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Biocontrol of *Botrytis cinerea* with essential oil and methanol extract of *Viola odorata* L. flowers

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ABSTRACT

The essential oil and methanol extract of Viola odorata L. were tested for antifungal activity against Botrytis cinerea in vitro on Petri plates and liquid culture, and under storage conditions. The essential oil composition of Viola odorata L. flowers were investigated by GC/MS with 63 identified volatile constituents. Phenyl butanone, linalool, benzyl alcohol, α-cadinol, globulol and viridiflorol were found to be the major components, respectively. The oil showed strong antifungal activity based on the inhibition zone and minimal inhibitory concentration values against the pathogen on Petri plates assays. The very low concentrations of them also reduced wet and dry mycelium weight of pathogen fungus in liquid culture. When the oils at 25, 12.5 and 6.25 µl/ml concentrations were applied to tomatoes before seven days of pathogen inoculation on storage conditions, the decay on fruits caused by the pathogen could be prevent completely. The results in this study showed that the essential oil of Viola odorata L. flowers had strong antifungal activity against pathogen fungi tested. So, the essential oil of Viola odorata L. could be used for management of this pathogen as a potential source of sustainable eco-friendly botanical fungicides.

Keywords: *Viola odorata* L. ; Essential oil ; Antifungal activity; *Botrytis cinerea ;* Grey mould disease.

INTRODUCTION

Grey mould, caused by *Botrytis cinerea* (Sclerotiniaceae family), is an important plant disease that affects a large number of plant species and is particularly important in greenhouse production of tomatoes in Mediterranean basin [1]. This pathogenic fungus can infect leaves, stems, flowers and fruits, either by direct penetration or through wounds caused by cultivation practices. Infection is amplified by high relative humidity, free moisture on the plant surface, and low temperatures [2,1]. Several chemical fungicides effectively control grey mould, but *B.*

cinerea can acquire resistance to some of these fungicides as benomyl, benzimidazoles and dicarboximides [3]. Furthermore, for environmental and health reasons, there is growing concern about indiscriminate use of chemical pesticides. This has been paralleled with an increase in the consumption of organic food and farming and has, accordingly, brought about a greater interest in the search for and development of natural and bioactive products for disease control [4]. Current research shows that natural antagonism of microorganisms against some plant pathogens has been extensively investigated for biological control and a number of fungal and bacterial species have proved effective against grey mould [5,6]. Similarly, some compounds as essential oils synthesized by plants have also been used as agricultural chemicals for the control of a range of important plant diseases [7,8]. During the last years, numerous studies have documented the antifungal effects of plant essential oils against post harvest fungal diseases [9, 10, 11, 12, 13, 14, 15]. *Viola odorata* is a species of the genus *Viola* native to Europe and Asia, but has also been introduced to North Africa. It is commonly known as Sweet Violet, English Violet, Common Violet, or Garden Violet. Medicinal and edible, the flowers and leaves of *Viola* are made into a syrup used in alternative medicine mainly for respiratory ailments associated with congestion, coughing, and sore throat. A decoction made from the root (dry herb) is used as a laxative. Tea made from the entire plant is used to treat digestive disorders and new research has detected the presence of a glycoside of salicylic acid (natural aspirin) which substantiates its use for centuries as a medicinal remedy for headache, body pains and as a sedative. There are many studies related to the extract of *V.odorata* , however, there is no report describing the antifungal activities of *V.odorata* flowers. In order to develop stable and safe antimicrobial sources, the first objective this study was to investigate the chemical composition of *V.odorata* flowers and to examine the antifungal activity of the essential oil and methanol extract obtained from *V.odorata* against *B. cinereae* on Petri plates and in liquid medium. Second, the minimal inhibition concentration of them against the growth of *B. cinereae* was determined. Third, to suppress or protect the growing of *B. cinerea* and under storage conditions on tomatoes was tested.

MATERIALS AND METHODS

Plant material

For the extraction of essential oil, *Viola odorata* L. was collected from the local area of Sfax (Tunisia, 35.23° N and 11.11° E). After the botanic identification of the species, a voucher specimen has been deposited in the herbarium of the laboraratory (Institut de l'Olivier de Sfax) for future reference.

Preparation of the methanol extract

Collected plant material was dried in the shade and ground in a grinder with 2 mm in diameter mesh. The dried and powdered plant materials (500 g) were extracted successively with 1 L of methanol (MeOH) by using Soxhlet extractor for 72 h at a temperature (65 °C) not exceeding the boiling point of the solvent [16]. The extracts were filtered using Whatman filter paper (no. 1) and concentrated in vacuo at 40 °C using a Rotary evaporator. The residues obtained were stored in a freezer at −80 °C until further tests. The extract yield was 7.88% (w/w).

Isolation of the essential oil

The air-dried and ground aerial parts of plants collected were submitted to water distillation for 3 h using a Clevenger-type apparatus. The obtained essential oil was dried over anhydrous sodium sulfate and, after it was filtered, stored in a sealed vial at 4 °C until tested. The essential oil yield was 2.3% (v/w).

Gas chromatography–mass spectrometry (GC/MS)

The chemical compositions of *V. odorata* L. flower was analyzed using a Hewlett Packard model HP6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with an HP-5MS (5% phenyl-polymethylsiloxane) capillary column (30 m×0.25 mm i.d., film thickness 0.25 µm; Agilent Technologies, USA) interfaced to an HP model 5973 mass-selective detector. The oven temperature was initially held at 50°C and then increased by 2°C/min to 180°C. The injector and detector temperatures were 250 and 280°C, respectively. Purified helium was used as the carrier gas at a flow rate 1 ml/min. EI mass spectra were collected at 70 eV ionization voltages over the range of m/z 29–300. The electron multiplier voltage was 1150 V. The ion source and quadrupole temperatures were set at 230 and 150°C, respectively. Identification of volatile components was performed by comparison of their Kovat retention indices, relative to C_8–C_{22} n-alkanes, and using a comparison of the mass spectra of individual components with the reference mass spectra in the Wiley 275 and NIST 98 databases. The quantity of all identified components was investigated by using a percent relative peak area.

Pathogen fungus

B. cinerea was kindly provided by Dr. Mohamed Ali Triki (Olive Tree Institute of Sfax, Tunisia. The fungal strain was originally isolated from infected tomatoes and was identified using molecular markers. A conidial suspension of the strain was prepared by culturing the fungus on Potato Dextrose Agar (PDA) medium until sporulation for 1 week at 25°C. The agar surface was then rinsed with 10 ml distilled water containing 8.5 g l^{-1} NaCl and 1 ml l^{-1} Tween80. The concentration of spores was determined using a hematocytometer (Thoma cell), adjusted to 3×10^6 spore ml^{-1} and used to infect tomato leaves.

Source of fruit materials

Tomatoes fruits of the cultivar Riogrande (Societa` Agricola Italiana Sementi, Centro Ricerca E Miglioramento Sementi Horticole E Foraggere, Cesena, Italia; 2006/2007). Fruits were selected free of wounds and rots and as much as possible homogeneous in maturity and size, and were stored at 4 °C for 2-4 days until use. Fruit were surface-disinfected by immersion for 1 min in a dilute solution of ethanol (70%), washed twice by immersion in distilled and sterile water, and left in a dry place to remove excess water on the surface until using for *in vivo* assays.

Screening for antifungal activity

Antifungal activity tests were carried out by disc diffusion method [17]. The tested fungus was grown on Potato Dextrose Agar (PDA) medium for sporulation on Petri dishes for 5–7 days. The concentration of spores was determined using a hemocytometer, adjusted to 1×10^6 spores/ml by appropriate dilution and used fresh as a stock suspension. Using 100 µl of suspension containing 10^6 spore ml^{-1} fungi spread using glass L-rod spreader on PDA. The dried plant extracts were dissolved in the some solvent (methanol) to a final concentration of 30 g ml^{-1}, and prepared stock solutions. The extracts and oils were sterilized by filtration through 0.45 µm Syringe Driven Filter Unit (MILLEX®-HA). The sterile filter papers discs (6 mm in diameter) were impregnated with 10 µl of the essential oil, or the 30 mg ml^{-1} extracts (300 µg/disk) were put in the middle of inoculated agar plates. Methanol and DMSO 10% were added on the discs to provide negative control. The inoculated plates were incubated at 37±2 °C and 72 h. At the end of the period, antifungal activity was evaluated by measuring the zone of inhibition (mm) against the test fungus. All treatments consisted of three replicates, and experiments were repeated three times, and determined the averages of the repeated experimental results.

Determination of the minimum inhibitory concentration (MIC)

The minimal inhibition concentration (MIC) value of the oil was determined by using the modified agar-well diffusion method [18]. The essential oils dissolved and prepared stock solutions in 10% dimethyl sulfoxide (DMSO), were first diluted to the highest concentration (500 µl ml^{-1}) to be tested, and than serial 2-fold dilutions were made in order to obtain a concentration range from 6.25 to 500 µl ml^{-1} in 2 ml sterile Eppendorf test tubes. A 100 µl amount from suspension contained 10^6 spore ml^{-1} of fungus spread on PDA plates. The discs were impregnated with 10 µl of the serial essential oils dilutions, and than were put in the middle of inoculated agar plates. The inoculated plates were incubated at 37±2 °C and 72 h. At this period, inhibition zones were determined. The least concentration of each the essential oil showing a clear zone of inhibition were taken as the MIC. All treatments consisted of three replicates, and experiments were repeated three times.

Determination of wet and dry mycelium weight

A 1000, 500 and 250 µl amount from the stock solutions of the essential oils (500 µl ml^{-1}) and extracts (30 mg ml^{-1}) was added in separately to each Erlenmeyer flask with 20 ml nutrient broth (NB). Three different final concentrations of the essential oil (25, 12.5 and 6.25 µl ml^{-1}) and methanol extract (30, 15 and 7.5 mg ml^{-1}) in liquid medium were prepared. A 100 µl amount from the fungal suspension containing 10^6 spore ml^{-1} of fungus suspension was inoculated in these flasks. The control contained NB medium plus 100 µl of fungal suspension. It was incubated at 37±2 °C until a strong mycelial growth was obtained at 10 days. The fungal myceliums were harvested by filtrating to separate from liquid culture and pre-weighed filter paper (Whatman no. 1), followed by washing twice with distilled sterile water. The wet weight of myceliums was determined. And then they were dried at 60 °C. The dry weight of mycelium was determined. All treatments consisted of three replicates, and experiments were repeated three times and determined the averages of the repeated experimental results.

In vivo assays on storage condition

The selected tomatoes for the experiments were washed in running, dipped in ethanol (70%) for 2 min, rinsed twice with double distilled sterile water (10 min each) and air-dried. Surface-sterilized tomatoes fruits were wounded with a flame sterilized nail to a uniform depth of 3 mm. The fungal inoculums containing 10^6 spore ml^{-1} was prepared by scraping spore material from the surfaces of the colonies with a wet cotton swab and resuspending the material in distilled water containing 0.5% Tween 80. Three different concentrations (25.0, 12.5 and 6.25 µl ml^{-1}) of the essential oils were prepared for testing antifungal activity against *B cinereae*. The essential oil of *V.odorata* at three different concentrations and fungal inoculum were sprayed on wounded tomatoes fruits. A total of eleven tomatoes fruits were tested for per concentrations groups. These experiments were arranged as three different applications. The oils and the pathogen inoculums were applied simultaneously, before 3 and 7 days of pathogen inoculation, and than fruits were stored at 10 °C in 70% relative humidity (rh) under a photoperiod of 12-h light and 12-h dark under storage condition. Fruits inoculated with only pathogen were used as positive control for each experiment. Not inoculated fruits with pathogen were used as negative control.

The tomatoes fruits were sealed in polyethylene-lined plastic boxes to retain 70% humidity and incubated at 10 °C storage condition. The diameters of decay on fruits were measured at 6, 8, 12 and 14th days after inoculation. It was accepted that the diameters of full decayed fruits were as 25 mm. All treatments consisted of three replicates, and experiments were repeated three times and determined the averages of the repeated experimental results.

Statistical analyses

An analysis of variance (ANOVA) and Duncan's multiple range test (at $P<0.05$) were performed to analyze statistical differences and to discriminate between means [19].

RESULTS AND DISCUSSION

Chemical composition of essential oil of *Viola odorata* L.

GC/MS analyses of the oil led to the identification of 63 different components, representing 83.05 % of the total oil. The volatile components identified by GC/MS, their relative area percentages and their retention indices are summarized in Table 1. The essential oil of *V. odorata* L. flowers contained high percentages of the group of monoterpenes and sesquiterpene. The dominant components were 1-phenyl butanone (22.43%), linalool (7.33% %), benzyl alcohol (5.65%), α-cadinol (4.91), globulol (4.32%) and viridiflorol (3.51%). Pulegone (3.33%), epi-α-cadinol (3.05%), terpinen-4-ol (2.31%), germacrene A (1.99%) and paramethyl anisole (1.09%) were also found to be minor components of the *V.odorata* L. flower oil.

Table 1 : Chemical constituents of *Viola odorata* L. flower oil with the percentage of content obtained by hydrodistillation

No.	Compound	LRI	%
1	Furfural	829	0.18
2	α Thujene	919	0.12
3	α-Pinene	925	t
4	Sabinene	964	0.13
5	Myrcene	982	0.15
6	α-Terpinene	1010	0.14
7	*para*-methyl Anisole	1016	1.09
8	β-Phellandrene	1021	0.37
9	δ-3-Carene	1028	0.15
10	Z-β-Ocimene	1039	0.52
11	Benzyl alcohol	1042	5.65
12	γ-Terpinene	1050	0.15
13	Acetophenone	1061	0.15
14	Z-Sabinene hydrate	1062	t
15	Z-linalol oxide (furanoid)	1066	0.16
16	Methyl benzoate	1088	0.14
17	Linalool	1098	7.33
18	1.3.8-*para*-Menthatriene	1109	0.43
19	1.3.8-*ortho*-Menthatriene	1113	0.55
20	Z-*para*-menth-2-en-1-ol	1118	0.65
21	1-Terpineol	1136	0.8
22	Ethyl benzoate	1163	t
23	Terpinen-4-ol	1171	2.31
24	Geraniol	1183	0.57
25	α-Terpineol	1188	1.26
26	Pulegone	1235	3.33
27	δ-Elemene	1326	0.22
28	α-Cubebene	1337	t
29	Isoledene	1361	0.37
30	α-Copaene	1366	0.22
31	β-Bournonen	1372	0.26
32	β-Cubebene	1378	0.24
33	α-Gurjunene	1397	0.22
34	Z-Caryophyllene	1411	0.84
35	β-Duprezianene	1416	0.14
36	α-Guaiene	1424	0.17

37	γ-Elemene	1425	0.26
38	Aromadendrene	1430	0.66
39	1-Phenyl butanone	1438	22.43
40	α-Humulene	1443	0.15
41	allo-Aromadendrene	1448	0.45
42	Germacrene D	1472	0.42
43	β-Selinene	1477	0.24
44	Bicyclogermacrene	1483	0.6
45	Viridiflorene	1485	3.51
46	Germacrene A	1491	1.99
47	E-β-Guaiene	1495	0.53
48	γ-Cadinene	1504	0.12
49	β-Sesquiphellandrene	1518	0.17
50	Germacrene B	1548	0.34
51	Ledol	1562	1.59
52	Germacrene D-4-ol	1564	0.12
53	Spathulenol	1575	0.47
54	Globulol	1581	4.32
55	Viridiflorol	1588	3.65
56	1.10-di-epi-Cubenol	1615	1.44
57	10-epi-γ-Eudesmol	1621	0.27
58	Eremoligenol	1625	t
59	epi-α-Cadinol	1634	3.05
60	α-Cadinol	1649	4.91
61	Z-methyl epijasmonate	1671	0.14
62	Z-α-bisabolene epoxide	1733	0.54
63	Benzyl benzoate	1755	1.67
Total			83.05

LRI, linear temperature program retention index on DB-5 column.
%, compound percentage; t, trace amounts <0.1%

Antifungal activity

The results of *in vitro* assays showed that the essential oil of *V. odorata* had a strong fungicidal effect against *B. cinereae* on Petri plate. The mean inhibition zone and the minimal inhibition concentration value of the oil were recorded as 68 mm and at 6.25 µl ml^{-1}, respectively. The methanol extract, DMSO 10% and methanol didn't show any antifungal activity.

Table 2 : Means wet and dry mycelium weight (g) of *B. cinereae* in liquid medium added the essential oil and methanol extract from *Viola odorata* at three different concentrations (25, 12.5, 6.25 µl ml^{-1})

Treatments	6.25 (µl ml^{-1})	12.5 (µl ml^{-1})	25 (µl ml^{-1})
Wet mycelium weight (g)*			
Essential oil	10.50 ± 0.23^{c}	8.52 ± 0.07^{b}	7.11 ± 0.25^{c}
Methanol extract	13.22 ± 0.29^{b}	11.62 ± 0.08^{a}	11.26 ± 0.12^{b}
Control (only pathogen)	12.60 ± 0.23^{a}	12.60 ± 0.23^{a}	12.60 ± 0.23^{a}
Dry mycelium weight (g)*			
Essential oil	1.76 ± 0.03^{c}	1.73 ± 0.08^{b}	0.30 ± 0.00^{b}
Methanol extract	5.10 ± 0.17^{b}	3.90 ± 0.10^{a}	3.26 ± 0.12^{a}
Control (only pathogen)	3.86 ± 0.16^{a}	3.86 ± 0.16^{a}	3.86 ± 0.16^{a}

**Values are means±standard deviation; data in columns with different letters are statistically different according to Duncan's multiple range tests at p<0.01 and all treatments consisted of three replicates, and experiments were repeated three times.*

Determination of wet and dry mycelium weight

The effects on wet and dry mycelium weight of pathogen fungus in liquid culture of the essential oil were showed in Table 2. All concentrations of the essential oil significantly reduced the wet and dry weight of mycelium of the pathogen. But, the extract did not reduce. Furthermore, 6.25

µl ml^{-1} concentration of the extract increased wet and dry weight of mycelium. The reason of this increase may be related to high incubation temperature and/or the chemical composition of *V. odorata* methanol extract. That is, they may contain high proportion of some components such as sugar used as a nourishment for microorganisms.

***In vivo* assays on storage condition**

The antifungal activity results of essential oils on tomatoes fruits under storage conditions were given in Table 3. According to the results of lesion diameters on the fruits, all concentrations (25, 12.5 and 6.25 µl/ml) of the essential oil applied before 8 days of pathogen inoculation showed strong antifungal activity even at the and of 14th days. Furthermore, there was no significant difference in lesion diameters among those treatments in comparison to the negative control even at the and of 14th days. But, other applications showed a weak effect on disease. It is widely accepted that higher concentrations of plant essential oils are required in foods than in laboratory media [20]. This may be a result of changes to the site of action of this oil at the lower temperature, or alterations in the fungal membrane, so reducing the penetration of the oil to the interior of the cell. But in this study, even though a 6.25 µl ml^{-1} concentration of the oil is applied before 7 days of pathogen inoculation, growing of *B. cinereae* on foods can protect. In the present study, the essential oil from *V. odorata* flower showed a strong antifungal activity against *B. cinereae* under *in vitro* and storage conductions. The inhibitory action of the essential oil could be attributed to the occurrence of high proportions of monoterpenes and sesquiterpenes in the oil as indicated by the study of [21, 22] due to their different chemical composition. Antifungal properties of action might be related to these compounds which have a high potential in strongly inhibiting microorganism pathogens. The following components present are believed to play an important role as antifungal agents, linalool, α-cadinol, globulol and viridiflorol, pulegone, epi-α-cadinol, terpinen-4-ol, germacrene A and *para*-methyl anisole corresponding to the amounts present in the essential oil. Conversely, [20] also reported that there was no significant correlation between the activity and the percentage of the identified compounds.

Table 2: Means of decay diameters (mm) which measured after 6, 8, 12 and 14 days on tomatoes fruits applied with 25, 12.5 and 6.25 µl ml^{-1}concentrations of essential oil from Viola odorata and inoculated *Botrytis cinereae* under standard storage condition

Treatments	Means of decay diameters (mm)*			
	6th days	8th days	12th days	14th days
Controls				
Negative (not pathogen was inoculated)	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
Positive (only pathogen was inoculated)	6.4±1.8^{bd}	8.6±2.4^{bc}	11.3±3.2^{b}	20.3±3.3^{bc}
Oil was applied before 7 days of pathogen				
6.25 µl ml^{-1}	0.0±0.0^{a}	0.6±0.6^{a}	2.3±1.6^{a}	3.5±2.4^{a}
12.5 µl ml^{-1}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.6±0.6^{a}
25 µl ml^{-1}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}
Oil was applied before 3 days of pathogen				
6.25 µl ml^{-1}	8.7±3.5^{cd}	11.5±3.8^{cd}	24.8±4.3^{cd}	26.4±4.1^{c}
12.5 µl ml^{-1}	7.5±3.5^{cd}	9.7±3.5^{bc}	24.8±4.3^{cd}	26.2±4.2^{c}
25 µl ml^{-1}	1.3±0.9^{ab}	3.3±1.9^{ab}	18.3±4.3^{bc}	18.3±4.17^{bc}
Oil was applied simultaneously with pathogen				
6.25 µl ml^{-1}	11.7±2.0^{d}	16.3±2.5^{d}	29.4±2.5^{d}	35.3±2.7^{d}
12.5 µl ml^{-1}	5.7±1.5^{ac}	9.7±1.7^{bc}	13.4±2.3^{b}	20.1±2.8^{bc}
25 µl ml^{-1}	5.3±1.7^{ac}	9.1±2.4^{bc}	12.8±2.9^{b}	15.0±3.3^{b}

**Values are means±standard deviation; data in columns with different letters are statistically different according to Duncan's multiple range tests at p<0.05 and all treatments consisted of three replicates, and experiments were repeated three times.*

CONCLUSION

This study showed that *V. odorata* L. oil has a strong antifungal activity against *B. cinereae*. So this essential oil can be used as a potential source of sustainable eco-friendly botanical fungicides to protect some stored food products from pathogen and saprophytic fungi. Further studies are needed to evaluate the *in vivo* potential of this oil as a natural disinfectant and its effect on seeds vigour response.

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