Australian Journal of Crop Science AJCS

AJCS 16(03):329-337 (2022) ISSN:1835-2707
doi: 10.21475/ajcs.22.16.03.p2874

Physiological and biochemical performance of lettuce (*Lactuca sativa* L.) seeds treated with essential oils used to control phytopathogens

Lilian de Souza Vismara[1,2,*], Thayllane de Campos Siega[1], Edson Bertoldo[1], Sérgio Miguel Mazaro[1,2], Edgar de Souza Vismara[2], Maristela dos Santos Rey[2], Pedro Valério Dutra Moraes[2]

[1]Postgraduate Program in Agronomy (PPGAG), Federal University of Technology - Paraná (UTFPR), Campus Pato Branco, Via do Conhecimento, km 1, CEP 85503-390, Pato Branco - PR, Brazil
[2]Federal University of Technology - Paraná (UTFPR), *Campus* Dois Vizinhos, CEP 85660-000, Dois Vizinhos, PR, Brazil

***Corresponding author: lilianvismara@utfpr.edu.br; lilianvismara@gmail.com**

Abstract

This work aimed to evaluate the physiological and biochemical performance of lettuce (*Lactuca sativa* L.) seeds treated with essential oils (EOs) of citronella (*Cymbopogon* sp.), guaçatonga (*Casearia sylvestris* Sw.), melaleuca (*Melaleuca* sp. L.), patchouli (*Pogostemon* sp. Benth), and pitangueira (*Eugenia uniflora* L.). The experimental design was completely randomized in double factorials (5 oils × 4 doses) at different doses (10, 20, 30, and 40 µL), with an additional treatment that served as growth control (without EOs, 0 µL). In other words, the experimental design entails 5 oils × 4 doses + 1 control, with 4 replicates of 100 Grand Rapid lettuce seeds without industrial chemical treatment. The response variables were: first germination count (FGC, %), last germination count (LGC, %), germination rate (GR), normal seedlings (NS, %), abnormal seedlings (AS, %), aerial part length (APL, cm), fresh mass (m_f, g), total soluble proteins content ($mg \cdot g^{-1}$), and enzyme activities of β-1,3-glucanase ($UA \cdot mg^{-1}$) and phenylalanine ammonia-lyase (PAL, $UA \cdot mg^{-1}$). The lettuce plant proved to be a good reference plant for evaluations related to physiological and biochemical performance when treated with EOs. However, although treatment of lettuce seeds with EOs did not cause undesirable damages, it positively altered the physiological parameters APL and m_f. All EOs affected the total proteins content and enzyme activities of PAL and β-1,3-glucanase. Therefore, EOs demonstrated the potential to activate the plant's defense mechanism to control phytopathogens. More specifically, 10 µL of citronella EO activated two plant defense mechanisms: PAL and β-1,3-glucanase activities. In addition, EOs of melaleuca (10 and 40 µL) and patchouli (20 and 30 µL) also activated PAL enzyme activity.

Keywords: allelopathy; citronella; guaçatonga; melaleuca; patchouli, pitangueira; plant extracts; resistance induction; seed vigor.
Abbreviations: AP_abnormal seedlings count; APL_aerial part length; BOD_Biochemical Oxygen Demand incubator; BSA_Bovine Serum Albumin protein; EO_essential oil; EOs_essential oils; FGC_first germination count; GC-MS_gas chromatography coupled to mass spectrometry; GR_germination rate; LGC_last germination count; m_f_fresh mass; NS_normal seedlings count; PAL_phenylalanine ammonia-lyase; PR-protein_proteins related to pathogenesis; PVPP_polyvinylpolypyrrolidone; RAS_rules for Seed Analysis; UTFPR_Federal University of Technology – Paraná.

Introduction

In terms of quantity and quality, pathologies are among the main challenges of crop production and productivity. The use of agrochemicals has remained the main practice for disease management in agriculture (Babychan et al., 2017) and is often characterized by low efficiency and lack of rational use (Ghini and Kimati, 2002), in addition to the problems of contamination of food, man, and nature. For these reasons, the use of natural methods has been widely investigated to enable the safe production of food based on sustainable agriculture practices (FAO, 2018; Shuping and Eloff, 2017).

The use of plant derivatives has consistently showed a great potential for the control of phytopathogens either by direct action to pathogens or by activation of the plant defense mechanisms. In that regard, essential oils (EOs) are substances of the secondary/specialized metabolism of plants and are rich in a great diversity of metabolites belonging to the classes of phenolics phytochemicals, nitrogen-containing phytochemicals, and terpene phytochemicals, with specific functions which include attraction of pollinating insects and plant defense or allelopathic action (Taiz et al., 2017; Sun et al., 2016; Dudareva et al., 2013). They may be simple, inexpensive, and non-toxic residual substances (Morais, 2009).

A lot of EOs has shown promising attributes for their usage in plant production. For instance, Mattos (2010),

demonstrated that EOs of *Citrus aurantifolia*, *Cymbopogon citratus*, *Eucalyptus* spp., *Lippia sidoides*, *Mentha arvensis*, *Ocimum basilicum*, *Piper aduncum*, *Pogostemon cablin*, *Romarinus officinalis*, and *Zingiber officinale*, in concentrations of 10.000 and 100.000 ppm, effectively controlled the green mold on orange fruits and inhibited the germination and mycelial growth of the fungus *Penicillium digitatum in vitro*. Lorenzetti et al. (2011) verified that EOs of *Cinnamomum zeilanicum*, *Citrus nobilis* var. *tangerinae*, *Citrus sinensis* var. *dulcis*, *Corymbia citriodora*, *Cymbopogon citratus*, *Cymbopogon martini*, *Cymbopogon nardus*, *Lavandula hybrida*, *Melaleuca alternifolia*, *Mentha pipertita*, *Romarinus officinalis*, and *Syzigium aromaticum* controlled the fungus *Botrytis cinerea*, which is the causal agent of gray mold disease in different cultures. For management of post-harvest phytopathogenic fungal diseases, EOs of *Acorus calamus*, *Agreratum conyzoides*, *Artemisia nilagirica*, and *Litsea cubeba* (Tripathi et al., 2016); as well as *Cinnamomum zeylanicum*, *Origanum majorana*, *Romarinus officinalis*, and *Thymus vulgaris* (Nikkhah et al., 2017) present a great potential for the control of diseases affecting fresh fruits and vegetables.

Apart from the direct action of EOs on pathogens, they have shown a good potential in the induction of plant resistance. In other words, they have the ability to activate the plant defense mechanisms, thus preventing or delaying infection in several pre-harvest pipeline systems (Alves and Perina, 2014; Schwan-Estrada et al., 2000) and during post-harvest of fruits, grains, and vegetables (Mengiste, 2014; Mazaro et al., 2008).

Although several studies have reported the potential of EOs, there is paucity of information regarding their mode of action and chemical composition, which reinforces the importance of research on the action of EOs constituent compounds in recipient plants (Miranda et al., 2015) and pathosystems (Lorenzetti et al., 2011). Such studies would also have an impact on the prospects of plant extract-based commercial products.

Considering the diversity of metabolites present in EOs of citronella, guaçatonga, melaleuca (tea tree), patchouli, and pitangueira, it is essential to study the physiological and biochemical performance of these metabolites in plants. Lettuce is a susceptible plant to allelopathic effects because it is very sensitive (Miranda et al., 2015; Pires and Oliveira, 2011; Souza Filho et al., 2010). Activation of plant defense mechanisms and non-harmful effects of lettuce seeds may open a new window for controlling plant pathogens using plant products, considering the challenges of pathogens in different crops.

Therefore, this work aimed to evaluate the physiological and biochemical performance of lettuce (*Lactuca sativa* L.) seeds exposed to different doses of EOs.

Results

Table 1 shows the results against the physiological performance of the seeds. The biochemical performance of the seeds are presented in Tables 2 and 3 and Figures 1, 2, and 3.

Chemical composition of EOs

Table A (Appendix A), adapted from Vismara (2019), shows the major chemical composition of EOs of citronella, guaçatonga patchouli, and pitangueira after subjection to gas chromatography-mass spectrometry (GC-MS) analysis, with exclusive consideration of substances of relative abundance greater than 5%. GC-MS analysis of the different essential oils (EOs) revealed that there is specificity in relation to the active ingredients as well as a high diversity of compounds (Table A, Appendix A). To consider diversity, the synergistic potential of the substances is explored; however, this opens up the possibility for specific studies to consider the fractionation of oils (Vismara, 2019).

Physiological performance

In terms of physiological performance, no significant effect was observed at the different doses of EOs, as well as for the factorial × control interaction. However, the treatment was significant in the contrast between the averages of the control and factorial in terms of aerial part length (APL, cm) and fresh mass (m_f, g) response variables, with the factorial having higher averages (Table 1).

However, Dunnett's test indicated that, for APL, pitangueira (30 µL) and guaçatonga (40 µL) differed significantly ($p < 0.05$) from control (without EO), with 0.34550 cm and 0.48124 cm difference in the observed means, respectively. For m_f, there was no statistical difference between the factorial (EO × dose) and control.

For first germination count (FGC, %), last germination count (LGC, %), germination rate (GR), normal seedlings (NS, %) and abnormal seedlings (AS, %), there was no statistical difference between the factorial (EO × dose) and control (without oil), thus showing no antagonistic effect (phytotoxicity) on the physiological performance of the seeds when treated with the products at the tested doses (Table 1). For first germination count (FGC, %), there was a significant difference between the different EOs, with a higher average for guaçatonga and pitangueira (96.063%) and lower average for melaleuca (94%), which was statistically significant. For aerial part length (APL, cm), the difference between factorial (1.81 cm) and control (1.45 cm) was significant. Also, m_f had a higher factorial average (0.936 g) compared to control (0.802 g).

Biochemical performance

For total soluble proteins content and enzyme activity of phenylalanine ammonia-lyase (PAL), at 5% level of significance, the difference between the factorial and control averages, as well as the EO × dose interaction was significant (Table 2). For total proteins content, control (0.675 $mg \cdot g^{-1}$) had a higher average than factorial (0.495 $mg \cdot g^{-1}$). For PAL, factorial (0.1096 $UA \cdot mg^{-1}$) had a higher average than control (0.0655 $UA \cdot mg^{-1}$).

For total proteins, the lowest average was recorded at 10 µL of citronella EO, while the highest was recorded at 30 µL of guaçatonga EO (Table 2). For PAL, the inverse occurred, such that the lowest average was recorded at 30 µL of guaçatonga EO and highest recorded at 10 µL of citronella EO (Table 2).

The proteins content of melaleuca and pitangueira were not dose-dependent ($p < 0.05$). Also, the PAL activity of guaçatonga, melaleuca, and pitangueira were not dose-dependent. The polynomial regression curves ($p < 0.05$) of the total proteins content and PAL, as a function of the dose-dependent EOs, are presented in Figure 1, 2, and 3.

For citronella EO, the model $\ln(y) = -1.7 + 0.0354x$ ($R^2 = 0.90$)

describes the presence of total soluble proteins content in the plant tissue as directly proportional to the dose (Figure 1a). While for PAL, the model $y = 0.216 - 0.00398x$ ($R^2 = 0.87$) describes the activity of the enzyme as inversely proportional to the dose (Figure 1b).

For patchouli EO, the model $\ln(y) = -0.0644 - 0.108x + 0.00217x^2$ ($R^2 = 0.99$) describes the presence of total proteins content in the plant tissue, with a minimum efficiency dose of 24.8848 µL (Figure 2a). While for PAL, the quadratic model $y = -0.0145 + 0.0141x - 000259x^2$ ($R^2 = 0.81$) had a maximum efficient dose for enzyme activity equal to 27.2201 µL (Figure 2b).

For guaçatonga EO, the model $\ln(y) = -1.28 + 0.0838x - 0.00149x^2$ ($R^2 = 0.81$) describes the presence of total proteins content in the plant tissue, with a maximum efficiency dose of 28.1208 µL (Figure 3a). At the doses of 20 and 30 µL, the total soluble proteins content was 0.7460 and 1.0636 $mg \cdot g^{-1}$, respectively.

For total soluble proteins content, Dunnett test indicated that the EOs of citronella (10 µL, 0.2260 $mg \cdot g^{-1}$), melaleuca (10 µL and 40 µL, 0.3068 $mg \cdot g^{-1}$ and 0.2950 $mg \cdot g^{-1}$, respectively), and patchouli (20 µL and 30 µL, 0.2605 $mg \cdot g^{-1}$ and 0.2668 $mg \cdot g^{-1}$, respectively) significantly ($p < 0.05$) differed from control (0 µL, 0.675 $mg \cdot g^{-1}$).

Analysis of variance (ANOVA) indicated that the EOs of citronella and melaleuca showed PAL activity at the lowest dose of 10 µL. The optimal dose estimated for patchouli was 27.22 µL, with the highest average observed at 20 µL. The Dunnett test indicated that EOs of citronella (10 µL, 0.1940 $UA \cdot mg^{-1}$), melaleuca (10 µL and 40 µL, 0.1518 $UA \cdot mg^{-1}$ and 0.1575 $UA \cdot mg^{-1}$, respectively), and patchouli (20 µL and 30 µL, 0.1830 $UA \cdot mg^{-1}$ and 0.1565 $UA \cdot mg^{-1}$, respectively) significantly ($p < 0.05$) differed from control (0 µL, 0.0655 $UA \cdot mg^{-1}$).

For enzyme activity of β-1,3-glucanase (Table 3), the average of control (0.023 $UA \cdot mg^{-1}$) and factorial (0.0341 $UA \cdot mg^{-1}$) did not differ statistically at 5% level of significance. The effect of EOs was significant ($p < 0.05$). Although citronella, patchouli, and melaleuca did not differ among themselves, they differed from pitangueira and guaçatonga. Melaleuca presented the highest average (0.0433 $UA \cdot mg^{-1}$) activity of the enzyme, while guaçatonga presented the lowest activity (0.0236 $UA \cdot mg^{-1}$). The EOs did not differ in terms of the tested doses (ANOVA, $p < 0.05$). However, the Dunnett test indicated that citronella EO (10 µL) significantly ($p < 0.05$) differed from control (0 µL), with a difference of 0.9640 $UA \cdot mg^{-1}$.

Discussion

A joint evaluation of the results obtained for the physiological parameters in lettuce seeds can affirm that the evaluated EOs can be explored in other plant species, considering that lettuce is a plant susceptible to physiological damage (a fact not observed in this study), especially when referring to abnormal plants. The short period of exposure of the seeds to the EOs (24 h before sowing) at different doses, with approximately 251 cm^3 of air, may have been homeopathic (Stangarlin et al., 2011) and, consequently, of synergistic action on initial development of the seedlings.

In addition, the results observed with gain in physiological parameters of agronomic interest are of extreme importance. For instance, the gain in APL and m_f result in plants with higher agronomic performance. In particular, the difference between the control and factorial (EO × dose) reflects a 19.93% and 14.32% increase in APL and m_f, respectively. Therefore, the EOs potentiated the initial development of the seedlings (Table 1), which indicates synergism. Such positive additions should be better explored in studies investigating the mechanisms involved in the process of germination and growth/development of plants.

It is known that *L. sativa* is sensitive to the effects of allelochemicals that demonstrate even little signs of phytotoxicity (Pires and Oliveira, 2011; Souza Filho et al., 2010). For instance, Miranda et al. (2015) reported that the EOs of *Cymbopogon citratus*, *Ocimum gratissimum* L., and *Ocimum basilicum* L. and/or their major compounds (citral, eugenol, and cineol), for seven days, was antagonistic of the germination and vigor of lettuce. However, in this work, no damage and undesirable changes were observed in the lettuce seedlings.

In fact, the observed divergence between the phytotoxicity of EOs of the same plant species can be justified by variations between the chemical compositions of the same EOs (Miranda et al., 2015). This is because the chemical composition of EOs is related to edaphoclimatic factors, location and time of collection, vegetative cycle of the material at harvest, method of extraction, among other factors (Morais, 2009).

However, the biological activity of allelochemicals depends on their concentration and mobility and not only of their chemical composition (Pires and Oliveira, 2011). Considering this context, since the seeds were treated by volatilization, monoterpenes present in the tested EOs would have a greater mobility (Table A). This is because their lipophilic and low molecular weight characteristics allow their release into the environment and through cell membranes (Dudareva et al., 2013).

In general, the action of the EOs (Table A) indicated synergism, with an increase of 40.24% of PAL activity when comparing the EOs (0.1096 $mg \cdot g^{-1}$) and control (0.0655 $mg \cdot g^{-1}$). For citronella EO (10 µL), PAL activity was 66.24% greater than that of control; whereas, for total soluble proteins content, there was a reduction of 66.52%. At 40 µL of citronella EO, PAL activity was 10.64% higher when compared to control and differed by 55.60% when compared to the 10 µL dose. The functional relationships confirm that, with the increase of dose, the total soluble proteins content increases (Figure 1a), while PAL activity decreases (Figure 1b) and vice versa. In the chemical composition of citronella EO (Table A), the major element was β-citronellal (34.15%), followed by geraniol (10.45%) and limolene (7.98%) (Vismara, 2019).

Chen et al. (2017) confirmed the inhibitory effect of clove and eugenol EOs on the enzyme activity of PAL, polyphenol oxidase, and peroxidase in lettuce, in view of preserving the intrinsic quality of post-harvest lettuce by avoiding leaf darkening. Therefore, it is possible that the volatile effect of citronella EO doses greater than 40 µL or longer time of exposure to EO volatiles may favor the inhibition of PAL.

The action of citronella and melaleuca EOs for 24 h showed similar behavior at the doses of 10, 20, and 30 µL in terms of total proteins content and PAL. In other words, with increasing dose, the total soluble proteins content increases, while PAL decreases and vice versa (Table 2). However, at 40 µL dose of melaleuca EO, PAL activity was 58.41% greater

Table 1. Averages of the physiological performance variables of *L. sativa* seeds - first germination count (FGC, %), last germination count (LGC, %), germination rate (GR), count of normal seedlings (NS, %) and abnormal (AP, %), length of the aerial part (APL, cm) and fresh mass (m_f, g) - of the completely randomized experiment, in double factorial with five essential oils (1) citronella, (2) guaçatonga, (3) melaleuca, (4) patchouli, and (5) pitangueira and four doses (10, 20, 30 e 40 µL) in the 100% concentration of the essential oil and an additional treatment as control (0 µL).

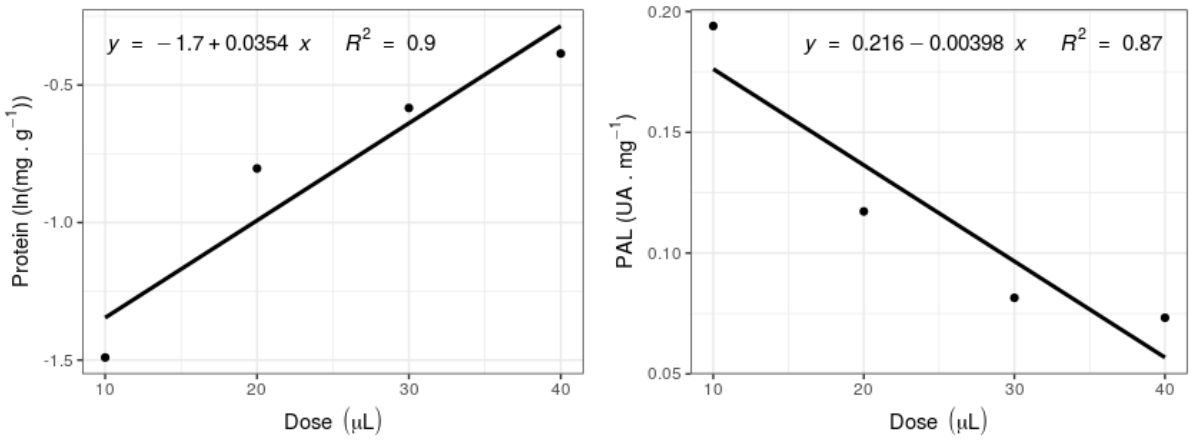
Factors	Levels	Averages of the physiological performance responses**						
		FGC (%)	LGC (%)	GR[I]	NS (%)	AP (%)	APL[II] (cm)	m_f (g)
Control*	-	95.75[ns]	96.75[ns]	73.00[ns]	92.50[ns]	7.50[ns]	1.45A	0.80A
Factorial	-	95.21	96.54	72.79	95.38	4.63	1.81B	0.94B
Essential oil (EO)	(1)	94.63ab	96.13[ns]	72.38[ns]	96.38[ns]	3.63[ns]	1.70[ns]	0.88[ns]
	(2)	96.06a	97.063	73.13	94.63	5.38	1.87	0.91
	(3)	94.00b	96.063	72.250	95.31	4.69	1.83	0.99
	(4)	95.31ab	96.625	72.94	95.63	4.38	1.78	0.94
	(5)	96.06a	96.81	73.25	94.94	5.06	1.86	0.96
Dose (µL)	10	95.15[ns]	96.30[ns]	72.60[ns]	95.70[ns]	4.30[ns]	1.74[ns]	0.97[ns]
	20	94.70	96.50	72.55	96.10	3.90	1.83	0.97
	30	95.05	96.30	72.70	94.65	5.35	1.83	0.91
	40	95.95	97.05	73.30	95.05	4.95	1.86	0.90

[ns]Not significant, at the 5% level of significance ($p < 0.05$). *Different uppercase letters in the columns determine the contrast of the additional treatment (control) with the factorial by the F test ($p<0.05$). Variable transformed by Box and Cox with [I]$\lambda=2$ and [II]$\lambda=0$.

Table 2. Averages of the total soluble proteins content ($mg \cdot g^{-1}$) and of the PAL enzyme activity ($UA \cdot mg^{-1}$) of *L. sativa* seedlings of the experiment completely randomized in double factorial with five essential oils (citronella, guaçatonga, melaleuca, patchouli and pitangueira) versus four doses (10, 20, 30 and 40 µL) in the 100% concentration of the essential oil and an additional treatment as control (0 µL).

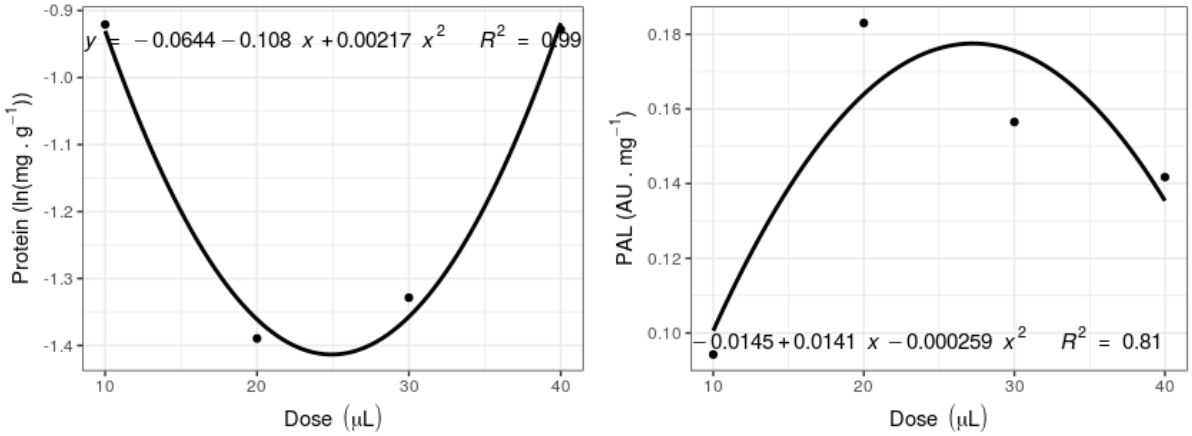
Factors	Levels	Averages of the biochemical performance responses**	
		Total proteins[III] ($mg \cdot g^{-1}$)	PAL ($UA \cdot mg^{-1}$)
Control*	-	0.675 A	0.0655 A
Factorial	-	0.495 B	0.1096 B
Dose** (µL)	Essential oil		
10	Citronella	0.2260 c	0.1940 a
	Guaçatonga	0.5875 a	0.0740 b
	Melaleuca	0.3068 bc	0.1518 a
	Patchouli	0.4008 ab	0.0943 b
	Pitangueira	0.4925 a	0.0868 b
20	Citronella	0.4753 b	0.1173 b
	Guaçatonga	0.7460 a	0.0778 b
	Melaleuca	0.4083 b	0.1160 b
	Patchouli	0.2605 c	0.1830 a
	Pitangueira	0.4195 b	0.0965 b
30	Citronella	0.5845 b	0.0815 bc
	Guaçatonga	1.0636 a	0.0510 c
	Melaleuca	0.4155 b	0.1090 ab
	Patchouli	0.2668 c	0.1565 a
	Pitangueira	0.4895 b	0.0900 bc
40	Citronella	0.6980 a	0.0733 b
	Guaçatonga	0.7223 a	0.0608 b
	Melaleuca	0.2950 b	0.1575 a
	Patchouli	0.4038 b	0.1418 a
	Pitangueira	0.6313 a	0.0803 b

*Different uppercase letters in the columns determine the contrast of the additional treatment (control) with the factorial by the F test at the 5% level of significance ($p < 0.05$). **Averages not followed by the same lowercase letter in the column differ statistically from one another by Duncan's Test ($p<0.05$). Variable transformed by Box and Cox with [III]$\lambda=0$.



(a) (b)

Fig 1. Relationship between the doses of the essential oil of citronella and the response variable: (a) total soluble proteins content and (b) activity of the enzyme phenylalanine ammonia-lyase (PAL).



(a) (b)

Fig 2. Relation between the doses of patchouli essential oil and the response variable: (a) total soluble proteins content at optimal dose 24.88 µL and (b) activity of the enzyme phenylalanine ammonia-lyase (PAL), with optimal dose 27.22 µL.

Table 3. Averages of β-1,3-glucanase enzyme activity (UA·mg^{-1}) of *L. sativa* seedlings of the experiment completely randomized in double factorial with five essential oils (citronella, guaçatonga, melaleuca, patchouli and pitangueira) versus four doses (10, 20, 30 and 40 µL) in the 100% concentration of the essential oil, and an additional treatment as control (0 µL).

Factors	Levels	Averages of the biochemical performance responses*
		β-1,3-glucanase^{IV} (UA·mg-1)
Control*	-	0.0230^{ns}
Factorial	-	0.0341
Essential oil (EO)	Citronella	0.0375 a
	Guaçatonga	0.0236 b
	Melaleuca	0.0433 a
	Patchouli	0.0401 a
	Pitangueira	0.0262 b
Dose* (µL)	10	0.0377^{ns}
	20	0.0379
	30	0.0294
	40	0.0315

^{ns}Not significant, at the 5% level of significance ($p < 0.05$). *Averages not followed by the same lowercase letter in the column differ statistically from each other by the Duncan Test ($p < 0.05$). Variable transformed by Box and Cox with $^{IV}\lambda=0$.

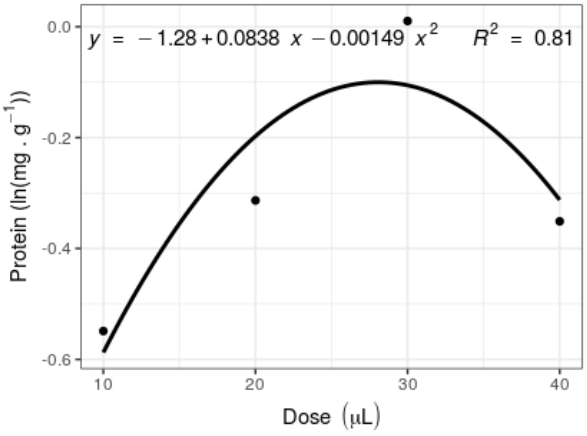


Fig 3. Functional relationship for total soluble proteins content of guaçatonga essential oil, with optimal dose 28.12 µL.

when compared to control. The major component of melaleuca EO was 1,8-cineole (72.31%), followed by α-terpineol (8.55%) and α-thujinene (6.1%) (Table A). According to Ponce et al. (2004), high antioxidant properties are exhibited by *Melaleuca alternifolia* EO, which indicates that this EO can be useful as a sanitizing agent.

The EO of guaçatonga stands out among others in terms of total proteins content in the plant tissue, since it presented up to 36.54% more total soluble proteins content and with a reduction of PAL activity by 22.14% when compared to control (Table 2). The functional relationship increased until the optimal dose (function maximum point) of 28.12 µL (Figure 3). The major compounds of guaçatonga EO (Table A) were γ- murolene (19.55%), followed by α-zingiberene (15.24%) and σ-amorphene (13.17) (Vismara, 2019).

The high concentration of proteins recorded by guaçatonga EO (30 µL) is advantageous from the point of view of induction of resistance. This is because it allows the route of several proteins related to pathogenesis, such as: β-1,3-glucanase, chitinase, peroxides, defensins, thionines, among others (Stangarlin et al., 2011). In addition, the 30 µL dose showed a 22.14% reduction in PAL activity when compared to control, which may benefit the post-harvest preservation of lettuce by inhibition of the enzyme (Chen et al., 2017).

In terms of total proteins content, patchouli EO has a behavior different from those of citronella and guaçatonga. The functional relationship decreased up to the optimal dose (function minimum point) of 24.88 µL (Figure 2a). Whereas, for PAL, it presents a maximum efficiency dose of 27.22 µL (Figure 2b). In chemical identification (Table A), patchoulol (21.99%) was the most abundant substance, followed by α-guaienum (18.32%), γ-patchoulene (16.44%), and seichhelene (10.6%) (Vismara, 2019).

For pitangueira EO, the 40 µL dose recorded the highest total proteins content (0.6313 $mg \cdot g^{-1}$), but with values close to that of control (0.675 $mg \cdot g^{-1}$). For PAL, the highest average activity (0.0965 $mg \cdot g^{-1}$) was obtained at the dose of 20 µL, which corresponds to an increase of 32.12% when compared to control (0.0655 $mg \cdot g^{-1}$). Following the chemical identification of this EO (Table A), calamen-10-one (20.21%) was the most abundant substance, followed by silfiperferol-6-em-5-one (10.06%) and Gemacreno B (6.24%) (Vismara, 2019).

The EOs of citronella, patchouli, and melaleuca presented a higher enzyme activity of β-1,3-glucanase; whereas, for guaçatonga and pitangueira, the averages were smaller and closer to control. It is verified that melaleuca showed up to 46.88% more β-1,3-glucanase activity compared to control (Table 3).

Indeed, the responses on how volatile compounds act (from the physiological to the environmental level) has remained the present focus of study (Dudareva et al., 2013). The use of RNA sequencing, as well as available plant and microbial genomes, will facilitate future identification of mechanisms that regulate metabolic pathways in plants (Sun et al., 2016).

A joint evaluation of the data can affirm that the lettuce plant proved to be a good reference plant for the proposed evaluations, since it was seen that the treatments interfered in the primary metabolism (proteins) and secondary activation specialized for PAL activity. It was observed that the EOs and their doses are specific in action, which can be observed by the non-egalitarian behavior exhibited by the different EOs or doses. Such behavior is expected, since the composition of the oils (Table A) have diverse in terms of their active compounds.

PAL is the enzyme of specialized secondary metabolism of plants most intensively studied, due to its importance in reactions involved in the metabolism of phenolic compounds, which exemplified in reactions involving the formation of esters, coumarins, flavonoids, and lignins (Stangarlin et al., 2011). The activation of PAL awakens to new works, since the EOs presented the potential of activating the route of phenylpropanoids, which is the main route of plant defense. This opens up the window for new works to explore the possible metabolites synthesized as final products in this group of phenolic compounds.

The results demonstrate that EOs have the potential to activate defense mechanisms related to PR-proteins, a protein of extreme importance in the process of plant defense (Fesel and Zuccaro, 2016). Medina et al. (2011) reported that extracts from various plant species have a link between antimicrobial activity and their antioxidant potential. This is because phenolic compounds can react with the cell membrane of the phytopathogen and, in this way, can inactivate the enzymes essential for its metabolism.

In plant defense process, one mode of action of β-1,3-glucanase is to hydrolyze the cell wall of pathogens.

Therefore, the results suggest the need for new studies with different pathosystems and pathogens rich in cell wall glucans, such as filamentous fungi. In addition, the identification of components presents in EOs, which may have an effect on the control of diseases, can become an auxiliary tool for evaluating prospective natural products, thus providing subsidies for alternative management practices and, consequently, promoting the advancement of organic agriculture (Dudareva et al., 2013; Lorenzetti et al., 2011).

Materials and methods

Experimental design

The study was carried out at the Laboratory of Seed Analysis and Biochemistry of the Federal University of Technology-Paraná (UTFPR), *Campus* Dois Vizinhos. The design was completely randomized in a double factorial (5 oils × 4 doses) and control (witness), with 4 replicates of 100 Grand Rapids TBR lettuce seeds without industrial chemical treatment. The model representing the experimental design is as follows:

$$Y_{ijk} = \mu + O_i + D_j + OD_{ij} + \epsilon_{ijk} \text{ and } Y_k = \mu + A + \epsilon_k. \quad (1)$$

Where Y is the observed value, μ is the average value inherent to all observations, O represents the effect of EO levels, D represents the effect of dose levels, OD represents the effect of combining the levels of EO and dose, A is the effect of the additional treatment that corresponds to growth control (positive control) and ϵ is the random error.

Plant materials

The EOs were extracted from fresh leaves of citronella, guaçatonga, melaleuca, patchouli, and pitangueira by hydrodistillation. A clevenger extraction device with cold finger graduated to 10 mL and 2 L glass balloon heating mantle was used for the extraction of oils denser than water. They were purified using membrane type syringe filter (Note 1) and reserved in Eppendorf® microtubes.

The density of each oil was determined with an analytical balance according to the Brazilian Pharmacopoeia (Brasil, 2010) as: citronella (0.8660 g·mL^{-1}); guaçatonga (0.8933 g·mL^{-1}); melaleuca (0.9188 g·mL^{-1}); patchouli (0.9386 g·mL^{-1}); and pitangueira (0.9707 g·mL^{-1}). The chemical identification of EOs was done by GC-MS at Empresa Brasileira de Pesquisa Agropecuária [Embrapa] Florestas (Table A, Appendix A) in the study of Vismara (2019).

Treatments

Dormancy of the lettuce seeds was prevented by cooling at 10°C for three days (Brasil, 2009). Afterward, the seeds were stored in stainless steel crucibles of 8 cm in diameter and 5 cm in height. Subsequently, 100% concentration the EOs were applied to 1 cm^2 of filter paper at the doses of 10, 20, 30, and 40 µL per paper/crucible. Finally, the crucibles were sealed for a period of 24 h in a cold chamber at 10°C. The sowing was done according to the Rules for Seed Analysis (RAS) over a substrate of blotting paper moistened with distilled water at 2.5 times its weight in Gerbox® taken to a Biochemical Oxygen Demand incubator camera at 25°C, with photoperiod of 12 h for 7 days (Brasil, 2009).

The traits measured

The response variables to infer the physiological performance of the seeds were collected according to RAS (Brasil, 2009). The first germination count (FGC, %), was performed on the fourth day after sowing. On the seventh day, the final germination count was performed (LGC, %). The emergencies between FGC and LGC were also counted to determine germination rate (*GR*), as adapted from Maguire (1962):

$$GR = \sum_{i=4}^{7} \frac{s_i}{d_i}. \quad (2)$$

Where s_i is number of germinated seedlings, d_i is number of days until the germination count i, with $4 \leq i \leq 7$.

The normal seedlings (NS, %) and abnormal seedlings (AS, %) were counted. With the aid of a ruler, length of the aerial part (APL, cm) of normal seedlings was measured (Nakagawa, 1999). Fresh mass (m_f, g) was weighed concomitantly with the assessment of APL using an analytical balance. In sequence, this plant material was reserved for the evaluation of the variables' response to biochemical behavior including: total soluble proteins content in plant tissue (mg·g^{-1}) and enzyme activities of β-1,3-glucanase (UA·mg^{-1}) and PAL, UA·mg^{-1}).

For extraction of proteins related to pathogenesis (PR-proteins), the methodology developed by Moerschbacher et al. (1988) and described by Guzzo and Martins (1996) was applied. In brief, the vegetable material of each treatment and repetition was weighed and macerated together with 0.2 g of resin Dowex1-X8 and 0.3 g of PVPP (polyvinylpolypyrrolidone). To the macerate, 5 mL of borate buffer (0.1 M, pH 8.8, EDTA 1 mM, DTT 1 mM, and ascorbic acid 50 mM) was added. The mixture was left to stand for 5 min. The vegetable extract was centrifuged at 20000 g and 4°C for 30 min. Supernatants were used to determine total soluble proteins content and β-1,3-glucanase.

For determination of total soluble proteins content (mg·g^{-1}) was transferred 40 µL of the supernatant extract, obtained in the extraction of proteins related to the pathogenesis, for test tubes, adding in the sequence 460 µL microliters of distilled water and 1000 µL of the Bio-Rad reagent. The tubes were vortexed and sequentially read in a spectrophotometer, at 630 nm. For quantification of the total proteins the absorbance was inserted in the mathematical equation obtained from the standard curve of Bovine Serum Albumin protein.

Determination enzyme activity of β-1,3-glucanase (UA·mg^{-1}) was based on the methodology developed by Wirth and Wolf (1988) and described by Guzzo and Martins (1996), with adaptations. In brief, 200 µL of diluted plant extract (obtained during the extraction of proteins related to pathogenesis) was transferred to microtubes containing 400 µL of sodium acetate buffer (50 mM, pH 5.0) and 200 µL of solution with enzymatic substrate (CM-Curdlan-RBB 4 mg·mL^{-1}). The samples reaction mix were vortexed and incubated in a water bath at 40°C for 1 h. The enzymatic reaction was stopped by adding 200 µL of HCl 2N, an exergonic reaction promoter. The samples were cooled on ice for 10 min and centrifuged at 10,000 g for 5 min. The supernatant obtained was read in a spectrophotometer at 595 nm.

The enzyme activity of PAL (UA·mg^{-1}) was determined

according to the methodology described by Rodrigues et al. (2006), with adaptations. PAL activity was evaluated based on the differences in absorbance resulting from its conversion of phenylalanine to trans-cinnamic acid. In brief, the plant material obtained from the germination test was weighed and transferred to a pre-chilled mortar and 6 mL of TRIS–HCL pH 8 buffers was added. After thorough maceration of the sample, it was packed in microtubes and centrifuged for 10 min at 6000 g and 4°C. An aliquot of 200 µL of the supernatant was transferred to a test tube and another 5.0 mL of the TRIS buffer was added. The samples were agitated in vortex again to obtain the enzymatic extract. Subsequently, 1.5 mL of the enzyme extract, 1 mL of the extraction buffer, and 0.5 mL of phenylalanine (49.6 $mg \cdot mL^{-1}$) were transferred to new test tubes and then vortex again. The tubes were incubated in a water bath for 1 h at 40°C. The reaction was stopped by placing the tubes in an ice bath for 10 min. The spectrophotometer readings were taken at 290 nm.

Statistical analysis

The computational tool R version 4.0.3 (R Core Team, 2020) was used for the data analysis and *ExpDes.pt* package version 1.2.0 was used for the variance analysis (Ferreira et al., 2018). When it was necessary to transform some variable, *MASS* package version 7.3-53 was used (Venables and Ripley, 2002). For the treatments (EO × dose) with control (without EO), DescTools version 0.99.38 was used (Signorell et al., 2020).

To verify the assumption of normality of the model residuals (1), Shapiro and Wilk (1965) test was applied at 5% level of significance. When this assumption was not met, Box and Cox (1964) transformation was applied; however, if obtained, the value of λ is matched to a known mathematical transformation, as proposed by Gaudry and Laferrière (1989).

In this way, the data were subjected to ANOVA and, when the F test was significant, Duncan (1955) post hoc comparison test was performed for the qualitative factor (EO), while polynomial regression was performed for the quantitative factor (dose). To compare the additional treatment (control) with the factorial, Dunnett's test was applied (Vaz, 2013; Dunnett, 1964, 1955). The bench mark for decision making in the tests was the p-value at 5% level of significance ($p < 0.05$).

Conclusion

The lettuce plant has been proven to be a good reference plant for assessments related to physiological and biochemical performance following treatment with EOs. The EOs used in the treatments of lettuce seed did not cause undesirable damages, but interfered positively with physiological parameters such as aerial part length and fresh mass.

Furthermore, the EOs have demonstrated the potential to activate the plant's defense process to control phytopathogens. More specifically, citronella EO (at the dose of 10 µL) activated two plant defense mechanisms: PAL enzyme activity route and β-1,3-glucanase enzyme activity route. The essential oils of melaleuca (10 and 40 µL) and patchouli (20 and 30 µL) demonstrated a successful activation of the PAL enzyme activity route.

References

Alves E, Perina FJ (2014) Extratos vegetais e óleos essenciais na indução de resistência em plantas contra patógenos. In: Schwan-Estrada KRF, Silva CM, Maia AJ, Faria CMDR, Colella JCT Indução de resistência em plantas a patógenos, Centro de Ciências Agrárias do Programa de Pós-Graduação em Agroecologia da Universidade Estadual de Maringá (MPA-UEM), Maringá, Brasil, p. 73-85.

Babychan M, Jojy ET, Syriac GM (2017) Bio-control agents in management of post-harvest diseases. Life Sci Int Res J. 4(1): 51-55.

Box GEP, Cox DR (1964) An analysis of transformations. J R Stat Soc Ser A Stat Soc. Series B (Methodological), 26(2): 211-243.

Brasil, Agência Nacional de Vigilância Sanitária [ANVISA] (2010) Farmacopeia brasileira. 5th ed., v. 1, Anvisa, Brasília, Brasil.

Brasil, Ministério da Agricultura, Pecuária e Abastecimento. Secretaria de Defesa Agropecuária (2009) Regras para análise de sementes. Ministério da Agricultura, Pecuária e Abastecimento (Mapa/ACS), Brasília, Brasil, 399 p.

Chen X, Ren L, Li M, Qian J, Fan J, Du B (2017) Effects of clove essential oil and eugenol on quality and browning control of fresh-cut lettuce. Food Chem. 214, 432-439.

Dudareva N, Klempien A, Muhlemann JK, Kaplan I (2013) Biosynthesis, function and metabolic engineering of plant volatile organic compounds. New Phytol. 198(1): 16-32.

Duncan DB (1955) Multiple range and multiple F tests. Biometrics. 11(1): 1-42.

Dunnett CW (1955) A multiple comparison procedure for comparing several treatments with a control. J Am Stat Assoc. 50: 1096-1121.

Dunnett CW (1964) New tables for multiple comparisons with a control, Biometrics. 20:482-491.

Ferreira EB, Cavalcanti PP, Nogueira DA (2018) ExpDes.pt: pacote experimental designs (portuguese). R package version 1.2.0, https://CRAN.R-project.org/package=ExpDes.pt.

Fesel PH and Zuccaro A (2016) β-glucan: crucial component of the fungal cell wall and elusive MAMP in plants. Fungal Genet and Biol. 90, 53-60.

Food and Agriculture Organization [FAO] (2018) Transforming food and agriculture to achieve the SDGs: 20 interconnected actions to guide decision-makers. Technical Reference Document, Rome, Italy, 116 p.

Gaudry M, Laferrière R (1989) The box-cox transformation: power invariance and a new interpretation. Econ Lett. 30(1): 27-29.

Ghini R, Kimati H (2002) Resistência de fungos a fungicidas. Embrapa Meio Ambiente, Jaguariúna, Brasil, 78 p.

Guzzo SD, Martins EMF (1996) Local and systemic induction of β-1,3-glucanase and chitinase in coffee leaves protected against *Hemileia vastatrix* by *Bacillus thuringiensis*. J Phytopathol. 144(9-10): 449-454.

Lorenzetti ER, Monteiro FP, Souza PE, Souza RJ, Scalice HK, Diogo Jr R, Pires MSO (2011) Bioatividade de óleos essenciais no controle de *Botrytis cinerea* isolado de morangueiro. Rev Bras de Pl Med. 13 (especial): 619-627.

Maguire JD (1962) Speed of germination — aid in selection and evaluation for seedling emergence and vigor. Crop Sci. 2(2): 176-177.

Mattos LPV (2010) Controle de *Guignardia citricarpa* e *Penicillium digitatum* em laranja com óleos essenciais e agentes de biocontrole. Tese (Doutorado) - Faculdade de Ciências Agronômicas da Universidade Estadual Paulista, Botucatu, Brasil, PR, 94 f.

Mazaro SM, Deschamps C, Mio LD, Biasi LA, Gouvea AD, Sautter CK (2008) Comportamento pós-colheita de frutos de morangueiro após a aplicação pré-colheita de quitosana e acibenzolar-S-metil. Rev Bras Frutic. 30(1): 185-190.

Medina AL, Hass LIR, Chaves FC, Salvador M, Zambiazi CR, Silva WP, Nora L, Rombaldi CV Araçá (*Psidium cattleianum Sabine*) fruit extracts with antioxidant and antimicrobial activities and antiproliferative effect on human cancer cells. Food Chem. v. 128, n. 4, p. 916-922, 2011.

Mengiste T (2014) Induced resistance to post-harvest plant pathogens. In: Schwan-Estrada, KRF, Silva, CM, Maia, AJ, Faria, CMDR, Colella, JCT Indução de resistência em plantas a patógenos, Centro de Ciências Agrárias do Programa de Pós-Graduação em Agroecologia da Universidade Estadual de Maringá (MPA-UEM), Maringá, PR, p. 141-161.

Miranda CASF, Cardoso MG, Carvalho MLM, Machado SMF, Gomes MS, Andrade J, Teixeira ML (2015) Allelopathic activity of medicinal plant essential oils on seed germination and vigor of lettuce achenes. Semina: Ciênc Agrár. 36(3Supl1): 1783-1798.

Moerschbacher BM, Noll UM, Flott BE, Reisener HJ (1988) Lignin biosynthetic enzymes in stem rust infected, resistant and susceptible near-isogenic wheat lines. Physiol Mol Plant Pathol. 33(1): 33-46.

Morais LAS (2009) Óleos essenciais no controle fitossanitário. In: Bettiol, W and Morandi, MAB Biocontrole de Doenças de Plantas: uso e perspectivas, Embrapa Meio Ambiente, Jaguariúna, Brasil, p. 139-152.

Nakagawa, J (1999) Testes de vigor baseados no desempenho de plântulas. In: Krzyzanowski FC, Vieira RD, França Neto JB (Ed.) Vigor de sementes: conceitos e testes, ABRATES, Londrina, Brasil, v.1, p. 20-31.

Nikkhah M, Hashemi M, Najafi MBH, Farhoosh R (2017) Synergistic effects of some essential oils against fungal spoilage on pear fruit. Int J Food Microbiol. 257: 285-294.

Pires NM, Oliveira VR (2011) Alelopatia. In: Oliveira Jr RS. Constantin J, Inoue MH Biologia e manejo de plantas daninhas, Omnipax, Curitiba, Brasil, p. 95-123.

Ponce AG, Del Valle CE, Roura SI (2004) Natural essential oils as reducing agents of peroxidase activity in leafy vegetables. LWT-Food Sci Technol. 37(2): 199-204.

R Core Team (2010) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org/.

Rodrigues AA, Bezerra Neto E, Coelho RS (2006) Indução de resistência a *Fusarium oxysporum* f. sp. *Tracheiphilum* em Caupi: eficiência de indutores abióticos e atividade enzimática elicitada. Fitopatol Bras. 31(5): 492-499.

Schwan-Estrada KRF, Stangarlin JR, Cruz MES (2000) Uso de extratos vegetais no controle de fungos fitopatogênicos. Floresta. 30(1/2): 129-137.

Shapiro SS, Wilk MB (1965). An analysis of variance test for normality (complete samples). Biometrika. 52(3/4): 591-611.

Shuping DSS, Eloff JN (2017) The use of plants to protect plants and food against fungal pathogens: a review. Afr J Tradit Complement Altern Med. 14(4): 120-127.

Signorell A et mult. al. (2020) DescTools: Tools for descriptive statistics. R package version 0.99.38., https://cran.r-project.org/package=DescTools.

Souza Filho ADS, Guilhon GMSP, Santos LS (2010). Metodologias empregadas em estudos de avaliação da atividade alelopática em condições de laboratório: revisão crítica. Planta daninha. 28(3), 689-697.

Stangarlin JR, Kuhn OJ, Toledo MV, Portz RL, Pascholati SF (2011) A defesa vegetal contra fitopatógenos. Sci Agrar Parana. 10(1): 18-46.

Sun P, Schuurink RC, Caissard JC, Hugueney P, Baudino S (2016) My way: noncanonical biosynthesis pathways for plant volatiles. Trends Plant Sci. 21(10): 884-894.

Taiz L, Zeiger E, Møller IM, Murphy A (2017) Fisiologia e desenvolvimento vegetal, 6rd ed, Artmed, Porto Alegre, Brasil.

Tripathi P, Yami H, Shukla AK (2016) Impact of essential oils on growth of phytopathogenic fungi responsible for rotting of fruits. J Adv Biol Biotechnol. 10(2): 1-10.

Venables WN, Ripley BD (2002) Modern applied statistics with S, 4th ed. Springer, New York.

Vaz MAB (2013) Estudo de delineamentos experimentais no esquema fatorial duplo com um tratamento adicional. Dissertação (Mestrado) - Universidade de São Paulo. Programa de Pós-Graduação em Estatística e Experimentação Agronômica, Piracicaba, Brasil, 102 f.

Vismara LS (2019) Óleos essenciais na indução de resistência em morangos ao mofo cinzento, à *Botrytis cinerea in vitro* e ação toxicológica. Tese (Doutorado) - Universidade Tecnológica Federal do Paraná. Programa de Pós-Graduação em Agronomia, Pato Branco, Brasil, 212 f.

Wirth SJ, Wolf GA (1992) Micro-plate colourimetric assay for endo-acting cellulase, xylanase, chitinase, 1,3-β-glucanase and amylase extracted from forest soil horizons. Soil Biol Biochem. 24(6): 511-519.

Notes

Note 1. Filter specifications for membrane type syringes: 0.45 µm pore size; 30 mm outer diameter.; filtration area of 4.3 cm^2; 100 mm processing volume; volume retained after filtration less than 100 µL and external polypropylene material.