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Original Research Article

Different susceptibility of two *Botrytis cinerea* strains to supercritical CO_2 plant extracts

Sabina Anžlovar [1], Jasna Dolenc Koce [1,*]

Abstract

Botrytis cinerea is an airborne plant pathogen with a necrotrophic lifestyle. As a generalist, *B. cinerea* has no host specificity and infects over 500 plant species. There are many studies about the phenotypic and genotypic diversity of *B. cinerea* strains worldwide. Two different morphological strains of *B. cinerea* were previously isolated also in Slovenia from buckwheat. The morphological diversity of *B. cinerea* is also reflected in different susceptibility to plant extracts. We tested the susceptibility of two *B. cinerea* strains derived from buckwheat grain to eleven extracts of plant species *Humulus lupulus, Nepeta cataria, Taraxacum officinale, Achillea millefolium, Calendula officinalis, Chamomilla recutita, Helichrysum arenarium, Hypericum perforatum, Juniperus communis, Sambucus nigra* and *Crataegus* sp. obtained by supercritical fluid extraction using CO_2 (SFE-CO_2). The resistance profiles showed that strain II of *B. cinerea* was generally susceptible to the action of these SFE-CO_2 extracts, whereas strain I was more resistant. The concentration-dependent antifungal activity of the chamomile extract and sandy everlasting indicates their possible use as a fungicide for both strains of *B. cinerea*.

Keywords

antifungal activity, *Chamomilla recutita*, fungal pathogen, *Helichrysum arenarium*, phytochemicals

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Različna občutljivost dveh sevov glive *Botrytis cinerea* za rastlinske izvlečke, pripravljene z ekstrakcijo s superkritičnim ogljikovim dioksidom

Izvleček

Botrytis cinerea je rastlinski patogen, ki se prenaša po zraku in ima nekrotrofni način življenja. Kot generalist nima specifičnega gostitelja in lahko okuži več kot 500 rastlinskih vrst. Obstaja veliko študij o fenotipski in genotipski raznolikosti sevov *B. cinerea* iz različnih regij sveta. Predhodno sta bila v Sloveniji iz ajde izolirana dva različna morfološka seva, ki kažeta različno občutljivost za rastlinske izvlečke. V raziskavi smo testirali občutljivost obeh sevov *B. cinerea* za enajst rastlinskih izvlečkov iz vrst *Humulus lupulus, Nepeta cataria, Taraxacum officinale, Achillea millefolium, Calendula officinalis, Chamomilla recutita, Helichrysum arenarium, Hypericum perforatum, Juniperus communis, Sambucus nigra* in *Crataegus* sp., pridobljnih z ekstrakcijo s superkitičnim ogljikovim dioksidom (SFE-CO_2). Iz rezulatov vidimo, da je bil sev II na splošno bolj občutljiv za delovanje izvlečkov SFE-CO_2, medtem ko je bil sev I bolj odporen. Koncentracijsko odvisna protiglivna aktivnost izvlečkov kamilice in smilja kaže, da sta rastlinska izvlečka lahko uporabna kot fungicida proti obema sevoma *B. cinerea*.

Ključne besede

protiglivna aktivnost, *Chamomilla recutita*, glivni pathogen, *Helichrysum arenarium*, rastlinske spojine

Introduction

Botrytis spp. are fungal pathogens that can cause pre- and postharvest disease in a wide variety of economically important crops. As a generalist, *B. cinerea* does not exhibit host specificity and infects over 500 plant species (Cheung et al., 2020). It causes grey mould disease in a wide range of crops, including vegetables, ornamentals, fruits, and cereals. The disease primarily affects flowers and buds, but infections can also occur on fruits, leaves, and stems. Grey mould rot is characterized by light brown to soft brown spots or blotches covered with a dusty mould that can cause seedlings, young shoots, and leaves to wilt and collapse and buds, flowers, and fruits to become blotchy and rotten. It is most destructive to mature or senescent tissues of dicotyledonous hosts, but it usually invades these tissues at a much earlier stage of plant development and remains dormant for a considerable period of time before rapidly decaying the tissues when the environment is favourable and the host physiology changes (Williamson et al., 2007).

There are numerous studies on the phenotypic and genotypic diversity of *B. cinerea* isolates from different regions of the world (Martinez et al., 2008; Isenegger et al., 2008; Kuzmanovska et al., 2012; Asadollahi et al., 2013; Kumari et al., 2014; Plesken et al., 2021). Two different morphological isolates of *B. cinerea* were also previously isolated from buckwheat in Slovenia (Kovačec et al., 2016). The genetic diversity and phenotypic characteristics of the different isolates may be the reason why *B. cinerea* can infect a large number of potential plant hosts (Corwin et al., 2016). As a result, it can counteract a wide range of plant defence chemicals. Control of diseases caused by *B. cinerea* has been largely dependent on the use of synthetic fungicides. Therefore, *B. cinerea* is a pathogen at high risk for developing resistance to fungicides (Hahn, 2014). High frequencies of isolates resistant to one or more chemically unrelated fungicides have been reported in *B. cinerea* populations from many crops (Zhao et al., 2010; Weber, 2011; De Miccolis Angelini et al., 2014; Rupp et al., 2017; Saito and Xiao, 2018). Therefore, due to the fungicide-resistant phenotypes in the *B. cinerea* population among crop hosts, an alternative control method, such as biological control of the fungus, is a better choice and must be integrated into existing rot management programmes to control this emerging disease (Ons et al., 2020). In addition, stricter regulations on the use of chemicals and the modern view of harmful residues have led to a renewed focus on natural products such as plant extracts and essential oils and their use as antifungal agents. Šernaite et al. (2020) reported

that cinnamon extract proved to be the most effective agent against apple grey mould. Behshti et al. (2020) confirmed the antifungal activity of the essential oils of anise, fennel, chamomile, and majaroam applied to fruits of grape both *in vitro* and *in vivo* after harvest. Among them, anise oil completely inhibited the growth of *B. cinerea* in vitro. Supercritical fluid extraction with CO_2 (SFE-CO_2) has gained attention as an environmentally friendly extraction method because CO_2 is a non-toxic, inert, and available solvent, and the extracts obtained are chemically more complex or different compared to conventional extracts (Fornari et al., 2012).

The aim of this study was to determine whether the morphological diversity of *B. cinerea* isolates from buckwheat is also reflected in the differential susceptibility to plant extracts. Although there is a report on the morphological and biochemical diversity of *B. cinerea* isolates from buckwheat (Kovačec et al. 2016), there are no reports on their pathogenic diversity. Given the different growth rates and morphology, different susceptibility of the two fungal strains to plant extracts is also expected.

Materials and Methods

Plant extracts

Supercritical CO_2 extracts of 11 plant species were obtained from IME Insol (Slovenia), using flowers but also whole herbs and fruits of wild-grown plants from the region of Bihač, Bosnia and Herzegovina (Table 1). The extracts were prepared with a BBES 2.0 extraction system (Waters, Milford, MA, USA) under 200-300 bar and 40-50 °C and analyzed for their content using a GC-MS system (GCMS-QP2010 Ultra, Shimadzu, Japan) as described in Schoss et al. (2022).

Fungal growth inhibition assay

The antifungal activities of the SFE-CO_2 extracts were tested against *B. cinerea* previously isolated from buckwheat grains (Kovačec et al., 2016) and deposited in the fungal bank of the Plant Physiology Laboratory (Biotechnical Faculty, University of Ljubljana, Slovenia). First, *B. cinerea* colonies were divided into two morphologically distinct groups based on mycelial growth rate and sclerotia formation. Subsequently, the fungal genomic DNA of each group was isolated using GenElute® Plant Genomic DNA Miniprep Kit (Sigma, USA) according to the manufacturer's instructions. DNA amplification was performed by PCR (Minicycler PTC 150, MJ Research) using Taq DNA polymerase and the primer pair ITS1F/ITS4 (Kovačec et al., 2016). The reaction mixtures and PCR conditions were the same as described in Likar and Regvar (2013). Purification and sequencing of PCR products were performed by Macrogen (The Netherlands). To identify the strains, the sequences were subjected to BLAST searches within the NCBI database (https://www.ncbi.nlm.nih.gov/).

The inhibitory effect of SFE-CO_2 extracts on the radial growth of *B. cinerea* mycelia was tested according to the

Table 1. The list of plants and plant parts used for the preparation of SFE-CO_2 extracts.

Tabela 1. Seznam rastlin in njihovih delov, uporabljenih za pripravo izvlečkov SFE-CO_2.

Plant	Latin name	Family	Plant part
Yarrow	*Achillea millefolium*	*Asteraceae*	flowering herb
Common marigold	*Calendula officinalis*	*Asteraceae*	flower
Sandy everlasting	*Helichrysum arenarium*	*Asteraceae*	flower
German chamomile	*Chamomilla recutita*	*Asteraceae*	flower
Common hops	*Humulus lupulus*	*Cannabaceae*	flower
Dandelion	*Taraxacum officinale*	*Cichoriaceae*	flower
Common juniper	*Juniperus communis*	*Cupressaceae*	fruit
St. John's wort	*Hypericum perforatum*	*Hypericaceae*	flowering herb
Catnip	*Nepeta cataria*	*Lamiaceae*	herb
Hawthorn	*Crataegus* sp.	*Rosaceae*	flower
Black elderberry	*Sambucus nigra*	*Sambucaceae*	flower

method described in our previous study (Anžlovar and Dolenc Koce, 2014). First, a 10% extract was prepared by mixing 0.1 g of each SFE-CO_2 extract with 1 mL of 70% ethanol (Merck, Germany) and stirring the extract on a vortex (IKA, USA) until it dissolved. Elderberry and dandelion extracts were dissolved in 100% acetone (Merck, Germany).

A volume of 50 µL of the 10% SFE-CO_2 extracts was spread with a Drigalski spatula in a Petri dish (2r = 90 mm; Golias, Slovenia) containing 2% (w/v) potato dextrose agar (Biolife, Italy). Disks of *B. cinerea* mycelia (2r = 5 mm) were cut from the margins of 7-day-old fungal cultures and aseptically inoculated by placing them in the centre of a fresh plate containing the extract. Control samples with 70% ethanol or 100% acetone and without extracts were prepared at the same time. fungal colonies were incubated for seven days at room temperature (23±2 °C) in the dark. Mycelial growth was assessed on the 7th day after the inoculation. The plates were photographed with a digital camera (EOS 1000D, Canon, Tokyo, Japan), and the area (cm^2) of fungal colonies was measured using the image analysis software ImageJ (Schneider et al., 2012). Inhibition of fungal growth was expressed as a percentage of growth reduction and calculated according to the formula of Anžlovar et al. (2020):

$$\text{Inhibition (\%)} = (AC - AT) / AC \times 100,$$

where *AC* is the area of mycelial growth of control colonies, and *AT* is the area of mycelial growth of treated colonies. Three replicates (N = 3) were performed for the controls and each treatment with SFE-CO_2 extract.

In addition, the two most active extracts (chamomile and sandy everlasting) were tested for antifungal activity at concentrations of 50%, 25%, 12.5%, 6.25%, and 3.13%.

Statistical analysis

The data were statistically analyzed to calculate mean values and standard errors, and the treatments were compared with a t-test (MS Excel). The level of significance was set at a P-value < 0.05.

Results and Discussion

Differences between morphological types of *B. cinerea* isolated from buckwheat have been observed previously (Kovačec et al., 2016). *Botrytis cinerea* strain I formed circular, white, and cotton-like colonies with filiform margins, whereas strain II had lobed margins, and the colonies were compact with black sclerotia (Figure 1). The growth of the two strains was also significantly different ($p < 0.0001$); the mean size of fungal colonies after seven days of growth was 47.98 ± 1.67 cm^2 for *B. cinerea* strain I and 12.33 ± 3.00 cm^2 for *B. cinerea* strain II (N = 12). The same type of differences in growth between *B. cinerea* strains has also been reported previously (Martinez et al., 2003; Kovačec et al., 2016).

The morphological diversity of the two *B. cinerea* strains was also reflected in different resistance profiles to the SFE-CO_2 extracts used in this study. Strain II was generally sensitive to these SFE-CO_2 extracts, whereas

Figure 1. Mycelium of *Botrytis cinerea* strain I (left) and strain II (right) on PDA 7 days after inoculation.

Slika 1. Micelij glive *Botrytis cinerea* sev I (levo) in sev II (desno) na krompirjevem agarju 7 dni po inokulaciji.

strain I was more resistant (Table 2). The growth of strain I was most strongly inhibited by extracts of chamomile and sandy everlasting. Extracts of hops, juniper and yarrow also significantly inhibited strain I growth, but their inhibition was much lower and close to control values. In contrast, the growth of strain II was significantly inhibited by almost all extracts (chamomile, hops, marigold, black elderberry, catnip, hawthorn), except for St. John's wort extract, which had a growth-promoting effect on it (Table 2). The yarrow extract showed markedly different strain-specific inhibition of *Botrytis* growth, with strain II being completely inhibited, whereas strain I was inhibited by less than 10% (Table 2). A similar but less pronounced pattern was also observed with SFE-CO_2 extracts of common juniper, common hops, common marigold, and catnip. An even more pronounced strain-specific pattern of *Botrytis* growth was observed with SFE-CO_2 extracts of black elderberry, dandelion, and hawthorn, where the growth of strain I was promoted by 1-6 %, while the growth of strain II was inhibited by 40-80% (Table 2).

The different resistance profiles of the two *B. cinerea* strains were the expected result considering the different morphological types and growth rates of the two strains. Kovačec (2016) reported that these two *B. cinerea* strains differed by orders of magnitude in their cellulase and polyphenol oxidase activities, indicating major differences in their biology. Because *B. cinerea* can be necrotrophic, cellulase activity is essential for the degradation of plant cell walls to obtain nutrients (Boddy, 2016) and is, therefore, important for the pathogenicity of the fungus. Anand et al. (2008) reported that virulent isolates of *Alternaria alternata* had higher production of cellulolytic enzymes compared to avirulent isolates. Kumari et al. (2014) found that higher concentrations of oxalic acid and higher activity of lytic enzymes were associated with the pathogenicity of *B. cinerea* isolates. On the other hand, the resistance profiles of the two strains are not correlated with the production of extracellular enzymes, as strain II, which was more susceptible to SFE-CO_2 extracts, has much higher cellulase and polyphenol oxidase activity than strain I (Kovačec 2016). Saito (2018) reported that of 200 *B. cinerea* isolates obtained from mandarin fruit, five fungicide-resistant phenotypes with triple resistance to azoxystrobin, pyrimethanil, and thiabendazole were detected. Five percent of phenotypes were resistant to one fungicide class, 23.5% to two fungicide classes, and 62% to three fungicide classes.

Since the strongest inhibition of the two *B. cinerea* strains was obtained with the extracts of chamomile and sandy everlasting, the antifungal activities were further evaluated using the 16-fold concentration range of these extracts, from 50% to 3.125%. In general, higher extract concentrations resulted in greater inhibition of fungal growth of the two *B. cinerea* isolates. Growth of the sensitive strain II and the resistant strain I was completely inhibited by the 50- and 25-per cent concentrations of

Table 2. Antifungal activity of 10% SFE-CO_2 plant extracts on mycelial growth of two strains of *Botrytis cinerea*. Data present means ± standard error (N=3). Statistically significant differences between control and extract treatment are marked with an asterisk (*).

Tabela 2. Protiglivna aktivnost 10% SFE-CO_2 rastlinskih izvlečkov na rast micelija *Botrytis cinerea* I in II. Podatki so povprečja ± standardne napake (N = 3). Zvezdica (*) prikazuje statistično značilno razliko (P < 0,05) med izvlečkom in kontrolo.

	Growth inhibition (%)	
SFE-CO_2 extract	***B. cinerea* strain I**	***B. cinerea* strain II**
German chamomile	95.77 ± 7.32*	100.00± 0.00*
Sandy everlasting	59.09 ± 4.10*	49.81 ± 8.36
Common hops	37.91 ± 10.56*	76.87 ± 14.47*
Common juniper	13.10 ± 3.47*	67.01 ± 7.85
Yarrow	7.37 ± 3.60*	100.00 ± 0.00*
Common marigold	3.98 ± 2.81	69.57 ± 6.59*
Black elderberry	-5.79 ± 5.60	81.13 ± 3.68*
Catnip	6.65 ± 5.28	87.57 ± 2.19*
St. John's wort	8.38 ± 4.03	-11.86 ± 3.47*
Dandelion	-1.61 ± 5.46	44.76 ± 12.83
Hawthorn	-5.40 ± 1.2	79.43 ± 5.14*

chamomile SFE-CO_2 extract, and both showed similar dose-dependent inhibition of fungal growth (Fig. 2). The sandy everlasting SFE-CO_2 extract had different effects on the growth of the two isolates: while the growth of strain I was significantly dose-dependent inhibited, the growth of strain II was strongly inhibited even by the lowest, 3.125% concentration of sandy everlasting extract (Fig. 2). The composition of the sandy everlasting SFE-CO_2 extract was chemically poorly identified and not comparable to what was previously reported for its essential oil (Schoss et al., 2022). The major constituent was γ-curcumin, which may be responsible for the antifungal activity of the sandy everlasting extract, as it was not found in other SFE-CO_2 plant extracts we studied (Schoss et al., 2022).

The chamomile flower SFE-CO_2 extract completely inhibited the growth of both strains (Fig. 2). The major components of the chamomile extract are α-bisabolol oxide A and α-bisabolol oxide B (Schoss et al., 2022). Bisabolol and its oxidized metabolites contribute to the therapeutic properties of chamomile. α-bisabolol has a Generally Regarded as Safe status (GRAS) and has been found to have antibacterial, antifungal, insecticidal, anti-inflammatory, and anti-ulcer activity (Avonto et al., 2013). While α-bisabolol is found in a wide variety of plants, bisabolol oxides are less common natural products, but all these compounds are of potential pharmacological importance (Avonto et al., 2013). The second major constituent of SFE-CO_2 extract from chamomile flower is tonghausu (Schoss et al., 2022), which is known as an antifeedant compound in the Chinese vegetable tonghao and other plants of the Asteraceae tribe Anthemideae (Chen et al., 2004). Polygodial, which exhibits antifungal activity against normal and resistant isolates of *B. cinerea*, also shows insect antifeedant activity (Carrasco et al., 2017). Similarly, tonghausu, which has antifeedant and antifungal activity, could be the reason for the wider antifungal activity of chamomile extract, as it exhibits inhibitory activity against both strains of *B. cinerea*. The strong antifungal activity of the chamomile extract could also be due to the α-bisabolol oxide (Lucca et al., 2011), as these were not detected in the other SFE-CO_2 extracts tested.

The two *B. cinerea* strains were resistant to St. John's wort extract (Table 2), whose main components are caryophyllene oxide, heneicosane, phytol, and caryophyllene (Schoss et al., 2022). St. John's wort is a chemically and pharmacologically well-studied medicinal plant. Extracts from the aerial parts are used internally to treat depression and externally to cure skin disorders (Tocci et al., 2018). The xanthone-rich extracts from the roots are being studied for their marked antifungal activity against human pathogens, including *Candida* species (Zubricka et al., 2015), and their activity against planktonic cells and biofilm of *Malassezia furfur* (Simonetti et al., 2016). Crockett et al. (2011) isolated three xanthones from St. John's wort root extract and tested them for growth inhibition of plant pathogenic fungi of the genera *Colletotrichum*, *Botrytis*, *Fusarium*, and

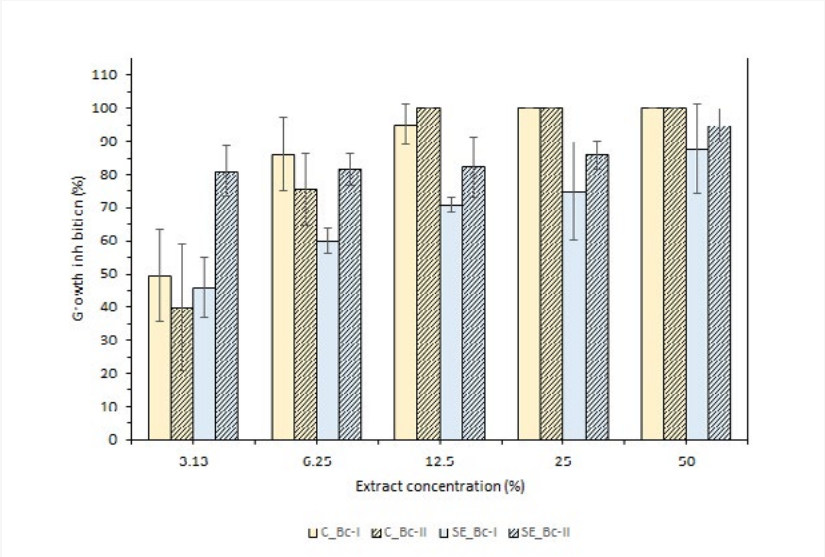

Figure 2. Concentration-dependent antifungal activity of chamomile and sandy everlasting SFE-CO_2 extracts. Data present means ± standard error (N = 3). Legend: C, chamomile; SE, sandy everlasting; Bc-I, *B. cinerea* strain I; Bc-II, *B. cinerea* strain II

Slika 1. Koncentracijsko odvisna protiglivna aktivnost SFE-CO_2 izvlečkov kamilice in smilja. Podatki so povprečja ± standardne napake (N = 3). Legenda: C, kamilica; SE, smilj; Bc-I, *B. cinerea* sev I; Bc-II, *B. cinerea* sev II

Phomopsis. Xanthone 1 was identified as a novel inhibitor of the plant pathogenic fungi *Phomopsis obscurans* and *P. viticola*, but no inhibition of *Colletotrichum*, *Botrytis*, and *Fusarium* species was observed. Since the SFE-CO_2 St. John's wort extract in this study was prepared from a flowering herb, the xanthone-rich roots would be a better choice for testing the antifungal activity of St. John's wort.

Control of diseases caused by *B. cinerea* depends largely on the use of fungicides. However, *B. cinerea* is a pathogen at high risk of developing resistance to fungicides (Hahn, 2014). The objective of this study was to test the resistance of two *B. cinerea* strains derived from buckwheat grain to eleven SFE-CO_2 plant extracts. The resistance profiles showed that strain II of *B. cinerea* was generally susceptible to the action of these SFE-CO_2 extracts, while strain I was more resistant. The extracts of chamomile and sandy everlasting have the potential to be effective as fungicides for both strains.

Author Contributions

Conceptualization, SA; methodology, SA; software, JDK; validation, SA, JDK; formal analysis, SA, JDK; investigation, SA..; data curation, SA, JDK; writing—original draft preparation, SA..; writing—review and editing, JDK; funding acquisition, JDK. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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