 days of incubation at 20 °C (Fig. 30). The curative effect of both antagonists was not enhanced by P1 application in mixture with Pss, whereas the addition of chitosan significantly enhanced the effect of both antagonists in mixture (green mold incidence of 65%). After 5 days of incubation the treatment with the mixture of microorganisms and chitosan still reduced severity of disease (green mold severity of 38%) whereas all the other treatments were ineffective (Fig.30). Only the fungicide totally inhibited the development of the disease. On the whole, all treatments tested provided poor curative activity against infections in wounds made and inoculated with the fungus before treatments.

20 °C **Curative activity**

Wounded, treated and 2 h later inoculated with *P. digitatum*



Curative activity of citrus fruit treated with: **1)** Thiabendazole (TBZ) and after 2 hours inoculated with *Penicillium digitatum* (P.d.), **2)** *Pseudomonas syringae* pv. *syringae* PVCT48SR2 (Pss) and after 2 hours inoculated with *P. digitatum*, **3)***Tricoderma atroviride* (P1) and after 2 hours inoculated with *P. digitatum*, **4)** Chitosan (Chi) and after 2 hours inoculated with *P. digitatum*, **5)** *Pseudomonas syringae* pv. *syringae* PVCT48SR2 after treated with *Tricoderma atroviride* and after 2 hours inoculated with *P. digitatum*, **6)** *Pseudomonas syringae* pv. *syringae* PVCT48SR2 after treated with *Tricoderma atroviride* after treated with chitosan and after 2 hours inoculated with *P. digitatum*, **7)** *Penicillium digitatum* used as control.

20 °C Curative activity

Wounded, treated and 2 h later inoculated with *P. digitatum*

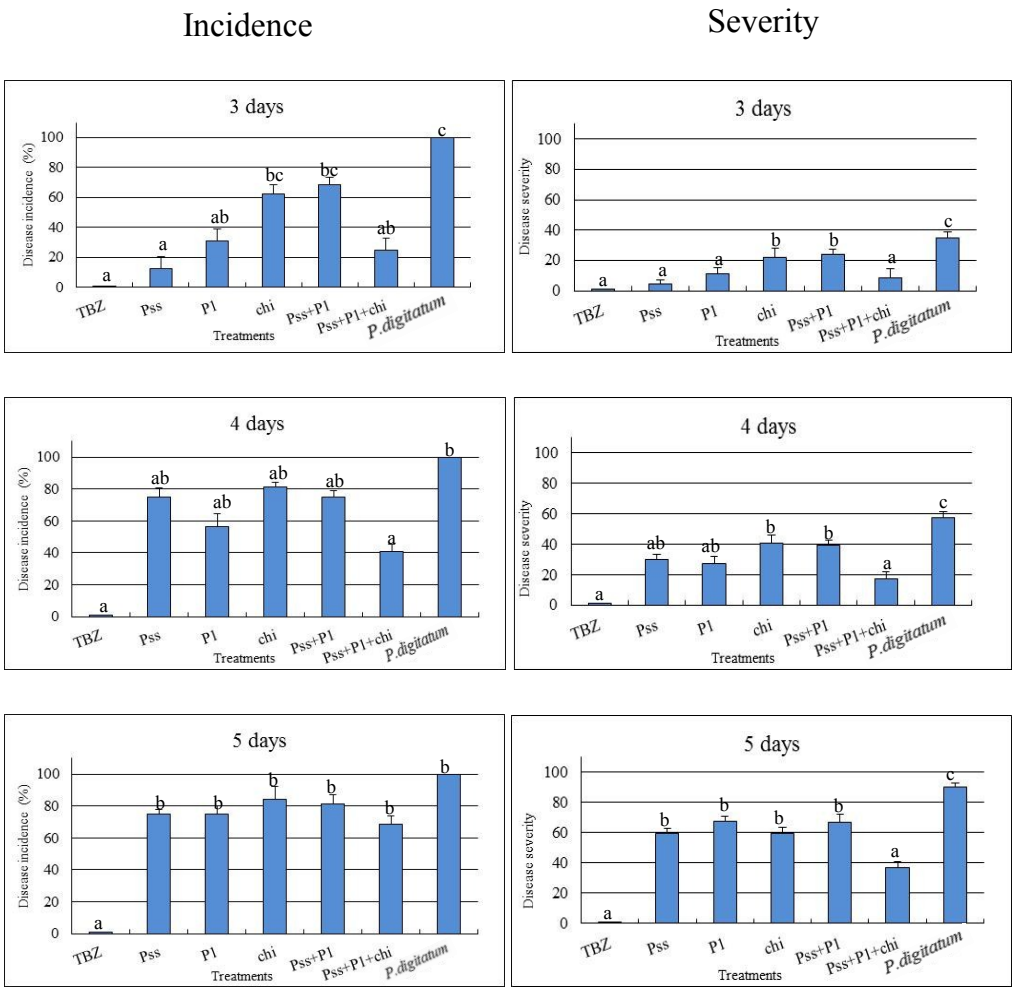
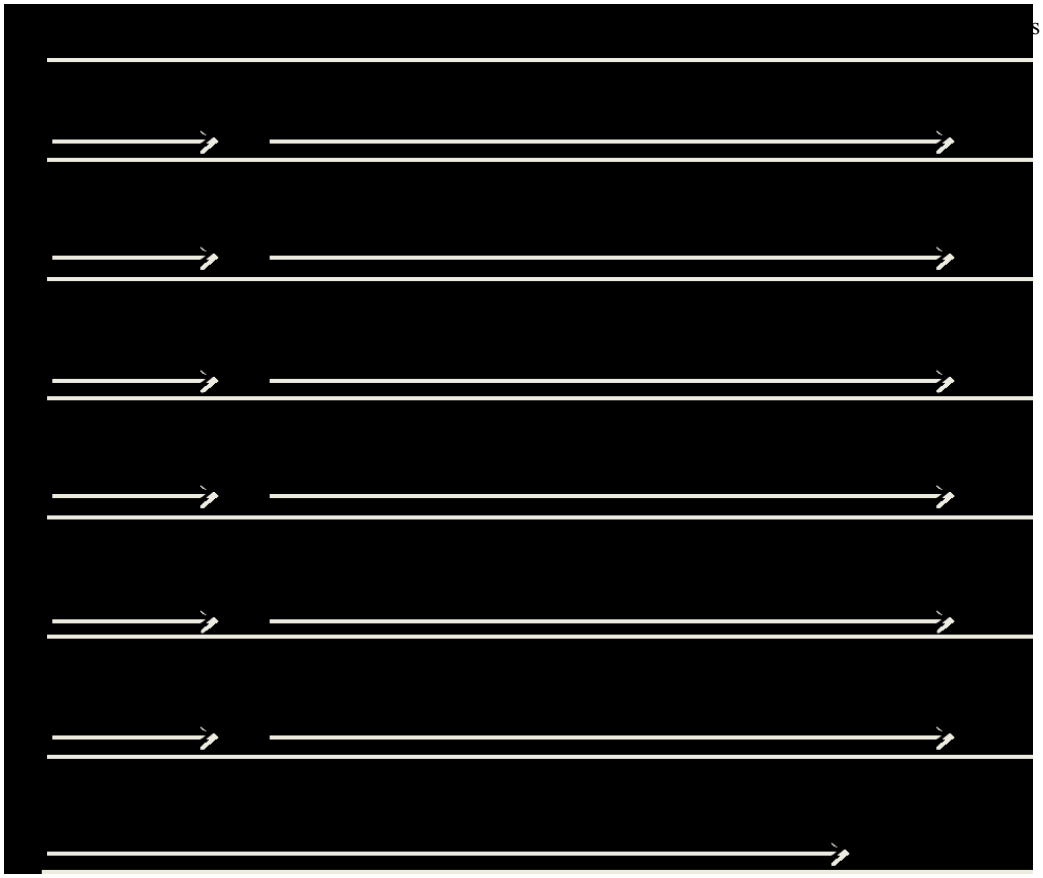


Figure 30. Disease incidence and severity on oranges cv. Tarocco inoculated with *P. digitatum* (P.d.) and with *P.syringae* pv. *syringae* 48SR2 (Pss), *T. atroviride* P1 (P1) and chitosan (chi) alone or in sequence to control green mold. Wounded fruit were immersed for 10 minutes in different solutions: Thiabendazole (TBZ) 0,1%, *P.s.*pv.*syringae* 48SR2 (Pss) 10^9 CFU/ml, *T. atroviride* P1 (P1) 10^8conidia/ml, chitosan (chi) 5 mg/ml, combined application of Pss+P1 and Pss+P1+chitosan. Control included fruit inoculated with *P. digitatum* 10^6spore/ml. Fruit were inoculated with *P. digitatum* 2 h after treatments. Disease incidence and severity were evaluated after 3-4-5 days of incubation at 20 °C. Values are the mean of 48 wounds per treatment. Columns with the same letters are not significantly different according to the Student-Newman-Keuls' one way ANOVA test at P ≤0.05. Disease severity was evaluated with an empiric scale (1=no visible symptoms; 2=initial soft rot; 3=presence of mycelium; 4=sporulation) data were converted percentage midpoint values where 1=0%; 2=35%; 3=65%; 4=90%).

The protective effect of treatments were notable on wounded and treated oranges that were inoculated 24 and 72 hours later with the pathogen. While green mold incidence was 20 and 80% respectively in Pss and P1 treatments made 24 h before *P. digitatum* inoculation (Fig. 31), it was 0 and 20 % in the same treatments made 72h before pathogen inoculation (Fig. 32). The protective effect was not enhanced by P1 and chitosan application in mixture with Pss (green mold incidence of 5 and 20% respectively in Pss+P1 and Pss+P1+chitosan) (treatments made 72h before pathogen inoculation) (Fig. 32). The protective effect of the treatment with the mixture of antagonists and chitosan significantly compensate for the lack of protective activity of chitosan (green mold incidence of 40 and 90% respectively when applied 24 and 72 h before *P. digitatum* inoculation) (Fig. 31-32).

Protective activity

Wounded, treated and 24 h later inoculated with *P. digitatum*



Protective activity of citrus fruit treated with: **1)** Thiabendazole (TBZ) and after 24 hours inoculated with *Penicillium digitatum* (P.d.), **2)** *Pseudomonas syringae* pv. *syringae* PVCT48SR2 (Pss) and after 24 hours inoculated with *P. digitatum*, **3)** *Tricoderma atroviride* (P1) and after 24 hours inoculated with *P. digitatum*, **4)** Chitosan (Chi) and after 24 hours inoculated with *P. digitatum*, **5)** *Pseudomonas syringae* pv. *syringae* PVCT48SR2 after treated with *Tricoderma atroviride* and after 24 hours inoculated with *P. digitatum*, **6)** *Pseudomonas syringae* pv. *syringae* PVCT48SR2 after treated with *Tricoderma atroviride* after treated with chitosan and after 24 hours inoculated with *P. digitatum*, **7)** *Penicillium digitatum* used as control.

20 °C **Protective activity**

Wounded, treated and 24 h later inoculated with *P. digitatum*

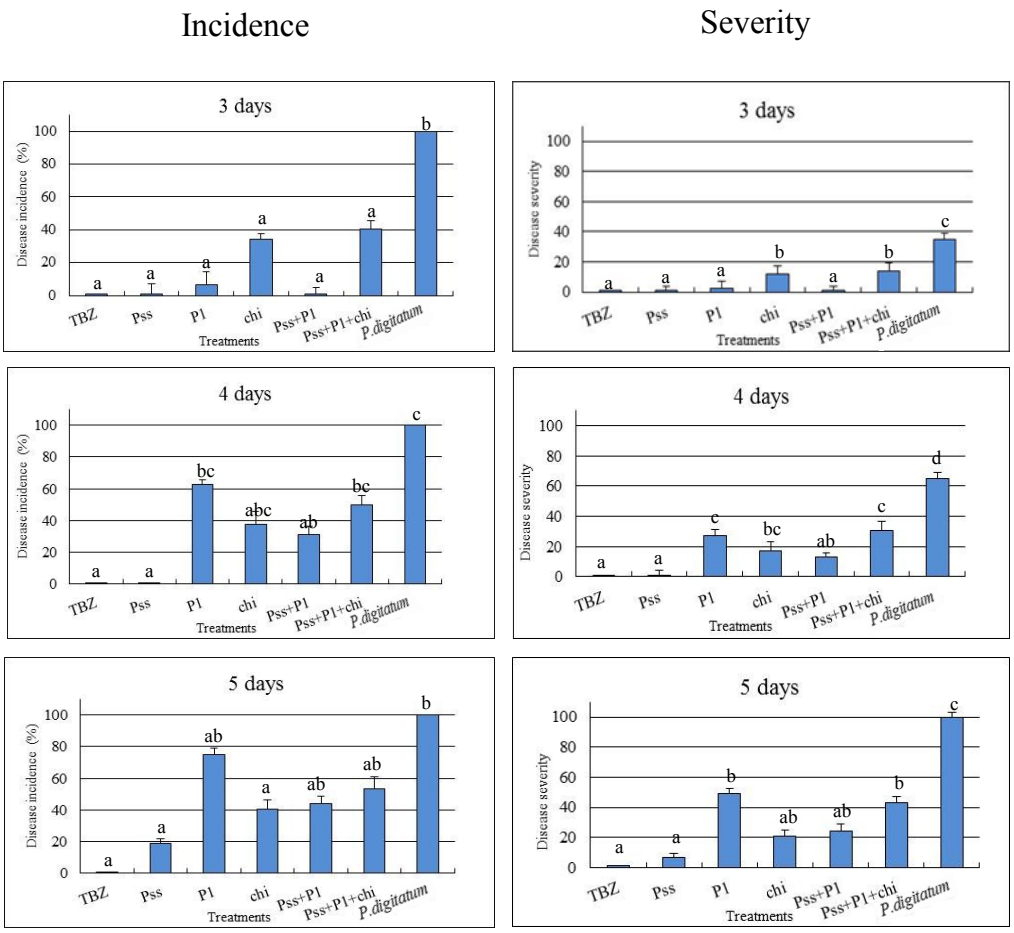
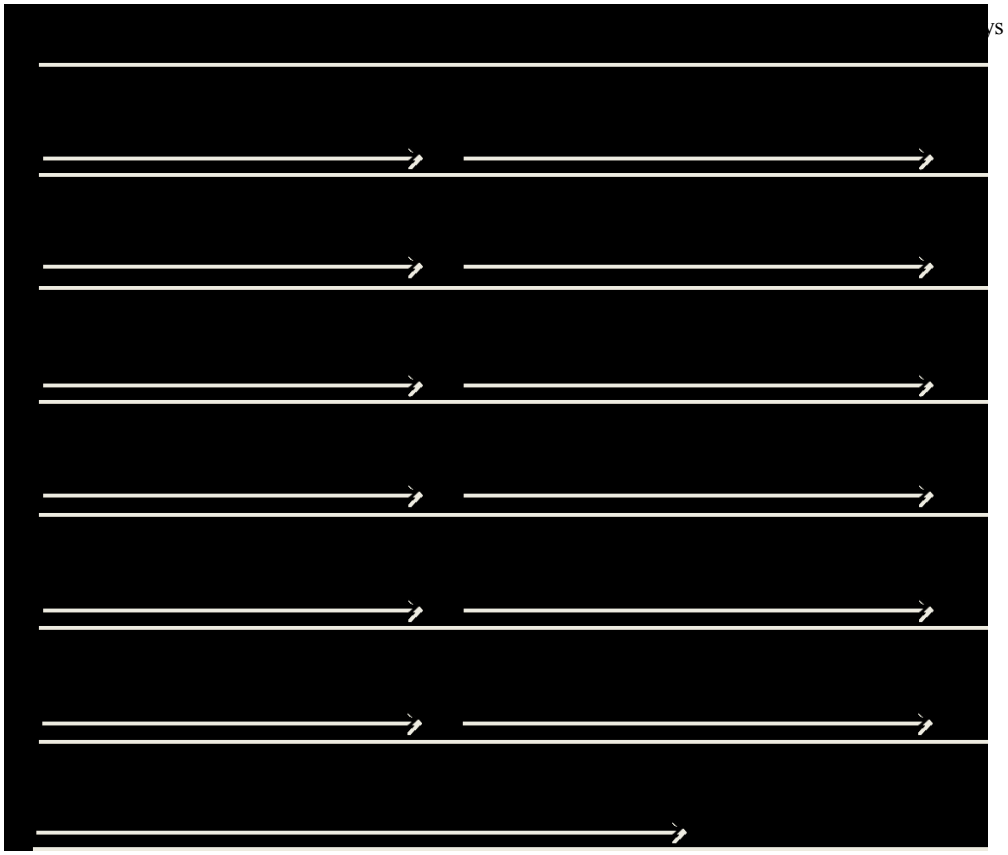


Figure 31. Disease incidence and severity on oranges cv. Tarocco inoculated with *P. digitatum* (P.d.) and with *P.syringae* pv. *syringae* 48SR2 (Pss), *T. atroviride* P1 (P1) and chitosan (chi) alone or in sequence to control green mold. Wounded fruit were immersed for 10 minutes in different solutions: Thiabendazole (TBZ) 0,1%, *P.s*.pv.*syringae* 48SR2 (Pss) 109 CFU/ml, *T. atroviride* P1 (P1) 10^8conidia/ml, chitosan (chi) 5 mg/ml, combined application of Pss+P1 and Pss+P1+chitosan. Control included fruit inoculated with *P. digitatum* 10^6spore/ml. Fruit were inoculated with *P. digitatum* 24 h after treatments. Disease incidence and severity were evaluated after 3-4-5 days of incubation at 20°C. Values are the mean of 48 wounds per treatment. Columns with the same letters are not significantly different according to the Student-Newman-Keuls' one way ANOVA test at P ≤0.05. Disease severity was evaluated with an empiric scale (1=no visible symptoms; 2=initial soft rot; 3=presence of mycelium; 4=sporulation) data were converted percentage midpoint values where 1=0%; 2=35%; 3=65%; 4=90%).

Protective activity

Wounded, treated and 72 h later inoculated with *P. digitatum*



Protective activity of citrus fruit treated with: **1)** Thiabendazole (TBZ) and after 72 hours inoculated with *Penicillium digitatum* (P.d.), **2)** *Pseudomonas syringae* pv. *syringae* PVCT48SR2 (Pss) and after 72 hours inoculated with *P. digitatum*, **3)** *Tricoderma atroviride* (P1) and after 72 hours inoculated with *P. digitatum*, **4)** Chitosan (Chi) and after 72 hours inoculated with *P. digitatum*, **5)** *Pseudomonas syringae* pv. *syringae* PVCT48SR2 after treated with *Tricoderma atroviride* and after 72 hours inoculated with *P. digitatum*, **6)** *Pseudomonas syringae* pv. *syringae* PVCT48SR2 after treated with *Tricoderma atroviride* after treated with chitosan and after 72 hours inoculated with *P. digitatum*, **7)** *Penicillium digitatum* used as control.

20 °C **Protective activity**

Wounded, treated and 72 h later inoculated with *P. digitatum*

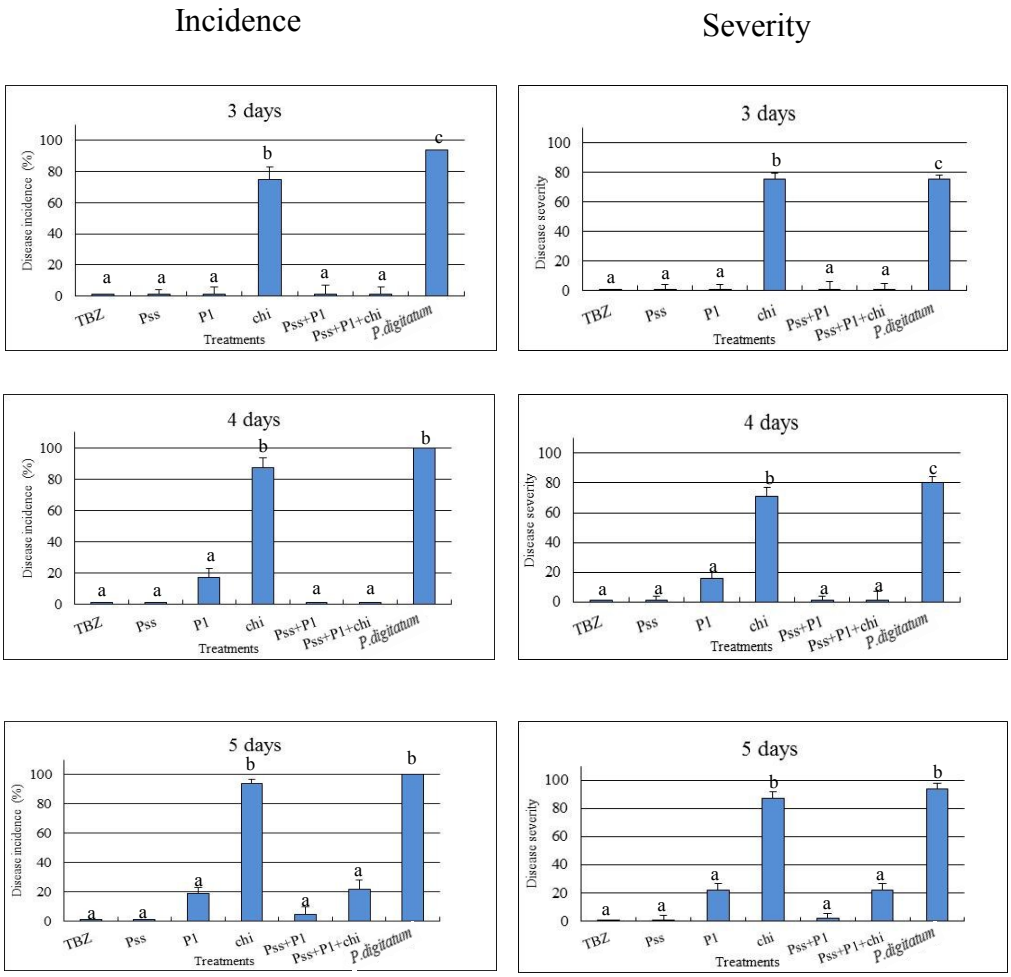


Figure 32. Disease incidence and severity on oranges cv. Tarocco inoculated with *P. digitatum* (P.d.) and with *P.syringae* pv. *syringae* 48SR2 (Pss), *T. atroviride* P1 (P1) and chitosan (chi) alone or in sequence to control green mold. Wounded fruit were immersed for 10 minutes in different solutions: Thiabendazole (TBZ) 0,1%, *P.s*.pv.*syringae* 48SR2 (Pss) 10^9 CFU/ml, *T. atroviride* P1 (P1) 10^8conidia/ml, chitosan (chi) 5 mg/ml, combined application of Pss+P1 and Pss+P1+chitosan. Control included fruit inoculated with *P. digitatum* 10^6spore/ml. Fruit were inoculated with *P. digitatum* 72 h after treatments. Disease incidence and severity were evaluated after 3-4-5 days of incubation at 20°C. Values are the mean of 48 wounds per treatment. Columns with the same letters are not significantly different according to the Student-Newman-Keuls' one way ANOVA test at P ≤0.05. Disease severity was evaluated with an empiric scale (1=no visible symptoms; 2=initial soft rot; 3=presence of mycelium; 4=sporulation) data were converted percentage midpoint values where 1=0%; 2=35%; 3=65%; 4=90%).

The efficacy of the treatments was evaluated after incubation at 6°C for 10-14-18 and 22 days. In fruit treated with Pss, P1, chitosan and with their mixtures 2 h before the inoculation with the pathogen, green mold incidence was significantly reduced (5-20% of disease incidence) after 10 days of incubation at 6°C, indicating a good curative activity long lasting at low temperatures (Fig. 33). The protective effect of the treatments was notable on wounded oranges that were inoculated 24 h later with the pathogen: since decay incidence was 10-35% in fruit treated with Pss, P1, chitosan and their mixtures (Fig. 34). When oranges were inoculated with the pathogen 72 h after treatments, green mold incidence was 25-45% with Pss, P1 and chitosan applied alone, and 18-24% with the mixtures (Fig. 35).

When the incubation time at 6°C was increased from 10 to 22 days, green mold incidence increased from 5-20% to 45-70% in fruit inoculated with *P. digitatum* immediately after treatments (curative activity) (Fig. 33); from 10-35 % to 30-80% in fruit inoculated with *P. digitatum* 24 h after treatments (protective activity) (Fig. 34); and from 20-45% to 50-85% in fruit inoculated with *P. digitatum* 72 h after treatments (protective activity) (Fig. 35).

6 °C Curative activity

Wounded, treated and 2 h later inoculated with *P. digitatum*

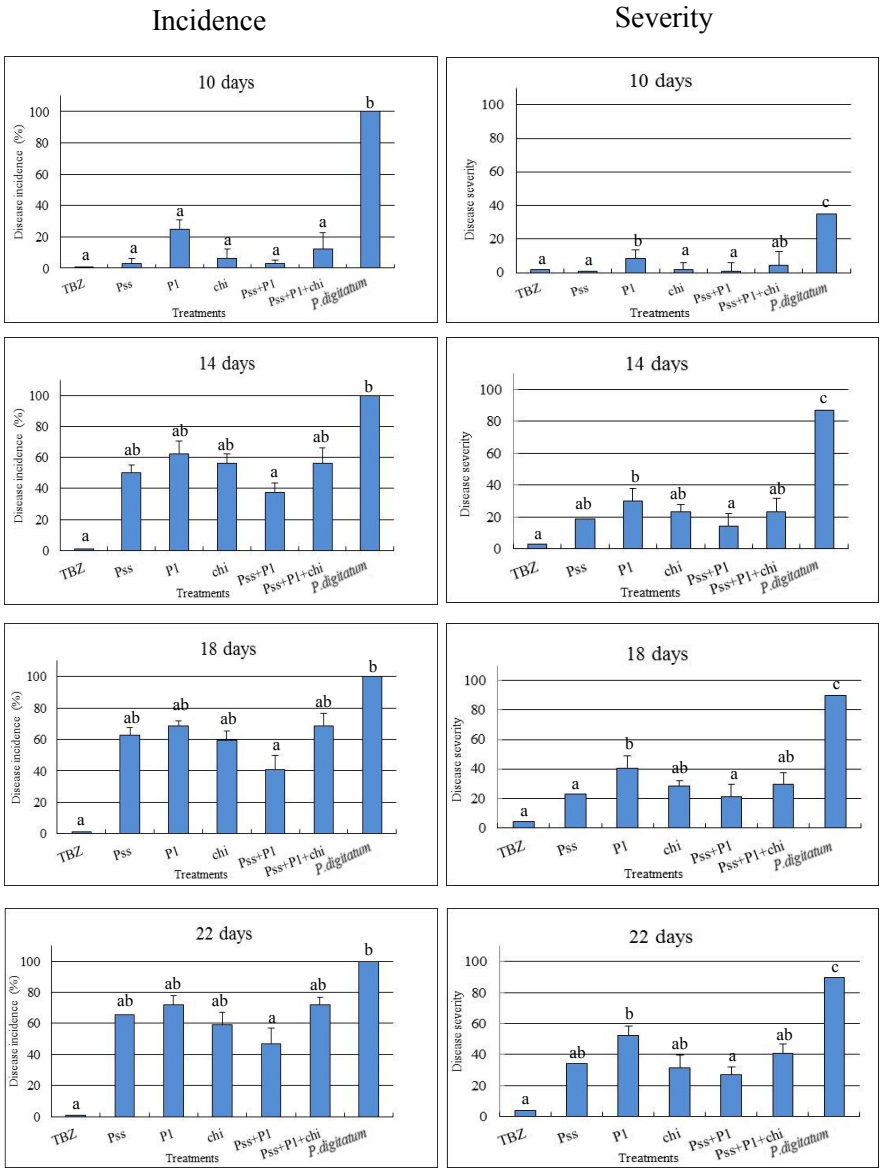


Figure 33. Disease incidence and severity on oranges cv. Tarocco inoculated with *P. digitatum* (P.d.) and with *P.syringae* pv. *syringae* 48SR2 (Pss), *T. atroviride* P1 (P1) and chitosan (chi) alone or in sequence to control green mold. Wounded fruit were immersed for 10 minutes in different solutions: Thiabendazole (TBZ) 0,1%, *P.s.pv.syringae* 48SR2 (Pss) 10^9 CFU/ml, *T. atroviride* P1 (P1) 10^8conidia/ml, chitosan (chi) 5 mg/ml, combined application of Pss+P1 and Pss+P1+chitosan. Control included fruit inoculated with *P. digitatum* 10^6spore/ml. Fruit were inoculated with *P. digitatum* 2 h after treatments. Disease incidence and severity were evaluated after 3-4-5 days of incubation at 6°C. Values are the mean of 48 wounds per treatment. Columns with the same letters are not significantly different according to the Student-Newman-Keuls' one way ANOVA test at P ≤0.05. Disease severity was evaluated with an empiric scale (1=no visible symptoms; 2=initial soft rot; 3=presence of mycelium; 4=sporulation) data were converted percentage midpoint values where 1=0%; 2=35%; 3=65%; 4=90%).

6 °C **Protective activity**

Wounded, treated and 24 h later inoculated with *P. digitatum*

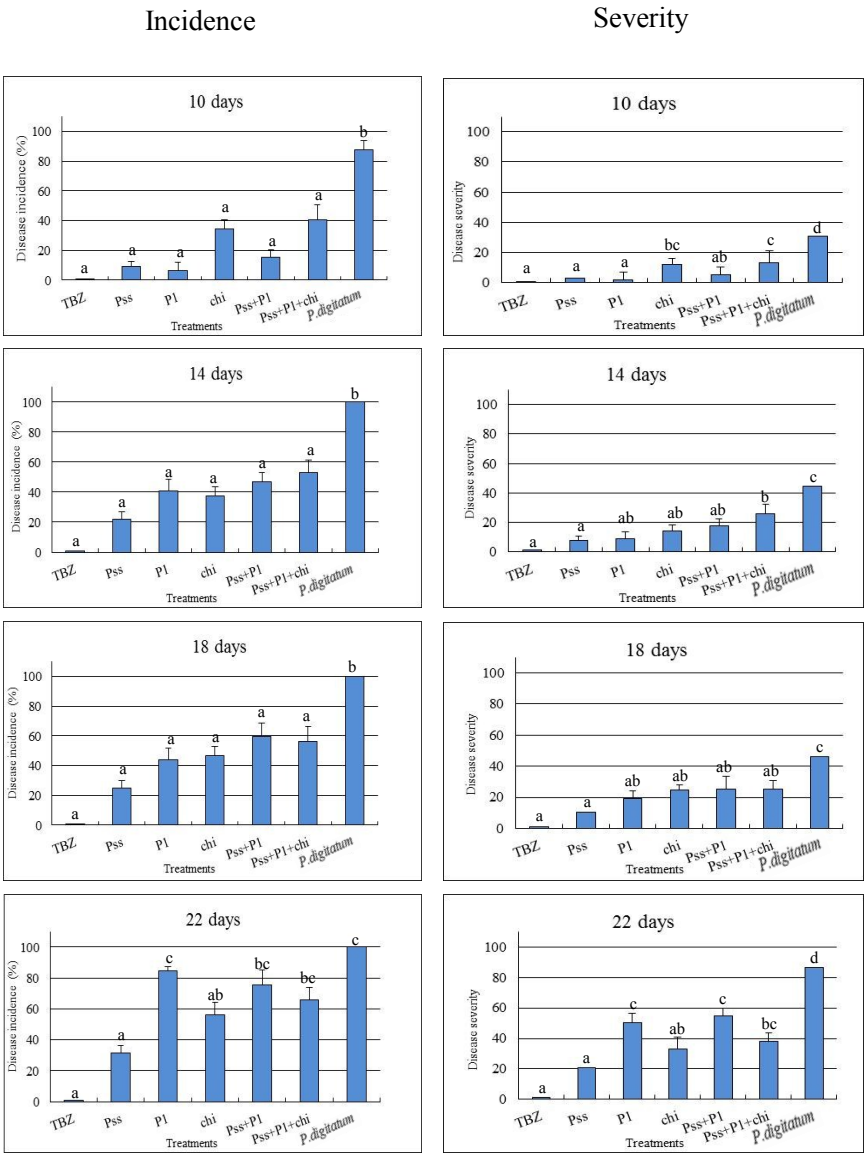


Figure 34. Disease incidence and severity on oranges cv. Tarocco inoculated with *P. digitatum* (P.d.) and with *P.syringae* pv. *syringae* 48SR2 (Pss), *T. atroviride* P1 (P1) and chitosan (chi) alone or in sequence to control green mold. Wounded fruit were immersed for 10 minutes in different solutions: Thiabendazole (TBZ) 0,1%, *P.s.pv.syringae* 48SR2 (Pss) 10^9 CFU/ml, *T. atroviride* P1 (P1) 10^8conidia/ml, chitosan (chi) 5 mg/ml, combined application of Pss+P1 and Pss+P1+chitosan. Control included fruit inoculated with *P. digitatum* 10^6spore/ml. Fruit were inoculated with *P. digitatum* 24 h after treatments. Disease incidence and severity were evaluated after 3-4-5 days of incubation at 6°C. Values are the mean of 48 wounds per treatment. Columns with the same letters are not significantly different according to the Student-Newman-Keuls' one way ANOVA test at P ≤0.05. Disease severity was evaluated with an empiric scale (1=no visible symptoms; 2=initial soft rot; 3=presence of mycelium; 4=sporulation) data were converted percentage midpoint values where 1=0%; 2=35%; 3=65%; 4=90%).

6 °C **Protective activity**

Wounded, treated and 72 h later inoculated with *P. digitatum*

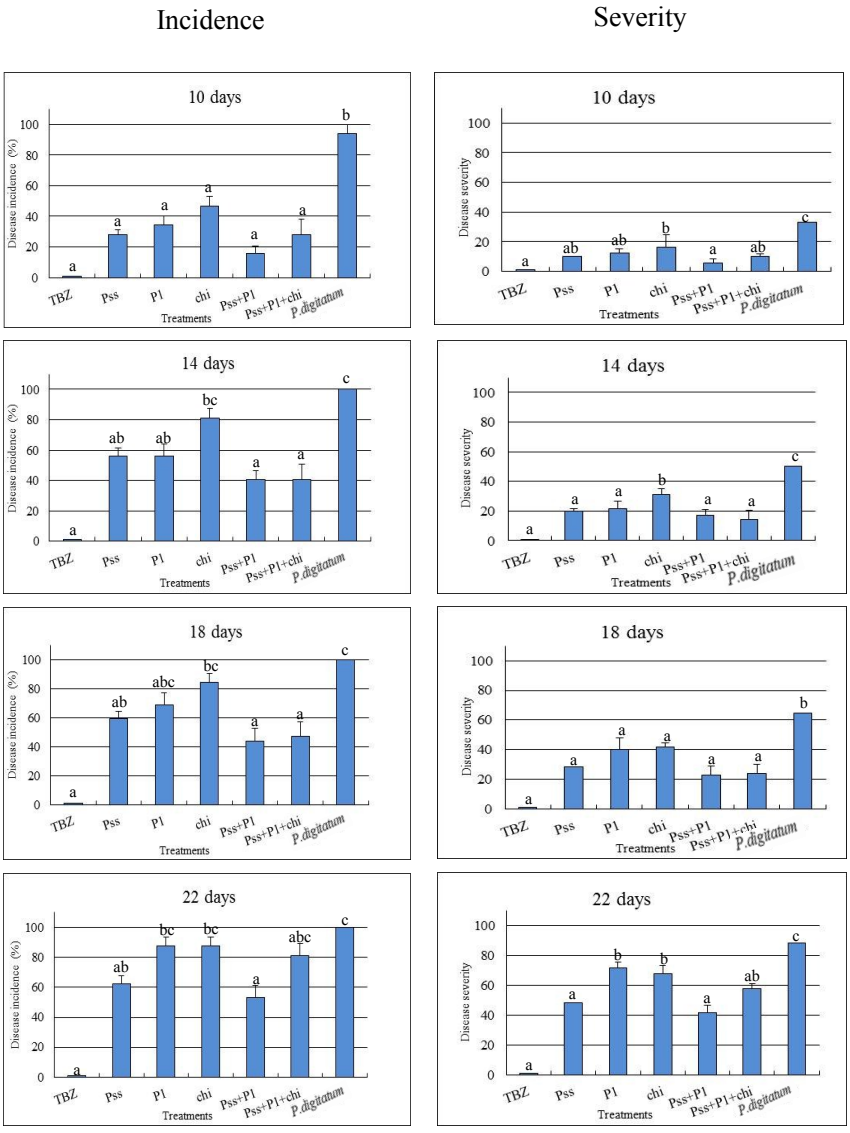


Figure 35. Disease incidence and severity on oranges cv. Tarocco inoculated with *P. digitatum* (P.d.) and with *P.syringae* pv. *syringae* 48SR2 (Pss), *T. atroviride* P1 (P1) and chitosan (chi) alone or in sequence to control green mold. Wounded fruit were immersed for 10 minutes in different solutions: Thiabendazole (TBZ) 0,1%, *P.s*.pv.*syringae* 48SR2 (Pss) 10^9 CFU/ml, *T. atroviride* P1 (P1) 10^8conidia/ml, chitosan (chi) 5 mg/ml, combined application of Pss+P1 and Pss+P1+chitosan. Control included fruit inoculated with *P. digitatum* 10^6spore/ml. Fruit were inoculated with *P. digitatum* 72 h after treatments. Disease incidence and severity were evaluated after 3-4-5 days of incubation at 6°C. Values are the mean of 48 wounds per treatment. Columns with the same letters are not significantly different according to the Student-Newman-Keuls' one way ANOVA test at P ≤0.05. Disease severity was evaluated with an empiric scale (1=no visible symptoms; 2=initial soft rot; 3=presence of mycelium; 4=sporulation) data were converted percentage midpoint values where 1=0%; 2=35%; 3=65%; 4=90%).

4.2.3. Combined treatment of *Pseudomonas syringae* pv. *syringae* PVCT48SR2, *Wickerhamomyces anomalus* BS91, hot water, sodium bicarbonate

Curative and preventive activity of combined treatments of hot water, sodium bicarbonates,* Pseudomonas syringae *48SR2 and* Wickerhamomyces anomalus *BS 91 to control postharvest green mold of oranges cv. Tarocco

Treatment of inoculated fruit with SBC reduced green mold incidence from 100% among the inoculated and untreated control fruit to 40% indicating modest curative activity (Fig. 36) The curative activity was not enhanced by biocontrol agent *Pss* following SBC treatment (compare treatment n°3 and 4). When inoculated alone, biocontrol agent *Pss* also showed curative activity (green mold incidence of 60%), but it was significantly inferior to that of SBC.

Curative activity

Wounded, inoculated with *P. digitatum* and treated

The following 4 treatments were applied on Tarocco oranges as curative activity: **(1)** fruit were wound inoculated with *Penicillium digitatum* (P.d.) and untreated (control treatment); **(2)** fruit were wound inoculated with *P. digitatum* then treated 2 h later in same wounds with the strain *Pseudomonas syringae* pv. *syringae* PVCT48SR2 (Pss); **(3)** fruit were wound inoculated with *P. digitatum* and treated 24 h later with sodium bicarbonate (SBC); **(4)** fruit were wound inoculated with *P. digitatum*, treated 24 h later with SBC, and treated 2 h later in same wound with *Pss*.

Curative activity

Wounded, inoculated with *P. digitatum* and treated

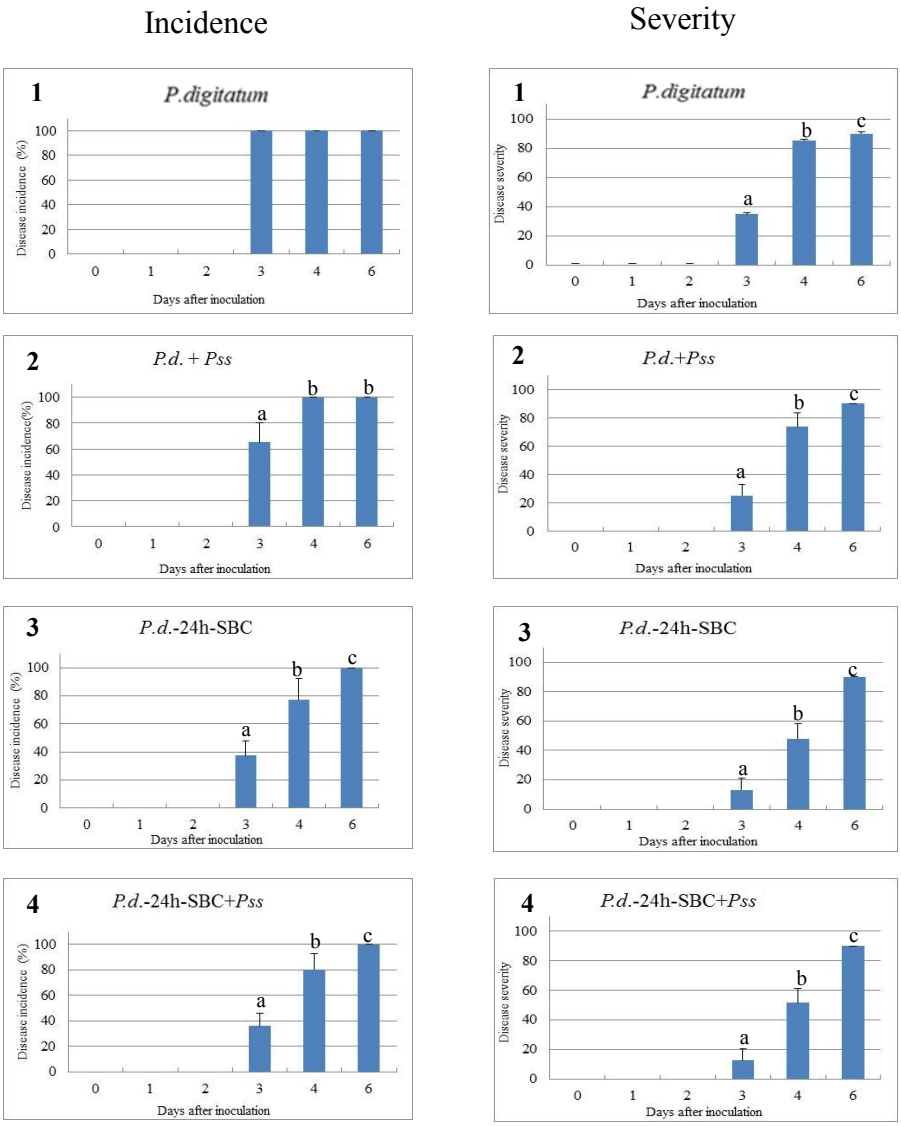


Figure 36. Disease incidence and severity on oranges cv. Tarocco treated with *P. digitatum* and with *P.syringae* 48SR2 (Pss), *W. anomalus* BS91 (Wa) and sodium bicarbonate (SBC) alone or in sequence to control green mold. (**1**) fruit were wound inoculated with *P. digitatum* and untreated (control treatment); (**2**) fruit were wound inoculated with *P. digitatum*, and treated 2h later in same wounds with the strain Pss 48SR2; (**3**) fruit were wound inoculated with *P. digitatum* and treated 24 h later with sodium bicarbonate (SBC); (**4**) fruit were wound inoculated with *P. digitatum*, treated 24 h later with SBC, and treated 2 h later in same wound with Pss. After every treatment, the fruit were incubated at 20°C for 5 d. Values are the mean of 3 experiments. Columns with the same letters are not significantly different according to the Student-Newman-Keuls' one way ANOVA test at P ≤0.05. Disease severity was evaluated with an empiric scale (1=no visible symptoms; 2=initial soft rot; 3=presence of mycelium; 4=sporulation) data were converted percentage midpoint values where 1=0%; 2=35%; 3=65%; 4=90%).

The protective effect of SBC treatment was notable on wounded oranges that were inoculated later with the pathogen (green mold incidence of 8%), and the addition of biocontrol agent *Pss* to this treatment significantly reduced green mold incidence (compare treatment n°5 and 6) (Fig. 37).

Protective activity

Wounded, treated and inoculated with *P. digitatum*

The following 2 treatments were applied on Tarocco oranges as preventive activity: **(5)** fruit were wounded, treated 24 h later with sodium bicarbonate (SBC), and inoculated 2 h later in same wound with *Penicillium digitatum* (P.d.); **(6)** fruit were wounded, treated 24 h later with SBC, inoculated 2 h later in same wound with *P. digitatum*, and treated 2 h later in same wound with Pseudomonas syringae pv. syringae PVCT48SR2 (Pss).

Protective activity

Wounded, treated and inoculated with *P. digitatum*

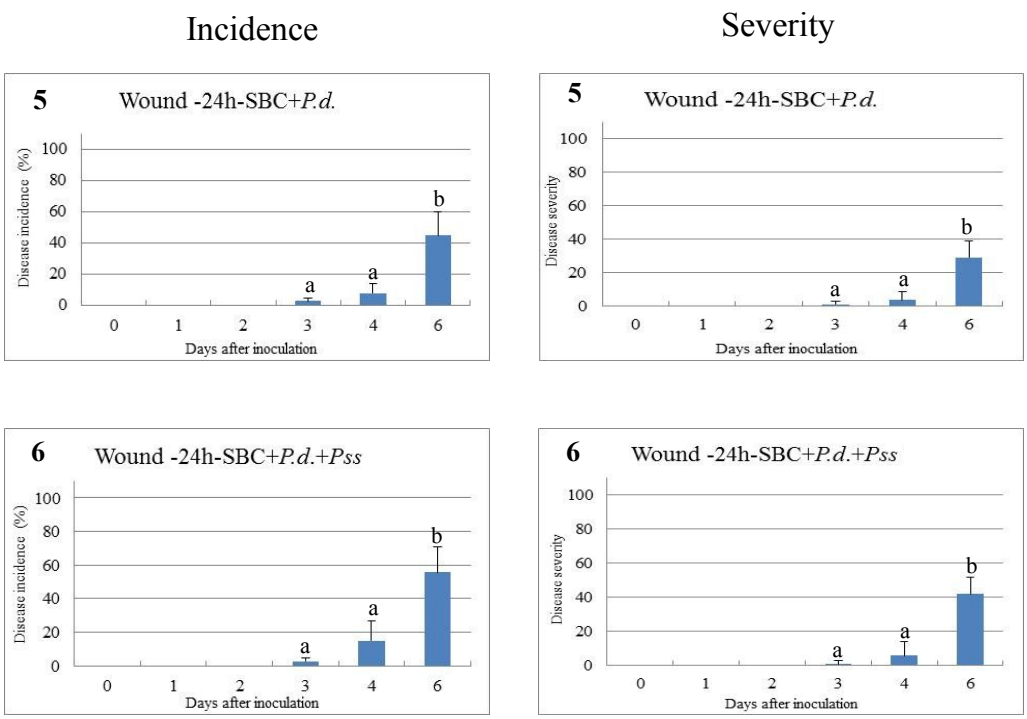


Figure 37. Disease incidence and severity on oranges cv. Tarocco wounded and treated with sodium bicarbonate (SBC), *P. digitatum*, *P.syringae* 48SR2 (Pss), in sequence to control green mold. (5) fruit were wounded, treated 24 h later with SBC, and inoculated 2 h later in same wound with *P. digitatum*; (6) fruit were wounded, treated 24 h later with SBC, inoculated 2 h later in same wound with *P. digitatum*, and treated 2 h later in same wound with Pss 48SR2. After every treatment, the fruit were incubated at 20°C for 5 d. Values are the mean of 3 experiments. Columns with the same letters are not significantly different according to the Student-Newman-Keuls' one way ANOVA test at P ≤0.05. Disease severity was evaluated with an empiric scale (1=no visible symptoms; 2=initial soft rot; 3=presence of mycelium; 4=sporulation) data were converted percentage midpoint values where 1=0%; 2=35%; 3=65%; 4=90%).

When oranges were inoculated with the pathogen after SBC treatment, green mold incidence was 40%, indicating that SBC treatment modestly protected the fruit from infection (treatment n°7: SBC treatment provided modest protection against infections in wounds made and inoculated with the pathogen after SBC treatment) (Fig. 38). When SBC treatment and subsequent fungal inoculation were followed by the application of biocontrol agent *Pss* (treatment n°8), decay incidence was reduced to 20%, showing that the treatment with the antagonist significantly compensated for the modest protective activity of SBC treatment. In this case, the addition of biocontrol agent to SBC-treated and *P. digitatum*-inoculated fruit significantly improved green mold control (Fig. 38).

The protective effect of SBC applied in sequence with biocontrol agents (*Pss*48SR2 and *W. anomalus* BS91) was also evaluated on oranges that were inoculated later with the pathogen (24 and 48 h, treatments 9-12). When oranges were inoculated with the pathogen 24h (treatment n°9) and 48 h (treatment n°11) after SBC-*Pss* treatments, green mold incidence was 0% (Fig. 39). The protective effect of SBC-biocontrol agent treatments on oranges that were inoculated later (24 and 48h) with the pathogen was also notable when antagonistic yeast *W. anomalus* BS 91 was used.

Protective activity

Treated SBC


ys

The following 6 treatments were applied on Tarocco oranges as preventive activity: **(7)** fruit were treated with sodium bicarbonate (SBC), wounded and inoculated 2 h later with *Penicillium digitatum* (P.d.); **(8)** fruit were treated with SBC, wounded and inoculated 2 h later with *P. digitatum,* and then treated 2 h later in same wound with *Pseudomonas syringae* pv. *syringae* PVCT48SR2 (Pss); **(9)** fruit were treated with SBC, wounded and treated 2 h later with *Pss*, and inoculated 24 h later with *P. digitatum;* **(10)** fruit were treated with SBC, wounded and treated 2 h later with *Wickerhamomices anomalus* BS91 (BS91), and inoculated 24 hours later with *P.d;* **(11)** fruit were treated with SBC, wounded and treated 2 h later with *Pss*, and inoculated 48 hours later with *P.d.*; **(12)** fruit were treated with SBC, wounded and treated 2 h later with BS91, and inoculated 48 hours later with *P.d.*

Protective activity

Treated SBC

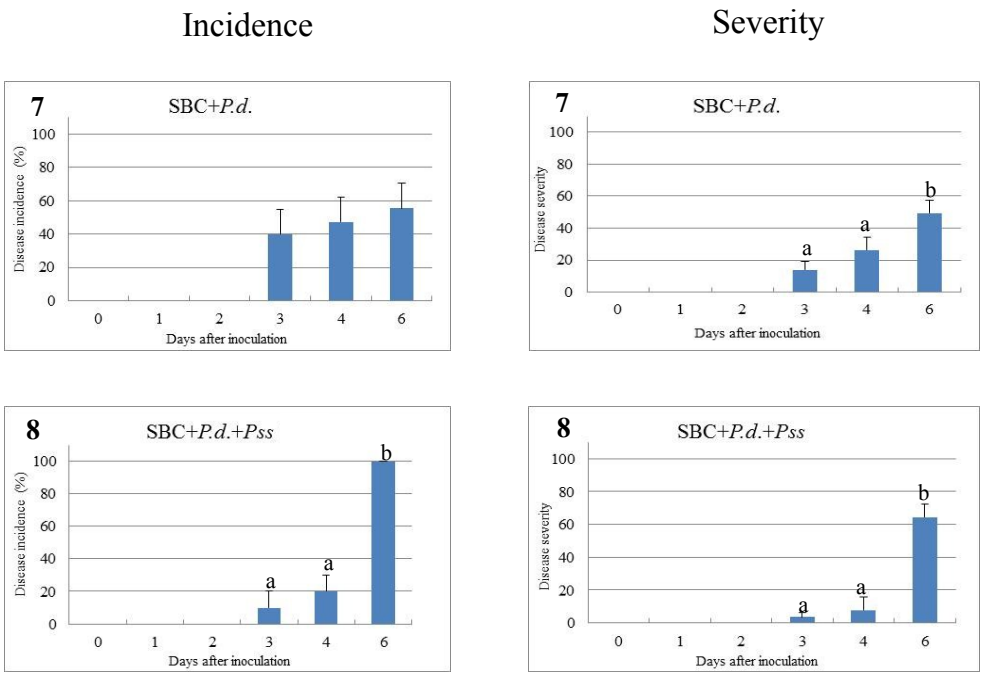


Figure 38. Disease incidence and severity on oranges cv. Tarocco treated with sodium bicarbonate (SBC) alone or in sequence with *P. digitatum* and *P.syringae* 48SR2 (Pss) to control green mold. (7) fruit were treated with SBC, wounded and inoculated 2 h later with *P. digitatum*; (8) fruit were treated with SBC, wounded and inoculated 2 h later with *P. digitatum*, and then treated 2 h later in same wound with Pss 48SR2. After every treatment, the fruit were incubated at 20°C for 5 d. Values are the mean of 3 experiments. Columns with the same letters are not significantly different according to the Student-Newman-Keuls' one way ANOVA test at P ≤0.05. Disease severity was evaluated with an empiric scale (1=no visible symptoms; 2=initial soft rot; 3=presence of mycelium; 4=sporulation) data were converted percentage midpoint values where 1=0%; 2=35%; 3=65%; 4=90%).

Protective activity

Treated with SBC

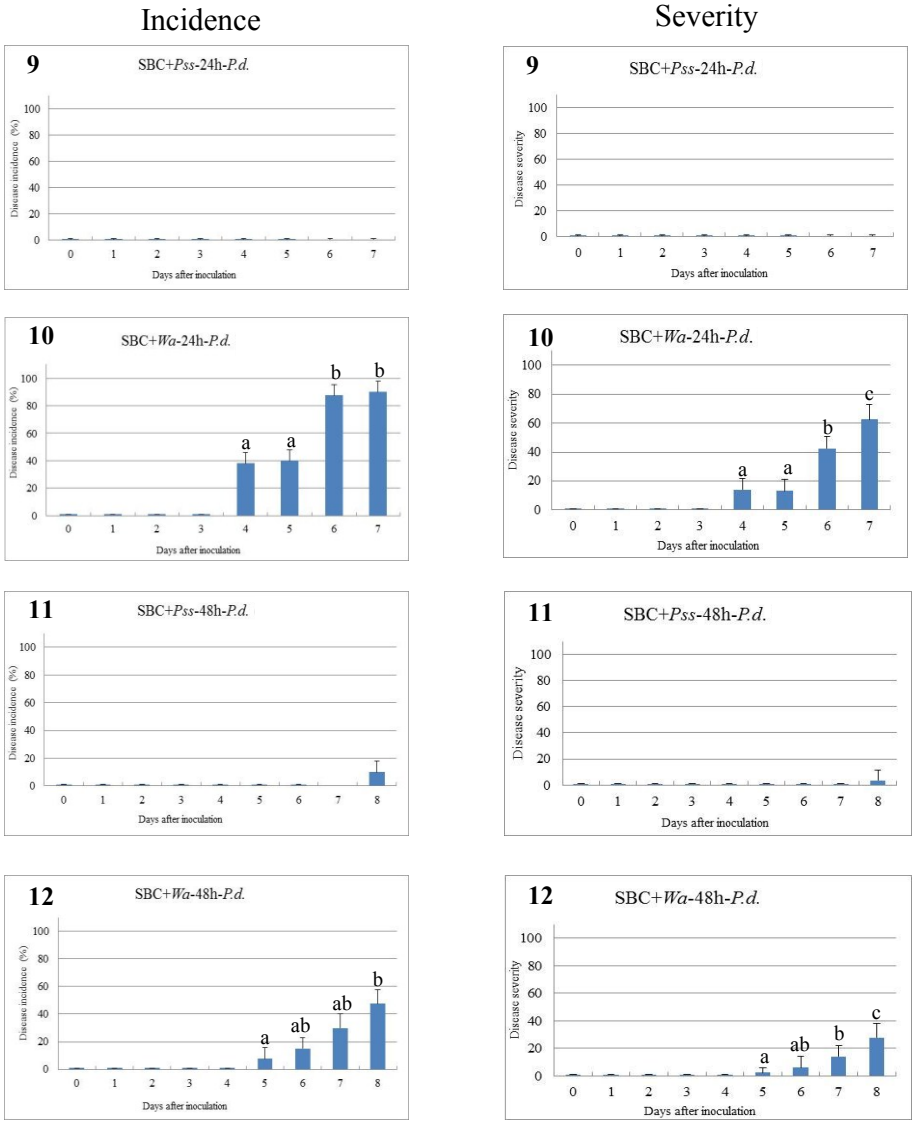


Figure 39. Disease incidence and severity on oranges cv. Tarocco treated with *P.syringae* 48SR2 (Pss), *W. anomalus* BS91 (Wa) and sodium bicarbonate (SBC) in sequence to control green mold. (9) fruit were treated with SBC, wounded and treated 2 h later with Pss 48SR2, and inoculated 24 h later with *P. digitatum*; (10) fruit were treated with SBC, wounded and treated 2 h later with *Wa* BS91, and inoculated 24 hours later with *P.d*; (11) fruit were treated with SBC, wounded and treated 2 h later with Pss 48SR2, and inoculated 48 hours later with *P.d*; (12) fruit were treated with SBC, wounded and treated 2 h later with *W.a*. BS91, and inoculated 48 hours later with *P.d*. After every treatment, the fruit were incubated at 20°C for 5 d. Values are the mean of 3 experiments. Columns with the same letters are not significantly different according to the Student-Newman-Keuls' one way ANOVA test at P ≤0.05. Disease severity was evaluated with an empiric scale (1=no visible symptoms; 2=initial soft rot; 3=presence of mycelium; 4=sporulation) data were converted percentage midpoint values where 1=0%; 2=35%; 3=65%; 4=90%).

RESULTS

4.3 Gene expression of defence-related genes and lipodepsipeptides synthetase genes in orange fruit in response to antagonist/elicitors–pathogen interaction using quantitative RT-PCR technique.

4.3.3 Quantitative RT-PCR of defence-related genes.

4.3.4 Quantitative RT-PCR of *syp*A and *syr*B1 genes.

4.3 Gene expression of defence-related genes and lipodepsipeptides synthetase genes in orange fruit in response to antagonist/elicitors–pathogen interaction using quantitative RT-PCR technique.

4.3.3. Quantitative RT-PCR of defence-related genes

To determine the amount of chitinase (CHI1), β-1,3-glucanase (GNS1), phenylalanine ammonia-lyase (PAL1) and phospholipid hydroperoxide gluthatione peroxidase (GPX1) transcripts involved in different resistance responses such as SAR, ROS and ISR, quantitative RT-PCR was applied. It was based on the specific transcription of the mRNA of each sample in cDNA followed by their amplification.

Gene transcript levels of CHI1, GNS1, PAL1 and GPX1 were measured for the following treatments: at 2h, 24h and 48 h after *P. digitatum* inoculation (treatment n°1, control), and at 2h, 24h and 48 h after treatments n° 4 (SBC+PSS 24 after *P. digitatum* inoculation), n°6 (SBC+Pss+P.d. 24h after peel wounding), n°11 (SBC+Pss 48h before *P. digitatum* inoculation), and n° 12 (SBC+BS91 48h before *P. digitatum*inoculation). All the examined treatments (n°4 with curative activity, and n° 6, 11, 12 with protective activity) showed the best results in terms of biocontrol of citrus green mold.

Real time PCR conditions were optimized for specific gene amplification so that melting curves profile for qRT-PCR analysis showed absolute specificity of each primer pair (CHI1, GNS1, PAL1, GPX) for its target sequence (Fig. 40).

CHI1 gene over-expression was always observed in citrus peel tissues when sampled at t=2h (Fig. 41). In non-inoculated wounded peel tissue chitinase gene was induced 4.4-fold more than in non-wounded peel tissue (Peel). In fruit tissues inoculated with *P. digitatum* (Treatment n° 1; t=2h), chitinase level increased 16-fold compared to the non-wounded peel tissue (Peel).

Such CHI1 gene over-expression significantly increased when the wounds were treated with biocontrol agents and sodium bicarbonate after *P. digitatum* inoculation (curative activity, treatment n°4) and before *P. digitatum* inoculation (protective activity, treatments n°6, 11, 12).

CHI1 gene expression in fruit exposed to treatment n° 4 showed a synergistic effect of SBC and *Pss* (89.4-fold) applied 24h after *P. digitatum* inoculation, but this response do not limit the infection (100% disease incidence 5 days after treatment) and no correlation was found with the lack effectiveness of the proposed curative activity of this treatment. The protective control method related to treatment n° 6 (wounds followed, 24h later, by SBC+ P.d.+ Pss) showed 20.16-fold CHI1 gene expression. This treatment controlled green mold disease with disease incidence values of 50% 5 days after treatment. The expression level of chitinase gene peaked (1351-fold) in citrus tissues exposed to protective treatment n° 11, corresponding to dipping treatment in SBC followed by inoculation with *Pss* and, 48h later, inoculation with the pathogen. CHI1 gene expression was high (57-fold) also after protective treatment n° 12, although with lower values compared to the treatment n°11, suggesting a weaker control activity of *W. anomalus* than *P. syringae* in presence of sodium bicarbonate. Both treatments (n°11 and 12) efficiently controlled disease incidence 5 days after treatment (disease incidence of 0% and 30%, respectively). On the opposite, no CHI1 gene over-expression was detected in tissue exposed to any treatment after both 24 and 48 hours.

There was no GNS1 gene expression in tissues exposed to any of treatments tested at any time of monitoring (Fig.42).

No PAL1 gene over-expression was observed in wounded tissues after 0 and 24h, whereas after 48h PAL1 gene was induced 8.5-fold more than no-wounded peel. There was no PAL1 gene expression in tissues inoculated with *P. digitatum* (treatment n°1) after 2, 24 and 48 h. Gene expression of PAL1 in fruit exposed to treatment n° 4 and n° 6 showed a synergistic effect of SBC and *Pss* (3.6-fold and 1.5-fold, respectively) at t=2h. Such over-expression induced by treatment n°4 and n° 6 expired after 24h, and again increased (4.0-fold and 2.4-fold, respectively) after 48 h. No PAL1 gene over-expression was observed in tissues exposed to treatments n° 11 and n° 12 at every time of analyses (2, 24 and 48 h) (Fig. 43).

Finally, the expression level of GPX gene peaked (10.5-fold) only in citrus tissue exposed to treatment n° 11 immediately after *P.d.* inoculation (t=2h), whereas no over-expression was detected after 24 and 48 h (Fig. 44).

Melting curve profiles of real time RT-PCR analysis

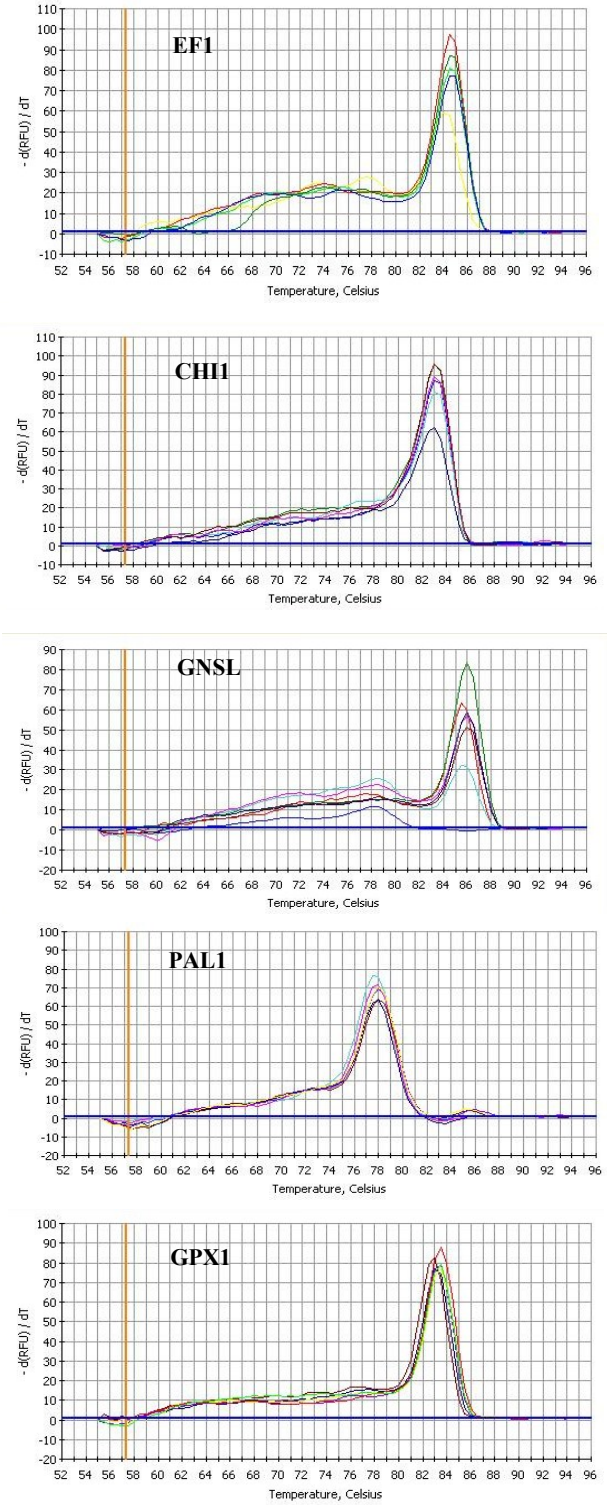
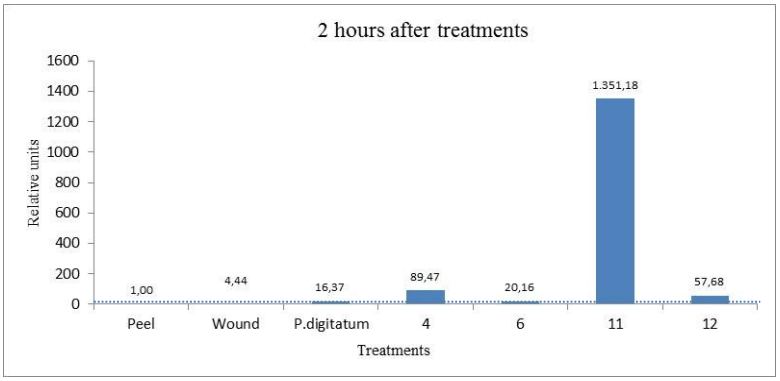
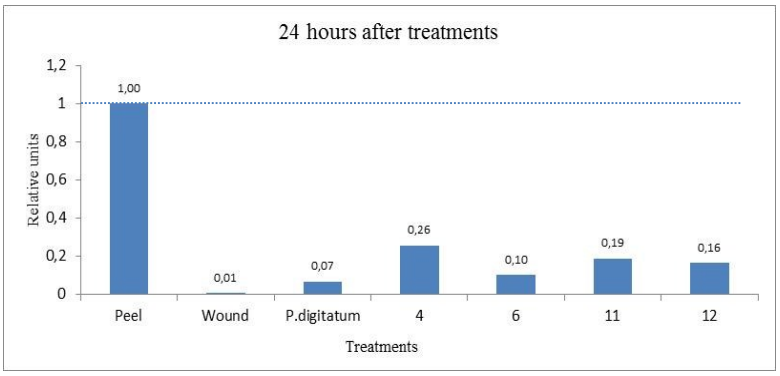


Figure 40. Melting curve profiles of real time RT-PCR analysis for the following genes: chitinase (CHI1), β-1,3-glucanase (GNS1), phenylalanine ammonia-lyase (PAL1), phospholipid hydroperoxide gluthatione peroxidase (GPX1), elongation factor 1-alpha (EF1). Each curve shows absolute specificity of each primer pair for its target sequence by a single peak.

Chitinase (CHI1) gene expression





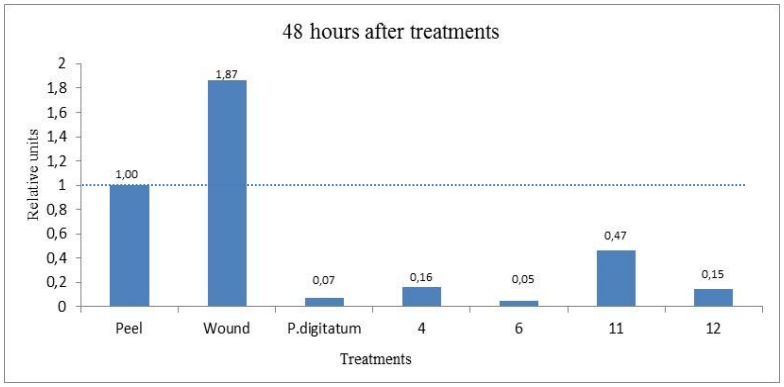


Figure 41. Q-retro transcriptase PCR analysis of chitinase gene involved in defence systems in non-wounded citrus peel (Peel); wounded citrus peel (Wound); wounded and inoculated with P. digitatum (*P. digitatum*); wounded, inoculated with *P. d.* and treated 24 h later with SBC+ Pss (4); wounded, treated 24 h later with SBC+Pss and inoculated with *P. d* (6); soaked in SBC, treated with *Pss* and after 48 hours inoculated with *P.d*. (11); soaked in SBC solution, treated with *W.a*. BS91 and after 48 hours inoculated with *P.d* (12). Tissue samples were collected 2, 24 and 48h after treatments. The ΔCt value of the control sample (peel time 2) was used as calibrator and fold activation was calculated by the expression: $2^{-\Delta\,\Delta Ct}$, where Δ(ΔCt) = ΔCt sample – ΔCt control. We have reported the data as relative units.

β-1,3-Glucanase (GNS1) gene expression

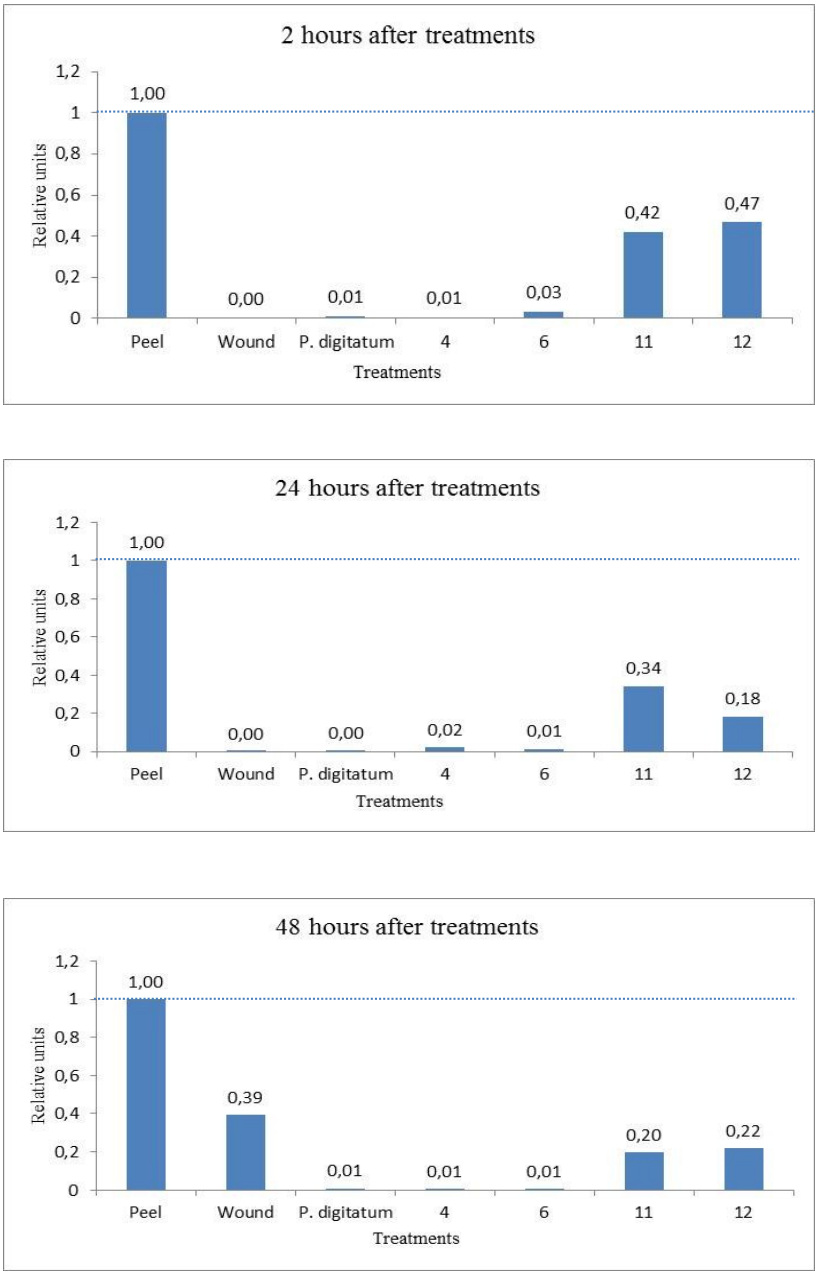


Figure 42. Q-retro transcriptase PCR analysis of β-1,3-Glucanase gene involved in defence systems in non-wounded citrus peel (Peel); wounded citrus peel (Wound); wounded and inoculated with *P. digitatum* (*P. digitatum*); wounded, inoculated with *P. d*. and treated 24 h later with SBC+ Pss (4); wounded, treated 24 h later with SBC+Pss and inoculated with *P. d* (6); soaked in SBC, treated with *Pss* and after 48 hours inoculated with *P.d*. (11); soaked in SBC solution, treated with *W.a*. BS91 and after 48 hours inoculated with *P.d* (12). Tissue samples were collected 2, 24 and 48h after treatments. The ΔCt value of the control sample (peel time 2) was used as calibrator and fold activation was calculated by the expression: $2^{-\Delta\Delta Ct}$, where Δ(ΔCt) = ΔCt sample – ΔCt control. We have reported the data as relative units.

Phenylalanine Ammonia-Lyase (PAL1) gene expression

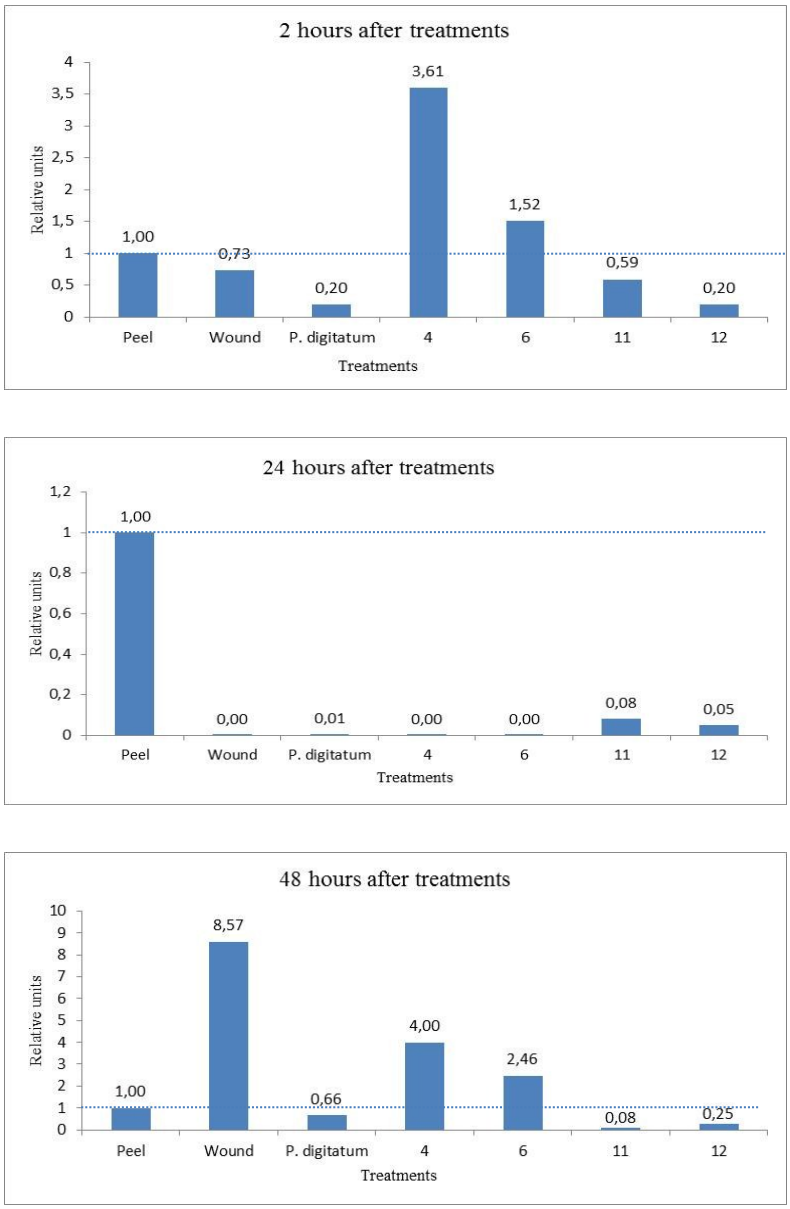
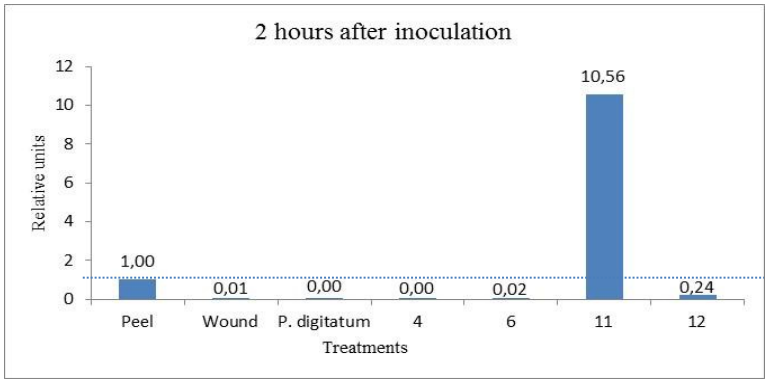
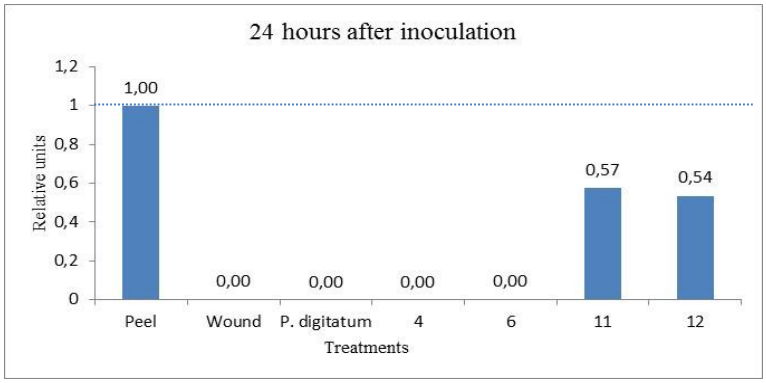


Figure 43. Q-retro transcriptase PCR analysis of Phenylalanine Ammonia-Lyase gene involved in defence systems in non-wounded citrus peel (Peel); wounded citrus peel (Wound); wounded and inoculated with *P. digitatum* (*P. digitatum*); wounded, inoculated with *P. d.* and treated 24 h later with SBC+ Pss (4); wounded, treated 24 h later with SBC+Pss and inoculated with *P. d* (6); soaked in SBC, treated with *Pss* and after 48 hours inoculated with *P.d*. (11); soaked in SBC solution, treated with *W.a*. BS91 and after 48 hours inoculated with *P.d* (12). Tissue samples were collected 2, 24 and 48h after treatments. The ΔCt value of the control sample (peel time 2) was used as calibrator and fold activation was calculated by the expression: $2^{-\Delta\Delta Ct}$, where Δ(ΔCt) = ΔCt sample – ΔCt control. We have reported the data as relative units.

Phospholipid hydroperoxide gluthatione peroxidase (GPX1) gene expression





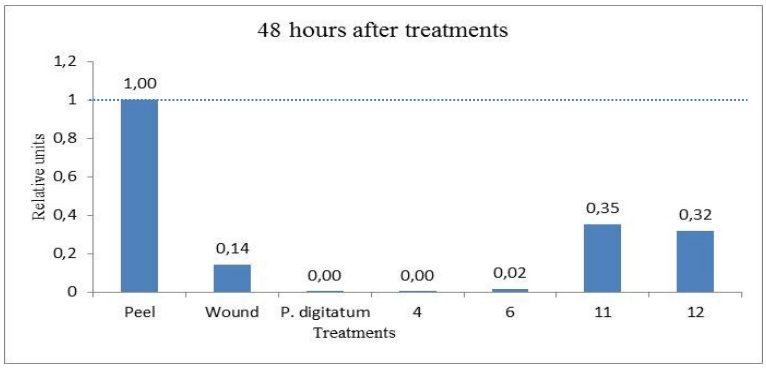


Figure 44. Q-retro transcriptase PCR analysis of Phospholipid hydroperoxide gluthatione peroxidase gene involved in defence systems in non-wounded citrus peel (Peel); wounded citrus peel (Wound); wounded and inoculated with *P. digitatum* (*P. digitatum*); wounded, inoculated with *P. d.* and treated 24 h later with SBC+ Pss (4); wounded, treated 24 h later with SBC+Pss and inoculated with *P. d* (6); soaked in SBC, treated with *Pss* and after 48 hours inoculated with *P.d.* (11); soaked in SBC solution, treated with *W.a.* BS91 and after 48 hours inoculated with *P.d* (12). Tissue samples were collected 2, 24 and 48h after treatments. The ΔCt value of the control sample (peel time 2) was used as calibrator and fold activation was calculated by the expression: $2^{-\Delta\Delta Ct}$, where Δ(ΔCt) = ΔCt sample – ΔCt control. We have reported the data as relative units.

4.3.4. Quantitative RT-PCR of *syp*A and *syr*B1 genes

To determine the amount of *syp*A and *syr*B1 transcript levels of *Pss* strain 48SR2 in citrus peel tissues treated with the antagonistic strain in mixture with SBC (treatment n°11), and their possible involvement in the biocontrol activity of this treatment, quantitative RT-PCR of bacterial RNAs was applied. It was based on the specific transcription of the mRNA of each sample in cDNA, followed by their amplification. Moreover, the treatment n°11 showed the highest over-expression of the defence-related genes analysed, as previously described.

The absolute specificity of each primer pair (Tab. 2) for their target sequences (*syr*B1, *syp*A, 16S) was demonstrated by the exclusive amplification of each template (Fig. 45), confirming that only specific product was amplified because only one specific peak was observed. Furthermore, no negative control samples included in this assays generated any amplification product.

The *syr*B1 and *syp*A transcript levels in citrus peel tissues exposed to treatment n° 11 were compared to *syr*B1and *syp*A transcript levels in citrus peel tissues treated with *Pss* alone. Samples were analysed at 2, 24 and 48 h after *Pss*+SBC treatment (before *P.digitatum* inoculation) and at 2, 24 and 48h after *P. digitatum* inoculation (Fig. 46-47).

The *syr*B1 gene transcript level in citrus peel tissue treated with SBC and Pss 48SR2 strain in absence of *P. digitatum* was no over-expressed after 2 and 24 h after their fruit inoculation, while a weak over-expression (1.6-fold) was observed only after 48h (Fig. 46). Similarly, *syp*A transcript level showed over-expression only after 48h (19-fold) whereas no over-expression was observed at 2 and 24 h from *Pss* inoculation (Fig. 46).

The fruit exposed to treatment n° 11 showed an over-expression of *syr*B1 gene 40.7-fold immediately (t=2h) after pathogen inoculation; such *syr*B1 transcript level decreased after 24 h (4.1-fold) and then showed no over-expression at 48 h (Fig. 47). The expression level of *syp*A was 1.8-fold after pathogen inoculation (t=2h) and peaked at 4.1-fold after 24 h. It decreased drastically after 48 h showing no over-expression (Fig. 47).

Melting curve profiles of real time RT-PCR analysis

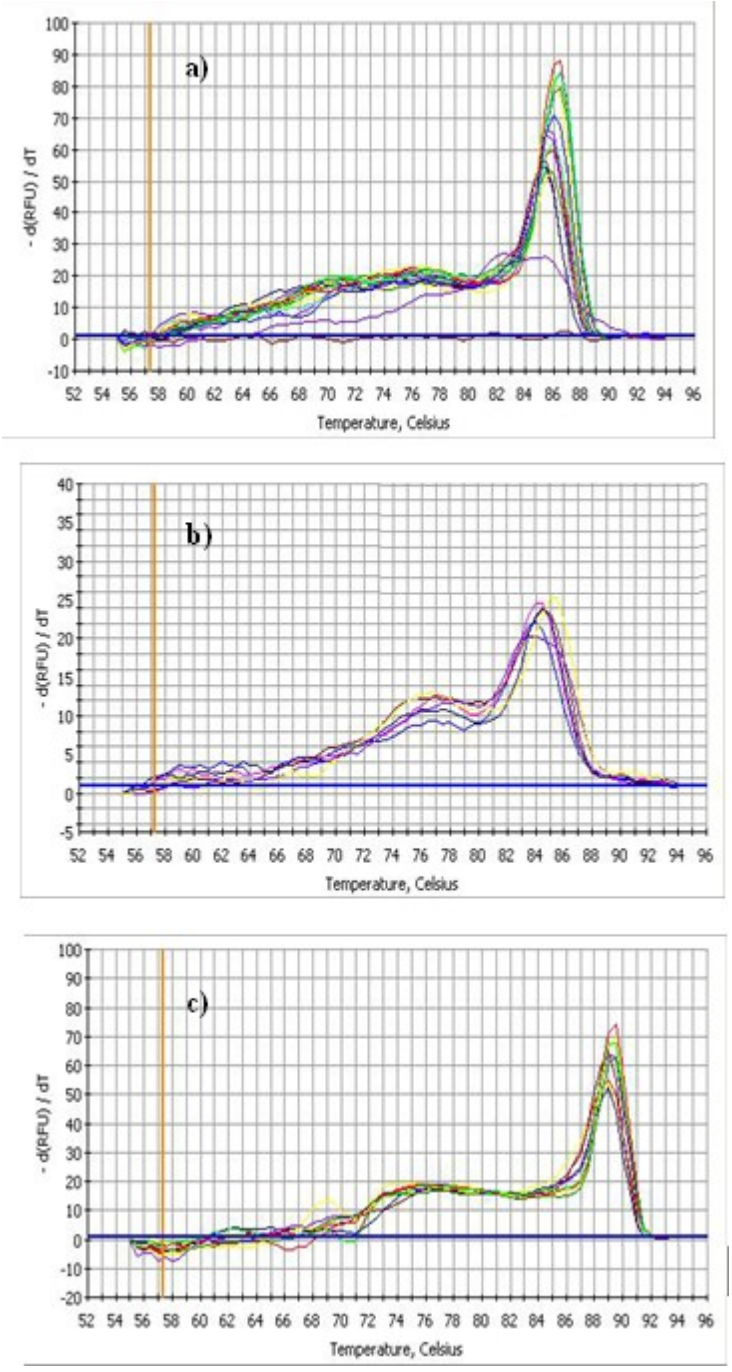


Fig. 45. Melting curve profiles of real time RT-PCR analysis for the following genes: **a**) syringopeptin synthetase gene (*syp*A); **b**) syringomycin synthetase gene (*syr*B1); **c**) 16S housekeeping gene. Each curve shows absolute specificity of each primer pair for its target sequence by a single peak.

In vivo syrB1 and _sypA_ gene expression at 2, 24, and 48 h after _SBC+Pss_ treatment (before _P. digitatum_ inoculation)

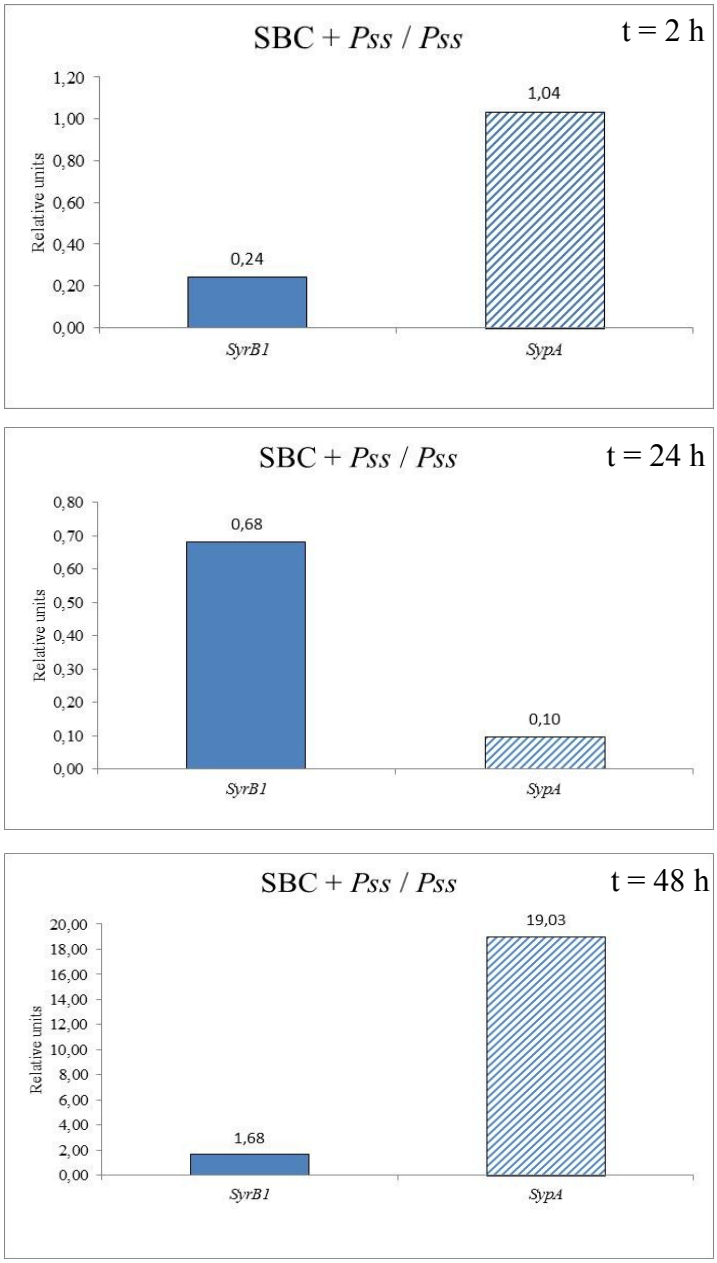


Figure 46. Expression levels of *syr*B1 and *syp*A genes were evaluated in citrus peel tissues exposed to treatment n° 11 compared to citrus peel inoculated with biocontrol agent *Pss* PVCT48SR2 strain.

In vivo syrB1 and _sypA_ gene expression at 2, 24, and 48 h after _SBC+Pss_ treatment (after _P. digitatum_ inoculation)

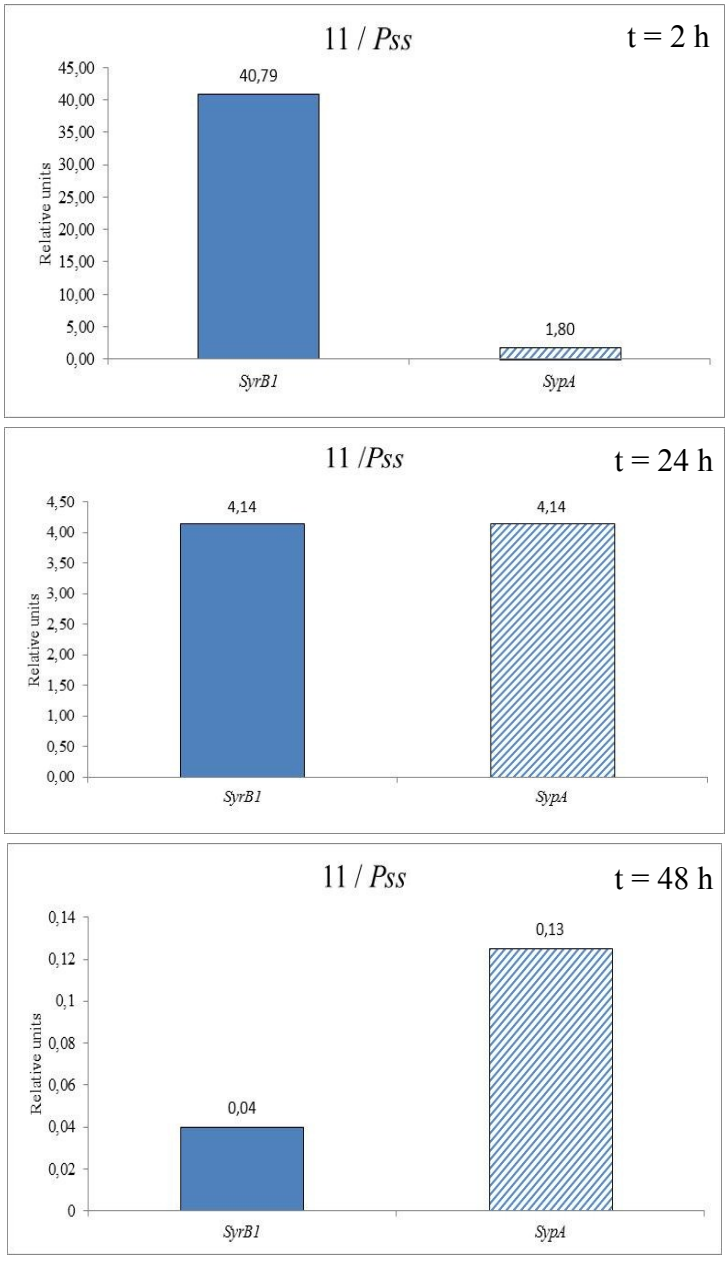


Figure 47. Expression levels of *syr*B1 and *syp*A genes were evaluated in citrus peel tissues exposed to treatment n° 11 compared to citrus peel inoculated with biocontrol agent Pss PVCT48SR2 strain.

Chapter 5.

CONCLUSION

Objective 1. Direct antifungal activities of elicitors and BCAs in controlling post-harvest green mold *in vitro* and on citrus fruit.

Injuries sustained by citrus fruit during harvest allow the entry of wound pathogens, including *Penicillium digitatum* Sacc (green mold) and *P. italicum* Wehmer (blue mold). These pathogens occur in almost all regions of the world where citrus is grown, and cause serious postharvest losses annually (Korsten *et al*., 2000; Palou *et al*., 2000). Synthetic fungicides have been the main method of control of citrus postharvest diseases including green and blue molds (Eckert, 1990). There is, however, a growing international concern over the often indiscriminate use of synthetic fungicides on food crops because of presence of residues in the food and in the environment, as well as the development of fungal resistance and the possible harmful effects on human health (Norman, 1988).

Over the last 20 years a large number of studies focused on the biological control of postharvest diseases. Recently, several elicitors of induced disease resistance have been studied and evaluated, and some of them have been shown to be effective to augment the biocontrol activity of antagonistic microorganisms against postharvest pathogens. Moreover, recent studies have demonstrated that some chemical inducers of resistance, commonly known for their lack of antimicrobial activity, can directly inhibit mycelial growth of fungal pathogens. ASM and BABA have been shown to inhibit *B. cinerea* growth *in vitro* at concentrations of 1 mM and 2.5 mM, respectively (Fischer *et al*., 2009; Munoz and Moret, 2010). BABA has also been shown to reduce germination and germ-tube elongation of *P. digitatum* at concentrations ranging from 1 to 100mM (Porat *et al*., 2003).

In the present study ASM, BABA and chitosan showed a different direct antimicrobial activity towards *P. digitatum*, *P. italicum* and *G. candidum* with some differences according to the pathogen and the agar media on which the antimicrobial activity was tested. Our results demonstrated that ASM exert direct antimicrobial activity *in vitro* and that this direct antimicrobial activity is more pronounced at the highest concentrations particularly against *P. digitatum,* whereas *P. italicum* and *G. candidum* mycelial growth was less affected and only at the highest concentrations. These results were similar to those observed by Eikemo *et al*. (2003) against *Phytophthora fragariae* var. *fragariae*, and by Cao *et al*., (2005) against *P. expansum.*

The antimicrobial activity of ASM against *P. digitatum* on PDA and on orange peel extract was not confirmed by *P. digitatum* conidial germination test: conidial germination was indeed unaffected at all the tested ASM concentrations.

ASM has been used as pre-harvest treatment for the protection of several fruits from postharvest diseases (Huang *et al*., 2000; Terry and Joyce, 2000; Willingham *et al*., 2002). In recent times, this chemical compound has also been applied effectively as a postharvest treatment to suppress the blue mould caused by *P. expansum* in peach (Liu *et al.,* 2005) and pear (Cao *et al.,* 2005), *Trichotecium roseum* in muskmelons (Wang *et al*., 2008) and the anthracnose rot caused by *Colletotrichum gloesporioides* in mango fruit (Zhu *et al.,* 2008). In our tests, none

of the ASM concentrations conferred protection against green mold when ASM was applied directly on wounded citrus fruit, thus demonstrating that ASM does not exert direct antimicrobial activity *in vivo* against *P. digitatum* when applied on fruit also at the highest concentrations.

Previuos works demonstrated that inhibitory effect of ASM on the postharvest disease is associated with its ability to enhance activities of defense enzymes in the fruit (Liu *et al.* 2005; Cao *et al.* 2005; Zhu *et al*., 2008). Treatments with ASM in orange fruit were able to increase accumulation of pathogenesis-related (PR) proteins, such as PR-2 and PR-3, but these defence responses were not effective for containing the development of disease caused by *P. digitatum.* (Panebianco, 2010).

In this study direct antifungal activity of chitosan was confirmed by *in vitro* tests. Mycelial growth of all tested fungi was significantly reduced. The antimicrobial activity against *P. digitatum* on PDA and on orange peel extract was confirmed by *P. digitatum* conidial germination test, in which germination of *P. digitatum* spores totally inhibited by all concentrations of chitosan. High control of green mould was also achieved *in vivo* when oranges, lemons and grapefruits were treated with chitosan at the lower concentrations. The most effective disease control occurred when chitosan was applied at 0.5 mg/ml on oranges cv. Washington Navel and at 0.2 and 0.5 mg/ml on grapefruits.

Chitosan and its derivated have been demonstrated to be able to inhibit the growth of several fungi, including *Penicillium* species (El-Ghouth *et al*., 2000; Yu *et al.*, 2007; Chien *et al*., 2007) and to induce host defence responses such as accumulation of the chitinases, β-1,3-glucanases and phenolic compounds, induction of lignification, synthesis of phytoalexins and inhibition of host tissue maceration enzymes, which help the host to be more resistant to subsequent pathogens attack (Fajardo *et al*., 1998; Reddy *et al*., 2000; Bautista-Baňos *et al*., 2006; Romanazzi, 2010).

In previous studies, BABA was reported ineffective in reducing spore germination and germ-tube elongation when tested against *Phytophthora infestans*, *P. capsici*, *Peronospora tabacina*, *Plasmopara helianthi* and *Alternaria alternata* (Cohen, 1994; Cohen *et al.*, 1994; Sunwoo *et al*., 1996; Tosi *et al*., 1998; Reuveni *et al.,*

2003). In those studies, however, the maximum BABA concentrations tested were 10-20 mM. More recent studies have instead demonstrated that BABA can act as toxic compound when employed at higher concentrations. Increasing concentrations of BABA increasingly reduced *P. digitatum* spore germination and germ-tube elongation, that were totally inhibited at concentrations of 100 and 1000 mM (Porat *et al*., 2003). Similarly, application of BABA at concentrations ranged from 50 to 200 mM significantly reduced *P. italicum* spore germination and growth *in vitro,* with a peak of efficacy at 200 mM (Tavallali *et al*., 2008). Also in the present study BABA significantly reduced the *in vitro* growth of *P. digitatum* and *P. italicum*, exhibiting highest antifungal activity at concentrations of 10 and 100 mM, whereas *G. candidum* was much less inhibited, especially on orange peel extract agar. Toxic effects of high BABA concentrations were confirmed by *P. digitatum* conidial germination tests.

Recently, Fisher *et al*. (2009) showed that direct antifungal effect of BABA was also dependent on the pH of the media, with enhanced fungicidal action at acid pH. In their experiments 100% inhibition of *Botrytis cinerea* was obtained with BABA at concentrations of 2 and 4 mM on PDA pH 4 and only 40% at pH 7. In our experiments a significant reduction of mycelial growth was obtained with higher concentrations (10 and 100 mM of BABA), and the efficiency of the growth reduction was only partially dependent on the pH of the substrates, since the experiments were carried out on PDA pH 7 and on orange peel extract media pH 5.

Postharvest applications of BABA have been previously reported effective in reducing disease incidence and severity in apple fruit against *A. alternata* (Reuveni *et al.*, 2003), in grapefruit against *P. digitatum* (Porat *et al*., 2003) and in sweet orange fruit against *P. italicum* (Tavallali *et al.*, 2008). In the present study the direct antifungal activity of BABA was confirmed on citrus assays. The greatest reduction of disease incidence and severity occurred when BABA was applied at the highest concentrations (10, 100, 1000 mM) on oranges cvs. Tarocco and cv. Valencia and on grapefruits, whereas BABA was less effective or totally ineffective on lemon and cv. Washington Navel, respectively.

In a previous study (Porat *et al*., 2003), high concentrations of BABA (200 mM) were less effective than low concentrations (20 mM) in the control of *P. digitatum* infections on grapefruit. Authors suggested as possible explanation that only a small portion of the compound is absorbed by the citrus tissue thus being active in inducing pathogen resistance. In the present study, on the opposite, different concentrations of BABA (10, 100 and 1000 mM) were equally effective and no significant differences were observed in the efficacy of the infection control.

Moreover, results of the present study showed that in lemon and grapefruit, but not on oranges, BABA was effective even when it was applied at a distance of 1 cm from *P. digitatum*-inoculated sites, thus confirming that, in addition to direct antifungal activity, the mode of action of BABA may involve activation of defense mechanisms, as reported in grapefruit by Porat *et al.* (2003).

In conclusion, these results suggests that use of BABA and chitosan at suitable concentrations should be considered an interesting approach to control citrus green mould. However, further extensive postharvest storage tests are needed to determine the feasibility of their application on a commercial scale.

Post-harvest biological control using antagonistic yeasts has been reported and has been considered one of the possible alternative strategies to synthetic fungicides (Geng *et al*., 2011). Data obtained from the present study showed that *W. anomalus* isolates exerted various levels of antifungal activity toward the tested pathogens. When assayed *in vitro* at pH 4.5, *W. anomalus* caused abnormal morphological changes and collapses in *P. digitatum* hyphae, as previously reported by Wisniewski *et al,* (1991) for *Pichia guillermondii*. The effect of *W. anomalus* on hyphae is crucial in terms of plant pathogenesis, as the loss of rigidity leads to unsuccessful penetration into plant cell wall (Mendgen *et al*., 1996).

In vivo tests also demonstrated that *W. anomalus* strains significantly reduced disease incidence and severity of orange fruits, and these results are in accordance with those obtained by Izgu *et al*, (2011) on lemon fruit. However, the ability to grow and survive in different and stressful environments (Walker, 2011) and the high level of efficacy evidenced in this study, lead us to affirm that β-glucanase

activity of *W. anomalus* could be more cheaply exploited without any expensive concentration and purification steps.

Furthermore, as the killer activity was evidenced *in vitro* at pH 4.5 and it is well known that surface pH of the injured or wounded citrus fruit has low values (around pH 5), this could be of particular importance in providing protection towards pathogens under acidic conditions (pH 3.5-5). It is well known, in fact, that changes in pH value of citrus fruit reduce the effectiveness of traditional fungicides (Lopez-Garcia *et al*., 2003).

The study on efficacy and mechanisms of action of new antifungal compounds is always desirable, because the access to several biocontrol yeast species offers the possibility of taking advantage of their different biological properties or just to avoid resistance phenomena, thus making biological control more effective.

The results of the present study highlight the potential application of killer yeast *W. anomalus* as biological agent for postharvest protection of Tarocco orange fruit against *P. digitatum*, both to increase the storage time of the fruit and to reduce the economic losses due to green mould decay.

Antifungal proteins have drawn attention for their potential application in plant disease control. In relation to both ecological and human health concerns, the selectivity of *W. anomalus* glucanases towards β-1,3-glucanase, which are not present in the structure of mammalian cells, together with the QPS status recently obtained by EFSA for this species, make killer toxin of this species an attractive candidate for future antifungal compound in fresh citrus protection.

Objective 2. Curative and preventive activity of biocontrol agents and elicitors in different combined treatments on orange fruit.

Biological control is becoming an effective alternative to the use of chemicals in plant disease control (Conway *et al*., 1999; El-Ghaouth *et al*., 2000; Korsten *et al*., 2000). Despite the effort that have focused on the development of microbial biocontrol agents, their widespread utility as a postharvest treatment has not been fully realized.

The level of control offered by microbial antagonists is often lower that offered by commercial fungicides currently in use. Since a levels of control comparable to that provided by fungicides is difficult to achieve with biological control systems, the use of an integrated rather than a single approach is advocated (Pusey, 1994).

Recent studies have indicated that integration of two or more alternative treatments might be a strategy to provide satisfactory control of postharvest diseases. Janisiewicz *et al*, (1992) reported the enhacement of *P. syringae* biocontrol activity of blue mold on apples by the addition of nitrogenous compounds. Wisniewski *et al*, (1995) achieved the enhancement of *C. oleophila* by the addition of calcium salts. El-Ghaouth *et al*, (2000) showed that the combination of *C. saitoana* and 0.2% glycolchitosan offered better control of decay of citrus and apple fruit than *C. saitoana* and glycolchitosan alone, thus conferming that glycochitosan is able to enhance the biocontrol activity of the antagonist. Nunes *et al*, (2002). achieved the enhancement of *C. sake* biocontrol activity on pear fruit by the addition of ammonium molybdate.

The present study evaluated several possible combinations *of P. syringae* 48SR2, 40SR4 and 46P, *T. atroviride* P1, *W. anomalus* SB91, ASM, chitosan, HW, SBS treatments in three orange cultivars, and in lemon and grapefruits, artificially inoculated with *P. digitaum* before (curative) or after (protective) the treatments, and incubated at 20°C or at 0°C.

1) In this work, we complemented the biocontrol activity of the antagonist *P. syringae* 48SR2 by applying it together with HW and SBC treatments.

P. syringae is one of the bacteria which is actually studied as a biological control agent on numerous cultivated plants in pre and post-harvest. Strains of *P. syringae* have been reported to be effective as biocontrol agents against *P. digitatum* on citrus (Bull *et al*., 1997; Cirvilleri *et al*., 2005), *P. expansum* and *B. cinerea* on pears and apples (Janisiewicz and Jeffers, 1997; Janisiewicz and Marchi, 1992; Sugar and Spotts, 1999; Zhou *et al*., 2001; Zhou *et al*., 2002), *V. inequalis* on apples (Zhou and DeYoung, 1996), *E. coli* 0157:H7 on apple (Janisiewicz *et al*., 1999), *M. fructicola* and *R. stolonifer* on peaches (Zhou *et al*., 1999), and as agent of induced resistance against *Plasmopara viticola* and *Uncinala necator* on grape (Kassemeyer *et al*., 1998). The beneficial effects of hot water treatments for

control of *Penicillium* rots in citrus have been reported (Porat *et al*., 2000), as well as the ability to inhibit spore germination and subsequent growth of *P. digitatum*. Carbonic acid salts are common food additives allowed with no restrictions for many applications by European and North American regulation. Treatments with sodium carbonate and sodium bicarbonate provided satisfactory control of green mould on lemons (Smilanick *et al*., 1995) and oranges (Smilanick *et al*., 1997; Palou *et al*., 2001). Recently, Palou *et al*, (2001) reported a reduction of up to 90% in the incidence of both green and blue molds after treating artificially inoculated fruit with different concentrations of SBC.

In this work, for the first time, *P. syringae* 48SR2 isolate was evaluated in combination with HW and SC to improve its efficacy as curative treatment. The temperature here investigated did not give control of the disease. Similarly, antagonist *P. syringae* 48SR2, applied 2h after *P. digitatum* inoculation, showed no curative activity. On the opposite, 3% SC treatments showed curative activity (40% disease incidence). The increased antagonistic activity observed when 48SR2 was applied following hot water treatment (20% disease incidence) or after heated SC treatments (30% disease incidence) may have been the result of the antagonist taking advantage of the disruption in the pathogen development to gain a competitive advantage over the pathogen at the wound site. The level of control (curative activity) conferred by the combination of *P. syringae* and heated 3% appears to be due to additive interactions between the bacteria, HW and SC. However, carbonate residues in inoculated wounds on the fruit could be tolerated by the bacterial antagonist, but non-tolerated by *P. digitatum.* The present strategy is compatible with already existing citrus packhouse practices and should be easy to adopt.

2) In this study, protective activity showed a good control of disease incidence and severity when fruit were wounded, 24 and 72hours before *P. syringae* inoculation; this could be related to the colonization of wounds and activation of pathogenesis signalling. When antagonistic *P. syringae* strains 40SR2, 48SR4 and 46P were applied 3 days before *P. digitatum* inoculation, they significantly reduced disease incidence and severity thus showing a good protective activity. All three *P. syringae* isolates on their own reduced significantly the incidence of

both green molds on artificially inoculated oranges cv. Tarocco, and isolates 40SR4 and 48SR2 controlled disease better than 46P.

ASM as dip-treatment of wounded oranges weakly controlled green mold (40-60% disease incidence), but the control was in any case higher compared to the ASM dip-treatment of unwounded fruit. The addition of ASM to the antagonist suspension didn't improve the biocontrol activity of isolates 40SR4 and 48SR2, whereas improved biocontrol activity of 46P.

In conclusion, appropriate combinations of biocontrol agents and physical or chemical elicitors resulted in a synergistic inhibition of the green mold in citrus fruit, offering high protection. Hot water, sodium carbonate and acibenzolar-S-methyl in combination with antagonists resulted promising for further large-scale evaluation in packinghouses. Combined application of treatments, SBC in heated water, biocontrol agents applied as preventive activity 24 or 72 hours before the pathogen attack may provide a reliable solution for control of post-harvest green mold of citrus fruit.

3) Generally, our results indicated that when *P. syringae* and *T. atroviride* on their own and the combined treatments provided an additive effectiveness in the control of green old in comparison with applying the treatments separately. A possible explanation for these results is that the mode of action of both treatments may have complemented each other

Our results also showed that when fruit were incubated for 20 days at 6°C, the additional effect to control *P. digitatum* provided by the combined treatments was not detected. The low efficacy of 48SR2 and P1 when fruit were incubated at 6°C could be explained because these antagonists grew poorly at 6°C. Although the mode of action of 48SR2 and P1 under our conditions is not known, at 6°C their physiological activity are delayed affecting all potential modes of action described for *P. syringae*, including nutrient competition and production of antibiotics (syringomycin).

In conclusion, our results showed that curative effect was confirmed at both low (6°C) and room (20°C) temperature of storage. However, when fruit were incubated for 20 days at 6°C, although treatments were still effective, the antagonists generally did not control disease development.

The mode of action of *P. syringae* 48SR2 against *Penicillium* spp. in citrus fruit can explain the limited curative activity of the antagonist when applied alone. The inhibitory effect due to competition for nutrients and space is considerably lower when the bacterium is applied after the pathogen is already established and actively growing within the infection site in the fruit peel. Because of this disadvantage in the competition, *P. syringae* 48SR2 and many other antagonistic microbes, mainly yeasts, which mode of action is not parasitism, are poor eradicants of pathogens infecting citrus fruit in the field or in rind wounds inflicted during or just after harvest (Droby *et al*., 1998; Spadaro and Gullino, 2004). We found that dips in SBC heated aqueous solutions and treatments with the biocontrol agent *P. syringae* 48SR2 didn't control green mold on citrus fruit that had been previously inoculated with the pathogen and that the combination of treatments was ineffective as either treatment alone.

The ability to control incipient infections by the integration of antagonistic B. subtilis or P. agglomerans strains with heated carbonate solutions has been reported, and these integrated treatmenst showed synergistic or additive inhibitory effects (Obagwu and Korsten, 2003; Teixido´ *et al*. , 2001; Torres *et al*. , 2007; Usall *et al*., 2008). On the opposite, in our case treatment of previously inoculated oranges with heated SBC followed by the application of P. syringae was totally ineffective, thus suggesting lack of curative action of combined treatments of SBC and *P. syringae* against citrus green molds. Moreover, in this work we found that 3% SBC dip treatments at 45 ◦C effectively protected pre-existing wounds from fungal infections that was inoculated about 2 h after SBC treatment (disease incidence lower than 10%). The protective activity of the treatment with SBC was not improved when it was followed by the application of *P. syringae* (green mold incidence of 18%).

This results were in agreement with those reported by Usall *et al*, (2008), in which the Authors discussed about unexpected results regarding the persistent effect of carbonate salts that, like other low-toxicity food additives or GRAS compounds, are believed to be more fungistatic than fungicidal and not very persistent.

It could be possible to suppose that the *in vivo* inhibitory effect of these compounds in infected citrus fruit is due to the unfavorable environmental conditions for fungal development that occur within the wound as a consequence of the presence of salt residues and of tissue reactions as a consequence of wounds and pathogen inoculation. This hypothesis could be confirmed by the tests we report hereafter in which chitinase activity, as defence reaction, was highly expressed in tissues that were wounded and, only after 24h, immersed in SBC solution plus *P. digitatum* inoculation.

In the experiments where pathogen inoculation followed after 2 h the application of the combined treatments (SBC + *P. syringae*), lack in preventive activity was observed (disease incidence of 60 and 100%). On the opposite, in the experiments where the period of time between combined treatments and fungal inoculation was 24 or 48 h, green mold incidence was significantly lower (0% disease incidence in combined treatments with SBC+*P. syringae*; 80-30% disease incidence in combined treatments with SBC+*W.anomalus*).

It can be concluded that the integration of carbonate treatments with *P. syringae* consistently provided good protective activity in pre-existing wounds, and that green mold reduction was lower in wounds that were inoculated with the fungus after the application of the treatments.

There are reports of improved performance of yeasts used as biocontrol agents when combined with calcium salts (Wisniewski *et al*., 1995; Droby *et al*., 1997; El-Ghaouth *et al*., 2000). No research has been reported to determine the compatibility of *W. anomalus* with SBC, and the possible improvement in biocontrol activity that might result from such integration, particularly in a post-inoculation treatment of citrus green and blue molds. This study represents the first evidence that *Wickerhamomyces anomalus* can reduce the incidence of disease caused by *P. digitatum* in citrus fruit, and the biocontrol efficiency cannot be enhanced by the addition of 3% SBC because *W. anomalus* as demonstrated before, grows in acid condition.

Fungi belonging to genus *Trichoderma* are the most effective BCAs and they have several mechanisms of action, including production a wide range of antibiotic substances, parasitism and competition with phytopathogens for nutrients and/or

space (Harman *et al*., 2004). Various strains of *T. harzianum* have been effective also against diseases caused by postharvest pathogens (Dubois, 1984; Elad *et al*., 1993; Sivakumar *et al*., 2000), and *T. viride* had shown to be effective in reducing the percentage of wounds infected by *P. digitatum* on orange Navaline (Dìaz and Vila, 1990). As observed by Dìaz and Vila (1990), the effectiveness of *Trichoderma* increased with the time between treatments with antagonists and *P. digitatum*; no reduction in incidence and severity of disease was observed in co-inoculations or when the pathogen was applied after 24 h. In our study, incidence and severity of citrus decay *in vivo* were consistently reduced when mixtures of fungal and bacterial antagonistic strains were applied 24 and 72 hours before challenging *P. digitatum*. Curative effect of *P. syringae* PVCT48SR2 or *T. atroviride* P1 was not improved by combined application of both antagonists in mixture, but the associated use of microrganisms and chitosan improved the antagonistic activity and reduced the disease incidence.

Strategies combining several alternative control methods can be promising to control postharvest green mold of citrus fruit and replace conventional fungicides used now in citrus packinghouses. The proposed sequence of treatments that showed the best results comprised initial treatment with heated solutions of SBC followed by the application of biocontrol agents. These results should be validated in commercial scale trials and in citrus packinghouses to demonstrate the value of these treatments that are compatible with existing facilities and postharvest handling practices and would imply minimal environmental and worker safety concerns. Currently, the main handicap to the adoption of such antifungal treatments by the citrus industry in Europe is the strict regulatory issues that exist regarding the registration of biological control commercial products, especially if compared to less restrictive policies of other countries such as the USA (Alabouvette *et al*., 2006).

Objective 3. Gene expression of defence-related genes and lipodepsipeptides synthetase genes in orange fruit in response to antagonistic/elicitors-pathogen interaction using quantitative RT-PCR technique.

Increasing natural resistance of fruit to pathogens is one of the alternatives being explored in the effort to reduce the dependency on chemical fungicides. Although many efforts have been invested in the practical implementation of induced resistance in mature citrus fruit against *P. digitatum* infection, we have only gathered partial information on the mechanisms responsible for this process, as no comprehensive study has been conducted.

Induced resistance has been associated with de novo synthesis of phytoalexins and induction of the PR proteins β-1,3-glucanase and chitinase in the flavedo of elicited fruits. Postharvest biological control using antagonistic bacteria and yeasts has been reported and has been considered to be one of the possible alternative strategies to synthetic fungicides (Cirvilleri *et al*., 2005; Zhang *et al*., 2009). The efficiency of a biocontrol agent can be enhanced by physical treatment such as hot water, or in combination with other substances generally recognized as safe (GRAS), such as sodium salts (Geng *et al*., 2011). However, interactions between postharvest biocontrol agents, salts and host tissue are poorly understood.

To gain a better understanding of the global changes in gene expression taking place in citrus fruit tissues following the application of biocontrol agents *Wickerhamomyces anomalus*, *Pseudomonas syringae* pv. *syringae* and sodium bicarbonate in control of green mold (*Penicillium digitatum*), we analyzed gene regulation of chitinase (CHI), glucanase (GNS), phenylalanine-ammonia lyase (PAL) and gluthatione peroxidase (GPX) by quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR). Special emphasis was placed on the molecular changes involved in defence-related genes occurring in citrus fruit tissue, particularly associated with early host resistance signalling. Plant defence-related genes chitinase and glucanase were up-regulated in grapefruit peel tissue following yeast treatment. An oxidative burst is known to induce a broad range of PR proteins. Plant PR proteins are represented by 17 protein families, including

GLUs, CHIs and PODs (Van Loon *et al*., 2006). PR proteins have been shown to be directly involved in plant immunity and associated with protective mechanisms (Pieterse and Van Loon, 1999). The expression levels of PR proteins can be regulated by various stress-related situations, including wounding, salinity, chemical elicitors, hormones and UV light (Van Loon *et al*., 2006). In postharvest systems, several reports have demonstrated the induction of chitinase or glucanase, including apple (El-Ghaouth *et al*., 1998), citrus (Ballester *et al*., 2010; Droby *et al*., 2002), peach (Xu *et al*., 2008) and cherry tomato fruit (Jiang *et al*., 2009), in response to the application of a biocontrol agent.

This work examined, for the first time, several combined applications of biocontrol agents (BCA), hot water (HW) and sodium bicarbonate (SBC) challenged with the pathogen *P. digitatum* (Pd) inoculated in citrus fruit tissues 24h before (curative, n°4) or 48h after (preventive, n°6, 11, 12) the treatments. Our results showed CHI1 over-expression for each treatment analysed (both curative and preventive type) only within 2h after treatments. Peak gene expression of CHI1 gene (relative units 1351) was observed in citrus tissues exposed to SBC, HW and *P. s.* pv. *syringae* 48SR2, and 48h later inoculated with the pathogen (treatment n° 11). This treatment significantly controlled green mold in vivo, with 0% of disease incidence after 6 days from the pathogen inoculation. Increases levels of CHI1 transcripts (relative units 57) were also observed in treatment including SBC, HW and *W. anomalus* BS91 (treatment n°12) which, as well as treatment n°11, efficiently controlled the green mold infection on oranges (disease incidence of 30% after 6 days of incubation), and treatment including SBC, HW and *P. s.* pv. *syringae* 48SR2 applied in mixture with the pathogen 24 h after wounding fruit tissues (disease incidence of 50% after 6 days of incubation). Surprysingly, high levels of CHI1 expression were also observed after treatment n°4, in which treatments SBC, HW and *P. s.* pv. *syringae* 48SR2 were applied 2h after inoculation, and that was totally ineffective in controlling green mold (100% disese incidence after 5 days of incubation). No CHI1 gene over-expression was registered 24h and 48h after tested treatments. Our data provide evidence that the higher chitinase gene expression was only observed shortly after *P. digitatum* inoculation, and that, despite CHI1 expression, only a

partial correlation is possible with the ability of these treatments to control green mold *in vivo*.

We found that either wounding alone, or treatment with sodium bicarbonate plus biocontrol agents, or inoculation by *P. digitatum*, induced no GNS1 gene expression in the peel tissue. Hereby, we showed that application of *Wickerhamomyces anomalus* and *Pseudomonas syringae* pv. *syringae,* alone or in combined application with sodium bicarbonate, did not increase the abundance of the glucanase mRNA message levels in the cells of the fruit peel tissue during first 48h after elicitation. These data are not in accordance with Porat *et al*, (2000) who, on the opposite, reported evident increasing in GNS1 gene expression 1-3 days after elicitation.

PAL is very important enzyme in plant disease resistance. It is involved in the formation of lignin and phytoalexins, which are related to plant resistance development (Graham and Graham, 1996). PAL is a key, rate-limiting enzyme in plant secondary metabolism, especially in the phenylpropanoid pathway. The process of lignification not only forms physical barriers against pathogen intrusion, but its precursor material can also be oxidized by polyphenol oxidase to generate quinines that act as strong poisons to pathogen (Ramiro *et al*., 2006). Thus, the variation of phenylalanine-ammonia lyase activity can indirectly reflect plant disease resistance. In citrus exposed to sodium bicarbonate and *P. syringae* pv. *syringae* PVCT48SR2 mixture, in curative and preventive treatments (treatment n° 4 and n° 6, respectively), the activity of PAL increased by 2h after treatment, and reached the peak value at 48h. These data confirm the involvement of PAL gene expression in the citrus fruit response to biocontrol agents and to citrus postharvest pathogen *P. digitatum*, as previously reported (Ballester *et al*., 2006; Gonzalez-Candelas *et al*., 2010).

AOS may induce expression of a variety of defence-related genes; one of the mechanisms contributing to oxidative stress is the activation of detoxification and protection genes such as SOD, GST and GPX (Alscher *et al*., 1997). The subcellular compartmentalization of antioxidative defence mechanisms seems to be essential for counteracting the potential oxidative damage at sites of enhanced AOS generation. Our findings showed enhanced GPX1 trancription level in citrus

peel tissues exposed to dipping treatment in sodium bicarbonate followed by inoculation with *P. syringae* pv. *syringae* PVCT48SR2 and 48h later inoculated with pathogen (treatment n° 11), thus confirming that GPX was shown to increase in plants under stress conditions, such as exposure to salt, H_2O_2 and pathogen infection (Kurama *et al*., 2002).

Antagonistic microorganisms protect fruits and vegetables against a number of postharvest pathogens. Although the biocontrol activity has been demonstrated on a variety of commodities, their mode of action has not been fully elucidated. The major component of the mode of action of antagonistic microorganisms is believed to be competition for nutrient and space. In the case of antagonistic *Pseudomonas syringae* strains, it has been suggested that their biocontrol activity may be partially associated with the production of lipodepsipeptides (Bull *et al*., 1998), with competition for nutrient and space, and with the ability to induce several biochemical defence responses (Scuderi *et al*., 2010).

Production of cyclic lipodepsipeptides has been also correlated with the ability of bacteria to control plant diseases caused by postharvest fungal pathogens. Syringomycin (syr) and syringopeptin (syp) toxins, whose production is a distinctive feature of *P. syringae* pv. *syringae* strains, are lipodepsipeptides with antimicrobial properties produced through a nonribosomal peptide synthetase system (Bender *et al*., 1999).

In this work we studied transcription levels of *syp*A and *syr*B1 genes in citrus peel tissues treated with SBC, HW and *P. s.* pv. *syringae* 48SR2 before and after *P. digitatum* inoculation (treatment n° 11).

Combined application of SBC, HW and *Pss* generated in citrus peel tissue 48h after treatment weak over-expression of *syr*B1 gene, with transcription levels 1,68-fold higher than those measured in citrus peel tissue treated with *Pss* alone, whereas undetectable *syr*B1 transcription levels were detected at 0 and 24h after treatments.

Similarly, combined application of and SBC, HW and *Pss* generated, 48h after treatment, *syp*A transcription levels 19-fold higher than those measured in citrus peel tissue treated with *Pss* alone, whereas very low *syp*A transcription levels were detected at 0 and 24h after treatments.

Two hours after *P. digitatum* inoculation, *syr*B1 gene was strongly over-expressed with transcriptional levels 41-fold higher than those measured in peel tissues treated with *Pss* alone; this over-expression 24h after *P. digitatum* inoculation was undetectable 48h after inoculation. The *P. digitatum* inoculation weakly induced *syp*A genes over-expression that reached his higher level 48h after *P. digitatum* inoculation. Pathogen inoculation resulted to be strongly stimulatory only to *syr*B1 expression, suggesting that at least *syr*B1 gene is involved in biocontrol activity.

In conclusion, our data provide further evidence that such responses were expressed earlier and with a much higher magnitude when treated citrus tissues were challenged with the pathogen *P. digitatum*, thus providing support to the concept that a signal produced by the pathogen is essential for triggering enhanced transcription levels of both defence-related and lipodepsipeptides synthetase genes.

Chapter 6.

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