Biocontrol of strawberry pathogen *Botrytis cinerea* using plant extracts and essential oils

Lina ŠERNAITĖ, Neringa RASIUKOVIČIŪTĖ, Edita DAMBRAUSKIENĖ, Pranas VIŠKELIS, Alma VALIUŠKAITE

Lithuanian Research Centre for Agriculture and Forestry, Institute of Horticulture
Kauno 30, Babtai, Kaunas dist., Lithuania
E-mail: lina.sernaite@lammc.lt

Abstract

Essential oils and plant extracts of the rosemary (*Rosmarinus officinalis* L.), bay-laurel (*Laurus nobilis* L.) and clove (*Syzygium aromaticum* L.) contain various valuable compounds that provide antimicrobial and antifungal properties. As a result, they have the potential to be used in plant-pathogen control. *Botrytis cinerea* is one of the main strawberry pathogens, which infects plants during various stages of growth. The study aimed to evaluate the efficacy of plant extracts and essential oils of rosemary, bay-laurel and clove, and their mixtures against *B. cinerea* in vitro. Rosemary, bay-laurel and clove extracts and essential oils were separately and in combinations mixed with potato dextrose agar media at different concentrations. Fractional inhibition concentration (FIC) indexes were calculated for the mixtures to determine synergistic, additive or antagonistic effects of the combinations of the investigated plant extracts and essential oils. Results of the experiment showed that clove extract had the highest inhibition (100%) of *B. cinerea*, and rosemary essential oil had the lowest inhibition (31.91%). The mixes, which managed to fully inhibit the growth of *B. cinerea*, were clove extract + clove essential oil, bay-laurel extract + clove essential oil and rosemary extract + clove essential oil. Synergy was found between bay-laurel extract and clove essential oil, and in the mixture of rosemary extract and essential oil, the FIC indexes were equal to 0.75 for both mixes. The mixture of bay-laurel extract and bay-laurel essential oil was antagonistic as the FIC index was 2.5.

Key words: antifungal effect, biocontrol, *Botrytis cinerea*, essential oil, plant extract, synergy.

Introduction

Plants are a natural source of bioactive compounds, which are responsible for their antifungal, aromatic, medicinal and other properties. Some of the essential oils are well known for their antifungal properties, which are expressed through various mechanisms: disruption of cell membrane or wall, mitochondria, reduction of cell growth, inhibition of biofilm development and other. Besides, by various extraction methods, essential oils and extracts can be obtained from plants (Nazzaro et al., 2017). Plant extracts and essential oils differ in chemical composition regarding the extraction method (Danh et al., 2013). Rosemary (*Rosmarinus officinalis* L.), bay-laurel (*Laurus nobilis* L.) and clove (*Syzygium aromaticum* L.) buds are widely used in food, medicine and cosmetic industries (Al-Kashimi, Mahmood, 2016; Oliveira et al., 2016). Antimicrobial activity and antifungal potential of rosemary essential oil have already been observed against foodborne pathogens (Fakoor, Rasooli, 2008; Soylu et al., 2010; Caputo et al., 2018). Foodborne pathogen *Aspergillus niger* is partly controlled by rosemary essential oil (Baghloul et al., 2017), besides it reduces *A. flavus* mycotoxin production (Mahmoud et al., 2014). Derwich et al. (2009) determined the inhibitory effect of bay-laurel essential oil on human pathogenic bacteria. Clove bud essential oil consists mainly of eugenol and its derivative eugenyl acetate (Hatami et al., 2019). In a comparison of extraction methods, clove oil obtained by supercritical CO₂ extraction was found to have a higher concentration of eugenol and eugenyl acetate than that obtained by steam distillation (Yazdani et al., 2005). Olea et al. (2019) determined the antifungal activity of eugenol and its derivatives against *Botrytis cinerea*.

Antifungal and antimicrobial activity of plant ingredients is getting increasingly more attention in plant protection (Nikkhah et al., 2017), as alternative plant protection products are needed due to high toxicity of the chemical pesticides to humans and the environment (Choudhury et al., 2018; Rosero-Hernandez et al., 2019). Biodegradable, non-toxic chemicals from plants with selective activity against pathogens could become safer disease control agents (Soylu et al., 2010). Biopesticides derived from plants are divided into extracts and essential oils depending on the method of extraction (Lengai, Muthomi, 2018). Due to the valuable composition and properties, rosemary, bay-laurel leaves...
and clove bud extracts and essential oils may be one of the options in this area. Higher efficacy of the biocontrol product could be achieved by combining several natural compounds. According to Mbunde et al. (2019), synergism occurring in herbal drugs improves efficacy, determines the faster therapeutic effect and also provides an alternative way to treat resistant microorganisms. However, a similar effect may be achieved in antifungal mixtures against plant pathogens. Several studies on the combinations of bioactive compounds can be found in the literature (Kocic-Tanackov et al., 2014; Milenkovic et al., 2018; Mbunde et al., 2019). Clove bud essential oil, together with eucalyptus essential oil, demonstrated synergistic antimicrobial activity against human pathogens Staphylococcus aureus, Escherichia coli and Microsporum gypseum (Kirui et al., 2014). Aguilar-Gonzalez et al. (2015) investigated clove bud essential oil for antifungal effect on B. cinerea together with mustard essential oil on strawberries in the vapour phase. 

B. cinerea is grey mould, a harmful disease with a wide range of hosts (Rasiukevičiūtė et al., 2018). It may infect plants at different growth stages and could result in high losses of the plants or whole yield (Roser-Hernandez et al., 2019). One of the host plants is strawberry, which can get infected through various parts, like leaves, fruits, flowers, petioles and stems (Rasiukevičiūtė et al., 2018). Although B. cinerea is controlled using integrated disease management, the most efficient way to control this pathogen is synthetic fungicides (Abbey et al., 2019). B. cinerea, determined as a high risk pathogen, is able to develop resistance to widely used fungicides (Minova et al., 2015) as a result of new, resistant fungal strains (Yildirim, Yapić, 2007). As the negative effects of the chemical fungicides on humans and environment are already known, new ways to control pathogens are under investigation (Rasiukevičiūtė et al., 2015; 2019; Mirmajlessi et al., 2016; Valiuškaitė et al., 2017).

Based on the demand for alternatives to chemicals for controlling grey mould, which is a common strawberry disease, the aim of this study was to investigate the efficacy of essential oils and extracts of rosemary and bay-laurel leaves and clove buds against B. cinerea in vitro and to examine, whether the mixtures of these extracts have synergistic effect.

**Materials and methods**

The research was carried out in 2018–2019 at the Laboratory of Plant Protection, Institute of Horticulture, Lithuanian Research Centre for Agriculture and Forestry. Bay-laurel (Laurus nobilis L.), rosemary (Rosmarinus officinalis L.) and clove (Syzygium aromaticum L.) were selected for this research according to chemical composition of their bioactive compounds and antiviral and antimicrobial properties (Fakoor, Rasooli, 2008; Derwich et al., 2009; Soyuşu et al., 2010; Baghhloul et al., 2017; Caputo et al., 2018; Olea et al., 2019), which have the potential to be effective against plant pathogens. Plant extracts and essential oils were produced using two methods; dried plant material was used in both of them to obtain higher yields of essential oil and to compare the efficacy of plant-based material from different extraction methods.

**Isolation of plant extracts.** High-pressure carbon dioxide (CO₂) extraction was used to obtain plant extracts. Dried bay-laurel and rosemary leaves and clove buds were purchased from the Cash and Carry Ltd., Lithuania. One kilogram of the plant material was extracted using a Clevegent distillation system (Glassco, India). The time of the essential oil distillation was 2 hours (AOAC, 1990). Rosemary essential oil was obtained after transferring plant extract from CO₂ extraction to the Clevegent system and collecting essential oil.

**Efficacy of individual plant extracts and essential oils.** Rosemary, bay-laurel and clove extracts and essential oils were separately poured into the potato dextrose agar (PDA) at these concentrations: 0, 200, 400, 600, 800, 1000, 1200, 1400, 1600, 1800 and 2000 μL⁻¹. Discs (6 mm diameter) of 7-day old strawberry pathogen Botrytis cinerea single-spore isolate (from Laboratory of Plant Protection, Institute of Horticulture, Lithuanian Research Centre for Agriculture and Forestry isolate collection) were cut and placed on each Petri dish with the PDA and the tested products. The fungus was used from the margin of the plate and put the mycelial side down in the centre of the Petri dish amended with PDA. Isolates were incubated for 7 days at 22 ± 2°C temperature in the dark. The radial growth (cm) of the B. cinerea colony (including the diameter of the disc) was measured after 2, 4 and 7 days. The efficacy of individual plant extracts and essential oils in vitro was expressed as mycelial growth inhibition (Cherkupally et al., 2017):

\[
\text{Mycelial growth inhibition} \text{ (%) } = \frac{C - T}{C} \times 100
\]

where C is radial growth of the pathogen colony in the control (cm), T – radial growth of the pathogen colony in the treatment (cm).

The lowest concentrations of each investigated plant extract or essential oil with no visible growth of the pathogen (mycelial growth inhibition equal to 100%) were considered to be minimal inhibition concentrations (MIC).

**Efficacy of the mixtures.** After evaluation of the efficacy of extracts and oils, MIC of the investigated plant extracts (PE) and essential oils (EO) were determined: clove EO MIC = 1200 μl L⁻¹, clove PE MIC = 600 μl L⁻¹, rosemary EO MIC = 2000 μl L⁻¹, rosemary PE MIC = 1600 μl L⁻¹, bay-laurel EO MIC = 2000 μl L⁻¹ and bay-laurel PE MIC = 1200 μl L⁻¹. The efficacy of 600 μl L⁻¹ concentration of rosemary PE differed from other investigated concentrations and was not chosen as MIC. The prepared mixtures were binary and contained plant extract and essential oil in five different ratios of the determined MIC of each component: ¼ MIC₀ + ¼ MIC₁, ½ MIC₀ + ½ MIC₁, MIC₀ + MIC₁, ¾ MIC₀ + ¾ MIC₁, ½ MIC₀ + ½ MIC₁, ¼ MIC₀ + ¼ MIC₁ and ½ MIC₀ + ½ MIC₁. The calculated amounts of plant extracts and essential oils were mixed with the PDA medium and further investigation of the efficacy of the mixtures was carried out in the same way as described for individual plant extracts and essential oils.

**Determination of fractional inhibition concentration (FIC) indexes.** A few steps were made to investigate, whether individual plant extracts and essential oils act synergistically in the mixtures. Firstly, the minimal inhibition concentrations (MIC) of the plant extract (PE) and essential oil (EO) in the mixture were...
determined. Then, fractional inhibitory concentration index (FIC\textsubscript{index}) was calculated using the formula, adapted by Stevic et al. (2014):

$$\text{FIC}_{\text{index}} = \frac{MIC_{\text{EO in mixture}}}{MIC_{\text{EO}}} + \frac{MIC_{\text{PE in mixture}}}{MIC_{\text{PE}}}$$

The mixture had a synergistic effect, meaning that it had higher efficacy than individual components: when evaluated FIC\textsubscript{index} < 1 the mixture had additive effect when the efficacy of the mixture was equal to that of its components and 1 ≤ FIC\textsubscript{index} ≤ 2; when efficacy of the mixture was lower than that of individual components, the mixture had an antagonistic effect and FIC\textsubscript{index} > 2 (Kocic-Tanackov et al., 2014).

**Statistical analysis.** The program SAS Enterprise Guide, version 7.1 (SAS Institute Inc., USA) was applied for the analysis of the results with the ANOVA procedure. The experiments were carried out in four replicates. Duncan’s multiple range test was used to determine significant differences between treatments (P < 0.05). Results in the figures are represented as the mean, vertical bars indicate standard deviation. Results in the table are represented as the mean ± standard error.

**Results and discussion**

**Efficacy of the plant extracts and essential oils.** The inhibition of mycelial growth of strawberry pathogen *B. cinerea* was determined by separately mixing plant extracts and essential oils with PDA at different concentrations. The results are presented in Figure 1. Clove extract, whose lowest concentration (200 μl L\textsuperscript{-1}) showed more than 80% inhibition, had the highest efficacy against the pathogen. This extract inhibited the pathogen growth 100% at 600–2000 μl L\textsuperscript{-1} concentration. However, clove essential oil had versatile inhibition concentrations (MIC) of each tested product: it demonstrated 31.91% of inhibition at the highest (2000 μl L\textsuperscript{-1}) concentration. Meanwhile, rosemary essential oil was least effective of all the tested plant-derived products: it demonstrated 31.91% of inhibition at the highest (2000 μl L\textsuperscript{-1}) concentration.

It can be assumed that rosemary essential oil contained the least amount of antifungal compounds or they were not entirely extracted using hydrodistillation method, because CO\textsubscript{2}-extracted rosemary demonstrated antimicrobial activity (El-Naggar et al., 2017). In our study, rosemary extract was moderately effective against *B. cinerea* with the highest inhibition of 52.21% at 600 μl L\textsuperscript{-1} and 43.97% at 1600 μl L\textsuperscript{-1}. Meanwhile, rosemary essential oil was least effective of all the tested plant-derived products: it demonstrated 31.91% of inhibition at the highest (2000 μl L\textsuperscript{-1}) concentration.

![Graphs](image1)

*Note.* Results are presented as the mean, vertical bars indicate standard deviation.

**Figure 1.** Inhibition (%) of *Botrytis cinerea* on PDA: clove essential oil (EO) and plant extract (PE), bay-laurel EO and PE and rosemary EO and PE.

Clove plant extract, as a more concentrated product of CO\textsubscript{2} extraction, resulted in a consistent growth of the efficacy. Meanwhile, clove essential oil exhibited high efficacy at 200–1000 μl L\textsuperscript{-1} concentration, though the composition of essential oil was not strong enough to fully suppress the pathogen growth.

Although clove products were not widely investigated on plant pathogenic fungi, there is evidence that ethanolic extract of clove 100% inhibited the growth of *Sclerotium rolfsii*, *Alternaria alternata*, *Fusarium moniliforme*, *Rhizoctonia solani* and *Aspergillus niger* (Singh et al., 2019). Additionally, clove essential oil was effective against phytopathogenic bacteria (Huang, Lakshman, 2010; Bozik et al., 2017).

El Alama et al. (2015) found that clove and rosemary essential oil is effective against *Candida albicans*. Methanol, ethanol and water extracts of rosemary demonstrated antimicrobial activity (El-Naggar et al., 2017). In our study, rosemary extract was moderately effective against *B. cinerea* with the highest inhibition of 52.21% at 600 μl L\textsuperscript{-1} and 43.97% at 1600 μl L\textsuperscript{-1}. Meanwhile, rosemary essential oil was least effective of all the tested plant-derived products: it demonstrated 31.91% of inhibition at the highest (2000 μl L\textsuperscript{-1}) concentration.

It can be assumed that rosemary essential oil contained the least amount of antifungal compounds or they were not entirely extracted using hydrodistillation method, because CO\textsubscript{2}-extracted rosemary exhibited higher efficacy. Bay-laurel leaf extract did not demonstrate the high inhibition (60.15% at 1200 μl L\textsuperscript{-1}). *B. cinerea* growth was reduced with increased concentrations of bay-laurel leaf essential oil at 1400–2000 μl L\textsuperscript{-1}, and the highest inhibition reached 55.88%. The active components of bay-laurel plant extract have also been extracted better with CO\textsubscript{2} extraction than hydrodistillation, because higher efficacy was observed at lower concentrations compared to bay-laurel essential oil. Investigation of higher concentrations of both bay-laurel plant extract and essential oil is needed, as they demonstrated potential to suppress the pathogen. Other studies determined that microemulsion with bay-laurel essential oil inhibited growth and conidia germination of *A. alternata* (Xu et al., 2017) and expressed bactericidal activity (Derwich et al., 2009). The determined minimal inhibition concentrations (MIC) of each tested product: clove EO MIC = 1200 μl L\textsuperscript{-1}, clove PE MIC = 600 μl L\textsuperscript{-1}, rosemary EO MIC = 2000 μl L\textsuperscript{-1}, rosemary PE MIC = 1600 μl L\textsuperscript{-1}, bay-laurel EO MIC = 2000 μl L\textsuperscript{-1}, bay-laurel PE MIC = 1200 μl L\textsuperscript{-1}, were used in the further assay with mixtures.

Overall, plant extracts were more effective against *B. cinerea* compared to essential oils. It may depend on the method of the extraction of antifungal compounds.

**Efficacy of the mixtures.** Mixtures of two components with each tested product were formed after evaluation of individual plant extracts in vitro. The efficacy of the mixture was determined by the different ratios of extracts and essential oils’ MIC from the previous experiment is presented in Table.

The most effective mixtures were clove bud extract and essential oil at all investigated ratios, also bay-laurel PE + clove EO and rosemary PE + clove EO, which gave 100% inhibition of pathogen growth at four mixture combinations. Clove PE + bay-laurel EO and clove PE + rosemary EO showed 100% inhibition at three tested combinations. However, other investigated mixtures increased inhibition of *B. cinerea* with higher ratio of the components in the mixture. The lowest
Table. Mycelial growth inhibition (%) of Botrytis cinerea by plant extracts (PE) and essential oil (EO) mixtures

<table>
<thead>
<tr>
<th>Ratio of MIC$<em>{PE}$ + MIC$</em>{EO}$ in the mixture</th>
<th>clove PE + clove EO</th>
<th>clove PE + rosemary EO</th>
<th>clove PE + bay-laurel PE + bay-laurel EO</th>
<th>bay-laurel PE + bay-laurel EO</th>
</tr>
</thead>
<tbody>
<tr>
<td>¼ + ¼</td>
<td>93.28 ± 7.13 ba</td>
<td>56.62 ± 3.67 k</td>
<td>53.53 ± 4.16 lk</td>
<td>73.97 ± 0.74 a</td>
</tr>
<tr>
<td>½ + ½</td>
<td>100.00 ± 0.00 a</td>
<td>82.50 ± 3.34 df</td>
<td>79.41 ± 3.81 gf</td>
<td>100.00 ± 0.00 a</td>
</tr>
<tr>
<td>1 + 1</td>
<td>100.00 ± 0.00 a</td>
<td>100.00 ± 0.00 a</td>
<td>100.00 ± 0.00 a</td>
<td>100.00 ± 0.00 a</td>
</tr>
<tr>
<td>1 + 1</td>
<td>100.00 ± 0.00 a</td>
<td>100.00 ± 0.00 a</td>
<td>100.00 ± 0.00 a</td>
<td>82.35 ± 2.84 df</td>
</tr>
</tbody>
</table>

Note. MIC – minimal inhibition concentrations; results are presented as the mean ± standard error; the same letter indicates no significant differences between treatments ($P < 0.05$).

Regarding this mixture, bay-laurel EO may significantly reduce the efficacy of the bay-laurel PE, as its inhibition was higher at many concentrations according to the results in vitro (Fig. 1). Rosemary PE + clove EO and clove PE + clove EO mixtures had the same effect as their components as determined FIC index was equal to 1 and was considered as additive.

The rest of the mixtures also had additive effect as their FIC index did not exceed 1.5. The results are similar to those of Mbunde et al. (2019), who reported that most of the investigated crude plant extract mixtures had additive effect. A few synergistic interactions between crude plant extracts against human pathogenic fungi were documented by Mbunde et al. (2019). The author emphasised that despite the low efficacy as an individual component, natural components may be useful when used in mixtures because of the synergy. For example, Milenkovic et al. (2018) examined essential oils and did not find them to have antimicrobial properties against several bacteria. However, in combination with antibiotics, the effect of mixtures varied from antagonistic to synergistic. We can partly agree with this statement, because rosemary PE + rosemary EO, not as effective as individual components, managed to have higher inhibition due to the synergy. However, further results are in contrast to those of other authors. In the case of mixture with clove PE + bay-laurel EO, again bay-laurel EO might be responsible for the lower efficacy of this extract, as clove PE was the most effective as an individual plant component. Overall, the efficacy of clove EO and PE was reduced by the second component of the mixture in many cases.

Conclusions

1. Individual plant extracts (PE) and essential oils (EO) of rosemary, bay-laurel and clove and their mixtures were investigated against strawberry pathogen Botrytis cinerea. Results revealed that the most effective was clove plant extract, and the lowest inhibition was exhibited by rosemary essential oil.

2. Binary mixtures of clove bud extract and essential oil, bay-laurel leaf extract and clove essential oil, and rosemary leaf extract and clove essential oil demonstrated the highest inhibition against B. cinerea and reduced the pathogen growth by up to 100%.
3. Mixtures of bay-laurel leaf extract and clove bud essential oil, and rosemary leaf extract and essential oil showed synergistic effect on the inhibition of B. cinerea, the fractional inhibition concentration (FIC) index was 0.75. The mixture of bay-laurel plant extract and essential oil had an antagonistic effect – FIC<sub>add</sub> = 2.5.

References


8. Cherkupally R., Kota R., Amballa H., Reddy B. N. 2017. The fractional inhibition concentration (FIC) index was 0.75. The mixture of bay-laurel plant extract and essential oil had an antagonistic effect – FIC<sub>add</sub> = 2.5.

9. Received 22 07 2019 Accepted 27 01 2020


Biocontrol of strawberry pathogen *Botrytis cinerea* using plant extracts and essential oils


ISSN 1392-3196 / e-ISSN 2335-8947

**Braškių patogeno *Botrytis cinerea* biokontrolė naudojant augalinius ekstraktus ir eterinius aliejus**

L. Šernaitė, N. Rasiukevičiūtė, E. Dambrauskinė, P. Viškelis, A. Valiuškaitė

Lietuvos agrarinių ir miškų mokslų centro Sodininkystės ir daržininkystės institutas

**Santrauka**