Studiy for inhibition of *Tomato spotted wilt virus* by ecologically pure product – extract of *Leuzea carthamoides*

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Abstract
The virus diseases cause yearly losses in the quantity and quality of the production of pepper. Ecologically clean means safe for human health that can inhibit plant viruses are necessary for keeping of clean environments and healthy people. Such means are extracts from medicinal plants, essential oils and others. The object of present study is leuzea - maral root [*Leuzea carthamoides* (Willd) DC]. Dried powdered root material from Leuzea was extracted with 80% methanol by maceration. After evaporation of the solvent the crude extract was subjected to subsequent analysis. The inhibiting effect of the extract of *L. carthamoides* was studied on pepper against economically important virus for vegetables, as *Tomato spotted wilt virus* (TSWV). The tests were carried out 2018 and 2019 in different concentrations of the diluted with distilled water extract – 1000 ppm, 5000 ppm, 10000 ppm, 15000 ppm и 20000 ppm by using immune enzyme method ELISA, variant DAS-ELISA. The results showed, that the dilution of the *L. carthamoides* extract in concentration 20000 ppm in the trials „in vitro" is suitable for use, because the extinction values for TSWV were negative i.e. inhibiting effect against TSWV was established. The tests „in vivo" in 2019 showed also 20000 ppm was a concentration with a good inhibiting effect against TSWV without one phytotoxic effect on the pepper plants. Metabolic profile of studied extract was analyzed by GC-MS. A variety of fatty, organic and phenolic acids, alkanes, fatty alcohols, mono- and disaccharides were identified. Among fatty acids palmitic acid (C16:0) and linoleic acid (C18:2) were the most abundant. Chlorgenic, syringic, quinic and caffeic acids were found as free phenolic acids. Additionally after alkaline hydrolysis of methanolic extract vanilic, protocatechuic and (p) hydroxycinnamic acids were established.

Keywords: *Leuzea carthamoides*; antiphytoviral effect; TSWV; pepper; GC-MS, phenolic acids

INTRODUCTION

Virus diseases decreased quantitatively the yield and deteriorated quality of the pepper fruit production. *Tomato spotted wilt virus* (TSWV) is one of ten most wide spread plant viruses in the world and it is a pathogen except for vegetables and for many other cultures. TSWV causes economically important diseases in tomato and pepper plants (Parella et al., 2003; Dikova, 2014, 2018, 2019). TSWV is thrips transmitted plant virus. Its vectors are thrips belonging to different species: *Frankliniella occidentalis*, *Frankliniella intonsa* and *Thrips tabaci* (Teulon & Nielsen, 2005).

The use of natural means for the control of the diseases and pests on the plants is a priority in contemporary research work. Different substances of natural and synthetic origin have been studied against viral phytopathogens, but none has a satisfactorily antiviral effect against plant virus diseases (Bishop,

1995; Yordanova et al., 1996; Shoman, 2002). 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 & Shoman, 2004). Chlorogenic, quinic and caffeic acids have been shown to exhibit antiviral properties (Wang et al., 2009; Ikeda et al., 2011; Li et al., 2013).

Leuzea [Maral root, *Leuzea carthamoides* (Willd) DC or *Rhaponticum carthamoides* (Willd) Iljin] is medicinal plant species, used as a stimulant of the immune system and vitality. The use of medicinal plant extracts, as ecologically appropriate means for inhibit of plant viruses is necessary for decrease the disease losses on vegetable cultures and simultaneously use of clean plant production with a view to protect the human health. *Leuzea carthamoides* extract was studied as eventual mean with antiphytoviral properties.

The objective of the research was to establish the influence of the *Leuzea carthamoides* extract on plant viral pathogen as *Tomato spotted wilt virus* (TSWV) and its possibility to inhibit viral infection.

MATERIAL AND METHODS

Plant material

The roots of *Leuzea carthamoides* was collected from cultivated area of the Institute of Roses, Essential and Medicinal Cultures (IREMC) near Kazanlak, Bulgaria

Extraction of plant material

Air-dried, ground plant material of *L. carthamoides* was extracted with methanol by classical maceration for 24 h three times. After evaporation of the solvent, the resulting crude extract was subjected to further analyzes.

Preparation of trials "in vitro" and "in vivo".

The trials " in vitro" were carried out in 2018 and 2019 in a glasshouse of the Department of Plant Protection, Institute of Soil Science, Agrotechnologies and Plant Protection (ISSAPP) „Nikola Poushkarov" in iron containers with length: width: height = 50: 50: 20. Per six plants of *Capsicum annuum* cv. Sofiyska kapia were planted in each container in sterilized soil (Fig.1a). Five variants of the "in vitro" trial were pepper plants infected with infectious TSWV inoculum, mixed with *L. carthamoides* extract, diluted with distilled water in the following concentrations: 1000 ppm, 5000 ppm, 10000 ppm, 15000 ppm and 20000 ppm. There were variants with only 20000 ppm concentration of the Leuzea extract without infectious TSWV inoculum and positive control – inoculated pepper plants with infectious sap, mixed with equal quantitiy distilled water and negative control – uninoculated (healthy) pepper plants. The manner of the tnoculation in the variants "in vitro" was mechanical inoculation i.e. hand inoculation of the solutions (diluted Leuzea extract in different concentrations with mixing or without mixing with infectious sap) with forefinger on the pepper leaves (Fig.1 a). The trial "in vivo" was carried out on soil area inside of glasshouse and the manner of inoculation was by spray of the solutions with sprayer. (Fig. 1 b).

Figure 1a

Figure 1b

Figures 1 a and b. The trials "in vitro" – **(a)** and "in vivo"– **(b)** carried out in glasshouse conditions

Methods of TSWV identification

Tomato spotted wilt virus (TSWV) was identified by the serological method DAS-ELISA (Clark & Adams, 1977) and by the indicator method according to the reaction of the test plants *Capsicum annuum* cv. Sofiyska kapia and *Nicotiana tabacum* cv. Samsun NN (Kovachevsky et al., 1995) in red pepper fruits, a variety long fleshy pepper, collected and purchased on the Bulgarian market in 2018.

Production of infectious sap containing *Tomato spotted wilt virus* (TSWV).

Raw leaf materials from *Nicotiana tabacum* cv. Samsun NN plants with symptoms of yellow concentric spots and line pattern figures were used as a source of the infectious TSWV sap (inoculum). *Nicotiana tabacum* cv. Samsun NN plants were infected by material of the red pepper fruits with symptoms of yellow spots used as a source of the infectious TSWV inoculum and previously tested by DAS-ELISA for TSWV proven. The infectious inoculum was made by grinding of raw material from red pepper fruits with an quantity of sodium - potassium phosphate buffer pH – 7.0 in a proportion 1:1.5 = g : ml. and with additions of 0.2% ascorbic acid and 0.2% sodium sulphite Crude infectious sap was prepared by filtering through gauze. The raw sap from *Nicotiana tabacum* cv. Samsun NN plants was prepared by the same manner as the sap from the red pepper fruits. Five milliliters of the infectious sap were mixed with equal quantities of distilled water, and plants of *Capsicum annuum* cv. Sofiyska kapia and *Nicotiana tabacum* cv. Samsun NN were inoculated as positive controls of the tests. Portions of the leuzea extract with concentrations 1000 ppm, 5000 ppm, 10000 ppm 15000 ppm and 20000 ppm were diluted in distilled water and mixed with equal quantities infectious TSWV sap (infectious inoculum). The mixtures for inoculation the pepper plants were incubated for 150 min in a refrigerator for increasing the influence of the extract against TSWV.

Dilutions of the *Leuzea carthamoides* extract in different concentrations.

The dilutions of the *L. carthamoides* extract in definite concentrations and their mixing with infectious sap (inoculum of TSWV) were made according to the following scheme:

1000 ppm - (0.1%) in 5 ml distilled water, mixed with 5 ml infectious sap.

5000 ppm - (0.5%) in 5 ml distilled water, mixed with 5 ml infectious sap.

10000 ppm - (1%) in 5 ml distilled water, mixed with 5 ml infectious sap.

15000 ppm - (1.5%) in 5 ml distilled water, mixed with 5 ml infectious sap.

20000 ppm - (2%) in 5 ml distilled water, mixed with 5 ml infectious sap.

DAS-ELISA tests for establishment of the antiviral effect of *L. carthamoides* extract against TSWV in pepper plants.

The pepper plants of each variant treated with the mixtures of diluted *L. carthamoides* extract and infectious sap, as well as the samples of the red pepper fruits used as a source of infectious inoculum of the *Nicotiana tabacum* cv. Samsun NN plants were analyzed by DAS-ELISA (Clark & Adams, 1977) with antiserum for *Tomato spotted wilt virus* (TSWV), purchased from the German company LOEWE, Biochemica. The examinations of the pepper plants for TSWV symptoms were done 14 - 19 days after treatment with infectious saps processed with diluted *L. carthamoides* extract. The ELISA tests were carried out after 20 days. The measurements of the optical density (OD) and the accounted extinction values were done 60 minutes after development of the ELISA reaction on a spectrophotometer SUMAL PE, Carl Zeiss, Jena, Germany at a wave length of 405 nm. The average extinction values from the extinctions of several pepper plants for each variant, showing the antiviral effect of the *L. cartha*moides extract are presented on Figures 4, 5 and 6.

Gas Chromatography-Mass Spectroscopy (GC–MS) analysis.

The methanolic extract (50 mg)_was processed as described by Berkov et al., 2017. The obtained fractions were silylated with 50 μL of N,O-bis-(trimethylsilyl)trifluoro-acetamide (BSTFA) in 50 μL of pyridine for 2 h at 50°C.

The GC–MS spectra were recorded on a Termo Scientific Focus GC coupled with Termo Scientific DSQ mass detector operating in EI mode at 70 eV. ADB-5MS column (30 m x 0.25 mm x 0.25 μm) was used. The temperature program was: 100-180 ^{o}C at 15 ^{o}C x min^{-1}, 180-300 20 at 5 ^{o}C x min^{-1} and 10 min hold at 300 ^{o}C. The injector temperature was 250 ^{o}C. The flow rate of carrier gas (Helium)

was 0.8 mL x min^{-1}. The split ratio 1:10 1 µL of the solution was injected. The metabolites were identified as TMSi derivatives comparing their mass spectra and Kovats Indexes (RI) with those of an on-line available plant specific database. The measured mass spectra were deconvoluted by the Automated Mass Spectral Deconvolution and Identification System (AMDIS), before comparison with the databases. RI of the compounds were recorded with standard n-hydrocarbon calibration mixture (C9-C36) (Restek, Cat no. 31614, supplied by Teknokroma, Spain) using AMDIS 3.6 software.

RESULTS AND DISCUSSION

Tomato spotted wilt virus (TSWV) is the most important viral pathogen, established in pepper fruits (Dikova, 2018). TSWV was established, as the basic and most widespread and as an agent responsible for the injuries on pepper fruits (Fig. 2 a and Fig. 2 b).

The reaction of TSWV on indicator (test) plants was very important for both trials -"in vitro" and " in vivo", because TSWV would be proven except by DAS-ELISA and by the certain symptoms on the leaves (Figures 3 a and b).

Figure 2 a

Figure 2 b

Figures 2 a and b. Symptoms of chlorotic spots, caused by TSWV on long fleshy pepper fruits with some injuries, as curve.

Figure 3 a. Symptoms of TSWV on N. tabacum cv. Samsun NN

Figure 3 b. Symptoms of TSWV on C. annuum cv. Sofiyska kapia

Figures 3 a and b. Symptoms of TSWV on indicator (test) plants, used as a source of infectious inoculum in the trials "in vitro" and "in vivo" and used as test plants for the influence of *L.carthamoides* extract.

The results from the DAS-ELISA tests from 2018 with the trials "in vitro" have shown that the concentration of the *L. carthamoides* extract 20000 ppm was suitable for use, because an inhibiting antiviral effect was established (Fig. 4). The extinction values for TSWV in the concentration 20000 ppm of the *L. carthamoides* extract are negative in comparison with the negative control. The extinction values for TSWV in the concentrations 1000 ppm, 5000ppm and 10000 ppm were determinately positive. The extinction values for TSWV in the concentration 15000 ppm of the *L. carthamoides* extract were equalized for the individual pepper plants, but were higher in comparison with the extinctions for TSWV in 20000 ppm (Fig. 4).

The average extinction values for each variant of the trial "in vitro" in 2018 are independently shown on (Fig. 5). The results from 2018 showed

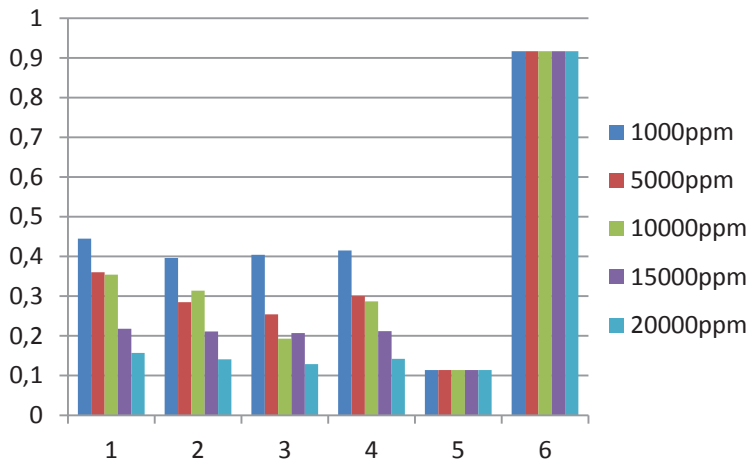

Figure 4. Results from DAS-ELISA tests of the trial "in vitro" in 2018

Legend:

On the abscissa -variants of the trial "in vitro" from 1 to 6. 1, 2 and 3 are data (extinction values) for the individual the pepper plants in each variant of the trial. 4 are average data (extinction values) for the pepper plants in each variant of the trial 5 – negative control; 6 – positive control

On the ordinate – Optical density (OD), presented by the measured extinction values for TSWV.

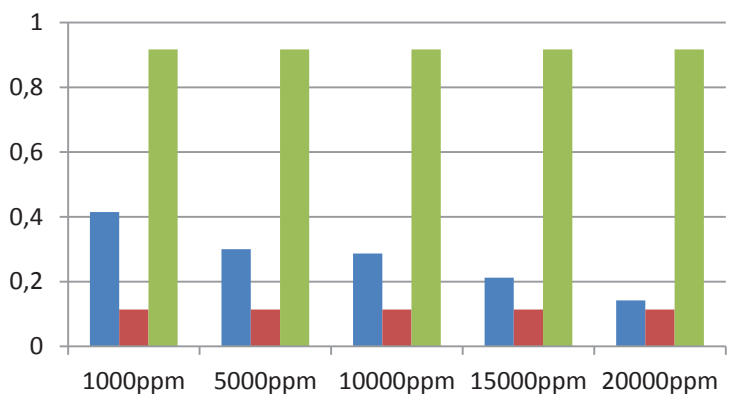

Figure 5. Average results (average extinction values for TSWV from the extinctions of the individual pepper plants) - DAS-ELISA test of the trial "in vitro" in 2018

Legend:

On the abscissa -variants of the trial 1 (blue) – average extinctions for the variants 2 (red) – negative control and 3 (green) – positive control

On the ordinate – Optical density (OD), presented by the measured extinction values for TSWV.

that the average extinction value for the concentration 20000 ppm of the Leuzea extract was near to the negative control and was a sure confirmation for an inhibiting antiviral effect against TSWV.

The results from the trials "in vitro" and "in vivo" in 2019 are presented on Fig. 6. Average extinction values for TSWV from the extinction values for the individual pepper plants in each variant of both trials showed, that the results are similar as established data in 2018. The average extinction values for TSWV in the concentrations 1000 ppm, 5000ppm and 10000 ppm distinguished in trial "in vivo" in comparison with trial "in vitro", but the average extinction values for TSWV in the concentrations 15000 ppm and 20000 ppm were equalized.

The concentration 20000 ppm of the *L. carthamoides* extract - L variant is a variant with spray only of the diluted Leuzea extract without TSWV infectious sap and showed none phytotoxic effect on the pepper plants (Fig. 7).

The Leuzea extract could be used, diluted in high concentrations as 20000 ppm without a phytotoxic effect, whereas some essential oils were given phytotoxic effect in lower concentrations (Dikova et al., 2017).

GC/MS analysis of the extract used in the experiments was performed. The presence of fatty, phenolic and organic acids, fatty alcohols, sterols, mono- and disaccharides was found. Among fatty acids palmitic acid (C16:0) and linoleic acid (C18:2) were the most abundant. Chlorgenic, syringic, quinic and caffeic acids were established as methanol soluble free phenolic acids. Vanilic, protocatechuic and (p)hydroxycinnamic acids were identified as methanol soluble alkaline hydrolysable phenolic acids. Malic, succinic acid glyceric acids were determined among organic acids. Fructose and sucrose were detected as main saccharides in the methanol extract. The phenolic acids are known to have antiviral properties (Wang et al., 2009; Ikeda et al. 2011,

Figure 7. Visual presentation of the absence of a phytotoxic effect of *L. carthamoides* extract-20000 ppm (L variant in trials "in vitro" in 2019)

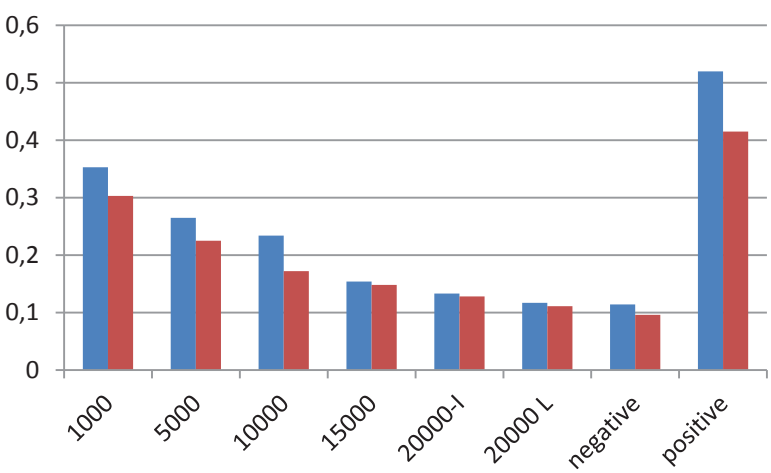

Figure 6. Results from DAS-ELISA tests for TSWV of the trials "in vitro" and "in vivo" in 2019

Legend:

On the abscissa -variants of the trials 1 (blue) – average extinctions for the variants in trial "in vitro" 2 (red) - average extinctions for the variants in trial "in vivo"

On the ordinate – Optical density (OD), presented by the measured extinction values for TSWV.

Li et al., 2013). Further research would show whether they are responsible for the inhibitory effect of the extract on studied virus. The phenolic acids are known to have antiviral properties.

CONCLUSION

The results for concentration 20000 ppm of the *L. carthamoides* extract showed an inhibiting effect against TSWV in both trials "in vitro" and "in vivo" in 2019 and these results were affirmative for the results in 2018 about *L. carthamoides* extract, used for a prevention of the eventual viral infection.

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