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ORIGINAL ARTICLE

Evaluation of *Pelargonium graveolens* essential oil to prevent gray mold in rose flowers

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Abstract

The main pathogen that deteriorates the quality of rose flowers during the postharvest stage belongs to the fungal genus *Botrytis*. The chemical products used to control the disease caused by this pathogen have been losing effectiveness due to the appearance of resistance. The present study describes the *in vitro* and *in vivo* fungicidal activity of *Pelargonium graveolens* essential oil and its chemical composition. The essential oil was obtained by hydrodistillation, and the *in vitro* fungicidal activity was determined by agar diffusion assays, showing 100% of fungal growth inhibition at 250 ppm. *In vivo* assays were performed on *Rosa grandiflora* flowers treated with 250 ppm of *P. graveolens* essential oil, using distillate water as a positive control and the commercial fungicide carbendazim as a negative one. No significant differences were obtained between the treatment with the essential oil and the treatment with the commercial fungicide. The chemical profile of the essential oil was determined by GC-MS. The main compounds detected were geraniol (24.89%), citronellol (19.50%), linalool (10.92%) and γ-eudesmol (8.93%). These results encourage the possible use of *P. graveolens* essential oil for the control of *B. cinerea* in rose flowers.

Keywords: *Botrytis cinerea*, flower production, natural products, post-harvest, terpenes

Introduction

Roses (*Rosa* spp.) are the most important ornamental plants, comprising 30% of the floriculture market worldwide (Kim *et al.* 2012). In recent years, the production of cut flowers and ornamental plants in the central-north area of Santa Fe province (Argentina) has increased due to local and regional market growth. One of the main cut flowers produced are roses, but the production volume fails to completely satisfy the demand; thus, this activity undergoes constant development (Morisigue *et al.* 2012; Zuliani *et al.* 2017).

Botrytis cinerea is the causal agent of gray mold infection, ranking second among the most dangerous phytopathogens worldwide with respect to its scientific and economic importance (Dean *et al.* 2012). It affects a wide range of crops with different modes of infection, such as the ability to develop under prevailing storage, shipping and commercialization conditions (Hua *et al.* 2018). This pathogen significantly reduces the ornamental value of cut roses, and determines the quality of their vase life (Bika *et al.* 2020). It has been demonstrated that vase life is negatively correlated with respiration after harvest, therefore, rapid cooling to the lowest and safest storage temperature is the key for long-distance transport (Reid and Jiang 2012). The spiral bouquet used by many producers makes the pre-chilling process even more difficult by increasing the risk of water condensation on the outer petals, which often results in gray mold infection (Bika *et al.* 2020).

The currently recommended postharvest fungicides are dicarboximide (iprodione) and chlorothalonil

formulations used during refrigeration, storage and shipment. Fungicides may fail to control the *Botrytis* disease due to the resistance developed in the pathogen population towards frequently used chemicals, such as benzimidazoles and dicarboximides (Herrera-Romero *et al.* 2017; Muñoz *et al.* 2019; Bika *et al.* 2020). For this reason, the search for new alternatives for their control has become necessary (Rivera *et al.* 2015).

In recent years, there has been an increasing interest in finding alternatives to synthetic fungicides for the control of postharvest diseases, both for fruit and flowers (Di Liberto *et al.* 2019; Stegmayer *et al.* 2021). Natural products from plants offer a set of structurally different fungicidal compounds that could represent an alternative to synthetic fungicides for the control of phytopathogenic fungi (Cheng and Cheng 2015; Guevara *et al.* 2015; Córdova-Guerrero *et al.* 2016; Alejo *et al.* 2019; Samara *et al.* 2021). These compounds are selectively active against different fungal species. Furthermore, they are biodegradable and potentially suitable for use as agrochemicals in integrated pest control programs (Mossa 2016).

Pelargonium graveolens L´Her. (v.n. geranium) belongs to the Geraniaceae family, which includes approximately 750 species. A thin hairy layer covers the entire plant where glandular trichomes are present and in which essential oil is biosynthesized (Blerot *et al.* 2016). This species has been widely evaluated against human fungal pathogens, but its effect on the control of phytopathogenic fungi has not yet been studied (Badawy and Abdelgaleil 2014; Diánez *et al.* 2018; Yan *et al.* 2021). Our research attempted to evaluate its fungicidal potential against *B. cinerea* isolated from infected *R. grandiflora* as an alternative to the use of chemicals in the postharvest stage of roses.

Materials and Methods

Plant material

The plant material used for the propagation of *P. graveolens* was provided by the Area of Intensive Crops (FCA-UNL, Esperanza, Santa Fe). The plants were reproduced asexually by using cutting techniques. During the month of October 2017, branches were cut into pieces of about 5 to 8 cm that contained 3 or 4 nodes. The cuttings were placed in plastic containers (No. 12, 200 cm^3) provided with commercial substrate (Grow Mix®-professional substrate). The plants obtained were transplanted to the greenhouse located in the Experimental Field of Intensive and Forest Crops (CECIF, route 6, km 7.5, 31°26' S; 60°56' W, Esperanza) in order to generate the plant material necessary for essential oil extraction. Once the vegetative parts were developed and before the appearance of floral organs, the apical leaves were harvested for distillation. A *voucher specimen* was deposited at the Herbarium of the FCA-UNL "Arturo Ragonese" (SF Herbarium), Kreder 2805-(3080HOF)-Esperanza, Argentina, with code 58.

Essential oil

Pelargonium graveolens leaves were collected and placed in a distillation flask adapted to a Clevenger-type apparatus, manufactured according to Pharmacopeial norms, which work by water steam distillation. The plant material was weighed in order to calculate the yield of essential oil obtained from 1 kg of processed fresh material. The obtained product was stored by undercooling in amber glass until it was used.

Microorganisms and media

Monosporic strains of *B. cinerea* were obtained from rose flowers that presented the corresponding symptoms of infection and were morphologically characterized by the Mycology Reference Center (CEREMIC, CCC, Rosario, Argentina). Strains of *B. cinerea* CCC-100 were grown on a Potato Dextrose Agar (PDA) medium using Petri dishes for 7 days at 13–15°C, and sub-cultured every 15 days to prevent pleomorphic transformations. Spore suspensions were obtained according to the Clinical and Laboratory Standards Institute (CLSI 2008) procedures, and adjusted to 1×10^4 Colony Forming Units (CFU) · ml^{-1}.

In vitro fungicidal assay

Petri dishes of 9 cm diameter were covered with 20 ml of PDA medium. When solidified, a conidia suspension of 10^4 CFU · ml^{-1} was inoculated inside a well located in the center. After the water from the inoculated conidia solution had evaporated, 10 µl of *P. graveolens* essential oil (treatment) or sterile water (control) was deposited on the center of the plate covers. This quantity of essential oil applied represented a concentration of 250 ppm. The Petri dishes prepared in this way were incubated upside down so that the essential oil, once evaporated, came in contact with the culture medium where the fungus was growing (methodology adapted from Álvarez-Castellanos *et al.* 2001). When the mycelium of the control plates completely covered the surface of the medium (approximately 7 days), the measurements of the mycelium diameter developed in each plate treated with essential oil were carried out by scanning the plates [LA2400 scanner with the WinRHIZO 2013 software (Regent Instrument Canada Inc. 2013) for later analysis with ImageJ® software (Rasband 1997)]. Assays were performed in triplicate, and the percentage of fungal growth inhibition was calculated according to the following equation: $I\% = 100(C–M)/C$, where: $I\%$ represented the inhibition percentage, C – the average of the three control plates mycelia area and M the average of the three treated plates mycelia area.

In vivo fungicidal assays

Red flowers of *R. grandiflora* produced in the CECIF greenhouse were used for the assays. Commercially mature, intermediate-sized and insect-free flowers were used, randomly separated into four groups of seven units each. The treatments were randomly assigned and consisted of: control (without inoculation or fungicidal application); treatment 1 (T1: inoculation with *B. cinerea* but without fungicidal application); treatment 2 (T2: inoculation with *B. cinerea* and subsequent application of commercial fungicidal carbendazim at the recommended commercial concentration); treatment 3 (T3: inoculation with *B. cinerea* and subsequent application of geranium essential oil at a concentration of 250 ppm). The inoculum of *B. cinerea* applied in all the treatments was about 10^4 CFU · ml^{-1}, and the inoculation mode was carried out by spraying with six repetitions of 0.05 ml each at the same time for each experimental unit in order to ensure the penetration of the fungus. The fungicidal treatments were carried out by spraying four times with 0.05 ml of the corresponding solutions, at the same time for each experimental unit. The time elapsed between fungus inoculation and treatment application was about 6 h.

A constant temperature of 20°C was maintained during the assay, with a level of humidity suitable for the development of fungal disease. After 5 days, trial observation and readings were performed. The scale used to record the visual symptoms and signs of the disease had three levels of classification: 0 – healthy flowers, 1 – flowers with the appearance of some lesions and 2 – totally injured flowers (Hazendonk *et al.* 1995). The scale used to record the visual symptoms and signs is presented in Figure 1, considering the level of infections 0 (A), 1 (B) and 2 (C).

Chromatographic analysis

The essential oil was subjected to gas chromatography coupled to mass spectroscopy (GC-MS) in order to identify its main components. The compounds were identified by comparing the mass spectra with the available 2011 NIST database. The sample was analyzed with a gas chromatograph (Agilent Model 7890B) coupled to a mass Spectrometer (Agilent model 5977, column: HP-5MS UI, 30 m × 0.25 mm with 0.25 µm film). The running conditions were as follows: 250°C Injector-Column temperature: 160°C maintained for 3 min, leading to 5°C · min^{-1} up to 30°C, running time: 31 min. Injection volume: 1 µl – split: 1 : 20, according to the Adams method (Adams 2007).

Statistical analysis

The results obtained were analyzed with the statistical software GraphPad Prism 5.0 (San Diego, CA, USA 2010) by using Tukey's multiple comparisons test with a level of significance $p < 0.05$.

Results

Essential oil

The essential oil yield obtained was 0.30 ml · 100 g^{-1} of fresh plant material (0.3%).

In vitro fungicidal assays

Pelargonium graveolens essential oil inhibited 100% *B. cinerea* growth during the *in vitro* assay. In Figure 2 *B. cinerea* growth inhibition in a concentration of 250 ppm can be seen.

In vivo fungicidal assays

In vivo flowers results are shown in Figure 3, which indicates the means (with their respective standard deviations) of the data obtained according to the experimental section. Each treatment presented an average disease severity caused by *Botrytis*, and it can be clearly observed that: 1 – the control units remained healthy throughout the trial (disease level 0); 2 – the units inoculated with fungus and not treated with fungicidal presented a disease

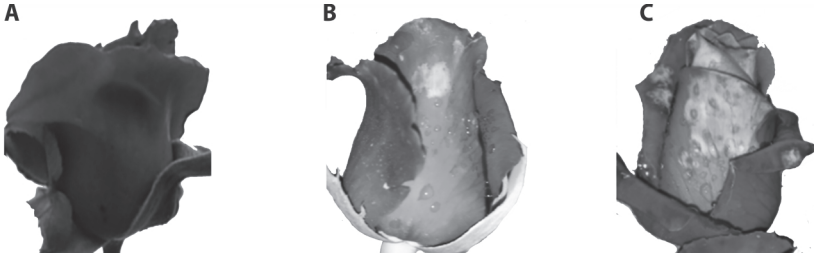

Fig. 1. Scale used to record the visual symptoms and signs of *Botrytis cinerea* infection in cut rose flowers after 5 days: A – level 0, B – level 1 and C – level 2

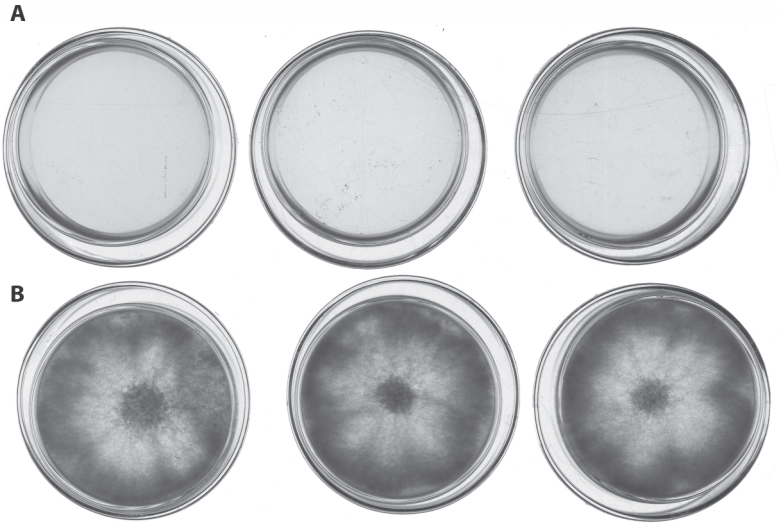

Fig. 2. *Botrytis cinerea* growth inhibition after 7 days: A – *Pelargonium graveolens* concentration 250 ppm; B – sterile water (control)

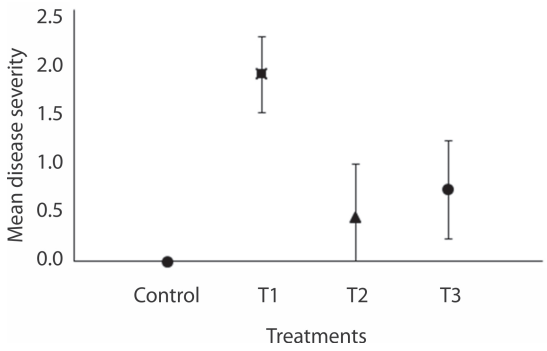

Fig. 3. Mean values (with their respective standard deviations) of disease severity levels in rose flowers submitted to: control – flowers without inoculation or fungicidal application; treatment 1 – flowers inoculated with *Botrytis cinerea* but without fungicidal application; treatment 2 – flowers inoculated with *Botrytis cinerea* and subsequent application of commercial fungicide carbendazim; treatment 3 – flowers inoculated with *Botrytis cinerea* and subsequent application of geranium essential oil at 250 ppm

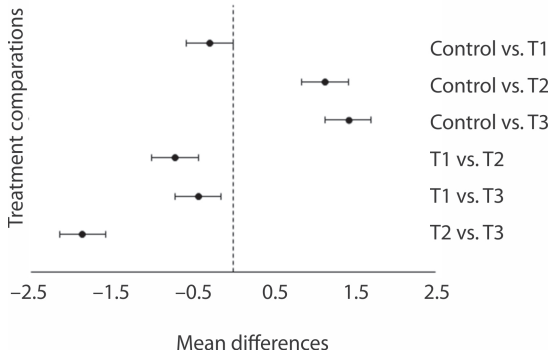

Fig. 4. Difference in disease severity means between groups with 95% confidence intervals. Control – flowers without inoculation or fungicidal application; treatment 1 – flowers inoculated with *Botrytis cinerea* but without fungicidal application; treatment 2 – flowers inoculated with *Botrytis cinerea* and subsequent application of commercial fungicidal carbendazim); treatment 3 – flowers inoculated with *Botrytis cinerea* and subsequent application of geranium essential oil at 250 ppm

severity close to level 2; 3 – the units treated with commercial carbendazim or geranium essential oil showed similar values of disease severity, both below level 1.

It can be observed that the control (without fungus inoculation) and treatment 1 (with inoculation but without fungicidal application) had greater differences in disease severity. This fact indicated that these groups showed the largest differences in injured experimental units. The differences in disease severity between the control and treatment 2 and the control and treatment 3 were very similar, as well as treatment 2 and treatment 3 groups. This analysis indicated that both treatments (commercial carbendazim and geranium essential oil) presented similar disease severity, a fact that was further corroborated when differences in means between treatments 1 – 3 and treatments 1 – 2 groups were observed (Fig. 4).

Chromatographic analysis

Quantification of the main compounds present in *P. graveolens* essential oil was performed by GC-MS. The chromatogram obtained is shown in Figure 5, indicating the major peaks corresponding to the nine more abundant compounds present in the sample. Furthermore, in order to identify the individual substances, each mass spectrum was compared with the NIST database. The most abundant compound (24.89%) was the one associated with peak 4, which was identified as geraniol; followed by citronellol (19.5%) associated with peak 3 and β-linalool (10.92%) associated with peak 1. The overall chemical composition, retention times of each compound and their percentages present in geranium essential oil are described in Table 1. The chemical structures of the main compounds are presented in Figure 6.

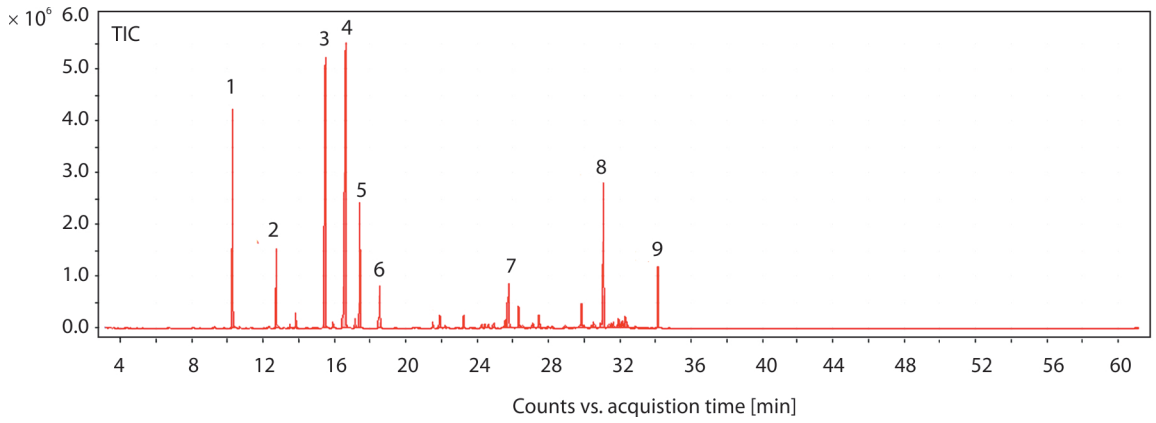

Fig. 5. Gas chromatogram of *Pelargonium graveolens* essential oil. Numbers represent the main compounds of the sample. TIC – total ion concentration

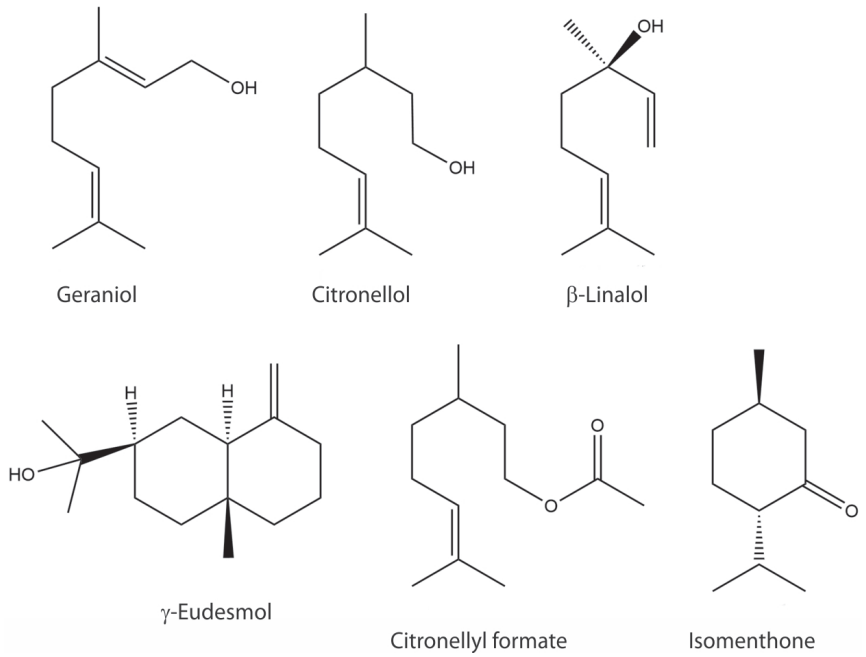

Fig. 6. Chemical structures of the six more important compounds present in geranium essential oil

Table 1. Retention time and percentage of the main compounds present in the geranium essential oil obtained in this work. Each row corresponds to a peak shown in Figure 5

Peak	Compound	Retention time [min]	Percentage [%]
4	Geraniol	16.602	24.89
3	Citronellol	15.469	19.50
1	*β*-Linalool	10.279	10.92
8	γ-Eudesmol	31.067	8.93
5	Citronellyl formate	17.392	6.30
2	Isomenthone	12.728	3.73
9	Geranyl tiglate	34.157	3.30
7	Germacrene D	25.740	2.55
6	Geranyl formate	18.496	2.14

Discussion

The geranium essential oil yield obtained in this work (0.3%) was slightly higher than those obtained in previous works (0.22–0.28%) (Doimo *et al.* 1999; Mossa 2016; Bika *et al.* 2020) but lower than the value reported by Bouzenna and Krichen (2013). However, it is widely known that the content of essential oils in plants is usually affected by the harvest time and season, among other conditions (Rao *et al.* 1996; Verma *et al.* 2013).

Pelargonium graveolens essential oil at a concentration of 250 ppm inhibited *B. cinerea* growth (100%) during the *in vitro* assay. This result was consistent with other studies cited in the literature in which phytopathogenic fungi such as *Alternaria alternata*, *Rhizoctonia solani*, *Fusarium* sp. and *Penicillium* sp. were inhibited by geranium essential oil at the same concentration (Bouzenna and Krichen 2013). Diánez *et al.* (2018) evaluated 12 essential oils, including *Pelargonium*, against the phytopathogens: *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Fusarium oxysporum*, *Phytophthora parasitica*, *Pythium aphanidermatum*, *Alternaria brassicae*, *Cladobotryum mycophilum* and *Trichoderma aggressivum* using the disc-diffusion method. These authors demonstrated the high antifungal effect of *Pelargonium* essential oil, but when analyzing its major components, citronellol and geraniol were about 50 and 3%, respectively. In our essential oil sample, the percentages of both compounds were about 20 and 25%, respectively. In addition, the control of *B. cinerea* isolated from contaminated fruits by using essential oils obtained from different plant sources, has been widely studied. Samara *et al.* (2021) evaluated the effect of eight Palestinian indigenous plant essential oils under *in vitro* and *in vivo* conditions against *B. cinerea* isolated from post harvested tomato and strawberry fruits and demonstrated that thyme, sesame and sage essential oils exhibited higher antifungal activity against *B. cinerea* on fruits than rosemary, mint and eucalyptus. Badawy and Abdelgaleil (2014) studied 18 Egyptian plants, among them *P. graveolens*, for their antimicrobial activity against the most economic plant pathogenic bacteria and fungi including *B. cinerea*. The chemical composition of the isolated oils was identified by gas chromatograph/mass spectrometer and the major constituent of *P. graveolens* oil was β-citronellol (35.92%). Moreover, this essential oil has been reported as an inhibitor of some human pathogens like *Aspergillus flavus*, *A. niger* and *Candida albicans*, among others (Džamić *et al.* 2014).

There are few references in the literature reporting studies related to the control of rose diseases that implement essential oils, but our results are in line with those developed by Pasini *et al.* (1997). These authors also employed natural products for the treatment of rose diseases, and demonstrated that synertrol and neem oils were effective against rose powdery mildew caused by the phytopathogen *Sphaerotheca pannosa*. Remarkably, we report for the first time the *in vitro* and *in vivo* effectiveness of geranium essential oil to prevent gray mold infection in roses.

The commercial value of geranium essential oil is determined by its citronellol content. The major components of our sample corresponded to geraniol and citronellol, but the ratio of these percentages was slightly inverted according to the characterization performed by Rana *et al.* (2002), Fayed (2009) and Džamić *et al.* (2014). This variation in the citronellol/geraniol relationship could be explained by the air temperature before harvest, since high citronellol ratios indicated cold stress in geranium plants (Doimo *et al.* 1999). Moreover, the essential oil obtained in this work by the Clavenger method turned out to be rich in geraniol, according to Babu and Kaul (2005) and Božović *et al.* (2017).

Conclusions

Pelargonium graveolens essential oil was properly obtained using water steam distillation while its chemical composition was determined by GC-MS, resulting in geraniol and citronellol being the major constituents of the sample. The *in vitro* fungicidal assay completely inhibited the growth of the phytopathogen *B. cinerea* isolated from infected flowers. Furthermore, an *in vivo* assay using cut rose flowers was performed. The infected flowers responded to the treatment with commercial carbendazim by showing a significantly lower disease severity than those inoculated but untreated flowers, thus ensuring the effectiveness of this product for the treatment of *Botrytis*. In addition, the infected flowers also responded to the treatment with geranium essential oil, showing a significantly lower disease severity than those inoculated but untreated flowers. These results suggest that the alternative fungicide geranium essential oil at a dose of 250 ppm is as effective as the commercial carbendazim for the treatment of gray mold disease in rose flowers. Moreover, the essential oil treatment did not affect the aroma of roses as it was applied at a very low concentration.

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