Pre- and postharvest application of alternative means to control *Alternaria* Brown spot of citrus

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ABSTRACT

Alternaria brown spot (ABS) is one of the main diseases of mandarins and their hybrids. Its control is based on fungicide applications, but their use is facing difficulties related to the development of resistant pathogen strains and adverse effects on animals, humans and the environment. The present study was undertaken to find feasible alternative solutions to control ABS, by testing the effectiveness of phenolic compounds and alternative formulations present on the market. They were tested *in vitro* and *in vivo* on a laboratory scale and then in semi- commercial conditions. Umbelliferone was the most effective phenolic compound in controlling by 86% ABS on mandarins cv. Fortune in semi-commercial conditions. Its effect seemed related to the ability of inducing resistance in the host. Whereas among the formulations, chitosan-based HendophytPS and salt-based FortisolCaPlus gave the best results in field-scale trials, with a reduction of disease incidence by 59–63 and 54–75% on fruit and leaves, respectively, as compared to the control, being the effect similar to that obtained by chemical controls. The formulations showed mainly to induce host resistance. The application of proposed alternative treatments, singly or in a combined strategy, might represent an interesting substitute to synthetic fungicides against ABS.

1. Introduction

Alternaria brown spot (ABS) is a highly destructive disease of mandarins (*Citrus reticulata* Blanco) and their hybrids (Akimitsu et al., 2003), whereas, satsumas (*Citrus unshiu* Mark. Marc.), and clementines (*Citrus clementina* Hort. ex Tan.) seem resistant at some extent. The causal agent is a pathotype of *Alternaria alternata* (Fr.) Keissl., which is prevalent in humid citrus-production areas as Florida, Brazil, Argentina, Colombia, Cuba, Peru, and China, but also in Mediterranean areas with cool, moist winters and hot, dry summers as Spain, Italy, Greece, Turkey, and Israel (Bassimba et al., 2014). In Italy, the disease was first reported by Bella et al. (2001), and recently by Garganese et al. (2016). Temperatures of 25–27 C with at least 10 h of wetness are optimal for infection. Conidia are produced on the lesion surface, and subsequently released by rainfalls (Timmer et al., 1998). The pathogen causes brown to black spots on young leaves and fruit, and twig dieback. Under optimal conditions, symptoms may appear within 24 h after infection (Timmer et al., 2003). In addition to a decrease in plant productivity, ABS symptoms cause loose of commercial value of fruit.

Where allowed, fungicides (e.g. dithiocarbamates, dicarboximides, strobilurins, and conazoles) are used for ABS control (Feichtenberger et al., 2005). Copper is also recommended in many countries (Timmer et al., 2000; Vicent et al., 2004). However, the management of ABS is particularly challenging in presence of recurrent rains and jumps in relative humidity (Timmer et al., 1998). In Italy, the containment of the disease is critical, and the number of needed treatments may change according to the weather conditions year by year. In a study conducted by Bella et al. (2012) during 2005–2011, two scheduled fungicide applications (late summer and in the fall) resulted inadequate to control ABS. Copper and, from late 2015, mancozeb and pyraclostrobin are the only fungicides allowed in Italy in conventional/integrated production, being the sole copper allowed in organic agriculture (http://m.bdfup.it/ bdfup/). Mancozeb and pyraclostrobin belong to a family of fungicides suspected for human health hazard (Bolognesi, 2003; Çayır et al., 2014). Moreover, the use of metallic copper in organic agriculture is limited to 6 kg per ha per year (European Commission Regulation N. 889/2008), and following the recommended spray schedules for citrus, this threshold is easily overcome (Vicent et al., 2009). Therefore, the search for safer and effective alternatives is gaining attention. Among them, 2–6% organic and inorganic salts were tested on a wide range of crops including citrus (Palou et al., 2008; Romanazzi et al., 2012; Wisniewski et al., 2016). Most of them belong to the GRAS (Generally Recognized as Safe) category by the US Food and Drug Administration (FDA) (Palou et al., 2016). Salts have been investigated both as pre- (Nigro et al., 2006; Teixido et al., 2010 ; Youssef et al., 2012) and postharvest treatments (Youssef et al., 2012, 2014; Montesinos-Herrero et al., 2016). Phosphite salts proved to both induce host resistance and control mycelial growth of oomycetes (Lobato et al., 2008; Thao and Yamakawa, 2009; Olivieri et al., 2012). Although marketed as fertilizers and plant strengtheners, they proved to be potential alternatives to conventional fungicides (Lobato et al., 2008; Olivieri et al., 2012) including citrus (Panabieres et al., 2016). Phosphites can have different application strategies according to the crop and the pathogen, but foliar spray is the commonest (Deliopoulos et al., 2010). Salts have been introduced in the market as formulations. For instance, FortisolCaPlus, a water-soluble mix of Ca, K and P, showed a protection against citrus postharvest disorders and fungicide-related phytotoxicity (Parra et al., 2014; Martínez-Hernandez et al., 2017). Similarly, the combined pre- and postharvest application of DeccoPhosk, a potassium phosphite formulation, was effective against green and blue mold of lemons, as well as chilling injury and aging (Strano et al., 2015). Furthermore, natural compounds of animal or plant origin showed good control performance of postharvest diseases. For example, chitosan, a linear polysaccharide from crustacean shells, proved to control numerous pre- and postharvest diseases on several vegetables and fruits (Romanazzi et al., 2003; Bautista-Banos et al., 2016; Palou et al., 2016) including green mold on citrus (Panebianco et al., 2014). Indeed, chitosan has direct antimicrobial activity, and, at the same time, triggers resistance mechanisms (El Hadrami et al., 2010). Among compounds of plant origin, phenolic substances are considered of a certain interest from a disease control perspective. They occur constitutively or are formed in response to pathogen attack (Jeandet et al., 2013). Accumulation of quercetin, umbelliferone, scopoletin, and scoparone in plant tissues, together with their antioxidant and antifungal properties (Sourivong et al., 2007; Sanzani et al., 2009, 2014) make them good “natural pesticide” candidates to increase the resistance of plants to fungal infections (Sanzani et al., 2010).

The aim of this study was to evaluate the effectiveness of selected phenolic compounds and alternative formulations against ABS, by testing their effectiveness in laboratory- and semi-commercial conditions.

2. Materials and methods

2.1. Phenolic compounds and alternative formulations

The phenolic compounds hesperidin (hesperetin7-rhamnoglucoside**)**, naringin (4^0,5,7-trihydroxyflavanone 7-rhamnoglucoside), quercetin (3,3^0,4^0,5,7-pentahydroxyflavone), and umbelliferone (7- Hydroxycoumarin**)** with a purity >95% were purchased from Sigma- Aldrich (Milan, Italy). Stock solutions of pure standards were prepared in a phosphate buffer (Sanzani et al., 2009) at a concentration of 50 mg/ml.

The tested alternative formulations were Fortisol®CaPlus (water- soluble mix of Ca, K and P, Citrosol, Valencia, Spain), DeccoPhosk® (potassium phosphite, Decco Italia, Belpasso, Italy), and Hendophyt®PS (chitosan, Iko-Hydro Ltd., Rutigliano, Italy).

The fungicides Ossiclor (copper oxychloride, 50% w/w, Manica, Rovereto, Italy) and CabrioWG (Pyraclostrobin, 20% w/w, BASF, Faenza, Italy) were used as positive controls at 175 g/hl and 0.06 g/hl a. i., respectively.

2.2. Alternaria alternata conidial suspension

A. alternata morphotype *alternata* strain A23, morphologically and molecularly identified (Garganese et al., 2016), and deposited in the “Fungal Culture Collection” of the Department of Soil, Plant, and Food Sciences, University of Bari Aldo Moro (Italy), was cultured on PDA (Conda, Spain) plates for 7 days at 24 1 C. To produce inoculum, colony surface was washed with 5 ml of a 0.05% (v/v) Tween 80 aqueous solution. The obtained suspension was filtered through a double layer of sterile gauze and counted by a Thoma counting chamber (HGB Henneberg-Sander GmbH, Lutzellinden, Germany). A 10^5 conidia/ml suspension was used for all *in vitro* and *in vivo* trials.

2.3. In vitro assays

The effect of selected treatments on the mycelial growth of *A. alternata* was determined on amended PDA. Briefly, a solution of each single compound/formulation was 0.22-µm filtered and added before pouring PDA into 90 mm Petri dishes, to obtain final concentrations of 10 and 100 µg/ml (100 and 1000 µg/dish, respectively) for the phenolic compounds, and 0.5, 0.5, and 1% (w/v) for Fortisol, DeccoPhosk, and Hendophyt, respectively. Dishes were seeded centrally with a 5 mm mycelial plug of the pathogen. Non-amended PDA plates were used as a control. For each treatment/concentration, five dishes were used as replicates. Colony growth (average of the two orthogonal diameters, mm) was recorded at 7 days post-inoculation (DPI). The experiment was repeated twice. *2.4. Laboratory in vivo assays*

2.4.1. Citrus samples

Mandarins cv. Fortune, a late ripening variety, were harvested at physiological maturity in a conventional farm located in the Bernalda area (Basilicata, Southern Italy). Fruit were uniform in size, colour, and ripeness and free of defects or injuries. The selected fruit were randomized, surface-sterilized in a 2% sodium hypochlorite solution, washed in tap water and left to dry at room temperature.

2.4.2. Testing of direct antifungal activity

Fruit were wounded twice (3 mm wide 3 mm depth) with a sterile nail at opposite sides in the area between the peduncle and the equatorial axis. Then, 1000 µg/wound of phenolic solutions, or 0.5% Fortisol/DeccoPhosk, or 1% Hendophyt were pipetted into each wound. Fruit whose wounds were treated with phenolic solving buffer or water served as controls. After drying, the same wounds were inoculated by 10 µl of *A. alternata* conidial suspension. Each treatment had three replicates, consisting of six mandarins each (18 fruit/36 wounds per treatment). Replicates were incubated at 24 1 C and 95–98% RH for 14 days. Disease incidence (infected wounds, %) and severity (lesion diameter, mm) were assessed at 14 DPI. The trial was performed twice.

2.4.3. Testing of products as resistance inducers

Surface-sterilized fruit were wounded and treated as reported above. Fruit amended with phenolic solving buffer or water were used as controls. After an incubation of 24 h at 24 1 C and 95–98% RH, another round of wound was made on each fruit about 5 mm from the previous one and inoculated with *A. alternata* conidial suspension (10 µl). Each treatment was made of three replicates with six fruit each (18 fruit/36 wounds per treatment). Replicates were incubated as reported above. Incidence of decay and disease severity were assessed at the end of incubation. The entire experiment was repeated twice.

2.5. Shelf-life experiments

The phenolic compounds were further tested as postharvest treatments on the freshly harvested mandarins cv. Fortune against natural infections by *Alternaria* spp. Briefly, 1000 µg of each selected compound was pipetted into each wound (two per fruit) as reported above. Fruit were incubated at 24 1 C and 95–98%. Each treatment comprised three replicates with twelve fruit each (36 fruit/72 wounds per treatment). At 14 DPI, disease incidence was assessed as previously reported. The entire experiment was repeated twice.

2.6. In the field experiments

Field trials with formulates were conducted in 2014 and 2015 by monthly treatments, from May to December. The experimental tests were conducted in an orchard of mandarin plants cv. Fortune, located in Pisticci area (Basilicata, Southern Italy). The plants were 8-year-old, grafted on Troyer citrange, and arranged in a completely randomized block design with 4 plots of 3 plants each. Plants were selected for canopy and fruit set homogeneity and lack of disorders. There were two- plant buffers between plots to reduce spray drift. A spray lance, powered by a walking-type motor pump, was used to apply substances at 20 atm into the canopy at a rate of 5 l/plant (20 hl/ha), approximately. The same number of plots was treated by the fungicides Ossiclor and CabrioWG as positive controls. Each year, a month after the last treatment, a survey was carried out before harvesting to assess the disease on leaves and fruit, randomly choosing 10 twigs and 10 fruit per plant, 120 fruit/ twigs per treatment, and counting the number of *A. alternata* lesions on five leaves per twig and on each fruit surface, respectively.

2.7. Data analysis

The statistical software package Statistics for Windows (StatSoft, Tulsa, OK, USA) was used. Percentage data of incidence of decay were arcsine-square-root transformed before ANOVA analysis. In case of homogeneity of variance, data from repeated experiments were combined. Significant differences (P 0.05) were identified by the General Linear Model (GLM) procedure with the Duncan's Multiple Range Test (DMRT). A control index (CI) expressed the extent of the control exerted by treatments (Sanzani et al., 2009).

3. Results

3.1. In vitro assay

None of the phenolic compounds at both tested concentrations reduced *A. alternata* growth (Table 1). Whereas, the three formulations significantly inhibited colony diameter (Table 1). Hendophyt provided the best results, inhibiting the growth of the colonies by 93%, followed by Fortisol and DeccoPhosk with a reduction of 80 and 77%, respectively.

3.2. Laboratory in vivo assays

3.2.1. Testing of direct antifungal activity

The incidence and severity of the decay on fruit treated by phenolic compounds and inoculated by *A. alternata* in the same wound are reported in Fig. 1A. Although none of the tested phenolic compounds reduced disease incidence, quercetin and hesperidin proved to decrease disease severity by 53 and 32%, respectively, as compared to the control.

Concerning the formulations, Fortisol resulted the best treatment reducing both disease incidence (58%) and severity (45%) as compared to the control (Fig. 1B). Whereas DeccoPhosk reduced only disease severity (57%). Hendophyt did not influence directly the disease development.

3.2.2. Testing of products as resistance inducers

In absence of a direct contact between treatment and pathogen, umbelliferone was the only phenolic compound able to reduce both disease incidence and severity by 47 and 90%, respectively, as compared to the control (Fig. 2A). Whereas, quercetin and naringin reduced only the severity by 74 and 73%, respectively. Concerning the formulations, Hendophyt and Fortisol reduced disease incidence by 40 and 47%, respectively. In Hendophyt-treated fruit even the disease severity was reduced by 42% (Fig. 2B).

3.3. Shelf-life experiments

Phenolic compounds were tested against *Alternaria* natural infections on harvested mandarin fruit at concentration 1000 μg/wound. Quercetin and umbelliferone reduced disease incidence by 35 and 86%, respectively as compared to the control (Fig. 3).

3.4. In the field experiments

In the field, the incidence of disease was recorded on citrus leaves and fruit treated from May to December each year in two consecutive years. Hendophyt and Fortisol were the most effective alternative treatments (Fig. 4). Compared to the control, the number of lesions on leaves and fruit treated with Hendophyt and Fortisol were reduced by 63 and 75%, and by 59 and 54%, respectively (Fig. 4A and B). DeccoPhosk was not effective in controlling lesion appearance on both tissues. Furthermore, no phytotoxicity was observed on fruit and leaves treated by the alternative formulations. Finally, Hendophyt and Fortisol results were similar to those obtained by fungicide treatments, with a reduction ranging between 89 and 92% for fruit, and 88 and 78% for leaves by Ossiclor and CabrioWG, respectively.

4. Discussion

ABS is a severe disease on susceptible mandarin and mandarin-like cultivars, so that their cultivation in absence of an effective control strategy might be unprofitable (Peres and Timmer, 2006; Vicent et al., 2009). Thus in several citrus-producing countries, chemical fungicide sprays are scheduled intensively during the growing season. However, the risks of residues on the product and in the environment has increased the demand for alternative substances less harmful for humans and animals (Li Destri Nicosia et al., 2016). Last alternative control strategies for pre- and postharvest decays are based on products with modes of action different from traditional fungicidal active ingredients, but also with multiple activities, e.g. direct effect plus induction of resistance (Wisniewski et al., 2016). Recently, the use of elicitors of either biotic or abiotic source to enhance host defenses has gained attention as approach of choice in integrated strategies to control postharvest diseases (Romanazzi et al., 2017). Following ripening, disease resistance of harvested fruit usually declines, increasing host susceptibility to pathogen attack. Thus, resistance inducers may be useful to reinstate a suitable level of resistance (Pangallo et al., 2017). Phenolic compounds are antimicrobial compounds produced by plants that accumulate or appear *de novo* in response to cellular damage; their production/increase in infected tissues is recognized as one of the main resistance mechanisms in plants (Lachhab et al., 2014). Their induction in challenged plant tissue or exogenous application have been proposed as alternative control approaches against blue and green mold of apple and citrus fruits, respectively (Afek et al., 1999; Sanzani et al., 2009, 2014). In the present investigation, a selection of phenolic compounds was applied against ABS. At the tested concentrations, they did not show a direct effect *in vitro* against the growth of *Alternaria*. Similar results were obtained, as far as quercetin and umbelliferone concern, against *P. expansum* (Sanzani et al., 2009) and *P. digitatum* (Sanzani et al., 2014). Whereas, on *Alternaria*-inoculated fruit, quercetin and hesperidin applied in the same wound inoculated by the pathogen proved to reduce disease severity as compared to the control. Overall, tested phenolics seemed to be more efficient *in vivo* than *in vitro,* in agreement with Sanzani et al. (2009, 2014). Better disease control results were obtained when phenolics were applied as resistance inducers. Indeed, all tested phenolics controlled disease severity, with umbelliferone reducing even ABS incidence. Thus, they seemed to exert their control effect indirectly. Several possible mechanisms might be involved: activation of host natural defenses including biochemical (e.g. PR-proteins) and physical (e.g. lignification) barriers; accumulation with endogenous phenolics, thus reaching a fungitoxic concentration; chemical interaction with pre-existing compounds, thus forming new toxic molecules (Sanzani et al., 2009). Quercetin ability to induce resistance phenomena in apples has been reported (Sanzani et al., 2010) as well as that of umbelliferone, which played a role in defense mechanisms of unripe reactive grapefruit against wound-pathogens as *P. digitatum* (Afek et al., 1999). Overall, in the present investigation *Alternaria* seemed less susceptible to treatments than other genera as *Penicillium* (Sanzani et al., 2009, 2014). This finding might be ascribed to the intense melanization of *Alternaria* structure, since melanin contributes to virulence of melanized microorganisms by decreasing their susceptibility to host defense mechanisms (Nosanchuk and Casadevall, 2003). Umbelliferone confirmed its efficacy even in shelf-life trials, and thus, although further larger scale assays are needed, this compound might be considered a good candidate to be applied after harvest within an ABS integrated control strategy, made up of pre- and postharvest application of alternative means, to prevent losses of citrus fruit.

Furthermore, in this study some commercial alternative formulations commercialized as citrus biostimulants were tested before harvest. In *in vitro* assays, they all proved to control *Alternaria* growth. However, when applied *in vivo* on fruit, only Fortisol, formulate based on sodium, calcium, and phosphorus salts, reduced disease incidence and severity. Furthermore, it proved to act even as resistance inducer, together with Hendophyt, a chitosan-based formulate. When applied preharvest monthly on fruit and leaves of mandarin cv. Fortune, these two formulations confirmed their reduction of the disease incidence on both organs. Chitosan is known to have multiple activities. For example, it can penetrate the plasma membrane and eventually kill the pathogen cells, as observed for *Neurospora crassa* (Palma-Guerrero et al., 2009). Furthermore, chitosan forms a physical barrier around the infection sites, preventing pathogen spread to healthy tissues (Bittelli et al., 2001). In numerous studies chitosan proved to prevent spore germination, germ tube elongation, and mycelial growth of pathogens as *Botrytis cinerea* (Monjil et al., 2013; Arasimowicz-Jelonek et al., 2014), *Fusarium solani* (Li et al., 2014), *Rhizopus stolonifer* (Monjil et al., 2013), *Penicillium* spp. (Olivieri et al., 2009; Arasimowicz-Jelonek et al., 2014), and *Sclerotium rolfsii* (Li et al., 2014). However, it is frequently used as a potent elicitor for controlling plant diseases rather than as antimicrobial agent (Romanazzi et al., 2017). Indeed, chitosan can chelate nutrients and minerals (i.e., Fe, Cu), and bind mycotoxins (Bornet and Teissedre, 2007), which may contribute in reducing damage of host tissues (Sanzani et al., 2012). The control activity of salt-based formulate Fortisol was remarkable too. Salts proved to have a multiple mode of action based not only on direct activity against fungi (Fallanaj et al., 2016), but also on resistance induction in treated tissues (Youssef et al., 2014). The activity of Fortisol and Hendophyt was equal or similar to that of chemical fungicides on leaves and fruit, respectively. In our study, the efficacy of copper and pyraclostrobin was analogous to that reported in literature (Vicent et al., 2007). The monthly application of treatments was scheduled based on data according which copper compounds can provide protection for around 1 month under rain-free weather conditions (Vicent et al., 2009). However, copper compounds, especially in summertime and as repeated sprays may become phytotoxic on citrus fruit (Albrigo and Timmer, 1997). Thus, reducing copper applications would lessen the phytotoxicity hazard. To this aim, pyraclostrobin is applied efficiently at beginning of the infection season, when fruit and leaves are small. However, since chemical fungicides are not free from drawbacks, an integrated pest management system with alternative formulations as Fortisol and Hendophyt could maintain a similar level of control without side effects. At this regard, the development and validation of predictive models that can estimate the real risk of infection and identify the optimal time for treatments according to the phenological stages of crop and climatic conditions encountered in the field might be particularly useful (Peres and Timmer, 2006).

5. Conclusions

In order to safeguard the quality and commercial value of the productions of mandarins cv. Fortune and others mandarins, it is necessary to widen the range of products approved for controlling ABS and to develop effective control programs. The good efficacy of alternative treatments, alongside the possibility to obtain healthier products, makes them a viable option to fungicides; the use of which, currently, has been reduced greatly for issues related to the presence of residues, operator safety, environmental pollution, and restrictions provided by the national legislations. The alternative products are gaining wide acceptance among consumers and operators, and as such might represent a valid mean to be used.

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Table 1. A*lternaria alternata* colony diameter (mm) on PDA amended with phenolic compounds (100 and 1000 µg/dish), and 0.5, 0.5, and 1% (w/v) Fortisol, DeccoPhosk, and Hendophyt, respectively. Dishes were incubated at 24 1 C for 7 days in the dark.

Treatments	Colony diameter SEM
Control	48.8 4.5
Quercetin_100	50.5 1.9
Hesperidin_100	42.5 5.7
Naringin_100	53.9 2.4
Umbelliferone_100	45.5 5.1
Control	65.3 5.4
Quercetin_1000	63.0 4.0
Hesperidin_1000	63.0 1.9
Naringin_1000	69.0 3.4
Umbelliferone_1000	59.5 4.3
Control	57.9 4.3
DeccoPhosk	13.1 2.3
Fortisol	11.4 1.0
Hendophyt	4.8 1.0

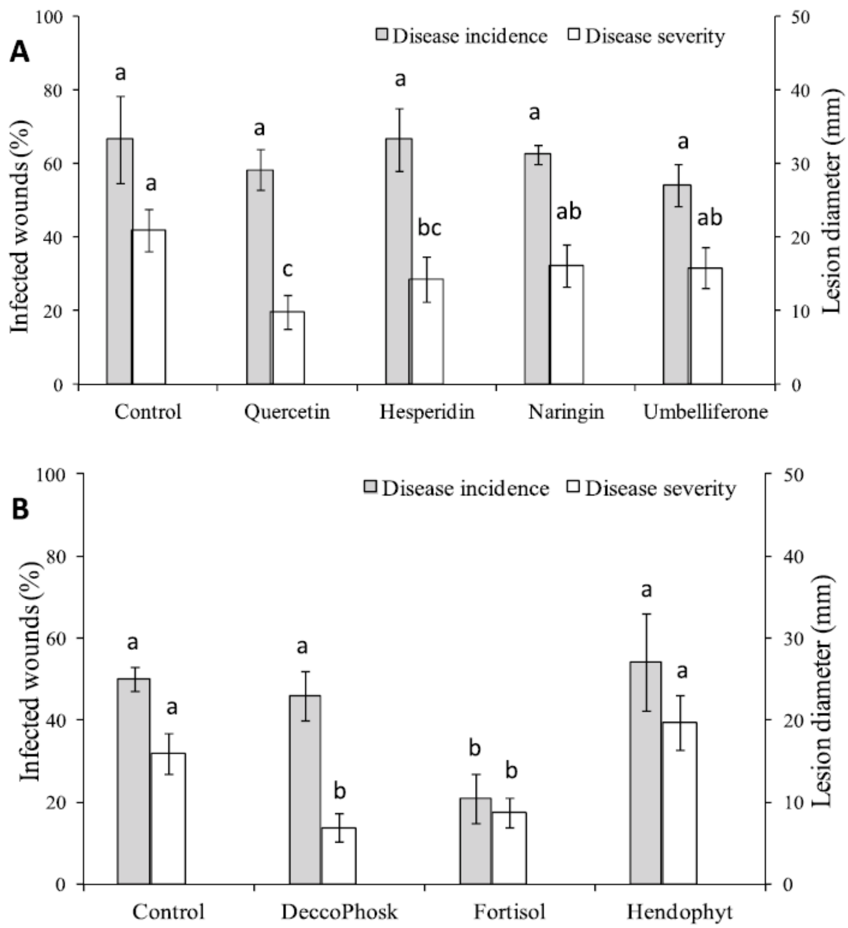

Fig. 1. Disease incidence (infected wounds, %) and severity (lesion diameter, mm) on harvested citrus fruit wound-treated by A) different phenolic compounds (1000 μg/wound) or B) DeccoPhosk/Fortisol (0.5%) and Hendophyt (1%), and inoculated by *Alternaria alternata.* Water- or buffer-treated fruit were used as a control. Data were recorded after 14 days of incubation at 241 C and 95–98% RH. Bars represent the standard error of the mean (SEM). For each parameter, columns with different letters are statistically different according to DMRT (*p* 0.05).

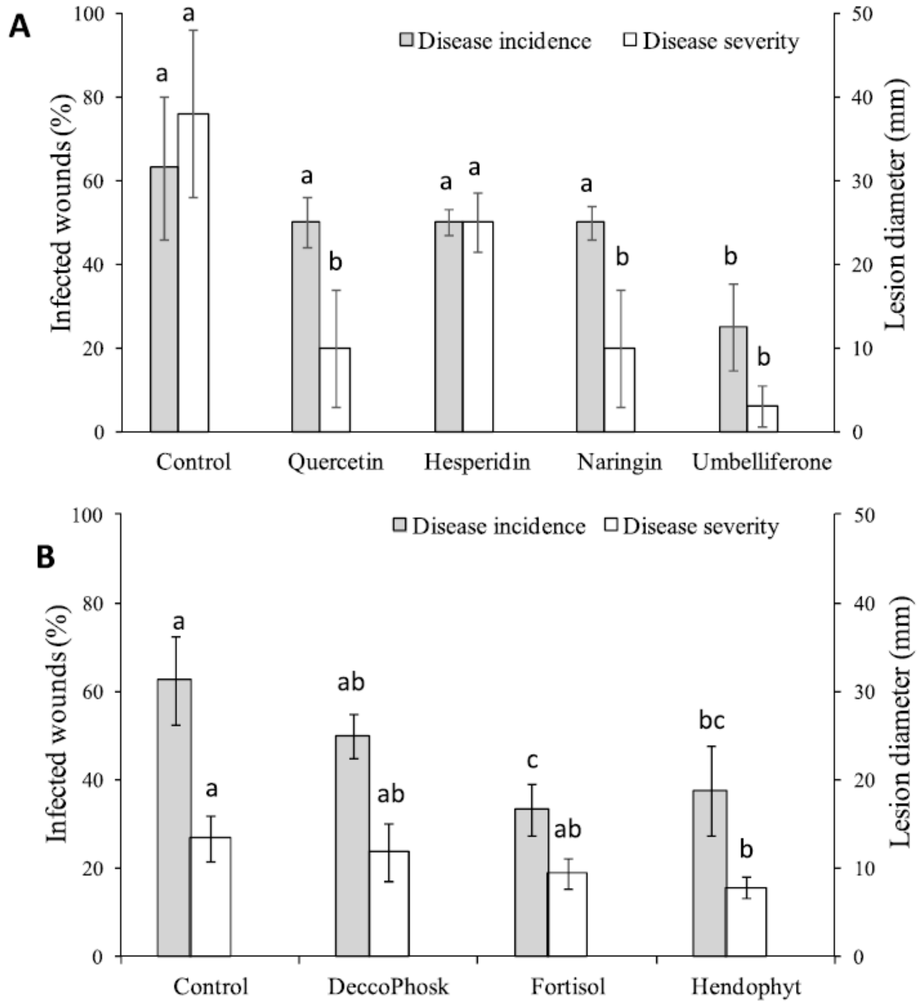

Fig. 2. Disease incidence (infected wounds, %) and severity (lesion diameter, mm) on harvested citrus fruit wound-treated by A) different phenolic compounds (1000 µg/ wound) or B) DeccoPhosk/Fortisol (0.5%) and Hendophyt (1%), and after 24 h inoculated by Alternaria alternata in different but close wounds. Water- or buffer-treated fruit were used as a control. Data were recorded after 14 days of incubation at 24±1°C and 95–98% RH. Bars represent the standard error of the mean (SEM). For each parameter, columns with different letters are statistically different according to DMRT ($p \leq 0.05$).

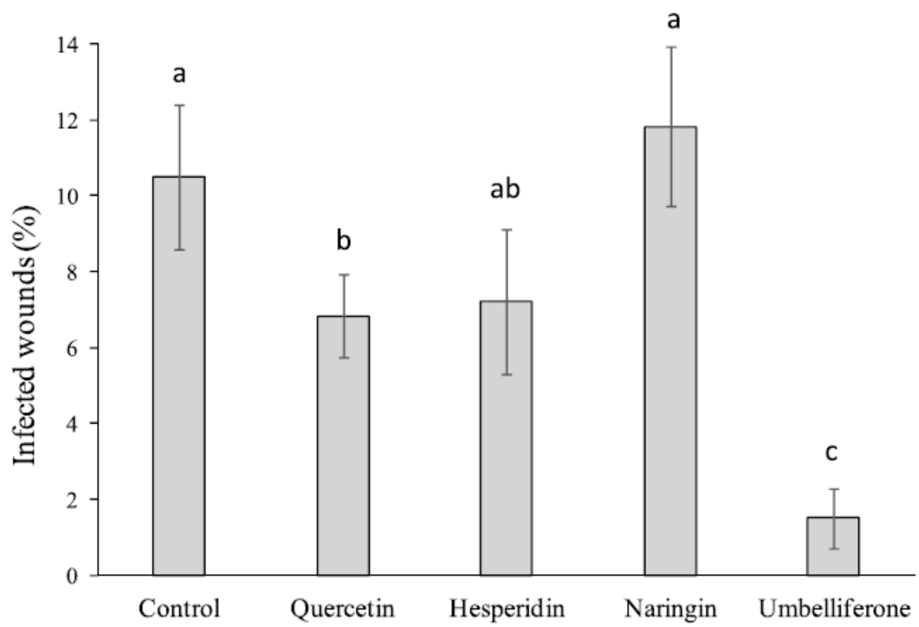

Fig. 3. Disease incidence (infected wounds, %) on harvested citrus fruit wound-treated by different phenolic compounds (1000 µg/wound) against *Alternaria* natural infections. Bars represent the standard error of the mean (SEM). Columns with different letters are statistically different according to DMRT (*p* 0.05).

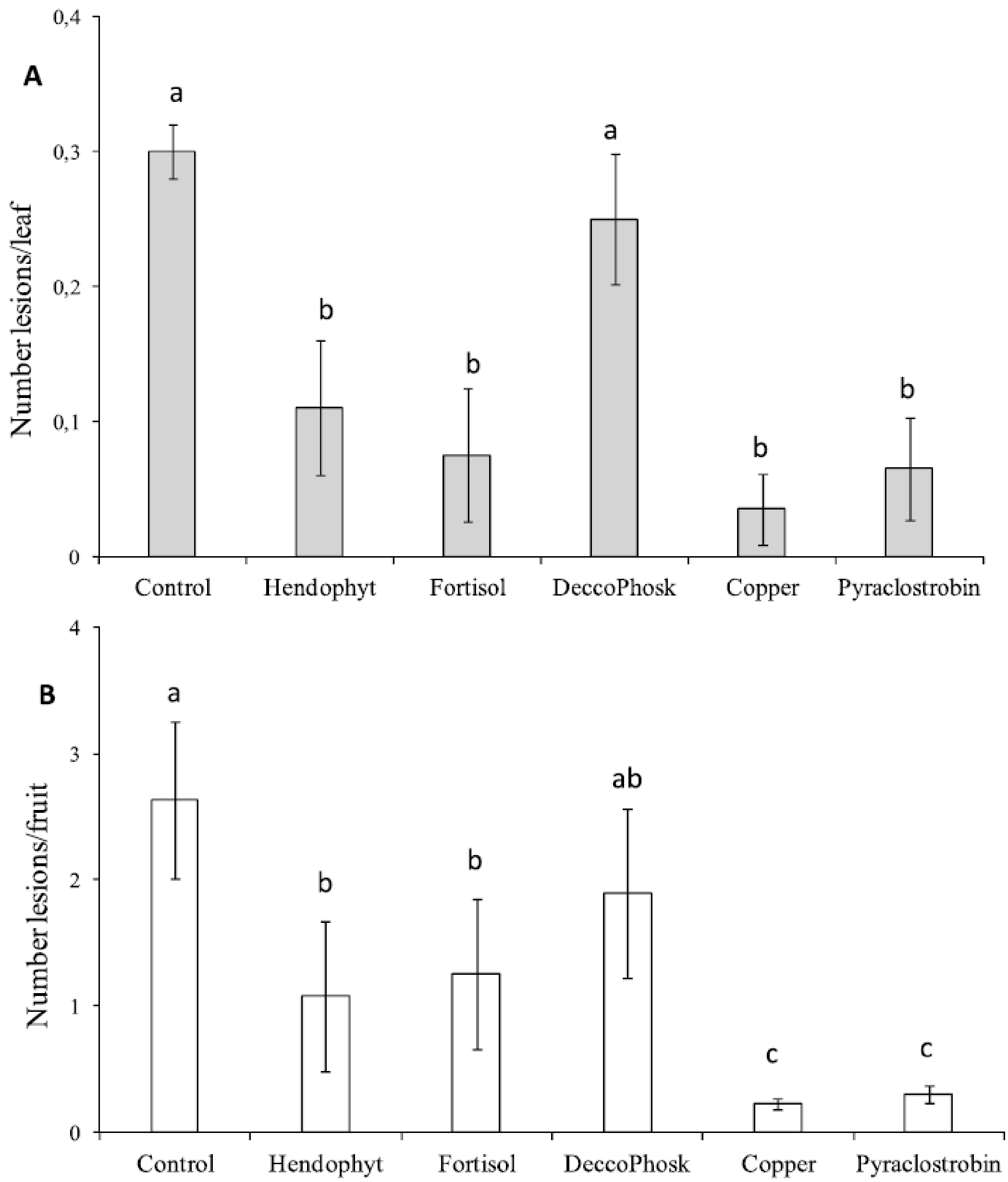

Fig. 4. Disease incidence (number lesions) on citrus leaves (A) and fruit (B) monthly treated in the field by alternative products DeccoPhosk and Fortisol (0.5%), and Hendophyt (1%) and two chemical fungicides Ossiclor and CabrioWG (commercial dose). Water treated samples were used as a control. Bars represent the standard error of the mean (SEM). Columns with different letters are statistically different according to DMRT (p 0.05).