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ORIGINAL ARTICLE

Tea tree and wintergreen essential oils in the management of the invasive species *Cortaderia selloana* and *Nicotiana glauca*

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Abstract

The chemical composition of tea tree (*Melaleuca alternifolia*) and wintergreen (*Gaultheria procumbens*) essential oils as well as their phytotoxic effects against two invasive species – *Cortaderia selloana* and *Nicotiana glauca* – were studied. Fifty-eight compounds accounting for 98.89–99.94% of the total commercial tea tree and wintergreen essential oils were identified by Gas Chromatography-Mass Spectrometry (GC-MS) analysis. Tea tree essential oil with terpinen-4-ol (28.37 ± 0.05%) followed by 1,8-cineole (15.81 ± 0.06%), γ-terpinene (15.60 ± 0.03%), α-pinene (10.92 ± 0.08%) and α-terpinene (8.52 ± 0.01%) as the main compounds did not produce significant effects against seed germination and hypocotyl growth of *N. glauca*, but showed significant effects in seed germination inhibition of *C. selloana* (34.69%) as well as in hypocotyl (60.96%) and radicle (62.55%) growth, at the highest dose (1 µl · ml^{-1}) assayed. High amounts of methyl salicylate (99.63 ± 0.02%) were found in *G. procumbens* essential oil with remarkable phytotoxic effects in *C. seollana*. Methyl salicylate inhibited seed germination (77.38%) and hypocotyl and radicle growth (96.38% and 96.65%, respectively) at the highest dose (1 µl · ml^{-1}) assayed. Wintergreen essential oil constitutes an eco-friendly alternative to control the high capacity of invasiveness of *C. selloana.*

Keywords: *Cortaderia selloana*, essential oils, *Nicotiana glauca*, phytotoxicity, tea tree, wintergreen

Introduction

The naturalization of invasive species brings serious consequences in ecosystems, from the replacement of endemic plant species, that could even become extinct, to changes in native fauna as well as alteration of soil chemistry, geomorphological processes, fire regime and hydrology (Cronk and Fuller 2013; Weber 2017). These very hazardous non-native species have been taken out of their native systems and are used as ornamental plants due to their exotic and attractive appearance (Simberloff *et al.* 2013). In this sense, *Hypericum canariense* L., a species native to the Canary Islands has been introduced and become naturalized in California with detrimental effects on surrounding original plants (Rejmánek 2015). Also, the invasive annual species, *Impatiens glandulifera* Royle native to the western Himalayas and introduced into Europe and North America in the middle of the 19th century, is able to affect soil fungal and bacterial communities and consequently alter soil properties (Gaggini *et al.* 2018). In addition, this species is able to interfere in networks between plant and pollinators, impairing native plant pollination (Vanbergen *et al.* 2018). It is estimated that at least 13,168 vascular plant species have become naturalized in at least one of the 843 regions (including 362 islands) covered by the GloNAF database (van Kleunen *et al.* 2015). *Sonchus oleraceus*, *Ricinus communis*, *Oxalis corniculata*, *Portulaca oleracea*, *Eleusine indica*, *Chenopodium album*, *Capsella bursa-pastoris*, *Stellaria media*, *Bidens pilosa*, *Datura stramonium* and *Echinochloa crus-galli* are the most widely distributed species (Pyšek *et al.* 2017). Information about alien species distribution is essential to determine their occurrence, status and impact as well as to prevent new incursions or reduce the impact of invasive species

(Latombe *et al.* 2017). Climatic change, among other factors, may contribute to the dispersion and tolerance of invasive plant species (Early *et al.* 2016). Regarding this, an important increase in naturalized plant numbers is expected in the next 20 years with more invasions in northern temperate countries and a reduction in tropical and sub-tropical regions (Seebens *et al.* 2015; Dullinger *et al.* 2017). In addition, environmental impacts of alien plant species also result in socioeconomic impacts. Out of 128 alien plant species screened in Europe, 96 negative impacts have been recorded in agriculture, animal and forestry production as well as in human infrastructure, health and social life (Rumlerová *et al.* 2016).

In relation to this, an interesting alien invasive species is pampas grass – *Cortaderia selloana*, Poaceae originally from Brazil, Argentina, Chile and Uruguay which has been traded extensively as an ornamental plant. As a consequence, it has become naturalized in the Macaronesia Islands, South Africa, Australia, New Zealand, the Hawaiian islands, the Pacific coast of the USA and southern Europe (Basnou 2006). Particularly, it is considered invasive in the Mediterranean Basin (Brunel *et al.* 2010) with a remarkably rapid and deep impact mainly in the north and along the eastern coast of the Iberian Peninsula and in the Canary Islands it is included in the Spanish Catalogue of Invasive Species (RD 630/2013) (Doménech-Carbó *et al.* 2018). *Cortaderia selloana* can tolerate moderate drought, winter frost, intense sunlight and warm summer temperatures as well as a broad variety of physiological soil conditions (Vourlitis and Kroon 2013). This wide tolerance for varying environmental circumstances is presumably the main reason why *C. selloana* is highly invasive together with the fact that it is a gynodioecious species with both female and hermaphrodite specimens (Doménech-Carbó *et al.* 2018), so it is able to self-fertilize its seeds without a pollinator, a typical characteristic of grasses, and regenerate a whole population from a single plant (Kroon 2007). Consequently, *C. selloana* changes soil properties and in this way, the areas affected by this species have lower total soil nitrogen and higher C/N values, with less species, families and life diversity (Domènech *et al.* 2006).

Another important example is tree tobacco (*Nicotiana glauca*, Solanaceae) which is an invasive species native to South America (Petanidou *et al.* 2012), and introduced in the first half of the 19th century as an ornamental plant. It is a naturalized species in Australia, California, Mexico, Hawaii, northern and eastern Mediterranean regions, the Canary Islands, and northern and southern Africa (Ollerton *et al.* 2012). It is included in the Global Invasive Species Database (Global Invasive Species Database 2019) due to its high invasion potential. *Nicotiana glauca* disturbs ecosystem structures and reduces native biodiversity by growing in a wide variety of open and disturbed habitats including roadsides and lakeshores and has become a problem in relatively dry areas (Thomas *et al.* 2016; Ayenew *et al.* 2018). Its capacity of water conservation by stomatal closure and osmotic adjustment, as well as the presence of leaf wax that reduces the absorption of excess radiation provide tree tobacco with the ability to invade disturbed areas subject to salinity, drought and high radiation (González *et al.* 2012). These characteristics together with its ability to reproduce sexually by pollination mainly through hummingbirds as well as by autogamy when these natural pollinators are missing increases its invasive capacity with high seed production and fast development. In addition it can also arise from roots which makes *N. glauca* more difficult to eradicate, improving its expansion. Therefore, *N. glauca* is able to impact the environment, socioeconomic factors as well as livestock and human health (Jones 2018) as a source of the alkaloid anabasine with teratogenic properties (Panter *et al.* 2011).

The management of alien species remains today an aspect of chief importance to guarantee the restoration of affected ecosystems. There are numerous ways to control invasive plants or weeds in natural areas, including prevention, mechanical, cultural, physical, biological and chemical methods (Tu *et al.* 2001; Melander *et al.* 2005). Herbicides are the most common remedy used to eradicate non-native species. It is estimated that in the USA half a million hectares of public wildlands were sprayed with herbicides in 2010, representing 201 tonnes, in which glyphosate was the most commonly used active ingredient (Wagner *et al.* 2016). Indeed, the application of glyphosate-based formulations has not stopped despite its consequent alteration of soil microorganisms, the notable increase in glyphosate-resistant weeds, the potential of direct and indirect health effects as well as environmental contamination (Anza *et al.* 2016; Duke 2018; Van Bruggen *et al.* 2018). So, it is necessary to find less hazardous natural alternatives to synthetic herbicides to control invasive plant species. Specifically, essential oils due to their short environmental half-life and less toxicity, represent a suitable option to synthetic herbicides. These secondary metabolites from mevalonic and shiquimic pathways are mainly recognised by their aromatic, antioxidant, antibacterial and antifungal properties, for which they have been widely used in several industries (Blázquez 2014). Among these mixtures of natural compounds, tea tree (*Melaleuca alternifolia*) essential oil is known for its antimicrobial properties against a broad spectrum of micro-organisms (bacteria, fungi, viruses and protozoa) as well as by its analgesic, anti-inflammatory and anticancer activities (Yadav *et al.* 2016). On the other hand, wintergreen (*Gaultheria procumbens*) essential oil is well-known, together with its antimicrobial properties, for its good antioxidant and antiradical profile. The analgesic activity of methyl salicylate can explain its topical use in the treatment of rheumatism (Garg 2005; Nikolić *et al.* 2013). So, the aims of this work were first to determine the chemical composition of *M. alternifolia* and *G. procumbens* essential oils through gas chromatography mass spectrometry (GC-MS) analysis in order to identify their main compounds. A second aim was to test the *in vitro* phytotoxic activity of these essential oils against seed germination and seedling growth of the problematic *C. selloana* and *N. glauca* in order to evaluate their herbicidal activity and consequently determine their potential use as natural alternatives to synthetic herbicides in the management of these invasive species.

Materials and Methods

Essential oils

Commercial samples of tea tree (*M. alternifolia*) (Batch 0051451) and wintergreen (*G. procumbens*) (Batch 0F18989) essential oils obtained from their leaves were purchased in Guinama and Pranarôm S.A, respectively. Both were stored at 4°C until chemical analysis and phytotoxic assays were carried out.

Invasive plant species

Mature seeds of the invasive plant species pampas grass (*C. selloana*) and tree tobacco (*N. glauca*) were supplied by the Botanical Garden of Valencia.

Gas chromatography mass spectrometry (GC-MS) analysis

GC-MS analysis was carried out with a mass spectrometer 5977A Agilent and a gas chromatograph (Agilent 7890B) apparatus, equipped with a capillary column (95 dimethylpolysiloxane-5% diphenyl), Agilent HP-5MSi (30 m long and 0.25 mm i.d. with 0.25 μm film thickness). The column temperature program was 60°C for 5 min, with 3°C/min increases to 180°C, then 20°C/min increases to 280°C, which was maintained for 10 min. The carrier gas was helium at a flow-rate of 1 ml · min^{-1}. Split mode injection (ratio 1 : 30) was employed. Mass spectra were taken over the m/z 30–500 range with an ionizing voltage of 70 eV. The resulting individual compounds were identified by MS and their identity was confirmed by comparison of their Kovat's retention index calculated using co-chromatographed standard hydrocarbons relative to C_8–C_{32} *n*-alkanes, and mass spectra with reference samples or with data already available in the NIST 2005 mass spectral library and in the literature (Adams 2007).

In vitro assays: *Cortaderia selloana* and *Nicotiana glauca* seed germination and seedling growth with tea tree and wintergreen essential oils

Sets of 20 seeds each with five replicates per treatment were homogenously distributed in Petri dishes (9 cm diameter) between two layers of filter paper (Whatman No. 1) moistened with 4 ml of distilled water and with 0 (control), 0.125, 0.250, 0.50, and 1 μl · ml^{-1} of tea tree and wintergreen essential oils. The Petri dishes were sealed with parafilm and incubated in a germination chamber Equitec EGCS 301 3SHR model, according to previous assays (Blázquez and Carbó 2015) alternating the humidity with 30.0 ± 0.1°C 16 h in light and 20.0 ± 0.1°C 8 h in dark. To evaluate the phytotoxic activity of the essential oils, the number of germinated seeds was counted and compared with those of untreated seedlings. Emergence of the radicle (≥1 mm) was used as an index of germination and seedling length (hypocotyl and/or radicle) data were recorded after 3, 5, 7, 10 and 14 days in each replicate.

Statistical analysis

Experiments were made with five replicates. Data were subjected to one-way analysis of variance (ANOVA) with SPSS statistics 24 software. Tukey's *post hoc* test was used when variances remained homogeneous (Levene's test) and T3 Dunnett's *post hoc* test was employed if not, assuming equal variances. Differences were considered to be significant at p ≤ 0.05.

Results and Discussion

Chemical composition of tea tree and wintergreen essential oils

Fifty-eight compounds accounting for 98.89–99.94% of the total commercial tea tree and wintergreen essential oils were identified by GC-MS analysis. Components were clustered (Table 1) in a homologous series of monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, aromatic compounds and others, and listed according to Kovat's retention index calculated in GC on apolar HP-5MS column.

Monoterpene compounds (95.13%), both hydrocarbons (45.20 ± 0.07%) and oxygenated (49.94 ± 0.05%) with 16 compounds and 14 identified compounds, respectively, were the main qualitative and quantitative fractions found in commercial tea tree essential oil. The monoterpene fraction was also the main phytochemical group in other previously analysed commercial essential oils, such as oregano (*Origanum vulgare*), marjoram (*Origanum majorana*) and *Thymus mastichina* reaching values of 93.05, 96.93 and 83.36%, respectively. These compounds played important roles in the phytotoxic activity against seed germination and seedling growth of weeds (*Portulaca oleracea*, *Lolium multiflorum* and *Echinochloa crus-galli*) (Ibáñez and Blázquez 2017).

Terpinen-4-ol (28.37 ± 0.05%) followed by 1,8-cineole (15.81 ± 0.06%) and the monoterpene hydrocarbons γ-terpinene (15.60 ± 0.03%), α-pinene (10.92 ± 0.08%) and α-terpinene (8.52 ± 0.01%) were the main compounds in *M. alternifolia* essential oil. Also, relatively large amounts of the oxygenated monoterpene α-terpineol (4.24 ± 0.03%) and the monoterpene hydrocarbons terpinolene (3.16 ± 0.03%), *p*-cymene (2.52 ± 0.01%) and β-pinene (1.87 ± 0.01%) were found. These results accomplish the standard ISO 4730 whose chromatography revealed the same characteristic compounds in tea tree essential oil obtained by steam distillation of the foliage and terminal branches (ISO

Table 1. Chemical composition of commercial tea tree from *Melaleuca alternifolia* and wintergreen from *Gaultheria procumbens* essential oils

RI	RI_{Ref}	Compound	Tea tree peak area [%]	Wintergreen peak area [%]
		Monoterpene hydrocarbons	45.20 ± 0.07	0.04 ± 0.00
926	926	Tricyclene	0.04 ± 0.00	–
932	930	α-Thujene	0.02 ± 0.00	–
940	939	α-Pinene	10.92 ± 0.08	0.02 ± 0.00
953	954	Camphene	0.20 ± 0.00	–
980	979	β-Pinene	1.87 ± 0.01	0.01 ± 0.00
987	987	3-*p*-Menthene	0.09 ± 0.00	–
993	990	Myrcene	0.68 ± 0.01	–
1006	1002	α-Phellandrene	0.32 ± 0.00	–
1012	1011	δ-3-Carene	0.47 ± 0.01	–
1019	1017	α-Terpinene	8.52 ± 0.01	–
1022	1024	*p*-Cymene	2.52 ± 0.01	T
1025	1026	1-*p*-Menthene	0.54 ± 0.00	–
1027	1029	Limonene	–	0.01 ± 0.00
1043	1037	*cis*-β-Ocimene	0.07 ± 0.01	–
1054	1050	*trans*-β-Ocimene	0.18 ± 0.01	–
1060	1059	γ-Terpinene	15.60 ± 0.03	–
1087	1088	Terpinolene	3.16 ± 0.03	–
		Oxygenated monoterpenes	49.93 ± 0.05	0.07 ± 0.01
1031	1031	1,8-Cineole	15.81 ± 0.06	0.03 ± 0.01
1071	1070	*cis*-Sabinene hydrate	0.03 ± 0.02	–
1076	1072	*cis*-Linalool oxide	0.29 ± 0.01	–
1098	1099	α-Pinene oxide	0.02 ± 0.00	–
1095	1096	Linalool	0.63 ± 0.01	0.03 ± 0.00
1137	1137	*cis*-*p*-Mentha-2,8-dien-1-ol	0.04 ± 0.00	–
1138	1139	*trans*-Pinocarveol	0.19 ± 0.01	–
1145	1146	Camphor	–	0.01 ± 0.00
1159	1160	Isoborneol	0.11 ± 0.01	–
1170	1169	Borneol	0.06 ± 0.01	–
1180	1177	Terpinen-4-ol	28.37 ± 0.05	–
1186	1186	Dill ether	0.08 ± 0.02	–
1190	1188	α-Terpineol	4.24 ± 0.03	–
1222	1220	α-Fenchyl acetate	0.05 ± 0.01	–
1270	1269	*trans*-Ascaridol glycol	0.02 ± 0.00	–

Table 1. Chemical composition of commercial tea tree from *Melaleuca alternifolia* and wintergreen from *Gaultheria procumbens* essential oils – *continuation*

RI	RI_{Ref}	Compound	Tea tree peak area [%]	Wintergreen peak area [%]
Sesquiterpene hydrocarbons			3.02 ± 0.03	0.01 ± 0.00
1351	1352	α-Longipinene	0.10 ± 0.01	–
1370	1371	Cyclosativene	0.02 ± 0.00	–
1372	1374	Longicyclene	0.06 ± 0.00	–
1389	1391	Sativene	0.04 ± 0.01	–
1403	1407	Longifolene	0.90 ± 0.01	–
1419	1419	β-Caryophyllene	0.02 ± 0.00	0.01 ± 0.00
1432	1433	β-Gurjunene	0.03 ± 0.00	–
1440	1441	Aromadendrene	1.44 ± 0.01	–
1454	1454	α-Humulene	0.02 ± 0.00	–
1461	1460	*allo*-Aromadendrene	0.19 ± 0.01	–
1488	1490	β-Selinene	0.02 ± 0.00	–
1495	1496	Viridiflorene	0.16 ± 0.00	–
1500	1500	α-Muurolene	0.02 ± 0.00	–
Oxygenated sesquiterpenes			0.17 ± 0.01	–
1577	1578	Spathulenol	0.02 ± 0.00	–
1591	1590	Globulol	0.06 ± 0.00	–
1593	1595	Cubenan-11-ol	0.03 ± 0.00	–
1601	1600	Rosifoliol	0.04 ± 0.00	–
1632	1632	γ-Eudesmol	0.02 ± 0.00	–
Aromatic compounds			0.58 ± 0.01	99.83 ± 0.01
959	960	Benzaldehyde	–	T
1173	1173	Ethyl benzoate	–	T
1195	1191	Methyl salicylate	–	99.63 ± 0.02
1199	1196	Methyl chavicol	0.58 ± 0.01	–
1269	1269	Ethyl salicylate	–	0.18 ± 0.00
1333	1337	Methyl *o*-anisate	–	T
1358	1359	Eugenol	–	0.01 ± 0.00
Others			-	T
1002	1002	3-Hexenyl acetate	–	T
TOTAL			98.89 ± 0.01	99.94 ± 0.01

RI – retention index relative to C_8-C_{32} *n*-alkane on HP-5MSi column; RI_{Ref} – Kovats index (Adams 2007); values are means ± standard deviation of three samples; T – trace amount less than 0.01

2017). Although the chemical composition of tea tree essential oil has been well defined by previous authors (Cox *et al.* 2001; Liao *et al.* 2017), there can be slight variations according to the origin of the plant species. High amounts of terpinolene (45.7%) have been found in samples from China that exceed even the usually major component terpinen-4-ol (44.7%) (de Groot and Schmidt 2016). These quantitative and qualitative variations in chemical composition could influence the biological activity of tea tree essential oil. According to this, it has been observed that terpinen-4-ol had more potent acaricidal effects than the following major compounds: 1,8-cineole and α-terpineol. Also, terpinen-4-ol had a significant synergistic effect with terpinolene, and an antagonistic effect with α-terpineol in killing mites (Tighe *et al.* 2013). In addition, terpinen-4-ol has been established to be the compound responsible for antimicrobial (Bordini *et al.* 2018), antitumor (Sobral *et al.* 2014) and herbicidal effects (Verdeguer *et al.* 2011). Phytotoxic effects have also been shown with 1,8-cineole, the second main component of tea tree essential oil analysed here. In this sense, *Eucalyptus* *globulus* Labill. essential oil (1,8-cineole 76.43 ± 0.35%), was able to inhibit the seedling growth of *E. crus-galli* and the radicle development of *L. multiflorum* when 1 μl · ml^{-1} and doses of 0.125, 0.25, 0.50 and 1 μl · ml^{-1}, respectively, of the essential oil were applied (Ibáñez and Blázquez 2018a).

Conversely, in wintergreen essential oil only four monoterpene hydrocarbons (0.04 ± 0.00%) α-pinene, β-pinene, *p*-cymene and limonene and three oxygenated monoterpenes (0.07 ± 0.01%): 1,8-cineole, linalool and camphor were identified.

Among the sesquiterpene fraction in tea tree essential oil (3.19%), 13 hydrocarbons were identified (3.02 ± 0.03%) from which only aromadendrene (1.44 ± 0.01%) and longifolene (0.90 ± 0.01%) reached percentages close to or greater than 1%, while none of the only five oxygenated sesquiterpenes identified reached 0.1%. Aromadendrene-like compounds can be detected in higher amounts in essential oils coming from *Melaleuca* species, like *M. styphelioides* in which isoaromadendrene epoxide (7.45%) and *allo*-aromadendrene have been found (1.18%) (Albouchi *et al.* 2017), as well as in other species, such as in the essential oil of *E. globulus* fruits, where aromadendrene (31.17%), the main compound, followed by 1,8-cineole (14.55%) showed antimicrobial and synergic properties against antibiotic-susceptible and antibiotic-resistant pathogens (Mulyaningsih *et al.* 2010). Regarding longifolene (22.0%), this sesquiterpene hydrocarbon was also together with the oxygenated monoterpene 1,8-cineole (22.9%). They are the main compounds of the essential oil of *E. oleosa* F. Muell leaves from Tunisia with antimicrobial properties, too (Hassine *et al.* 2012).

From the sesquiterpene fraction, β-caryophyllene (0.01 ± 0.00%) was the only sesquiterpene hydrocarbon detected in a slight percentage in wintergreen essential oil analysed here.

On the other hand, aromatic compounds both C_6–C_3 and C_6–C_1 from the shiquimic pathway was the principal fraction of *G. procumbens* essential oil. Methyl chavicol (0.58 ± 0.01%), not detected in wintergreen, was the only aromatic compound found in tea tree essential oil. This fraction (99.82 ± 0.01%) with six compounds identified represented the main qualitative and quantitative groups of wintergreen essential oil analysed here. Methyl salicylate (99.63 ± 0.02%) was by far the principal compound of *G. procumbens* essential oil and of the fraction with benzaldehyde, ethyl benzoate ethyl salicylate, methyl *o*-anisate and eugenol, that did not reach 0.20%. These results are according to previous research that confirms methyl salicylate as being the main compound in essential oil wintergreen leaves (Kujur *et al.* 2017; Singh and Ali 2017). Also, methyl salicylate is popularly known for its analgesic, astringent, carminative, diuretic, stimulant, antispasmodic and antiseptic properties (Cock 2015). Furthermore, it has been included in a pesticidal composition together with rosemary essential oil. At doses with pesticidal effects there is no phytotoxicity or dermal sensitivity (Bessette and Lindsay 2016). However, methyl salicylate has been observed to exert a certain phytotoxicity against *P. oleracea,* inhibiting its seed germination 31.40–44.19% as well as radicle and hypocotyl elongation 37.78–47.75% and 52.63–52.34, respectively, at different doses (0.125, 0.25, 0.50 and 1 μl · ml^{-1}) (Ibáñez and Blázquez 2018b).

In vitro phytotoxic activity of tea tree and wintergreen essential oils against seed germination and seedling growth of *Cortaderia selloana* and *Nicotiana glauca*

Several studies have tested the phytotoxicity of essential oils among the most widely naturalized species, showing a relationship between composition, dosage, soil and weeds. Although monoterpene hydrocarbons may have great herbicidal effects against certain weeds (Blázquez 2014), in general the results showed that oxygenated monoterpenes as well as aromatic compounds are responsible for the main phytotoxic effects. So, the phytotoxic effect of tea tree and wintergreen essential oils with 49.93% and 99.83% of oxygenated monoterpenes and aromatic compounds, respectively, was tested against the seed germination and seedling growth of *C. selloana* and *N. glauca*.

The invasive *C. selloana* species could be controlled by both *M. alternifolia* and *G. procumbens* essential oils. The highest dose (1 μl · ml^{-1}) of tea tree essential oil assayed, significantly inhibited their seed germination by 34.69% in comparison to the control (Table 2). The high percentage of the aromatic compound methyl salicylate in wintergreen essential oil (Table 1) was able to inhibit in a dose dependent manner the seed germination of *C. selloana* with a significant effect regarding the control (reductions of 33.34% and 77.38%) at 0.50 and 1 μl · ml^{-1} (Table 2). *Nicotiana glauca* was a more resistant species to these essential oils. Seed germination of this invasive species was not affected at either dose (0.125, 0.25, 0.50 and 1 μl · ml^{-1}) tested of tea tree essential oil, with lower phytotoxic effects than with wintergreen essential oil. Only an inhibition percentage of 15.96% was reached with the application of the highest dose (1 μl · ml^{-1}) of *G. procumbens* essential oil.

As occurs with the application of synthetic herbicides, in some cases expected results are not obtained. Glyphosate and imazapic only reduced the belowground biomass of the invasive species *Liriope spicata* Lour. by 43 and 45%, respectively, at 180 days after treatment (Enloe *et al.* 2015). Preliminary studies have shown that herbicide mixes

Table 2. *In vitro* effects of tea tree from *Melaleuca alternifolia* and wintergreen from *Gaultheria procumbens* essential oil against *Cortaderia selloana* and *Nicotiana glauca* seed germination

Essential oil	Controlled species	Control	Doses [µl · ml^{-1}]			
			0.125	0.25	0.5	1
Tea tree	*C. selloana*	98.00 ± 2.00 a	92.00 ± 2.55 a	92.00 ± 2.00 a	86.00 ± 4.30 a	64.00 ± 7.81 b
	N. glauca	91.00 ± 3.32 a	60.00 ± 7.58 a	65.00 ± 11.29 a	72.00 ± 11.02 a	65.00 ± 7.91 a
Wintergreen	*C. selloana*	84.00 ± 2.45 a	83.00 ± 2.00 a	77.00 ± 6.04 a	56.00 ± 3.67 b	19.00 ± 6.21 c
	N. glauca	94.00 ± 4.00 a	90.00 ± 3.54 a, b	87.00 ± 2.55 a, b	91.00 ± 1.87 a, b	79.00 ± 3.32 b

Values are mean percentages of five replications ± error deviation after 14 days of incubation; means followed by different letters in the same line indicate that they are significantly different at $p < 0.05$ according to T3 Dunnett and Tukey tests

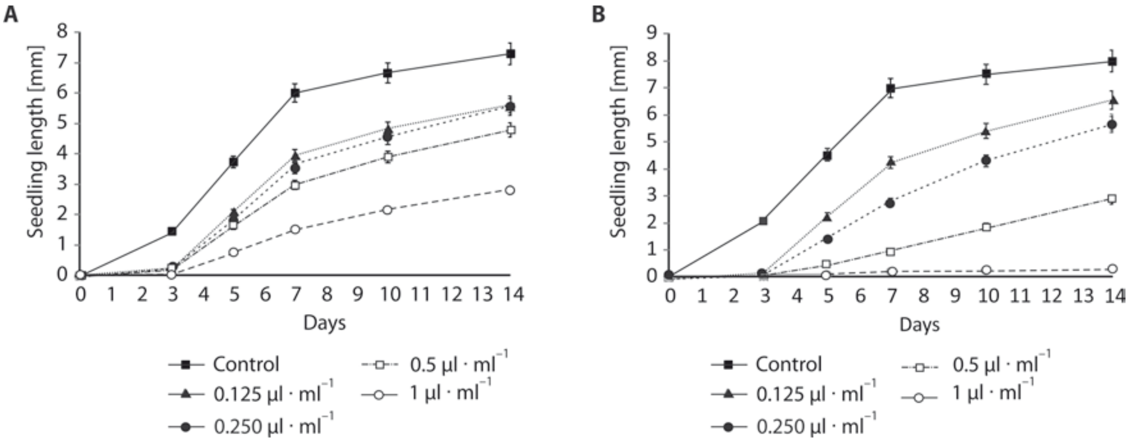

Fig. 1. Values of seedling length (mean ± SE) of *Cortaderia selloana* control and treated with tea tree essential oil (A), and wintergreen essential oil (B) at 0.125, 0.25, 0.50 and 1 µl · ml^{-1}

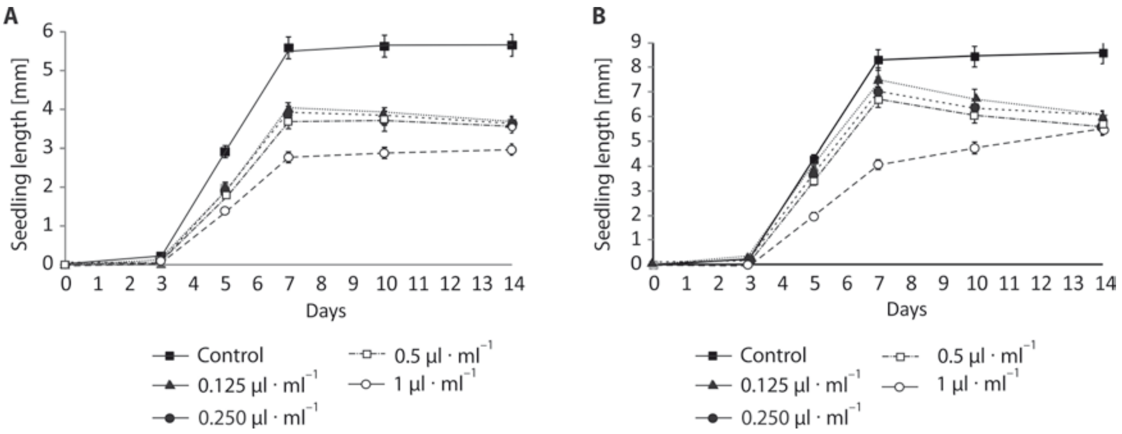

Fig. 2. Values of seedling length (mean ± SE) of *Nicotiana glauca* control and treated with tea tree essential oil (A), and wintergreen essential oil (B) at 0.125, 0.25, 0.50 and 1 µl · ml^{-1}

could improve the control of certain weeds (Rolando *et al.* 2011). It would be quite interesting to obtain significant results at very low doses of essential oils and it would not be recommended to test higher doses of these essential oils but to apply mixtures of them. Previous studies with winter savory, oregano or peppermint essential oils with other oxygenated monoterpenes different from their main compounds were able to produce at these doses a complete inhibition of the seed germination of other problematic weeds (Ibáñez and Blázquez 2017; Ibáñez and Blázquez 2018c).

The parallel study of the seedling growth corroborated that *C. selloana* is the most susceptible invasive species to tea tree and wintergreen essential oils (Fig. 1) and methyl salicylate, the main principle of wintergreen essential oil, is the most phytotoxic compound in the reduction of both hypocotyl and radicle growth of the tested invasive species (Table 3).

Table 3. *In vitro* effects of tea tree from *Melaleuca alternifolia* and wintergreen from *Gaultheria procumbens* essential oil against *Cortaderia selloana* and *Nicotiana glauca* seedling growth

Essential oil	Dose [µl · ml^{-1}]	*Cortaderia selloana*		*Nicotiana glauca*	
		hypocotyl [mm]	radicle [mm]	hypocotyl [mm]	radicle [mm]
Tea tree	Control	4.56 ± 0.17 a	2.75 ± 0.20 a	2.53 ± 0.24 a	3.14 ± 0.27 a
	0.125	3.79 ± 0.45 a, b	1.85 ± 0.18 b	1.46 ± 1.19 a	2.18 ± 0.15 a, b
	0.25	3.70 ± 0.08 a, b	1.85 ± 0.16 b	1.71 ± 0.45 a	2.17 ± 0.47 a, b
	0.50	3.28 ± 0.25 b	1.51 ± 0.23 b, c	1.41 ± 0.49 a	2.25 ± 0.37 a, b
	1	1.78 ± 0.32 c	1.03 ± 0.14 c	1.24 ± 0.33 a	1.73 ± 0.24 b
Wintergreen	Control	4.14 ± 0.56 a	3.88 ± 0.36 a	4.72 ± 0.30 a	3.87 ± 0.23 a
	0.125	4.14 ± 0.13 a	2.42 ± 0.27 b	2.95 ± 0.35 b	3.00 ± 0.36 a, b
	0.25	3.86 ± 0.43 a	1.89 ± 0.44 b	2.94 ± 0.14 b	3.00 ± 0.03 a, b
	0.50	1.55 ± 0.41 b	1.31 ± 0.33 b	2.79 ± 0.31 b	2.76 ± 1.16 b
	1	0.15 ± 0.07 c	0.13 ± 0.08 c	2.78 ± 0.18 b	2.74 ± 0.26 b

Values are mean percentages of five replications ± error deviation after 14 days of incubation; means followed by different letters in the same column indicate that they are significantly different at $p < 0.05$ according to T3 Dunnett and Tukey B tests

Especially noteworthy was the phytotoxic effect of wintergreen essential oil on *C. selloana.* Very high values of up to 96.38 and 96.65% of reduction of both hypocotyl and radicle growth, respectively, were recorded at the highest dose (1 µl · ml^{-1}) assayed in comparison to the control (Table 3, Fig. 1B). Moreover, a considerable decrease in hypocotyl and radicle development of *C. selloana* was observed after the application of a lower dose (0.50 µl · ml^{-1}) of wintergreen essential oil reaching 62.56 and 66.24%, respectively (Table 3). Comparable percentages of reduction were obtained with the highest dose (1 µl · ml^{-1}) of tea tree essential oil in *C. selloana* reducing the hypocotyl and radicle length by 60.96 and 62.55 mm, respectively (Table 3, Fig. 1A).

On the other hand, *N. glauca* was more resistant to treatment with tea tree and wintergreen essential oils (Table 3, Fig. 2). Tea tree essential oil did not affect the hypocotyl growth of *N. glauca* at any dose (0.125, 0.25, 0.50 and 1 µl · ml^{-1}) tested (Table 3) and only slight differences between 30.57 and 44.9% of reduction were observed in the radicle development of *N. glauca* with tea tree essential oil (Table 3). Wintergreen essential oil was able to inhibit significantly both hypocotyl and radicle enlargement (Fig. 2B) with percentages of inhibition comparable with the control of 37.50 to 41.02% for the hypocotyl and from 22.48 to 29.20% for the radicle elongation at all doses applied.

In relation to these results, tea tree essential oil could be used as a post-emergent bioherbicide to control *C. seollana,* whereas wintergreen essential oil showed powerful pre and post-emergent herbicidal effects against this invasive species. Bioherbicides represent less than 10% of all biopesticides (fungicides, bactericides, insecticides and nematicides) and most of them are constituted of fungus and bacteria. A methyl salicylate-like pelargonic acid (Beloukha®, Katoun®), a bioherbicide obtained from rapeseed oil (Cordeau *et al.* 2016), provides an interesting alternative in the formulation of new herbicides derived from plant products. In this sense, it is interesting to study the effect of methyl salicylate on food crops to ensure its beneficial effects and harmlessness. Regarding this, previous works have studied the influence of methyl salicylate on rice seeds obtaining an increase of rice seedling emergence, development and disease resistance of rice (Kalaivani *et al.* 2016). In fact, methyl salicylate is involved in plant defence and can act as a key airborne signal, which activates disease resistance and the expression of defence-related genes in healthy neighbouring plants (Shulaev *et al.* 1997).

In summary, tea tree and wintergreen essential oils with terpinen-4-ol (28.37 ± 0.05%) and methyl salicylate (99.63 ± 0.02%) as the main compounds, respectively, could be used in the control of invasive plant species. *Nicotiana glauca* was more resistant to tea tree and wintergreen essential oils than *C. selloana* with respect to seed germination and seedling growth. Wintergreen essential oil showed more phytotoxic effects in both non-native species. Methyl salicylate effectively controlled seed germination and seedling growth of *C. seollana* in a dose-dependent manner. Together with its pharmacological properties, this aromatic compound from wintergreen essential oil represents a potential source of bioherbicides.

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