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In Vitro and *in Planta* Activity of Some Essential Oils against *Venturia inaequalis* (Cooke) G. Winter

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Abstract

The effect of the essential oils of thyme (*Thymus vulgaris*), cinnamon (*Cinnamomum verum*) and sweet orange (*Citrus sinensis*) on conidial germination of *Venturia inaequalis* was investigated *in vitro*. Moreover, the effect of cinnamon oil was further studied by analyzing its preventive and curative activity as well as its rainfastness in an apple seedling-Venturia pathosystem under growth chamber and greenhouse conditions. The essential oils suppressed the germination of conidia at concentrations of 0.01% and 0.1% effectively. The highest inhibition level was achieved with thyme oil. Cinnamon oil (0.2%) with Silwet Star adjuvant (0.02%) was protective and curative against *Venturia inaequalis* when applied 72 h, 24 h, 1 h before or 24 h, 72 h after inoculation of plants. Effective control was achieved when essential oil was applied by 24 h, 1 h or 24 h pre- or post-inoculation, respectively. The 72 h application was less effective, however, it still showed decent control level. Although the simulated rainfall of light (1 mm), moderate (3 mm) and heavy (5 mm) rain decreased the control efficacy of cinnamon oil, the water amounts did not influence control level. The oil application resulted in a moderate and long lasting control (92% efficacy without rainfall, 65% efficacy with rainfall). The results of this study show that the tested essential oils are appropriate candidates for the development of biofungicides against apple scab.

Keywords: apple scab, conidia, essential oils, preventive and curative activity, rainfastness

Introduction

Apple scab (*Venturia inaequalis* (Cooke) G. Winter) is a widespread and common disease around the world wherever apples are grown (Vaillancourt and Hartman, 2000). The pathogen causes significant loss in the Hungarian apple production. In epidemic years, the number of fungicide applications against the disease often exceeds twenty sprayings in a vegetation period. In recent years, the use of fungicides against apple scab is strictly regulated by EU legislation (eg. Directive 2009/128/EC, Commission regulations No 834/2013, No 1004/2013, No 1138/2013, No 61/2014). In organic production the use of copper fungicides is limited due to soil accumulation as well (Commission Regulation No. 473/2002, Council Regulation (EEC) No. 2092/91). Therefore, the research of alternative solutions to synthetic or copper based fungicides is particularly needed due to the potential risks of the environment pollution and of fungicide resistance. Essential oils of medicinal and aromatic plants are suitable candidates for this purpose. Their antimicrobial and antifungal activity has been known for centuries and it is still an intensively studied field. Since essential oils contain several chemical compounds, the inhibition is based on different biological modes of action. The primary target of essential oils is the fungal plasma membrane. Active compounds cause lesions and reduce ergosterol biosynthesis resulting in severe morphological changes of the hyphal wall (Pinto *et al*., 2006; Soylu *et al*., 2010; Tian *et al*., 2012).

Although there are numerous data available on the research of the effect of plant extracts and essential oils on plant pathogens, fewer results can be found about the effect on *Venturia* species. The plant extracts from *Citrus* sp., *Galega officinalis, Inula viscosa*, *Quillaja saponaria*, *Polypodium vulgare* and *Saponaria officinalis* inhibited conidial germination of *Venturia inaequalis* (Cooke) G. Winter effectively and gave good control of apple scab on seedlings in greenhouse studies, however, simulated rainfall decreased the efficacy significantly (Pfeiffer, 2002; Pfeiffer *et al*., 2004). Plant extracts containing high contents of active ingredients of artemisinin (*Artemisia annua*), chelidonine (*Chelidonium majus*), menthol (*Mentha piperita*), populin (*Populus nigra*), linalool + linalyl acetate (*Salvia sclarea*) and thymol (*Thymus vulgaris*) were tested against the ascospore and conidia germinaton of *Venturia inaequalis* (Cooke) G. Winter. Almost all extracts showed high inhibition of ascospore germination. Artemisinin, thymol and populin extracts effectively inhibited conidial germination as well. The 1% *Populus* extract gave similar control efficacy against apple scab on apple trees as synthetic fungicides in a 2 years field study (Bálint *et al.*, 2014; Theisz *et al.*, 2007). The preventive application of six plant oils (canola, corn, grapeseed, olive, soybean and sunflower) reduced scab infection on apple leaves significantly under controlled condition, however, orchard application of canola and soybean oil with emulsifier did not give better control level than that of the application of emulsifier alone (Northover and Schneider, 1993). The essential oils of *Phellodendron chinense, Russowia sogdiana* and *Zanthoxylum bungeanum* inhibited *in vitro* the mycelial growth of *Venturia pirina* and other fungi effectively. The wide antifungal activity could be attributed to the high contents of monoterpene compounds (Gong *et al.*, 2009, 2011; Tan *et al.*, 2007). Berngtsson *et al.* (2006, 2009) compared the effect of plant extracts and the resistance inducer acibenzolar-S-methyl on conidial germination with their effect on scab infection on apple seedlings. Several extracts inhibited effectively conidial germination, however, showed no or moderate *in vivo* efficacy. They pointed out that besides the direct fungistatic or fungicide effect of the plant extracts, induction of plant resistance might also play an important role in their mode of action.

Although the effect of plant extracts on *Venturia inaequalis* (Cooke) G. Winter has been actively investigated, the effect of essential oils is little-known. This is particularly true to their *in vivo* mode of action, duration of efficacy and rainfastness, which latter highly influence the control efficacy in outdoor conditions.

The effect of the essential oils of thyme (*Thymus vulgaris*) and cinnamon (*Cinnamomum verum*) in different plant – pathogen relationships has been investigated in the Department of Plant Pathology, Corvinus University of Budapest since 2005 (eg. Hochbaum and Nagy, 2013; Horváth *et al.*, 2013; Petróczy *et al.*, 2006).

The objectives of this study were to (i) investigate the effect of thyme, cinnamon and sweet orange oil on the germination of the conidia of *Venturia inaequalis* (Cooke) G. Winter, (ii) reveal *in vivo* mode of action of cinnamon oil against scab on apple seedlings, (iii) assess the length of efficacy of cinnamon oil and (iv) evaluate the effect of rain amount on the efficacy of essential oil treatment.

Tab. 1. Composition (%) of the selected essential oils

Component	RT	LRI	Cinnamon (%)	Thyme (%)	Orange (%)
α-pinene	5.56	938	-	0.67	0.31
camphene	5.95	952	-	1.44	-
sabinene	6.52	976	-	-	0.19
β-myrcene	6.99	995	-	1.47	1.25
α-terpinene	7.79	1018	-	2.15	-
ρ-cymene	8.09	1026	1.77	24.58	-
limonene	8.19	1029	0.86	-	97.64
1,8-cineol	8.38	1034	3.58	6.54	-
γ-terpinene	9.2	1056	-	7.42	-
linalool	10.76	1097	3.65	5.5	0.22
borneol	13.43	1162	-	0.63	-
terpinen-4-ol	13.96	1175	-	0.93	-
α-terpineol	14.55	1189	1.12	-	-
phenylethyl alcohol	14.83	1201	1.64	-	-
thymol	18.81	1290	-	40.64	-
carvacrol	19.2	1300	-	3.6	-
cinnamyl aldehyde	20.8	1351	67.9	-	-
eugenol	21.44	1361	6.83	-	-
geranyl acetate	22.43	1388	-	0.52	-
β-caryophylene	23.68	1420	2.5	3.08	-
methyl trans-cinnamate	24.14	1436	2.09	-	-
ethyl cinnamate	24.2	1440	0.75	-	-
α-humulene	25.07	1454	-	0.32	-
cinnamyl-acetate	26.13	1488	6.62	-	-
caryophylene oxide	30.2	1590	-	0.21	-
benzyl benzoate	35.67	1738	0.33	-	-
Total			99.64	99.7	99.61

Note: RT - Retention time, LRI - Linear retention index relative to C8-C23 n-alkanes on a HP-5 column

Materials and methods

Essential oils and adjuvant

The essential oils of cinnamon (*Cinnamomum verum*) (Aromax Inc.), thyme (*Thymus vulgaris*) (Aromax Inc.) and sweet orange (*Citrus sinensis*) (Naturol Ltd.) were selected for *in vitro* and *in vivo* assays. Chemical composition was determined by GC 6890N Gas Chromatograph equipped with 5975 Inert mass selective detector (Tab. 1). According to previous studies (Hochbaum and Nagy, 2013), Silwet Star adjuvant was added in 0.02% concentration to aqueous essential oil dilutions in order to facilitate the dispersion of the oils. The applied concentration did not influence conidium germination. In the wash-off test the adjuvant was not applied.

Test plants and fungal inoculum

Apple seedlings with 4-6 fully expanded leaves were used for *in vivo* assays. The seedlings were produced from seeds of apple cultivars 'Gala' and 'Jonagold'. Plants were grown in a light-room at 18-24 °C with 12 h photoperiod in vegetable trays and were later transferred into individual pots.

Diseased apple leaves were collected from non-treated orchards and home gardens from different cultivars. Leaves were stored in paper bags in refrigerator (4 °C) until inoculation. Conidia were harvested from the leaves by a small brush into sterile distilled water to obtain conidial suspension of a mixed population. Inoculum concentration was measured by haemocytometer.

In vitro *assay of the inhibition of conidial germination*

Two-fold dilutions of essential oils dissolved either in water or in 0.1% apple leaf ('Jonagold') brew were prepared. Oil dispersion was facilitated by Silwet Star (0.02%). 60-60 µl of conidium suspensions (7.2×10^5 conidia/ml) was measured into the wells of single concave microscope slides. 60-60 µl essential oil suspensions were added to the conidium suspensions. Final concentrations of essential oils were 0.001%, 0.01% and 0.1%. The wells were covered by cover slips to prevent evaporation of the oils. Conidia suspensions were incubated on 20°C in the dark. Germination was assessed microscopically after 96 h by counting 3 × 30 spores per treatment and compared to the germination in water, in apple leaf brew and in 0.02% Silwet Star solution. Conidia were considered germinated when the germ tube was at least twice as long as the one of the conidia. The trial was performed in triplicates. Efficacy of essential oils was calculated by Abbott's formula (Abbott, 1925).

In vivo plant assays

The essential oil of cinnamon was selected for plant assays. According to our earlier *in vitro* studies (Cseh *et al.*, 2014), the essential oil of thyme could be harmful to plants under forced conditions. Orange oil does not show significant *in vivo* inhibition against *Venturia inaequalis* (Cooke) G. Winter in ongoing orchard trials.

Assessment of preventive and curative activity of cinnamon oil

The experiment was carried out in a greenhouse during the winter of 2013-2014. Potted 'Gala' seedlings were placed into plastic boxes. Plants were kept between 10 °C and 27 °C under natural photoperiod supplemented with 12 h artificial light. Relative humidity ranged between 70% and 97%. Artificial inoculation was performed by spraying conidium suspension ($4.9\text{-}5.4 \times 10^5$ conidia/ml) onto the upper and lower side of the leaves. Seedlings were sprayed with 0.2% cinnamon oil diluted in 0.02% Silwet Star 72 h, 24 h and 1 h prior to inoculation as protective treatments and 24 h or 72 h after inoculation as curative treatments. Conidium and essential oil suspensions were sprayed onto the plants by hand sprayer until "run-off". After inoculation, relative humidity was maintained above 90% for three days by covering boxes by a plastic lid in order to facilitate conidial germination. Evaluation was carried out on the 14th and 21st days after inoculation by the assessment of 30 leaves per treatment in six blocks according to their infected leaf area. Inhibition of essential oil treatments was compared to inoculated control plants.

Wash-off study of cinnamon oil

The experiment was carried out in a growth room (14-16 °C, 12 h photoperiod, 80-95% relative humidity). Potted 'Jonagold' seedlings were sprayed until "run-off" by a single-nozzled hand sprayer with 0.2% cinnamon oil diluted in distilled water without adjuvant. In order to ensure dilution homogeneity sprayer was being shaken continuously. Treated plants were left to dry for 4 hours (15 °C, 80% RH) before rainfall simulation. To assess the effect of natural rainfall on the rainfastness of the essential oil different quantity of distilled water was sprayed onto the seedlings for simulating light (1 mm), moderate (3 mm) and heavy (5 mm) rainfall. Treated plants were left to dry for 4 hours (15 °C, 80% RH) before artificial inoculation. Untreated, no-water-exposed (0 mm) and water-exposed seedlings were inoculated with conidium suspension (4.9×10^5 conidia/ml) in the same manner as described earlier. Evaluation of rainfastness was carried out on the 14th and 21st days by the assessment of 30 leaves per treatment in six blocks according to their infected leaf area.

Statistical analysis

In order to normalize the data and homogenize the variances, in the case of the rate of germinated conidia, arcsin transformation; in the cases of infected leaf area data, $\ln(x+0.0001)$ transformation were used. Normality was proved by skewness and kurtosis (Tabachnick and Fidell, 2006). The effect of essential oil treatment on conidial germination was evaluated by univariate ANOVA model having fixed factors as treatments (essential oil types cinnamon, thyme and orange in concentration of 0.001%, 0.01% and 0.1%, Silwet Star and Untreated control) as well as germination media (water, apple leaf brew). The preventive and curative effect of cinnamon oil was evaluated by univariate ANOVA with random block design having fixed factors as treatments (Control, 1 h, 24 h, 72 h or Control, 24 h, 72 h, respectively) as well as the length of time period after inoculation (days 14 and 21) and blocks as different plants. The effect of cinnamon oil under simulated rainfall was evaluated by univariate ANOVA with random block design having fixed factors as treatments (Control, 0 mm rain, 1 mm rain, 3 mm rain and 5 mm rain) as well as time period after inoculation (days 14 and 21) and blocks as different plants. Homogeneity of variances and the normality of the residuals of the ANOVA models run with the transformed data was yet slightly violated, however, considering the homogeneity of sample sizes and the robustness of ANOVA in case the ratio of maximal and minimal variances is under 2, we accepted the results as reliable. In cases of significant factor effects Games-Howell's post hoc test was run.

Results and discussion

Effect on conidial germination

Germination was more intensive in water than in apple leaf brew, however, similar tendency was observed in the inhibition of the germination by the essential oils in both types of applied germination media. In the lowest applied concentration (0.001%), none of the essential oils suppressed germination significantly in comparison with Silwet Star control. Inhibition level was more remarkable in 0.01% concentration. Thyme and sweet orange suppressed germination in apple brew significantly at 94.3% and 82.9% efficacy, respectively. In water, only the inhibition of thyme (96.0% of efficacy) proved to be significant. In 0.1% concentrations, all essential oils suppressed germination in apple brew effectively ($p<0.05$) at 98.9-100.0% efficacy, however, cinnamon oil did not show significant inhibition in water. Both the effects of essential oil treatment and germination media were significant on conidial germination ($F_{tr}(10;44)=46.35$ with $p<0.001$; $F_{med}(1;44)=12.84$ with $p<0.001$, resp.). The interaction was also significant ($F_{tr*med}(10;44)=2.71$ with $p<0.05$). The treatment effect size was high, meanwhile the media effect size was low (partial $\eta^2=0.91$ and 0.27, resp.). The observed power was above 0.9 in all cases (Fig. 1).

All tested essential oils suppressed conidial germination effectively. The highest inhibition level was achieved with thyme oil. Bálint *et al.* (2014) and Theisz *et al.* (2007) found thyme extracts effective as well. The high contents of phenolic monoterpens, especially thymol (40.6%) and its precursore p-cymene (24.6%), might be responsible for the antifungal effect of thyme oil (Gong *et al.*, 2009), however, the synergistic effect of minor oil components might also contribute to the antifungal activity (Zambonelli *et al.*, 2004). According to Tan *et al.* (2007), the high content of limonene (97.6%) might have caused the good inhibitory effect of orange oil.

In apple leaf brew the germination rates of conidia were lower than in water in most treatment, which could be explained by the accumulation of antifungal substances in old apple leaves (Agrios, 2005).

Preventive and curative activity of cinnamon oil

The preventive and curative applications of 0.02% cinnamon oil decreased the disease severity on inoculated plants significantly compared to inoculated control plants. Oil application 1 h or 24 h before inoculation showed almost complete inhibition (100 to 99% efficacy) by the first while an outstanding inhibition (91 to 92% efficacy) by the second evaluation. Cinnamon oil applied 72 h before inoculation decreased infection level at a rate of 65% (first evaluation) and 51% (second evaluation) efficacy.

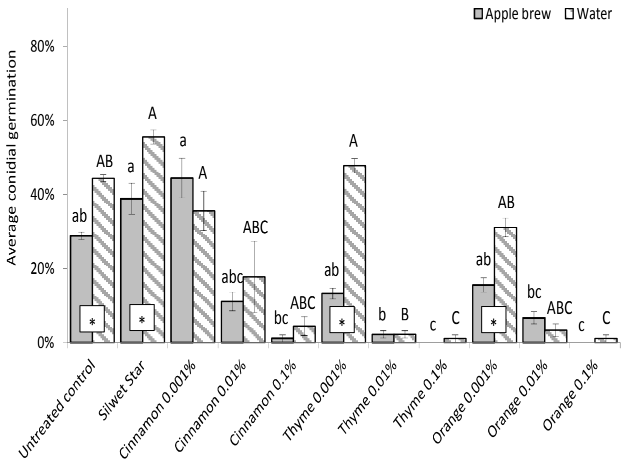

Fig. 1. Mean and standard deviation of conidium germination (%) of *Venturia inaequalis* (Cooke) G. Winter in 0.05% apple brew and in water under different treatments . Different letters are for significantly different groups (apple leaf brew: lower case; water: upper case; $p<0.05$; Games Howell's test). Asterisk is for significantly different types of media per treatment level.

Curative application of cinnamon showed good disease control even when applied 72 h after inoculation. At the first evaluation, efficacy ranged from 97% (24 h curative application) to 83% (72 h curative application). By the second evaluation, efficacy of cinnamon almost remained the same (96.8%) when applied 24 h after inoculation and decreased by 20% when applied 72 h after inoculation.

Both the effects of preventive and curative application were significant (preventive: $F_{tr}(3;165)=37.03$ with $p<0.001$; curative: $F_{tr}(2;123)=65.13$ with $p<0.001$) while both the effect of time length after inoculation and the interaction were insignificant in both cases (preventive: $F_{time}(1;165)=3.73$ with $p=0.06$; $F_{tr*time}(3;165)=0.04$ with $p=0.99$, resp.; curative: $F_{time}(1;123)=0.093$ with $p=0.76$; $F_{tr*time}(2;123)=0.28$ with $p=0.76$, resp.). The treatment effect size was moderate (partial $\eta^2=0.51$ and 0.40 for preventive and curative treatments, respectively) with observed power above 0.99 (Fig. 2).

Foliar application of cinnamon oil inhibited scab infection on apple seedlings effectively under controlled conditions. Interestingly, curative applications gave similar control efficacy as that of the preventive applications. Since curative activity of active substances is based on the inhibition of mycelial growth rather than spore germination, it can be concluded, that the main compounds of the essential oil (cinnamyl aldehyde (67.9%), cinnamyl-acetate (6.6%) and eugenol (6.8%)) effect both spore germination and mycelial growth (Soylu *et al.*, 2010).

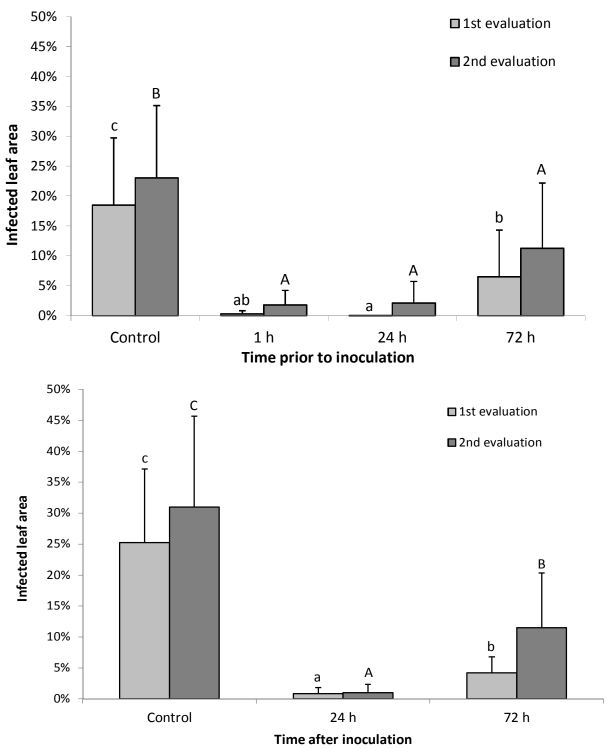

Fig. 2. Mean and standard deviation of infected leaf area (%) of apple leaves affected by preventive (above) and curative (below) treatments of 0.2% cinnamon oil, compared to inoculated untreated control. Different letters are for significantly different groups (1st evaluation: lower case; 2nd evaluation: upper case; $p<0.05$; Games Howell's test).

According to Velluti *et al.* (2004) cinnamyl aldehyde and eugenol are responsible for the antifungal activity. A strong curative effect of cinnamon oil was observed against Fusarium head blight of winter wheat in previous field experiments as well (Horváth *et al.*, 2013; Nagy *et al.*, 2013). However, Berngtsson *et al.* (2006) found curative application of plant extracts less effective.

The oil components eugenol, cinnamyl aldehyde, cinnamyl-acetate belong to phenylpropanoid molecules, which play an important role in plant defense mechanisms (Bhattacharya *et al.*, 2010; Dixon *et al.*, 2002). Therefore, according to Berngtsson *et al.* (2006, 2009), beside direct antifungal activity cinnamon oil may influence plant defense as well.

The application of cinnamon oil within 24 h pre- or post-inoculation resulted in very effective and long lasting disease control. Decent control level was achieved with its application within 72 h, especially by the curative timing (Fig. 3). This observation emphasizes the importance of disease forecasting for outdoor application of essential oils.

Further research is needed for the determination of the translocation of cinnamon oil within the plant.

Rainfastness of cinnamon oil

Without simulated rainfall cinnamon oil inhibited scab infection on apple seedlings effectively. Control efficacy decreased as a result of rain. However, significant difference in disease severity was not observed among the different rain amounts. The effect of treatment was significant ($F_{treatm}(4;149)=5.96$ with $p<0.001$) that is to say, untreated control revealed significantly different results that those of treated ones. Both the effect of time length after inoculation and the interaction were insignificant ($F_{time}(1;149)=1.32$ with $p=0.25$; $F_{treatm*time}(4;149)=0.48$ with $p=0.75$, resp.). The treatment effect size was low (partial $\eta^2=0.14$) with

Fig. 3. Scab symptoms on the leaves of inoculated control plant (above) and that of curatively (72 h) treated plant by cinnamon oil (below) on the 21[th] day

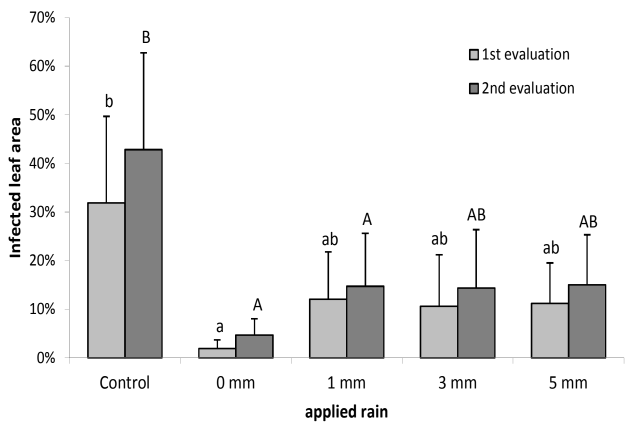

Fig. 4. Mean and standard deviation of infected leaf area (%) of apple leaves affected by cinnamon oil treatment followed by different amount of simulated rainfall, compared to inoculated untreated control. Different letters are for significantly different groups (1[st] evaluation: lower case; 2[nd] evaluation: upper case; $p<0.05$; Games Howell's test).

observed power above 0.98 (Fig. 4).

Although control efficacy decreased by simulated rainfall, the water amounts did not influence control level. It can be concluded that part of the active compounds of cinnamon oil remains on the leaf surface due to their lipophilic properties. However, Pfeiffer *et al.* (2004) found that larger amount of rain decreased the efficacy of plant extracts on apple seedlings more considerably.

In spite of simulated rainfall, oil application resulted in moderate (approximately 65% efficacy) and long-lasting disease control in laboratory conditions.

Conclusions

The tested essential oils, especially *Thymus vulgaris* suppress the conidial germination of *Venturia inaequalis* effectively. Cinnamon oil applied with adjuvant has preventive and strong curative effect against apple scab on plants which could be observed within 3 days prior- or post-inoculation. Although the efficacy of cinnamon oil application decreased by simulated rainfall, a moderate control effect was still exhibited even after the plants were exposed to relatively high amount of rain. The results of this study show that the tested essential oils may represent bases for the development of biofungicides against apple scab. However, further field experiments are needed for the investigation of control efficacy in outdoor growing conditions. Moreover, the translocation of essential oils in plant should also be tested in laboratory.

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