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Patentable Formula Based on Essential Oils with a Protective Effect Against *Rhizobium Vitis*

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RESEARCH ARTICLE

Abstract

The crown gall of grapevines is an economic importance bacteriosis caused by *Rhizobium vitis* strains. Five essential oils (EO's) were tested against the autochthonous 2btm strain of *R. vitis* for their capacity to inhibit the phytopathogen growth, by *in vitro* and *in planta* tests. The measurements regarding *in vitro* inhibition zone were performed. Subsequently, the ability of patentable formula based on essential oils to prevent/reduce tumor formation in tomato and vine plants has been tested. After 30 days for tomato plants, the evaluation of disease incidence and effectiveness of treatments applied at two points in time (T_0 and T_{30}), were determined. In vine plants, tumorigenesis is initiated up to 9 months after inoculation, so the results are being processed. Efficacy values of 94.7% at both T_0 and T_{30} and, a very low disease incidence of 5% compared to positive control were recorded. Also, the tumor of EO's treated tomato plants were smaller in size and did not form a complete ring as positive control plants. The use of patentable formula as prevented treatment, reduced the number of plants developing crown gall symptoms and tumor size compared to positive control plants.

Keywords: biocontrol, crown gall, essential oils, *Rhizobium vitis*

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INTRODUCTION

Plants are often exposed to various pathogens that cause diseases determining significant reductions in crop yield and severe production losses worldwide. Crown gall is one of the most important bacteriosis of plants, caused by the bacteria of the *Agrobacterium* genus (Sawada et al., 1993). The tumorigenicity of agrobacteria is determined by the presence of a large plasmid (Ti) carrying tumor-inducing virulence genes. Part of the Ti plasmid (T-DNA) is transferred and integrated into the plant genome during the infection process, leading to an abnormal proliferation of plant cells (Lacroix and Citovsky, 2019; Kawaguchi et al., 2017), (Figure 1). Grapevine is highly susceptible to this disease (Burr et al.,1998), caused mainly by strains of *Agrobacterium vitis*, recently named *Rhizobium vitis* (Mousavi et al.,2014; Mousavi et al.,2015). The bacterium can be spread through asymptomatic and apparently healthy planting material as it systematically survives in grapes. Frostbite and injury are important factors in the infection process, providing a mode of entry for the pathogen. Symptomatic plants are both older vines that usually survive the infection but especially young vines that develop tumors at the grafting point and often die (Süle and Burr, 1998). In both cases, the damage could threaten grape yield and quality in the absence of proper crop

management (Burr et al., 1998)"). Management of crown gall relies on cultural practices to reduce the adverse effects of large-scale lesions if the trunk surface is affected by 50% or more. However, the increasing resistance of microorganisms to traditional chemicals and drugs is a worldwide problem so, the acceleration of research to identify new broad-spectrum biocides is necessary. Also, there are no effective chemical treatments available to combat the disease.

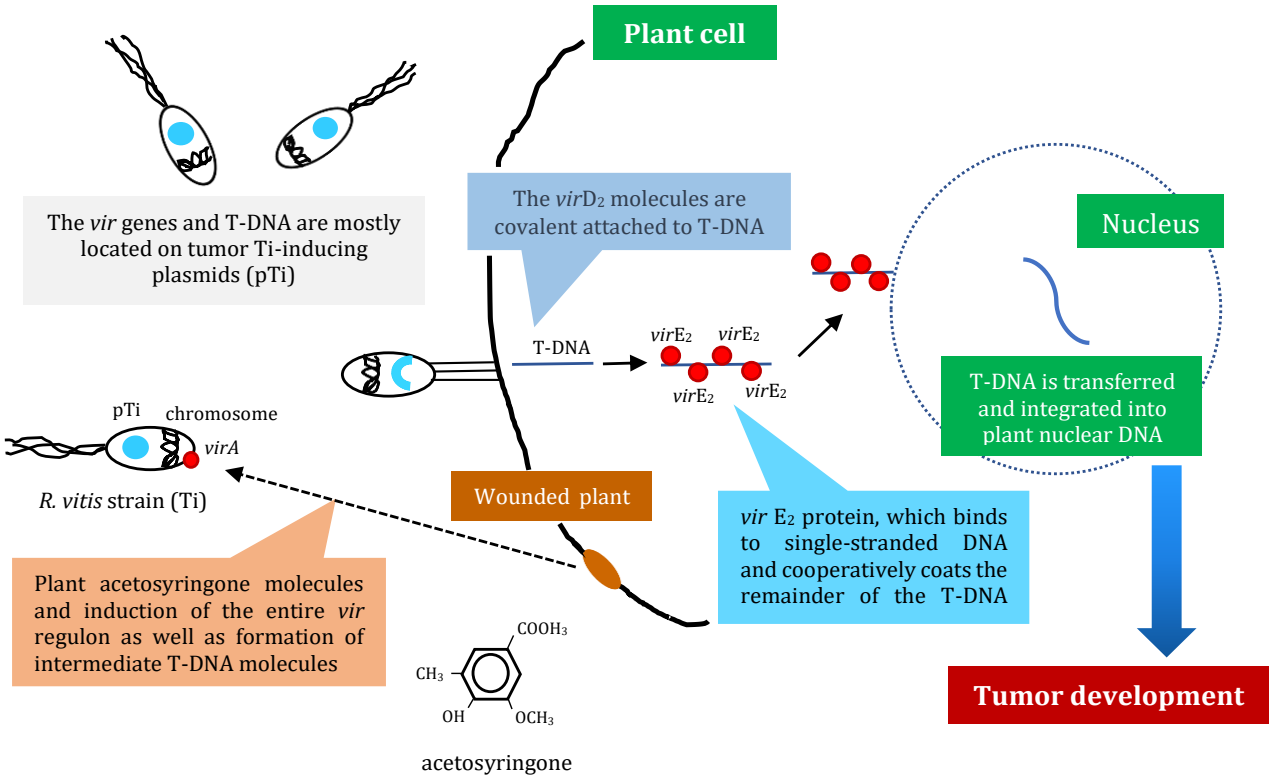

Figure 1. Mechanism of crown gall in plant tissues. Pathogenic strains of *Rhizobium* transfer single-stranded T-DNA and several virulence proteins through a bacterial type IV secretion system into host plant cells (source: Kawaguchi et al., 2017, modified by Dinu).

Until now, prophylaxis against *Rhizobium vitis* has been based on the use of certified plant material, free of any pathogens (Armijo et al., 2016). Since the treatments with copper-based compounds or antibiotics are not sufficiently effective against these bacteria, the current trend is directed towards the use of natural antimicrobial microorganisms or biopesticides, non-toxic and non-polluting, which could be valuable for the control of agricultural diseases (Cantrell et al., 2012). Studies carried out in this regard have shown that the essential oils obtained from medicinal and aromatic plants are effective in limiting the development of various tumorigenic agrobacteria strains, taxonomically classified in different genera (El-Zemity et al., 2008). The action mechanism of essential oils involves the destruction of the cell membrane system of tumor-inducing agrobacteria as a result of the formation of reactive oxygen species (Lee et al., (2020).

The use of plant essential oils with the potential to control phytopathogens including bacteria like *Rhizobium vitis*, would be a promising and ecological alternative for agriculture (Bajpai et al.,2010; Moghaddam et al., 2014; Nazzaro et al.,2013).

The goal of this study was to assess the antibacterial activity of essential oils against *Rhizobium vitis*, by *in vitro* and *in vivo* performed tests.

MATERIAL AND METHOD

In vitro testing of antimicrobial activity of essential oils (EO's)

Bacterial strain and culture conditions

The pathogenic autochthonous strain 2btm of *Rhizobium vitis*, isolated from tumor tissue collected from vine cuttings *cv*. Fetească Neagră was tested. To obtain biomass, the bacterial pathogen was cultivated in liquid YEM

medium (g/L: yeast extract…1 g; mannitol…10 g; KH_2PO_4…0.5 g; $MgSO_4 \cdot 7H_2O$…0.2 g; NaCl…0.1 g; distilled water up to 1000 ml; pH 7.2±0.2), incubated at 28°C, with shaking at 120 rpm, for 72 h. Subsequently, a spectrophotometrically calibrated aqueous suspension, with a density of 10^8 cfu/ml was prepared.

Plant material

The essential oils (EO's), are obtained by distillation method of different plant parts: St. John's wort flowers (*Hypericum perforatum*), garlic cloves (*Allium sativum*), ginger root (*Zingiber officinale*), aerial parts of thyme (*Thymus vulgaris*) and cinnamon bark (*Cinnamomum verum*), known for their effects antibacterial, were purchased as commercial products.

Disc diffusion method

The test was performed on YEM agar medium, in Petri dishes (85 mm diameter). Thus, 50 µL of bacterial inoculum were dispersed on the agar surface using a sterile glass loop. Sterile filter paper discs (5mm diameter) were soaked with 10 µL EO's and placed in the center of the inoculated agar plate. Petri dishes were incubated at 28°C for 48 h. Antimicrobial activity was assessed by measuring EO's inhibition zone (mm) around the disk. Streptomycin sulphate was used as a positive control and distilled water as a negative control. All varians were performed in triplicate.

EO's vapor phase method

A 50 µL sample of 2btm strain of R. *vitis* were inoculated by flooding the YEM agar surface. Squares of 0.5 cm of adhesive sponge were placed on the lid of each Petri dish and, subsequently were impregnated with 10 µL of each EO's tested. The inoculated plates were incubated at 28°C for 48 h. After incubation,the inhibition zone (mm) was measured. Streptomycin sulphate was used as a positive control and distilled water as a negative control. All varians were performed in triplicate.

Statistical analysis.

To determine if there was a statistically significant difference between the essential oil applied variants, in terms of the diameter of the inhibition zone, standard analysis of variance was performed using ARM-9 Software program. Significant differences between means were determined by Duncan's Multiple Range Test and values with $p<0.05$ were considered significantly different.

***In vivo* testing of EO's yield**

Inoculum

Strain 2btm of *Rhizobium vitis* was used for *in planta* test. The bacterial biomass was prepared according to the description mentioned above.

Plant material

The tests were carried out on one-old-month tomato plants (*Lycopersicon esculentum* L.) cv. Siriana, and young vine plants (*Vitis vinifera*) cv. Fetească Neagră sensitive to crown gall. Tomato plants were grown in a protected area, in 12- cm- diameter pots to contain a mixture of Florimo soil and peat (2:1 by volume) and watered daily with tap water.

The patentable formula based on a mixture of cinnamon (*C. verum*) and thyme (*T. vulgaris*) essential oils, has been tested for its ability to prevent/reduce *R. vitis*-induced tumor formation in tomato plants and grapevines.

2.3 *Inoculation method* consisted in decapitating the apical tissue of plants and then, 20 µL of the treatment formula and bacterial suspension respectively, were injected into the injured stem. In tomato plants, the pathogenic inoculum was applied at two points in time (soon after biological treatment application – T_0 and 30 min after the treatment – T_{30}). The wounded sites were covered with sterile cotton pads that were removed after 24 hours. Simultaneously, control samples of untreated and inoculated plants with *R. vitis* strain, as a positive control, and inoculated plants with sterile distilled water, as negative control were done. The treatment variants included 4 replicates of 5 plants each replicate and were arranged randomly.

The observations were accomplished after 30 days, the presence of tumors being visible 3 weeks after inoculation. In vine plants, tumorigenesis is initiated up to 9 months after inoculation as a result, the data are processing.

The inhibition effect (E%) of EO's applied treatments was calculated according to Xue formula (Xue et al.,2009), as follows:

$$E\% = \frac{DIc - DIt}{DIc} \times 100 \quad \text{where,}$$

DIc – disease incidence in positive control and *DIt* – disease incidence in the EO's treatment variant.

Statistical analysis

The data were statistically analysed using ARM-9 Software program. Significant differences between means

were determined by Duncan's Multiple Range Test and values with $p<0.05$ were considered significantly different.

RESULTS AND DISCUSSIONS

In vitro testing of antimicrobial activity of essential oils (EO's)

Essential oils of thyme and cinnamon, tested by *disc diffusion method were* highlighted as an inhibitory effect and the diameter of the lysis zone clearly superior to streptomycin sulphate used as positive control Table 1, Figure 2.

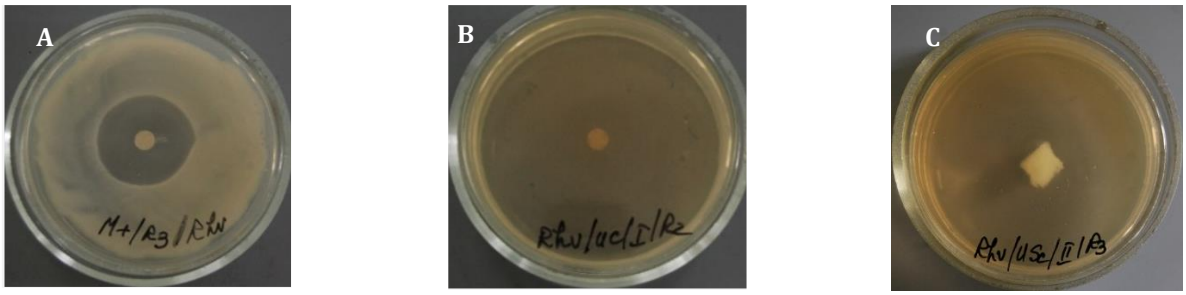

Figure 2. Antibacterial effect of EO's vs. *R. vitis*, compared to streptomycin sulphate (10mg/ml) as positive control. **A**–control; **B**–inhibitory effect of thyme EO by *disc diffusion method*; **C**–inhibitory effect of cinnamon EO by *vapor phase method.*

Smaller inhibition zones and a low inhibitory effect were observed in the treatment variant with garlic essential oil, compared to positive control.

Regarding the EO's bioactivity against *R. vitis* strain by *vapor phase method*, stood out thyme and cinnamon essential oils again, which recorded a total inhibitory effect on the growth of the pathogen. Also, the garlic essential oil induced both smaller lysis zones and a weak inhibition effect compared to the other treatment variants.

Table 1. Inhibitory effects of EO's against *R. vitis* 2btm strain (mm of inhibition zone diameter)

Variant	*Disc diffusion method*	*Vapor phase method*
T. vulgaris	85.0^{a}	85.0^{a}
C. verum	85.0^{a}	85.0^{a}
A. sativum	10.0^{c}	6.0^{c}
Z. officinale	0.0^{d}	0.0^{d}
H. perforatum	0.0^{d}	0.0^{d}
Streptomycin	26.0^{b}	26.0^{b}

Note:Values expressed are mean of three replicates. Means followed by same letter or symbol do not significantly differ ($p<0.05$) according to Duncan's test.

Based on results obtained in the *in vitro* tests, an emulsified composition consisting of a mixture (1:1 volume) of thyme and cinnamon essential oils, supplemented with chitosan, honey and fatty acids was realized, to test the ability to reduce/prevent tumor forming induced by *R. vitis* 2btm strain, in tomato plants and grapevines. It should be mentioned that this composition is the subject of patent application A/00514/25.08.2022, registered at OSIM, Romania.

The protective effect of EO's *in planta*

In vitro antimicrobial activity of EO's was confirmed *in planta* experiments. The use of EO's mixture as preventing treatment reduced not only the number of plants with crown gall symptoms but also the size of tumors compared to positive control plants.

Thus, the effectiveness values of 94.70% both at time T_0 and T_{30} and a very low disease incidence of 5%, compared to positive control were recorded Table 2, Figure 3.

Table 2. EO's treatments efficacy against *R. vitis* on tomato plants

Variant	Number of plants with tumors
V1 – positive control	4.7[a]
V2 – EO's treated plants at T_0 moment	0.2[b]
V3 – EO's treated plants at T_{30} moment	0.2[b]

Note: Values expressed are mean of four replicates. Means followed by same letter or symbol do not significantly differ ($p<0.05$) according to Duncan's test.

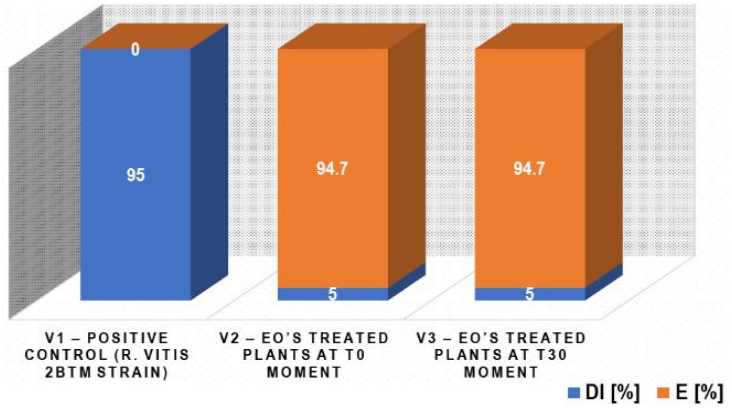

Figure 3. Diagram of the antimicrobial effect of EO's treatment on tomato plants

Also, the tumors of EO's treated tomato plants (figure 4 E, F) were smaller in size and did not form a complete ring as positive control plant (Figure 4 C, D).

Figure 4. Protective activity of based EO's patentable composition *in planta* (tomatoes). **A** – plants before inoculation; **B** - inoculated plants: detail; **C, D** – inoculated plants with 2btm strain of *R. vitis* (positive control); **E** – inoculated plants with *R. vitis* at T_0 time (soon after applied EO's treatment); **F** – inoculated plants with *R. vitis* after 30 min (T_{30}) from applied EO's treatment. The arrows show the presence of tumors on inoculated surface.

The control efficacy of some essential oils against *Agrobacterium* strains has also been reported (Badawy and Abdelgaleil, 2013; Gormez et al.,2015; Mikicinski et al.,2012). It has been scientifically proven that the active components of thyme essential oil, namely thymol and carvacrol, affect the cell membranes system of gram-negative phytopathogenic bacteria such as *Rhizobium* spp. The antimicrobial activity of carvacrol would be due to the presence of a hydroxyl group that transports H^+ ions in the cell cytoplasm and K^+ ions in the opposite direction, disrupting cation exchanges at the external cell membrane level. Also, carvacrol can inhibit the synthesis of flagellin, a protein with a role in bacterial motility, which becomes low. The antimicrobial activity of thymol can cause structural and functional changes that disturb the intern and external cytoplasmic membranes. These compounds have been reported as inhibiting *A. tumefaciens* growth (El-Zemity et al.,2008) and suggested to be effective against *R. vitis* (Altundag et al.,2011).

Cinnamon essential oil mainly contains cinnamaldehyde and eugenol which are components with antifungal and antibacterial properties, active against Gram-positive and Gram-negative bacteria and several fungal phytopathogens (Sanla-Ead et al.,2011; Perdones et al.,2014).

In this study, EO's of *Thymus vulgaris* and *Cinnamomum verum* proved to be effective both *in vitro* and *in planta* experiments, and also reducing the number and size of tumors development *in vivo*. This correlation between *in vitro* and *in vivo* bioactivity of antimicrobial plant components was reported in a study performed with a methanolic crude extract (but not essential oil) of *Schinus terebinthifolius* leaves for *A. tumefaciens* (Ghanney and Rhouma, 2015).

CONCLUSIONS

As a result of this study, essential oils of thyme and cinnamon showed the best *in vitro* antibacterial activity against *R. vitis*, evaluated by both methods, compared to streptomycin as a reference control and the other variants. Also, the *in vivo* use of patentable formula as prophylactic treatment reduced the number of tomato plants developing crown gall symptoms and tumor size compared to positive control plants.

Accordingly, the interactions between essential oils and their active components require more studies to improve agricultural applications, as they are effective, non-toxic and non-polluting products for the control of plant crown gall.

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Conflicts of Interest

The authors declare no conflict of interest.

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