In vitro antifungal activity of the essential oils from *Artemisia* spp. L. on *Sclerotinia sclerotiorum*

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Abstract

Ten *Artemisia* L. taxa grow in different Romanian areas, as spontaneous flora (*A. santonica*, *A. pontica*, *A. annua*, *A. austriaca*, *A. lerchiana*, *A. vulgaris*, *A. scoparia*) and also cultivated species (*A. abrotanum*, *A. dracunculus*, *A. dracunculus var. pilosa*) were chosen as experimental materials. The aim of this study was to investigate the antifungal effects of *Artemisia* spp. essential oils obtained by hydro distillation, against fungal pathogen *Sclerotinia sclerotiorum* (Lib.) de Bary, from carrots roots stored in the refrigerator. The essential oils chemical composition was identified by GC-MS. The inhibition of the fungus mycelium growth was evaluated under *in vitro* conditions at the concentrations of 0, 300, 600, 1200 and 2400 μL L⁻¹. The mycelium growth inhibition depended on species and was directly proportional to the concentration increase of *Artemisia* essential oils. The summarizing results showed that after five days, the minimum inhibitory concentration (MIC) was found to be 2400 μL L⁻¹ for *A. santonica*, *A. pontica*, *A. annua*, *A. austriaca*, *A. dracunculus*, *A. lerchiana*, *A. vulgaris* and *A. vulgaris var. pilosa*. In the case of *A. abrotanum* MIC was 1200 μL L⁻¹, while *A. scoparia* did not totally inhibit the mycelium growth in any of the used concentrations.

Keywords: *Artemisia*, botanical preservative, antifungal activity, soft rot, essential oil, carrot

Introduction

In recent years, the environmental problem caused by excessive use of pesticides has been a major topic in debates conducted for both scientists and public (O. KOUL & al. [1]). Pesticides reduce biodiversity of flora and fauna, their residues are present in all compartments of agro-ecosystems and serious risk to humans is due to consumption of these residues in food as vegetables and fruits (C. PRICE [2]). The use of chemicals for pest control in agricultural products is becoming increasingly restricted by various authorities, and by consumers who prefer technologies which are safe to humans and the environment (S. GAN-MOR & al., [3]). Explicit requirements for food safety and environmental protection have led to the need to create new and safe plant disease control strategies (O. ADEBAYO & al. [4]). Natural products are a suitable alternative to synthetic pesticides as a mean to reduce negative impacts on human health and the environment (S.N. WEGULO & al. [5]). The move toward *green chemistry* processes and the continuing need for developing new crop protection tools with novel modes of action makes discovery and commercialization of natural products as green pesticides an attractive and profitable pursuit that is commanding attention (O. KOUL & al. [1]). In this context, the evaluation of antifungal activity of essential oils extracted from plants, in view of their potential applications as botanical fungicide is a way which is very useful in finding alternatives to synthetic fungicides (A.C. UGO & al. [6]).
Soft rot caused by *Sclerotinia sclerotiorum* (Lib.) de Bary is a polyphagous parasite distributed worldwide in the temperate zones (G.S. SAHARAN & N. METHA [7]), among the most non-specific, global and devastating plant pathogens (L.H. PURDY [8]). During post-harvest, carrot (*Daucus carota* L.) is susceptible to many pathogens attack and the incidence of some physiological disorders (A. GALATI & al. [9]).

From an economic perspective, one of the most important diseases of carrot is caused by the fungus *S. sclerotiorum* (A.J. FOSTER & al. [10]), which causes epidemics in field conditions, and during post-harvest, or during storage (B.G. LEVIS & B. GARROD [11]; C.KORA [12]; C. KORA & al. [13]). Moreover it is one of the fungal pathogens that during mature carrot roots storage may limit the storage life (A. VIKRAM & al. [14]).

Higher plants belonging to the genus *Artemisia* (family Asteraceae), a very large and diverse genus which includes about 500 species mainly found in Asia, Europe and North America (M.J. ABAD & al. [15]) contain important medicinal plants, nowadays routinely subject of interest in terms of phytochemical, biological and chemical diversity, and essential oil production ( M.L. BADEA [16]; L.P. PONOMARENKO & al. [17]).

To our knowledge, in our country, the action of essential oils extracted from various species of *Artemisia* against *S. sclerotiorum* fungus causing soft rot of carrot has not been studied so far. Therefore, the purpose of this experiment was to test the antifungal activity of essential oils extracted from various *Artemisia* spp, collected from Romania, on the fungus *S. sclerotiorum* isolated in the Laboratory of Plant Physiology of the Faculty of Horticulture Bucharest, from the infected carrot roots stored in the fridge.

**Materials and methods**

*Plant materials*

Aerial parts of ten *Artemisia* L. taxa collected from different Romanian areas, as Romanian spontaneous flora (*A. santonica, A. pontica, A. annua, A. austriaca, A. lerchiana, A.vulgaris, A.scoparia*) and also cultivated species (*A. abrotanum, A. dracunculus, A.dracunculus var.pilosa*) during the flowering period of the year 2010 were chosen as experimental materials.

*Extraction of essential oil and their characterization*

As a fresh material, herbals were hydro distillated by using a Singer-Nickerson -type apparatus (H.P. SINGH & al. [18]). Briefly, 250 g fresh plant material was mixed with distilled water (1 L) in a round bottom flask (2 L) fitted with condenser and boiled 3 h. Anhydrous sodium sulfate was used to remove water after extraction. After this time, the clear yellow-colored oil was collected from the nozzle of the condenser and was dried over anhydrous Na$_2$SO$_4$ and preserved in sealed plastic tubes in refrigerator at 4°C. The composition of the essential oils was determined using gas chromatography (GC) and mass spectrometry (GC-MS) analysis. The separation and identification of components was carried out on an Agilent GC, equipped with quadruple MS detector. A capillary column DB-5: (25 m length x 0.25 mm i.d.; 0.25 μm film thickness) was used. The carrier gas was helium; with a linear velocity of 33.2 cm/s. Initial oven temperature was 60°C, then rising to 280 °C at a rate of 4°C /min. The injection and ion source temperatures were 270 and 250 °C, respectively. The injection volume was 1 μL in the splitless mode. The NIST spectra bank was used to identify the volatile compounds, which were verified with the Kovats indices.

*Chemicals*

Potato dextrose agar (PDA) was prepared after A.A. ANWAR [19], by using European Bacteriological Agar (SO.BI.GEL – Hendaye- France). Ethanol, sodium sulfate and Tween 20 were products of Sigma-Aldrich.
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**Fungal culture**

The pytopathogenic fungus *Sclerotinia sclerotiorum* (Lib.) de Bary was isolated from *Daucus carota* L. infected roots, stored in the refrigerator. The stock fungus culture were maintained and grown on Potato Dextrose Agar (PDA) (R.M ATLAS [20]).

**In-vitro antifungal activity test**

The essential oils antifungal activity was carried out by using a contact assay (in vitro), which produces mycelium growth inhibition, as described by E.M. SOYLU & al [21], S.K. SHAHI & M.P. SHAHI [22], M. AMINI & al. [23] and S. MOHammADI & M.H. AMINIFARD [24], by with slight modifications, in Petri dishes (9 cm in diameter) containing PDA. When temperature of the medium (PDA) reached about 45 °C, specific initial concentrations (Control- 0 μL L⁻¹ on simple PDA; 300 μL L⁻¹; 600 μL L⁻¹; 1200 μL L⁻¹ and 2400 μL L⁻¹) of plant essential oils were added to PDA and mixed thoroughly. Different concentrations were prepared by dissolving various amounts of *Artemisia* essential oils in 1 mL of ethanol 98 % (0.5%) and Tween 20 (0.1%). The controls received the same quantity of ethanol and Tween 20 to mixture with PDA and only sterile distilled water. Afterwards, mycelium discs of 5 mm diameter cut from the periphery of 7 days old *S. sclerotiorum* cultures were used to aseptically inoculate the centre of each plate. Inoculated Petri plates were incubated in the dark at 22°C for 5 days. For each treatment and concentration, two replication plates were used. The evaluations of mycelium growth consisted of daily measurements of colony diameter, every 24 hours for five days. Percentage of mycelium growth inhibition (MGI) was calculated after S. JAVED & al. [25] as follows: MGI (%) = (dc-dt) x 100 / dc, where, dc = mycelium growth diameter in control, dt = mycelium growth diameter in essential oil treated Petri dish. Statgraphics software was used to compare the obtained data.

**Results and discussions**

**Composition of the Essential Oils**

The chemical composition of *Artemisia* sp. essential oils used determined by GC-MS analysis emphasized the presence as different major compounds (Table 1) (the entire essential oil composition data are not shown).

The obtained results also can confirm the idea expressed earlier by S. BAYKAN EREL & al. [26], that geographic origin has an important effect on the chemical compositions of *Artemisia* species. For instance, the essential oil isolated from Turkish tarragon (A. *dracunculus*) contained as the predominant components in the oil (Z)-anethole (81.0%) (S. KORDALI & al. [27]), as compared with those from Romania (sabinene – 51.06 %). Also, it had weaker antifungal activity as compared with other *Artemisia* oils tested, possible due to its different major components and chemical composition.

**Table 1.** The major chemical constituent of *Artemisia* spp. essential oils

<table>
<thead>
<tr>
<th><em>Artemisia</em> species</th>
<th>Origin</th>
<th>Compound</th>
<th>Amount (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. santonica</td>
<td>Slănic-Prahova</td>
<td>α - thujone</td>
<td>44.80</td>
</tr>
<tr>
<td>A. pontica</td>
<td>Ulmeni-Teleorman</td>
<td>eucaliptol</td>
<td>62.41</td>
</tr>
<tr>
<td>A. annua</td>
<td>Bucu- Ialomița</td>
<td>artemisia ketone</td>
<td>31.31</td>
</tr>
<tr>
<td>A. austriaca</td>
<td>Corbii Mari- Dâmbovița</td>
<td>camphor</td>
<td>35.75</td>
</tr>
<tr>
<td>A. abrotanum</td>
<td>București</td>
<td>eucalyptol</td>
<td>25.27</td>
</tr>
<tr>
<td>A. dracunculus</td>
<td>București</td>
<td>sabinene</td>
<td>51.06</td>
</tr>
<tr>
<td>A. lerciana</td>
<td>Cap Doloșman -Tulcea</td>
<td>eucaliptol</td>
<td>44.00</td>
</tr>
<tr>
<td>A. vulgaris</td>
<td>Pașcani- Iași</td>
<td>germacrene D</td>
<td>27.69</td>
</tr>
<tr>
<td>A. dracunculus var. pilosa</td>
<td></td>
<td>București</td>
<td>28.29</td>
</tr>
<tr>
<td>A. scoparia</td>
<td>Mahmudia- Tulcea</td>
<td>capillene</td>
<td>66.20</td>
</tr>
</tbody>
</table>
The composition of the essential oil is characterized by significant variation depending on the ecological niche occupied. Additionally, sample cytotype is vital in determining essential oil characteristics. Major components of Russian tarragon are reported to be terpinen-4-ol, sabinene, and elemicin. However, estragole is one of the predominant compounds in French tarragon essential oil, with up to 82% presence (D. OBOLSKY & al. [28]). M. FARZANEH & al. [29] noticed that the major compounds in the essential oil of *A. scoparia* from Iran, Bajestan (Khorasan province) at flowering stage was alpha-thujone (81.7%).

As M. PÂRVU & M.E. PÂRVU [30] noticed, antifungal effects of plant extracts, including those against *S. sclerotiorum* (they did not mention its origin) depend on the pathogenic species, on the type of plant extract and on the content of biologically active compounds. It is well known that after harvest, vegetables are characterized by intense metabolic activity and their quality does not improve. Among the many factors that adversely affect their quality are pathogens attack and saprophytes associating agents (P. NARAYANASAMY [31]. Although many plants belonging to different angiosperm families have been screened for their antifungal activity, *Artemisia* sp. from Romania is reported for its antifungal activity against *S. sclerotiorum* post harvest fungal pathogens of carrots probably for the first time.

Amongst the fungal isolate, *S. sclerotiorum* was found to be highly sensitive to volatile and contact phase of the essential oil, with a minimum fungicidal concentration of the volatile phase of 1.6 µg mL⁻¹ air, and in the contact phase the minimum fungicidal concentration was 6.4 µg mL⁻¹. The major components were identified as sabinene in *A. absinthium* (17.56%), camphor in *A. arborescens* (33.39%), 1,2-dehydroacenaphthylene in *A. campestris* and *A. scoparia* (20.71%) and 11.80%, respectively), and alpha-thujone in *A. vulgaris* and *A. santonicum* (56.13% and 39.46%, respectively). *A. scoparia* were the most active plants against *Candida albicans* (BAYKAN EREL & al. [26]).

In a recent review, M.J. ABAD & al. [15] mentioned that the quality and yield of essential oils from *Artemisia* species is influenced by the harvesting season, fertilizer and pH of soils, the choice and stage of drying conditions, the geographic location, chemotype or subspecies, choice of plant part or genotype, or extraction method. There are not mention any Romanian data in this review. For some plant materials used as green pesticide is absolutely necessary to consider some additional information, so be strictly adhered safe for humans, other mammals, as well as any other forms life.

**Antifungal activities of essential oils on mycelium growth**

The growth pattern of *S. sclerotiorum* can be observed in Figure 1 and effects of different concentrations of the essential oils on mycelium growth expressed as percentage mycelium growth inhibition are shown in Figure 2, 3, 4 and 5.
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**Fig. 1.** Growth pattern of *Sclerotinia sclerotiorum* (A): on PDA, 7 days old; B): on PDA amended with different concentration of *A. santonica* essential oil – a) 1200 μL L\(^{-1}\); b) 600 μL L\(^{-1}\); c) 300 μL L\(^{-1}\); 9- Control- without drugs at the 5\(^{th}\) day; C) - the second day after inoculation (1200 μL L\(^{-1}\)).

Generally, the second day after inoculation, all of the essential oils inhibited the growth of *S. sclerotiorum* in a dose dependent manner. The most active one was *A. abrotanum* essential oil, followed by *A. austriaca* and *A. vulgaris*. On the opposite side was the essential oil from *A. scoparia* which had the lowest antifungal effect.

**Fig. 2.** Percentage of *S. sclerotiorum* mycelium growth inhibition at different *Artemisia* spp. essential oils concentrations (the second day after inoculation)

At the third day, the species *A. abrotanum* antifungal effect was maintained and there was a percentage of mycelium growth inhibition close to 100%. A quite similar effect was recorded in *A. austriaca*, *A. vulgaris*, *A. dracunculus*, *A. dracunculus* var. pilosa and *A. lerchiana*, for the two higher concentrations. As regard as the two lower concentrations, it can be observed that there are significantly differences between experimental variants. It can be noticed that *A. scoparia* essential oil did not inhibit the mycelium growth at the concentration of 300 and 600 μL L\(^{-1}\), so, it can be said that it had only a fungistatic effect.
Four days after inoculation, the fungus mycelium growth followed a similar trend. Further proves fungitoxic effect of essential oils extracted from species A. abrotanum (all concentrations used), followed by A. vulgaris and that of A. dracunculus, A. dracunculus var. pilosa and A. lerchiana (the two higher concentrations). Effects of volatile oil extracted from A. scoparia species was almost zero.

The concentration of 2400 µL L⁻¹ inhibited mycelium growth for all observation period, which proves fungitoxic effect of essential oils, except from species A. pontica and A. annua with 100.00 % inhibition only after the second day, followed by a progressive decrease until 23.00 % (4th day) and 0.00 % (5th day) respectively in the case of A. pontica (1200 µL L⁻¹). In the same time, it can be mention that at a concentration of 1200 µL L⁻¹, the most active essential oils were those extracted from A. lerchiana, A. abrotanum and A. dracunculus var. pilosa, where after 72 hours, there were registered a totally mycelium growth inhibition (for all the period A. lerchiana), after which slowly continued mycelium growth occurred (A. abrotanum, A. dracunculus var. pilosa).

Although growth in essential oils amended plates had a significantly lower mycelium diameter, complete growth inhibition was achieved at the highest tested concentration (2400 µL L⁻¹) in the case of A. santonica, A. austriaca, A. vulgaris, A. dracunculus, A. dracunculus var. pilosa and A. lerchiana. Among the essential oils extracts, those of A. abrotanum was the most promising for reducing mycelium growth, with a fungitoxic effect of 98.23 % inhibition at 1200 µL L⁻¹, and 88.23 % at 600 µL L⁻¹ (after 5 days).
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Fig. 5. Percentage of *S. sclerotiorum* mycelium growth inhibition at different *Artemisia* spp. essential oils concentrations (the fifth day after inoculation)

From the above presented results, after five days, the minimum inhibitory concentration (MICs) defined as the lowest concentration of the leaf essential oil that results in complete growth inhibition of *S. sclerotiorum* was found to be 2400 μL L⁻¹ for *A. santonica, A. austriaca, A. dracunculus, A. lerchiana, A. vulgaris* and *A. vulgaris var. pilosa*. In the case of *A. abrotanum* MIC was 1200 μL L⁻¹, while *A. scoparia* did not totally inhibited the mycelium growth in any of the used concentrations.

Conclusions

The essential oil of *Artemisia* species is one potential and promising antifungal agent, which could be used as botanical fungicide or green pesticide in the postharvest protection of carrots against *S. sclerotiorum*.

The mycelium growth inhibition depended on *Artemisia* species (origin, composition) and was directly proportional to the concentration increase of the essential oils.

Essential oils can be recommended to use for development of new and safe fungicides, but, as recently M. AMINI & al. [23] noticed, further formulation and vivo experiments are necessary to achieve this target.

Acknowledgements

Taxonomic identification was confirmed by botany specialist (Prof. Vasile Ciocârlan) from the Faculty of Horticulture Bucharest.

References


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