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Essential Oils of Lavender and Fennel for Inhibiting *Tomato Spotted Wilt Virus* in Pepper Plants

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Abstract

Commercial batches of pepper fruits, a variety of the long red fleshy pepper with serious deformities were established in the period 2014-2016. Analyses of spotted and deformed fruits purchased from supermarkets in the summer months were carried out by the serological method ELISA, variant DAS-ELISA and artificial inoculation of test (indicator) plants. The studies showed that the deformities in the pepper fruits were caused mainly by *Tomato spotted wilt virus* (TSWV). The latter is an important viral pathogen of pepper. We tested essential oils from lavender (*Lavandula angustifolia* Mill.) and fennel (*Foeniculum officinale* All. var. *dulce*) by dilutions in concentrations of 1000 ppm, 2000 ppm, 3000 ppm, 5000 ppm and 10000 ppm as a possible means of control against TSWV. The diluted essential oils were mixed with TSWV infectious sap (inoculum) and pepper plants of the variety Sofiyska kapiya were inoculated in each variant. The results showed that lavender essential oil inhibited TSWV *in vitro* in the pepper plants in the following concentrations: 3000 ppm, 5000 ppm and 10000 ppm. Fennel essential oil did not show an inhibiting effect in any of the five concentrations. The initial results suggested that the studies on lavender essential oil as a possible means with an inhibiting effect against TSWV should be continued by an *in vivo* trials.

Key words: TSWV, pepper, antiphytoviral effect, essential oils.

Резюме

В периода 2014-2016 година бяха установени търговски партиди пипер, червена капия, със симптоми на вирусно заболяване - сериозни деформации, влошаващи пазарния вид на плодовете. На напетнените и деформирани плодове, купени през летните месеци от супермаркети бяха проведени анализи чрез серологичния метод ELISA, вариант DAS-ELISA и изкуствена инокулация на тестови (индикаторни) растения. Проучванията показаха, че деформациите на пиперовите плодове са причинени главно от вируса на доматената бронзовост (*Tomato spotted wilt virus,* TSWV). Последният се явява икономически важен вирусен патоген за пипера. Като възможно средство за контрол на заболяването изпитахме етерични масла от лавандула (*Lavandula angustifolia* Mill.) и резене (*Foeniculum officinale* All. var. *dulce*) при разреждания в концентрации 1000 ppm, 2000 ppm, 3000 ppm, 5000 ppm и 10000 ppm. Разредените етерични масла бяха смесени с инфекциозен сок, получен от инфектиран с TSWV растителен материал (инокулум) и за всеки вариант пиперови растения от сорт Софийска капия бяха инокулирани. Резултатите показаха, че етеричното масло от лавандула инхибира TSWV *in vitro* в пиперовите растения при разреждания в следните концентрации: 3000 ppm, 5000 ppmи 10000 ppm. Етеричното масло от резене не показа инхибиращ ефект при използваните концентрации. Тези първоначални резултати показаха, че проучванията за инхибиране на TSWV с етерично масло от лавандула би трябвало да продължат в опит *in vivo*.

Introduction

Tomato spotted wilt virus (TSWV) from *Tospovirus* genus *Bunyaviridae* family causes economically important diseases in tomato and pepper plants, decreasing their yield (Parella *et al.,* 2003). TSWV has a large host range that includes different plant species: vegetables, ornamentals, essential

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oil-bearing, medicinal and wild plants (Parella *et al.,* 2003; Dikova, 2011, 2014a; 2015a).

The use of natural means for the control of the diseases and pests on the plants is a priority in contemporary research work.

Numerous literature sources are available on essential oils or their main compounds acting as an effective means of pest control. They are the so-called "Green pesticides" (Isman, 2000; Koul *et al.,* 2008). Such are the essential oils from carnation, savory, thyme, eucalyptus, basil, cumin, tansy, starry anise, vetiveria, nepeta and many others (Yildirim *et al.,* 2011). Biofungicides based on plant oils and extracts are widely applied in agricultural production. Nowadays, there exist commercial preparations, produced in the international agripharmaceutical net such as Timorex on the basis of tea-tree (*Melaleuca alternifolia*) essential oil, approved for application on the oil-bearing rose (Lambev, 2009). Usually, this property is connected with a powerful anti-microbial and anti-fungal activity (Kokoškova *et al*., 2006; Soylu *et al.,* 2006; Schuster *et al.,* 2010; Rashidi *et al.,* 2012).

Different substances of natural and synthetic origin have been used against viral phytopathogens, but none has a satisfactorily selective influence for the management of plant virus diseases (Allam *et al*.,1979; Verma and Baranwel, 1983; Barakat, 1988; Hansen, 1989; Takanami *et al.,* 1990; Othman *et al.,* 1991; Meyer *et al.*, 1995; Yordanova *et al.,* 1996; Eldougdoug, 1997; Shoman, 2002). There are data on the inhibiting effect of fennel oil on plant viruses when it is used at a dilution of 3000 ppm (Shukla *et al.,* 1989). Essential oil of tea-tree gave good results in the treatment of *Tobacco mosaic virus* in *Nicotiana glutinosa* (Bishop, 1995).Viral symptoms, as lesions caused by *Tobacco mosaic virus* and *Cucumber mosaic virus* , were reduced to 29.2% and 24%, respectively, by mountain savory essential oil (Dunkićet *et al.,* 2010). Extracts of different plants such as *Plectranthus tenuiflorus* had antiphytoviral activity against TSWV attacked *in vitro*. When this extract was mixed with the virus inoculum for three hours it inhibited the local lesion development in the test plant *Chenopodium amaranticolor* by 100% (Othman and Shoman, 2004). The use of essential oils and plant extracts is one of the possible means of achieving a phytoviral inhibiting effect against TSWV. The antiphytoviral effect could be explained with an appointed component, leading in the composition of the essential oil.

Cyclic monoterpenes – α-terpineol, terpinen-4-ol, evgenol, thymol or borneol, and some others, have been determined as the most toxic (Isman, 2000; Koul *et al.,* 2008). Thereby, essential oils are complex mixtures of many components and the final result could be affected by the synergism or the antagonism of the remaining ingredients. The technological factors in the process of distillation could be also affected by the chemical profile of the essential oil and thus by its properties (Schmidt, 2010).

The selection results of the lavender varieties (*Lavandula angustifolia* Mill.) in Bulgaria showed that the chemical composition of the essential oil varied over a large range. The Bulgarian variety "Yubileyna" contains a high level (8-10%) of terpinen-4-ol,–an undesirable component because of the odour of the essential oil, but indicated as one of the most active ingredients with a pesticidal effect (Stanev, 2008). Terpinen-4-ol is the main component of the tea-tree. The essential oils of the tea-tree and lavender have commensurable potency and spectrum of antimicrobial properties (Settineri *et al.,* 2003; Evandri *et al.,* 2005). Lavender specimens with a higher content of terpinen-4-ol, created by selection in Bulgaria, provide raw materials for essential oils – potential natural substances with an antiphytoviral effect. Literature data on fennel essential oil has also encouraged research in this direction.

The objective of the study was to test the inhibiting effect of lavender and fennel essential oils against TSWV by dilutions in different concentrations. Ecologically pure substances were used, as essential oils which could decrease and even prevent the damage to pepper fruits caused by TSWV, and therefore contribute to the bioproduction of this important vegetable crop.

Material and Methods

Essential oil-bearing production

The raw material for the essential oils were plants, representing populations of: *L. angustifolia* Mill. and *Foeniculum officinale* All. var. *dulce*, cultivated in the industrial plantations of the Institute of Roses, Essential and Medical Cultures near Kazanlak, Bulgaria. Essential oils were produced by steam distillation on a semi-industrial installation with capacity of the apparatus 10 dm^2, with the following parameters: Lavender essential oil: speed – 8 - 10%; continuance – 90 min; temperature of the distillate – 30 – 35° C. Fennel essential oil: after grinding of the seeds; speed – 7 – 8%; continuance – 600 min; temperature of the distillate – 40-45° C. Distillates were drained and treated with Na_2SO_4

(sodium sulfate) to remove water. The essential oils were kept in closed glasscontainers without refrigeration. The lavender essential oil was produced in 2008, and the fennel essential oil in 2012.

Methods of TSWV identification

TSWV in red pepper fruits, a variety of the long fleshy pepper, collected and purchased on the Bulgarian market in 2016, was identified by the serological method DAS-ELISA (Clark and Adams, 1977) and by the indicator method according to the reaction of the test plants *C. annuum* cv. Sofiyska kapiya and *Nicotiana tabacum* cv. Samsun NN (Noordam, 1973).

Production of infectious sap containing TSWV

Samples of the eight red pepper fruits with symptoms of yellow spots used as a source of the infectious TSWV inoculum were previously tested by DAS-ELISA (Fig. 1). The infectious inoculum was made by grinding 17 g of raw material from eight red pepper fruits with 25 ml of sodium - potassium phosphate buffer pH – 7.0 with additions of 0.2% ascorbic acid and 0.2% sodium sulphite in a proportion 1:1.5 = g:ml. Thirty-two milliliters of crude infectious sap was prepared by filtering through gauze. Thirty milliliters of the infectious sap were used for mixing with the diluted essential lavender and fennel oils in different concentrations. Two milliliters of the infectious sap were mixed with equal quantities of distilled water, and plants of *C. annuum* cv. Sofiyska kapiya and *Nicotiana tabacum* cv. Samsun NN were inoculated as positive controls of the tests. The mixtures for the inoculums were incubated for 150 min in a refrigerator and per 2 plants of *C. annuum* cv. Sofiyska kapiya for each variant were artificially inoculated.

Dilutions of the essential oils in different concentrations

The dilutions of the essential lavender and fennel oils in definite concentrations and their mixing with infectious sap (inoculum of TSWV) were made according to the following scheme:

1000 ppm (0.1%) – 5 µl in 5 ml distilled water, mixed with 3 ml (60%) infectious sap.

2000 ppm (0.2%) – 10 µl in 5 ml distilled water, mixed with 3 ml (60%) infectious sap.

3000 ppm (0.3%) – 17 µl in 5 ml distilled water, mixed with 3 ml (60%) infectious sap.

5000 ppm (0.5%) – 25 µl in 5 ml distilled water, mixed with 3 ml (60%) infectious sap.

10000 ppm (1%) – 50 µl in 5 ml distilled water, mixed with 3 ml (60%) infectious sap.

DAS-ELISA tests for establishment of the antiviral effect of lavender and fennel oils against TSWV in pepper plants

The pepper plants of each variant treated with the mixtures of diluted essential oils and infectious sap, as well as the samples of the eight red pepper fruits used as a source of infectious inoculum were analyzed by DAS-ELISA (Clark and Adams, 1977) with antiserum for TSWV, purchased from the German company LOEWE, Biochemica. The examinations of the pepper plants for TSWV symptoms were done 18 days (29.08 - 15.09. 2016) and 38 days (29.08. – 05.10.2016 - approximately 40 days) after treatment with infectious saps processed with essential oils. The measurements of the optical density and the accounted extinction values were done 30 minutes after development of the ELISA reaction on a spectrophotometer DTX 880 Detector at a wave length of 405 nm. The average extinction values from the extinctions of both pepper plants for each variant, showing the antiviral effect of the lavender and fennel oils are presented on Figures 6 and 7.

Results and Discussion

The DAS-ELISA results for TSWV existence in the eight fruits of long red fleshy pepper with symptoms are presented in Fig. 1. The viral concentration of TSWV in these fruits was very high. The extinctions over 2.0 OD (optical density) were reached for most of them. A part of these fruits are presented on Board I, Fig. 2. The symptoms of the disease were chlorotic (yellow) rings or concentric spots, as well as chlorotic areas of irregular shapes, occupying a large part of the fruits (Fig. 2). The symptoms of TSWV after artificial inoculation on the leaves of the test (indicator) plants – *N. tabacum* cv. Samsun NN and *C. annuum* cv. Sofiyska kapiya were similar.

The local reaction was latent infection on the inoculated leaves, rarely chlorotic spots that became necrotic. The systemic reaction was expressed as numerous chlorotic spots, turning necrotic. The necrotic spots, appearing on the middle stages of the leaves caused typical "bronzing" Severe mosaic spotting on the apical leaves is a cause of deformation of the leaf laminas (Fig. 3 and 4).

The results on the application of essential oils are presented graphically in Fig. 6 and Fig. 7. We have established an inhibiting effect against TSWV of the lavender oil after comparing charts 6 and 7 by dilutions in concentrations of 3000 ppm, 5000 ppm and 10000 ppm. A symptomless pepper plant (without symptoms of the disease caused by TSWV) is

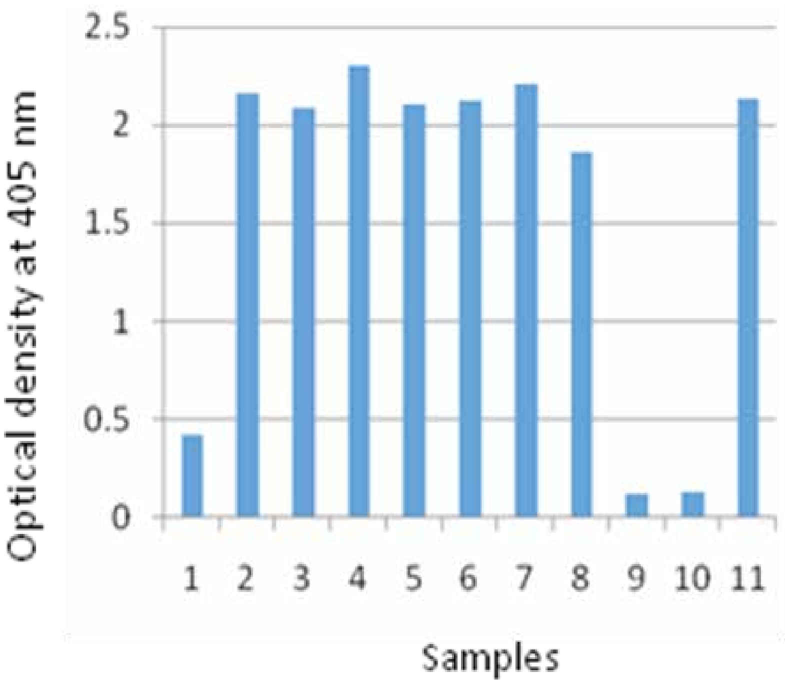

Fig. 1. Establishment of TSWV in fruits of pepper by DAS-ELISA. Samples: from 1 to 8 – red pepper fruits with symptoms of TSWV; 9-Buffer; 10 - Control negative-uninoculated by TSWV (healthy) pepper plant cv. Sofiyska kapiya; 11 - Control positive – infected with TSWV pepper plant cv. Sofiyska kapiya

Board I Symptoms of TSWV on fruits of red pepper and on test (indicator) plants

Fig. 2. Symptoms of TSWV on fruits of commercially purchased red pepper.

Fig. 4. Systemic symptoms of TSWV as chlorotic spots turned necrotic, on the leaves of test plant C. annuum cv. Sofiyska kapiya.

Fig. 3. Systemic symptoms of TSWV as chlorotic spots turned necrotic, on the leaves of test plant species *Nicotiana tabacum* cv. Samsun NN.

Fig. 5. Lack of symptoms of TSWV on test plant C. annuum cv. Sofiyska kapiya, inoculated with infectious sap, mixed with lavender essential oil diluted in a concentration of 3000 ppm

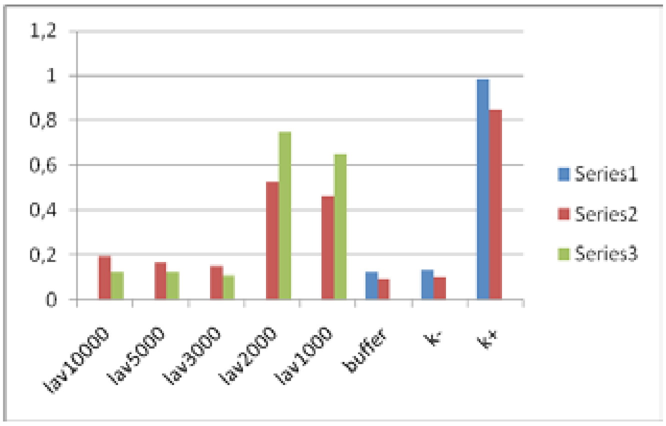

Fig. 6. Results from DAS-ELISA show the effect of LAVENDER essential oil against TSWV in *C. annuum* cv. Sofiyska kapiya

Series 1 (in blue)- **Extinction values for controls** (buffer, k-, k+): Buffer-solution for grinding the plant samples; Negative controls (k-) – pepper plants uninfected with TSWV; Positive controls (k+) – pepper plants infected with TSWV

Series 2 (in red) –**Average extinction values after 18 days** for the pepper plants infected with TSWV in variants of the mixtures of the infectious sap with diluted lavender essential oil in different concentrations.

Series 3 (in green) - **Average extinction values after 38 days** for the pepper plants infected with TSWV in variants of the mixtures of the infectious sap with diluted lavender essential oil in different concentrations.

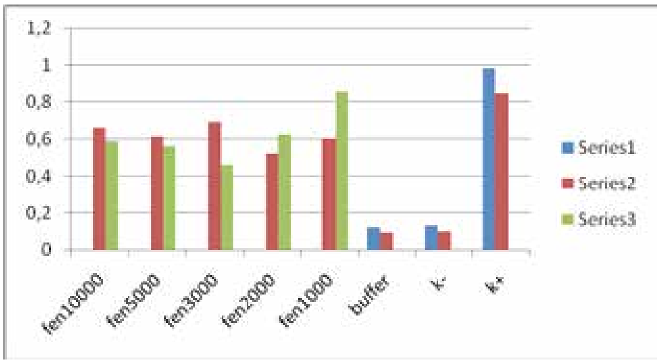

Fig. 7. Results from DAS-ELISA show the effect of FENNEL essential oil against TSWV in *C. annuum* cv. Sofiyska kapiya

Series 1 (in blue) - **Extinction values for controls** (buffer, k-, k+) –: Buffer-dilution for grinding of the plant samples; Negative controls (k-) – pepper plants uninfected with TSWV; Positive controls (k+) – pepper plants infected with TSWV

Series 2 (in red) –**Average extinction values after 18 days** for the pepper plants infected with TSWV in variants of the mixtures of the infectious sap with diluted fennel essential oil in different concentrations.

Series3 (in green) - **Average extinction values after 38 days** for the pepper plants infected with TSWV in variants of the mixtures of the infectious sap with diluted fennel essential oil in different concentrations.

shown in Fig. 5 of Board I. This plant was inoculated with infectious sap mixed with lavender essential oil diluted in a concentration of 3000 ppm. The extinction values for the DAS-ELISA tested samples from that pepper plant were: 0.148 OD after 18 days and 0.099 OD after 38 days (Table 1). Similar and the nearest to the negative controls were the extinctions for the pepper plants in dilutions of the lavender essential oil of 5000 ppm and 10000 ppm (Table 1).

The comparison of the average extinction values obtained from the extinctions of both pepper plants for variants 3000 ppm, 5000 ppm and 10000 ppm showed the same tendency (Table 1). The average extinction value for the plants for dilution in a concentration of 3000 ppm was the lowest in both tests: after 18 days (0.151 OD) and after 38 days (0.107 OD) in comparison with the extinctions for concentrations 5000 ppm and 10000 ppm. The inhibiting effect of the lavender essential oil against TSWV increased at dilution in a concentration of 3000 ppm, 5000 ppm and 10000 ppm for the pepper plants in the tests after 38 days from the inoculation in comparison with the tests after 18 days (Table 1).The concentration of the dilution of 3000 ppm was advisable with a view to the consumption of the lavender essential oil. The dilutions of lavender essential oil in concentrations of 2000 ppm and 1000 ppm did not contribute to the inhibition of the TSWV infected sap (Table 1 and Fig. 6). No inhibiting effect against TSWV in the pepper plants was established for the dilutions of the fennel essential oil in concentrations of 1000 ppm, 2000 ppm, 3000 ppm, 5000 ppm and 10000 ppm (Table 1 and Fig. 7).

The results on the inhibiting effect against TSWV should be connected by the compositions of the essential oils. The lavender essential oil represented a mixture of terpenehydrocarbons and their derivatives. It complies with the ISO 3515:2002 standard and has time 3 years shelf life. The analysis of the lavender essential oil used in this trial showed that the chemical profile was typical and significant deviations from the standard were absent (Shellie *et al.,* 2000). The basic compounds over 1% were: linalool – 30.5%; linalyl acetate – 22.0%; ß-cariophyllene -6.5%; farnesene – 6.4%, ß-ocimene – 5.8%; lavandulyle acetate – 4.4%; α – terpineol – 3.8%; 3-octanone – 2.3% and terpinen-4-ol – 1.8%. An exception was the ester linalyl acetate, which was out of the norm for lavender essential oil originating from Bulgaria (30 – 42%), but was near the lower limit for this essential oil

Table 1. Results from DAS-ELISA for pepper plants (*C. annuum* cv. Sofiyska kapiya), treated with mixtures of essential oils diluted in distilled water and TSWV infectious sap.

Pepper plants	10000 ppm	5000 ppm	3000 ppm	2000 ppm	1000 ppm
First, Lav. after 18 days	0.198	0.171	0.153	0.499	0.555
First, Lav. after 38 days	0.118	0.134	0.114	0.749	0.759
Sec. Lav. after 18 days	0.185	0.160	0.148	0.557	0.368
Sec. Lav. after 38 days	0.126	0.107	0.099	●	0.547
First, Fen. after 18 days	0.783	0.643	0.660	0.563	0.585
First, Fen. after 38 days	0.571	0.581	0.456	0.661	0.812
Sec. Fen. after 18 days	0.541	0.583	0.722	0.476	0.626
Sec. Fen. after 38 days	0.607	0.547	0.464	0.588	0.910
Average Lav. after 18 days	0.192	0.166	0.151	0.528	0.462
Average Lav. after 38 days	0.122	0.121	0.107	0.749	0.653
Average Fen. after 18 days	0.662	0.613	0.691	0.520	0.606
Average Fen. after 38 days	0.589	0.564	0.460	0.625	0.861

Negative control (uninoculated, symptomless pepper plant: 0.195 OD after 18 days and 0.101 OD after 38 days.
Buffer – 0.137 OD after 18 days and 0.092 OD after 38 days.
● – lack of data because of loss of pepper plant.

originating from France (25 – 45%). The component was unstable and its dissociation led to an increase in the quantity of free linalool. It was the latter that was connected with the antimicrobial activity of lavender essential oil (Perrucci *et al.,* 1996). Probably a synergism was achieved, giving the accounted antiphytoviral effect against TSWV in combination with terpinen-4-ol and the reactive ketons (camphor, 3-octanon and cripton).

The composition of the fennel essential oil was the following (compounds over 1%): trans-anetol - 59%; lymonen - 15,1%; fenchon - 12,8%; α-pinen - 2,7%; β felandren - 2,5%; p-zimen - 2,3% and methylhavikol - 0,9%. The quantity of trans-anetol was only slightly under the usual quantity for sweet fennel (65 – 91%) and inclined to the data for bitter fennel (55 – 70%), but fenchon, the component giving bitterness, was in the established limits for *F. officinale* var. *dulce* (1 – 12%). It is difficult to make an analogy with the results of Shukla *et al.* (1989) about the inhibiting effect of fennel essential oil against *Potato virus X* (PVX), *Tobacco mosaic virus* (TMV) and *Tobacco ring spot virus* (TRSV). The composition of their essential oil is not commented and the viruses and test plants are different. The potentialities of fennel essential oil used in our trial should be optimized by testing more concentrations of dilutions.

Our results showed that the concentration of diluted lavender essential oil should not be lower than 3000 ppm. The inhibiting effect proven in previous research on essential oils of lavender, fennel and oregano against TSWV depends on the chemical composition of the essential oils, the concentrations of dilution, the conditions of storage and the species of the infected test plants (Dikova, 2014b; 2015b). Fennel essential oil has been shown to have the same inhibiting effect against TSWV as lavender essential oil, when as the test plants used belonged to the species *Chenopodium quinoa* and *Petunia hybrida* (Dikova, 2014b).

Conclusion

The results of the *in vitro* trial allow us to recommend a dilution of the lavender essential oil, produced in IREMC in 2008, in a concentration of 3000 ppm for inhibition of TSWV in pepper plants. These initial results should encourage further studies against the important pathogen - TSWV *in vivo* in glasshouse or field conditions. The amount of lavender essential oil in water dilution of 10 liters should be 34 ml, and in 100 liters – 340 ml. The viral pathogen TSWV can be transmitted by thrips, as insect vector in nature. The influence of lavender essential oil could be studied also for possible thrips control in a *in vivo* trial. The *in vivo* trials by spraying the pepper plants with lavender essential oil diluted in a concentration of 3000 ppm must show its real use in practice as a biopesticide. More and different concentrations of dilution of fennel essential oil should be studied for a possible inhibiting effect against TSWV in pepper plants.

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