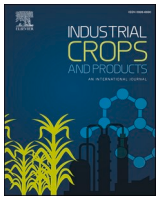
Industrial Crops & Products 187 (2022) 115317

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop



Composition and biological activity of essential oils from *Artemisia roxburghiana* Besser and *Elsholtzia fruticosa* Rehder cultivated in Italy

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ARTICLE INFO

Keywords:
Aphicidal activity
Bactericidal activity
Bioactivity
Essential oil

ABSTRACT

The plants *Artemisia roxburghiana* Besser and *Elsholtzia fruticosa* Rehder were evaluated as innovative crops for the production of essential oils with valuable biological activity. For both species, Clevenger distillation resulted in high essential oil contents of 1.08 and 1.79 mL·100 g^{-1} dry weight for *A. roxburghiana* and *E. fruticosa*, respectively. According to the results of GC/FID and GC/MS analyses, the composition of essential oil of *A. roxburghiana* was characterized by *cis*-thujone (23.05%), 1,8-cineole (21.56%), and camphor (13.82%), while *E. fruticosa* oil was rich in 1,8-cineole (50.06%) and γ-terpinene (14.11%). The bioactivity of the oils of the two species was evaluated in vitro against both pathogenic Gram-positive and Gram-negative bacterial strains by disc diffusion assay and determination of the minimal inhibitory concentration. They were also tested as natural insecticides by carrying out mortality and fecundity analyses against the aphid *Mizus persicae*. *E. fruticosa* oil showed higher antibacterial activity compared to *A. roxburghiana*, particularly against *Staphylococcus aureus* (MIC 2.0 and 62.5 mg·mL^{-1}) and *Escherichia coli* (MIC 7.8 and 62.5 mg·mL^{-1}). Both plant species revealed high aphicidal activity against the polyphagous pest *M. Persicae* according to the nymph mortality and fecundity reduction, and their efficacy was comparable to that of azadyrachtin. This study shows the prospects of the two investigated species as possible innovative crops for the production of essential oils to be employed in agro-industry.

1. Introduction

Essential oils have been extracted from a multitude of plant species, and over the years, many studies have focused on their biological properties (Bakkali et al., 2008; Raut and Karuppayil, 2014), particularly their antibacterial activity (Burt, 2004; Teixeira et al., 2013) and insecticidal activity (Rajendran and Sriranjini, 2008; Boulogne et al., 2012). Moreover, their potential use as alternative products to synthetic compounds has been highlighted by various studies in the pharmaceutical (Zielińska-Błajet and Feder-Kubis, 2020), agricultural (Raveau et al., 2020; Godlewska et al., 2021), and food sectors (Diniz do Nascimento et al., 2020). Most of the studies on the essential oils' composition and their biological activity focus on plants in their natural site of growth, where different environmental factors may influence the composition of essential oil and mask the genetic influence of the plant species (Li et al., 2016a, 2016b). In the same way, when transferring a plant from its natural ecosystem to a different area of cultivation, the content and composition of the secondary metabolites might be affected (Canter et al., 2005; Carlen, 2016; Yang et al., 2018). The domestication and cultivation of plants is an efficient method to control the environmental factors that affect the growth and composition of secondary metabolites, thus standardizing the quality of the raw material demanded by the business trades. Furthermore, it could help to avoid the risk of endangering the plants' long-term survival in areas where they are collected (Schippmann et al., 2006).

The Asteraceae and Lamiaceae families are well known for containing essential oils (Raut and Karuppayil, 2014). Asteraceae is the largest family of plants, comprising 1535 genera, including *Artemisia* L., which has about 390 species that are mainly distributed in the northern hemisphere (Bremer, 1994). Within this genus, *Artemisia roxburghiana*

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https://doi.org/10.1016/j.indcrop.2022.115317
Received 7 February 2022; Received in revised form 29 June 2022; Accepted 3 July 2022
Available online 8 July 2022

Besser is a perennial herb distributed in the Himalayas from Pakistan to Central Nepal at altitudes ranging from 1000 to 4300 m a.s.l. It is characterized by its creeping rootstock, simple stem, deeply dissected wooly or nearly hairless leaves, and purplish flower heads in terminal and lateral spikes (Polunin and Stanton, 1990). In various Asian countries, the species is used as a folk remedy for the treatment of malaria, skin disorders, and intestinal worms, although its medicinal properties have not yet been scientifically validated (Joshi et al., 2016; Kumar et al., 2019) except in one recent report on its antidiabetic properties (Kumar et al., 2022).

Few data are available on the chemical composition of *A. roxburghiana*. Bicchi et al. (1998) reported the essential oil composition of cultivated plants of the species. Phan et al. (2012) identified sesquiterpene lactones and other constituents from methanolic extracts of the species. Other studies investigated the essential oil composition of two different *A. roxburghiana* varieties, var. *hypolenca* (Mathela et al., 1994) and var. *purpurascens*. Oil was extracted from plants collected in the wild in the Himalayan region (Haider et al., 2009; Pandey et al., 2015). Two studies focused on the essential oils' insecticidal activity against some Coleoptera pest species that are responsible for damage to stored tobacco (Cheng et al., 2019) and foodstuffs (Liang et al., 2017). However, apart from these studies, no reports have been published on the biological activities of *A. roxburghiana* essential oil to date.

The Lamiaceae family (formerly Labiatae) is the sixth largest family of flowering plants (Li et al., 2016a, 2016b) and one of the most important in terms of aromatic properties. It is also one of the most important families as a source of essential oils with antimicrobial properties (Nieto, 2017; Napoli et al., 2020). It contains 236 genera and about 7173 species (Harley et al., 2004). Within this family, the genus *Elsholtzia* Willd. comprises 43 accepted species, and the majority occur in the mountain ranges of East Asia (Li et al., 2017). *Elsholtzia fruticosa* Rehder is a shrubby plant that is distributed from Pakistan to Southwest China at altitudes ranging from 1800 to 3300 m a.s.l. It is characterized by narrow, elliptical, lanceolate leaves; saw-toothed, glandular, finely hairy leaves; and long narrow spikes of white hairy flowers that are strongly aromatic (Polunin and Stanton, 1990). Various species of *Elsholtzia* are used as domestic folk medicine, and the chemical composition of their essential oils and their biological activity have been reported, including antibacterial activity (Guo et al., 2012).

Thappa et al. (1999) reported the essential oil composition of different species of *Elsholtzia* growing wild in the Himalayan region, including *E. fruticosa*. Saini et al. (2010) reported the chemical composition of the essential oil extracted from fresh leaves of *E. fruticosa* by different methods. Recently, Liang et al. (2020) reported the chemical composition of essential oil extracted from *E. fruticosa* and its biological activity against the plant pathogenic nematode *Ditylenchus destructor*. To the best of our knowledge, no report has been published on the composition of essential oil extracted from cultivated plants thus far, nor on the antibacterial and insecticidal activity of extracts obtained from *E. fruticosa*.

Aphids are major insect pests for many crops cultivated worldwide. One of the most polyphagous is the peach potato aphid, *Myzus persicae* (Sulzer), in the family Aphididae and order Hemiptera. It feeds on more than 500 species in 40 plant families and causes significant losses of many vegetable and fruit crops. The damages caused by this pest may be direct by phloem feeding and indirect by the transmission of more than 180 different viruses affecting the host plant (Blackmann and Eastop, 2000). Conventional pesticides may cause environmental pollution, negative side effects, and resistance in target pests, so it is necessary to develop efficient and eco-sustainable methods as alternatives (Foster et al., 2000). In examples of this approach, the aphicidal activity against *M. persicae* was recently reported for essential oils extracted from *Achillea millefolium* L., *Santolina chamaecyparissus* L., *Tagetes patula* L. (Asteraceae) (Czerniewicz, and et al., 2018, 2021), *Piper nigrum* L. *(Piperaceae),* and *Melaleuca alternifolia* Cheel (Myrtaceae) (Ahmed et al., 2021).

The aim of this study was to evaluate the production and the composition of essential oil obtained from cultivated plants of *A. roxburghiana* and *E. fruticosa*. Furthermore, their antibacterial and aphicidal activities were examined. It is possible that the two species could be used as innovative crops for the production of essential oils to be employed in the food and agro-industries.

2. Material and methods

2.1. Plant material

Aerial parts of *A. roxburghiana* and *E. fruticosa* were respectively harvested on the 9th and 16th of July, 2015, from cultivated plants in a vegetative stage at the experimental farm of CREA, Research Center for Forestry and Wood located in Trento, Italy (coordinates WGS 84: 46°02′52′' N, 11°08′49′' E; altitude: 370 m a.s.l.). The climate of the site is temperate sub-continental (annual average minimum and maximum temperatures: 7.3 and 16.8 °C; annual average rainfall: 1130.8 mm; annual average rainy days: 91.1 days; reference period: 1979–2015). The soil of experimental site is classified as a Hyperskeleti-rendzic leptosol (IUSS Working Group WRB, 2015) and is composed of sand (27.8%), silt (62.2%), and clay (10%) at pH 7.92. It is characterized by a high total $CaCO_3$ content (47.4%), a normal content of active $CaCO_3$ (0.6%), and high contents of organic matter (3.5%), total N (0.24%), assimilable P_2O_5 (117 mg·kg^{-1}), and exchangeable K_2O (294 mg·kg^{-1}).

Cultivated plants of both species originated from seeds collected in 1992 from wild plants growing in the Dudh Kosi and Khumbu valley, Nepal, at altitudes ranging from 2600 and 4250 m a.s.l. A previous report provides detailed information regarding the origin and the main agronomic features of cultivated plants of the two species (Lucchin et al., 1994). Plants used in this study were transplanted to an open field in 1994 and have been conserved since then in the in vivo germplasm collection of CREA of Trento (formerly ISAFA, Istituto Sperimentale per l'Assestamento Forestale e l'Alpicoltura). Conventional cultivation practices were used without fertilizers and pesticides, and the plants were vegetatively propagated when necessary.

To determine the aerial part yields, 14 individuals of *A. roxburghiana* and 30 of *E. fruticosa* were manually harvested by cutting them at 10 cm from the ground. The plants had been cultivated at densities of 1.04 plants·m^{-2} (1.2 m between rows and 0.8 m between plants) and 1.38 plants·m^{-2} (1.2 m between rows and 0.6 m between plants), respectively. The fresh weight of aerial parts of each harvested plant was recorded, and the dry matter content was determined by drying three replications using 100 g fresh weight samples per plant in a thermostatic ventilated oven at 105 °C until steady weight was obtained. Three plants per species were immediately separated after harvest and distilled to obtain essential oil. For this study, a voucher specimen of *A. roxburghiana* was authenticated by Prof. Kazumi Fujikawa of the Kochi Prefectural Makino Botanical Garden, Kochi, Japan, while *E. fruticosa* was authenticated by P. Fusani of CREA of Trento, Italy, where voucher specimens of both species are deposited (AR#20150709 and EF#20150716).

2.2. Isolation and analysis of essential oils

2.2.1. Extraction of essential oils

For the extraction of the essential oils, fresh whole aerial parts of *A. roxburghiana* and *E. fruticosa* were used. Portions of about 500 g of plant material were separately steam distilled in a Clevenger apparatus for 1 h. The obtained oils were dried over anhydrous sodium sulfate (Carlo Erba, Milano, Italy) and kept in sealed vials with a Teflon cap at − 20 °C until analysis. The resulting oils were then diluted with diethyl ether and analyzed by GC/FID and GC/MS. For both species, samples from three different plants were separately distilled, and the results are reported as the mean of three independent analyses ± the standard deviation (SD).

2.3. GC/FID and GC/MS analysis

GC/FID analyses were carried out using a Perkin Elmer Clarus 500 GC equipped with a 30-m × 0.32-mm Elite-5MS capillary column (0.32-μm film thickness). Samples were injected (0.5 μL) in the "split" mode (1:30) with a column temperature program of 40 °C for 5 min, an increase to 280 °C at 4 °C min^{-1}, and finally holding at this temperature for 10 min. The injector and detector were set at 250 °C and 300 °C, respectively, and the carrier gas was He with a head pressure of 12.0 psi.

GC/MS analyses were carried out using a Perkin Elmer Clarus 500 GC equipped with a Clarus 500 mass spectrometer using the same capillary column and chromatographic conditions as for the GC/FID analyses. Mass spectra were acquired over a range of 40–500 amu at 1 scan/sec with ionizing electron energy of 70 eV and ion source at 200 °C. The transfer line was set at 300 °C, while the carrier gas was He at 1.0 mL min^{-1}. The oil components were identified by determination of their retention indices (RIs) by comparison with authentic reference compounds and published mass spectra (Adams, 2007), as well as a peak-matching library search (NIST/EPA/NIH, 2000). RIs were calculated using an *n*-alkane series (C_6-C_{25}) under the same GC conditions as for the samples. The relative amount (%) of individual components of the oil was expressed as the percent peak area relative to the total peak area from the GC/FID analyses of the whole extracts.

2.4. Evaluation of antimicrobial activity

2.4.1. Bacterial strains

Six bacterial strains were selected to carry out the study. The Gram-positive bacteria comprised *Staphylococcus aureus* ATCC 12600, *Enterococcus faecium* ATCC 19433, and *Listeria innocua* LIN11 (all tested strains belong to CREA bacterial collection), and the Gram-negative bacteria comprised *Escherichia coli* ATCC11775, *Pseudomonas fluorescens* ATCC13525, and *Salmonella* spp. SAN39 (CREA collection). Bacterial strains were cultivated in Tryptone Soya Broth (TSB, Thermo Scientific Oxoid, Basingstoke, UK) at 37 °C for 18 h.

2.4.2. Determination of antibacterial activity by disc diffusion assay

A disc diffusion assay was used for preliminary screening of the essential oils for antimicrobial activity (Aghraz et al., 2018). The oils were dissolved in dimethyl sulfoxide (DMSO) to obtain a final concentration of 500 mg mL^{-1} and used for the assay. Each bacterial culture was diluted and inoculated in melted (45 °C) Tryptone Soya Agar (TSA, Oxoid) to obtain a final concentration of 10^6 colony forming units (CFU) mL^{-1}. Volumes of 15 mL were distributed in sterile plates and allowed to solidify. An aliquot of 15 μL of each oil was applied onto sterile filter paper discs of 6-mm diameter and then placed on the agar surface.

Sterile solutions of ampicillin and gentamicin (100 μg mL^{-1}; 15 μL) were used as positive controls for Gram-positive and Gram-negative bacteria, respectively, and DMSO (15 μL) was used as a negative control. The plates were then placed at 4 °C for 2 h and incubated for 24 h at 37 °C. The inhibition zones around the discs were visually examined, and the diameter was measured (including the diameter of the disc). Each test was performed in four replicates, and all results have been expressed as the mean ± SD (mm).

2.4.3. Determination of minimal inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of each oil was determined using a broth microdilution method (Chaimanee et al., 2017). Twofold dilutions of each oil in TSB were made in 96-well microtiter plates with concentrations ranging from 0.25 to 125 μg·mL^{-1}. Overnight bacterial cultures were used to prepare the cell suspensions adjusted to 10^6 CFU in TSB. Each well containing 100 μL of twofold sample dilution was inoculated with 100 μL of the bacterial suspension. A negative growth control (medium alone) and a positive growth control (100 μL of medium with 100 μL of inoculum suspension) were included to each microdilution plate. The MIC was determined as the lowest concentration of essential oils at which no bacterial growth was detected, and each test was performed in duplicate.

2.5. Evaluation of aphicidal activity

Experiments were performed using *M. persicae* asexual lineages maintained on pea seedlings (*Pisum sativum* L.) in a thermostatic room (18 ± 1 °C) at the Department of Life Sciences of the University of Modena and Reggio Emilia in Italy. A regime of 16 h of light and 8 h of dark was applied. The *M. persicae* strains #1 and #64 described by Nardelli et al. (2017) were used in this study. *M. persicae* asexual lineage 1 was originally collected on peach *Prunus persica*, whereas lineage 64 was sampled from *Nicotiana tabacum*. Seeds of *Pisum sativum* L. cv. 'Meraviglia d'Italia' were germinated at room temperature and then sown in plastic jars (120 mL) containing agriperlite Agrilit® 3 (Perlite Italiana s.r.l., Italy) with particle size of 2–5.6 mm, pH 7.5, density of 90 kg·m^{-3}, and EC of 0.1 dS·m^{-1}.

In order to perform mortality and fecundity analyses, a single parthenogenetic female was inserted in each jar, which was immediately closed using a net with a mesh of 0.2 mm. The experiments were performed under constant environmental conditions (18 °C, 16 h light: 8 h dark photoperiod). Nymph mortality and adult fecundity analyses were carried out on synchronized aphid specimens. To synchronize aphid lineages, 10 adult females were put in jars containing 3 pea shoots. After 24 h, they were removed so that a single newborn nymph with known age with 24-h accuracy was left in each jar.

Two experiments were carried out. In the first experiment, essential oils were assessed at 1 mL·L^{-1} (test 1), while in the second one, 2 mL·L^{-1} was assessed (test 2). Tap water was used as a solvent, and emulsification was performed prior to spraying. For both experiments, two controls were used: one using only tap water and another using the commercial product OIKOS (azadirachtin at 2.40%, SIPCAM Spa, Milan, Italy). Before the spraying, one leaf was retained per seedling, and afterwards the aphid fitness was quantified every day.

Five replications were used for each asexual lineage, with each replicate consisting of a single synchronized aphid maintained on a plant for each jar. Newborns (nymphs) were removed daily, and their numbers were noted. Oil spraying was done with a 0.5-L hand pressure sprayer (Classic LUX-TOOLS, OBI, Reggio Emilia, Italy). The experiment was developed in accordance with previous studies performing similar evaluations (Mordue and Blackwell, 1993; Pavela et al., 2004; Kraiss and Cullen, 2008; Shannag et al., 2014).

2.6. Data analysis

A heteroscedastic ANOVA with Welch's test was applied to the data on antibacterial activity from the disc diffusion assay, those concerning the activity against *E. faecium* and *L. innocua* having been previously transformed by ranks. The non-transformed data are shown in the text and tables. Data on aphicidal activity were subjected to one-way ANOVA, and means were compared using a Tukey test at 5% significance. All computations were carried out using the XLSTAT software package (Addinsoft, New York, US).

3. Results and discussion

3.1. Plant yields and essential oil yield and composition

Preliminary agronomical data on the cultivation of *A. roxburghiana* and *E. fruticosa* revealed that the yields of aerial parts were quite variable in both species [1798 ± 1359 and 659 ± 321 g fresh weight (f.w.) per plant for *A. roxburghiana* and *E. fruticosa,* respectively]. The dry matter content was 28.5% for *A. roxburghiana* and 25.8% for *E. fruticosa.* Both species revealed high and stable essential oil contents of 1.08 ± 0.06% (*A. roxburghiana)* and 1.79 ± 0.27% (*E. fruticosa)*, which were evaluated by volume on a dry weight basis. Despite the high variability

of the weight of a single plant's aerial parts, for a cultivated surface of 100 m^2 as refer, the obtainable yields would be estimated as about 173 kg of fresh aerial parts and 260 mL of essential oil in the case of *A. roxburghiana* and about 48 kg of fresh aerial parts and 220 mL of essential oil in the case of *E. fruticosa*. These results are based on a small number of plants, so they are applicable to a limited cultivated area, but they represent the first useful practical indication for possibly starting the cultivation of the species.

Regarding the *A. roxburghiana* essential oil content, comparison with available literature data is difficult because the only report on the same taxon is that of Bicchi et al. (1998), who reported 0.26% essential oil content calculated on a fresh weight basis. Our result was higher than that reported for a closely related taxon, *A. roxburghiana* var. *purpurascens,* for which Pandey et al. (2015) reported an essential oil content ranging from 0.77% to 0.9% calculated on a dry weight basis. Haider et al. (2009) reported 0.2–0.85% content, depending on the altitude of growing of plants. With regard to *E. fruticosa,* Thappa et al. (1999) reported 0.8% essential oil content on a dry weight basis (a lower value compared to this study), while Saini et al. (2010) reported 0.15% from fresh leaves, which is not comparable to our results.

Volatiles identified in the aerial parts of both the plant species are reported in Table 1 in order of elution on a DB-5 column, while their GC/FID traces are shown in Fig. 1. In total, more than 60 compounds were identified in the essential oil of the two investigated species, and their relative amounts were calculated. Chemical investigations of the obtained essential oils indicated quite different composition between *A. roxburghiana* and *E. fruticosa*, particularly in terms of major oil constituents (Table 1 and Fig. 1).

Oxygenated monoterpenes were the most abundant chemical class of components, and their relative amounts were 71.99 ± 4.26% and 56.62 ± 1.35% in the volatile fractions from *A. roxburghiana* and *E. fruticosa*, respectively. The second major chemical class was monoterpene hydrocarbons (15.17 ± 4.45% and 33.50 ± 1.37% in *A. roxburghiana* and *E. fruticosa*, respectively), followed by sesquiterpene hydrocarbons (8.83 ± 1.66% and 6.32 ± 0.72% in *A. roxburghiana* and *E. fruticosa*, respectively). Minor relative amounts of oxygenated sesquiterpenes were also identified at 0.55 ± 0.03% and 0.36 ± 0.08% of the total oil in *A. roxburghiana* and *E. fruticosa*, respectively.

1,8-cineole was the main component identified in the essential oils obtained from *E. fruticosa*, accounting for 50.06 ± 2.06% of the total. Other major constituents detected in this species were γ-terpinene (14.11 ± 1.50%), β-pinene (5.48 ± 0.15%), and p-cymene (4.37 ± 0.08%). The main components identified in the volatile oil of *A. roxburghiana* were *cis*-thujone (23.05 ± 2.78%) and 1,8-cineole (21.56 ± 2.11%), followed by camphor (13.82 ± 1.84%) and *trans*-thujone (5.17 ± 1.51%). The presence of camphor, *cis*-thujone, and *trans*-thujone represents a characteristic feature of *A. roxburghiana* as these compounds were not detected in the *E. fruticosa* essential oil (see Table 1).

The results for *A. roxburghiana* agree with those of Bicchi et al. (1998), who reported camphor, 1,8-cineole, and *cis*-thujone as the main components. In contrast, data on different taxa of *A. roxburghiana* from the Himalayan region reported that *trans*-thujone was the major component of *A. roxburghiana* var. *hypolenca* (Mathela et al., 1994), while *cis*-thujone was reported as one of the main constituents of *A. roxburghiana* var. *purpurascens* essential oil (Haider et al., 2009; Pandey et al., 2015). Literature data on essential oil from *E. fruticosa* reported that perillene was the most abundant compound, in contrast to our findings, followed by 1,8-cineole, terpinen-4-ol, and caryophyllene oxide from plants collected in the Himalayan region (Thappa et al., 1999; Saini et al., 2010). However, 1,8-cineole and γ-terpinene were reported as the major constituents of this plant species collected in China (Liang et al., 2020), in accordance with our results.

Table 1
Essential oil composition of *A. roxburghiana* and *E. fruticosa* (mean ± standard deviation; N = 3).

No.	RI tab[a]	RI[b]	Compound	Percentage (%)	
				A. roxburghiana	*E. fruticosa*
1	847	847	*cis*-Salvene	0.10 ± 0.03	–
2	858	858	*trans*-Salvene	0.01 ± 0.00	–
3	906	906	Santolina triene	3.03 ± 0.88	–
4	921	919	Tricyclene	0.04 ± 0.02	–
5	924	924	α-Thujene	0.05 ± 0.03	1.22 ± 0.04
6	932	931	α-Pinene	1.03 ± 0.48	1.36 ± 0.06
7	945	945	α-Fenchene	1.65 ± 0.48	–
8	946	947	Camphene	1.74 ± 0.55	0.07 ± 0.01
9	953	953	Thuja-2,4(10)-diene	–	0.29 ± 0.05
10	969	971	Sabinene	3.72 ± 1.51	0.32 ± 0.13
11	974	975	β-Pinene	2.01 ± 0.87	5.48 ± 0.15
12	979	981	Octan-3-one	–	0.10 ± 0.08
13	988	990	Dehydro-1,8-cineole	0.17 ± 0.06	tr
14	1002	1006	α-Phellandrene	–	0.10 ± 0.01
15	1014	1016	α-Terpinene	0.17 ± 0.08	1.42 ± 0.05
16	1020	1024	p-Cymene	0.16 ± 0.07	4.37 ± 0.08
17	1024	1028	Limonene	0.76 ± 0.28	1.30 ± 0.06
18	1026	1032	1,8-Cineole	21.56 ± 2.11	50.06 ± 2.06
19	1032	1037	*Z*-β-Ocimene	tr	1.12 ± 0.45
20	1044	1018	*E*-β-Ocimene	0.19 ± 0.10	1.63 ± 0.55
21	1054	1058	γ-Terpinene	0.45 ± 0.19	14.11 ± 1.50
22	1065	1072	*cis*-Sabinene hydrate	0.98 ± 0.06	–
23	1086	1086	Terpinolene	0.08 ± 0.03	0.31 ± 0.02
24	1089	1093	*trans*-Sabinene hydrate	0.34 ± 0.01	–
25	1101	1109	*cis*-thujone	23.05 ± 2.78	–
26	1112	1120	*trans*-thujone	5.17 ± 1.51	–
27	1095	1103	Linalool	–	0.78 ± 0.25
28	1102	1100	Perillene	–	0.39 ± 0.04
29	1137	1143	*trans*-Sabinol	0.49 ± 0.04	–
30	1141	1148	Camphor	13.82 ± 1.84	–
31	1160	1164	Pinocarvone	0.78 ± 0.08	–
32	1162	1169	δ-Terpineol	–	0.38 ± 0.18
33	1165	1174	Borneol	0.84 ± 0.08	–
34	1174	1182	Terpinen-4-ol	0.57 ± 0.13	1.35 ± 0.07
35	1186	1198	α-Terpineol	–	1.51 ± 0.36
36	1192	1191	Dihydrocarveol	–	0.32 ± 0.10
37	1215	1213	*trans*-Carveol	0.07 ± 0.01	–
38	1226	1223	*cis*-Carveol	tr	–
39	1287	1286	Bornyl acetate	3.86 ± 0.20	0.07 ± 0.03
40	1289	1291	*trans*-Sabinyl acetate	0.31 ± 0.08	–
41	1298	1297	*trans*-Pinocarveyl acetate	–	0.35 ± 0.07
42	1311	1311	*cis*-Pinocarveyl acetate	–	1.79 ± 0.18
43	1326	1326	Silphiperfol-5-ene	0.11 ± 0.03	–
44	1345	1345	Silphiperfol-5-ene-7-epi	1.28 ± 0.36	–
45	1358	1358	Silphiperfol-4,7(14)-diene	0.21 ± 0.06	–

(*continued on next page*)

Table 1 (*continued*)

No.	RI tab[a]	RI[b]	Compound	Percentage (%)	
				A. roxburghiana	*E. fruticosa*
46	1374	1377	*α*-Copaene	–	0.16 ± 0.01
47	1417	1421	*β*-Caryophyllene	1.33 ± 0.13	2.85 ± 0.62
48	1430	1431	*β*-Copaene	–	0.18 ± 0.03
49	1440	1441	*Z*-*β*-Farnesene	0.06 ± 0.01	–
50	1452	1457	*α*-Humulene	–	0.47 ± 0.10
51	1454	1453	*E*-*β*-Farnesene	0.65 ± 0.23	–
52	1478	1477	*γ*-Muurolene	–	0.27 ± 0.07
53	1481	1478	*γ*-Curcumene	–	0.60 ± 0.31
54	1484	1482	Germacrene D	3.59 ± 0.62	0.36 ± 0.05
55	1489	1490	*β*-Selinene	0.80 ± 0.11	–
56	1500	1497	Bicyclogermacrene	0.57 ± 0.14	0.25 ± 0.04
57	1500	1500	*α*-Muurolene	–	0.15 ± 0.01
58	1500	1504	*β*-Himachalene	0.25 ± 0.02	–
59	1513	1515	*γ*-Cadinene	–	0.27 ± 0.02
60	1522	1520	*δ*-Cadinene	–	0.70 ± 0.11
61	1537	1539	*α*-Cadinene	–	0.06 ± 0.01
62	1582	1585	Caryophyllene oxide	0.21 ± 0.03	0.13 ± 0.05
63	1630	1627	*γ*-Eudesmol	–	0.24 ± 0.04
64	–	1782	Methyl eremophila-1 (10),11(13)-dien-12-oate	0.19 ± 0.06	–
65	–	1803	Methyl-*β*-costate	0.15 ± 0.06	–
			Monoterpene hydrocarbons	15.17 ± 4.45	33.50 ± 1.37
			Oxygenated monoterpenes	71.99 ± 4.26	56.62 ± 1.35
			Sesquiterpene hydrocarbons	8.83 ± 1.66	6.32 ± 0.72
			Oxygenated sesquiterpenes	0.55 ± 0.03	0.36 ± 0.08
			Total	96.53 ± 1.88	96.90 ± 0.21

tr: trace (<0.01%)

[a] RI tab, Retention index from Adams (2007).

[b] RI calculated by GC using n-alkane series under the same conditions as for samples.

3.2. Antimicrobial activity

The two essential oils showed different antimicrobial activity against some selected Gram-positive and Gram-negative pathogenic bacterial strains according to the disc diffusion assay. The negative control DMSO had no inhibitory activity against the strains, so it was removed from the statistical analysis. *A. roxburghiana* oil showed a certain antimicrobial activity against *S. aureus* ATCC12600 (10.5 ± 1.0 mm) and *E. coli* ATCC11775 (10.0 ± 0.8 mm), indicating lower activity than the positive control. No inhibition zones were observed for the other pathogens (see Table 2).

E. fruticosa oil had higher inhibitory activity than *A. roxburghiana* against all tested pathogens with the exception of *E. coli* ATCC11775. At the tested concentration, the essential oil of *E. fruticosa* showed the same activities as the controls (ampicillin and gentamicin) against *S. aureus* ATCC12600, *E. faecium* ATCC 19433, *Salmonella sp.* SAN39, and *E. coli* ATCC11775 (Table 2). The MIC values obtained for the two oil samples against the pathogenic bacteria confirmed the higher inhibitory activity of *E. fruticosa* oil compared to *A. roxburghiana*, particularly against *S. aureus* ATCC12600 (2 mg mL^{-1}) and *E. coli* ATCC11775 (7.8 mg mL^{-1}) (Table 3).

The effective antimicrobial activity of the *E. fruticosa* essential oil could be related to the higher relative amounts of 1,8-cineole and *γ*-terpinene (50.06 ± 2.06% and 14.11 ± 1.50% of the total oil, respectively) as these metabolites are well known for their antimicrobial activities (Sato et al., 2007; Oyedemi et al., 2009; Salehi et al., 2019; Cai et al., 2020). Nevertheless, in *A. roxburghiana* essential oil, 1,8-cineole is also present at a relative high amount (21.56%), together with other compounds with known antimicrobial efficacy, such as *cis*-thujone (23.05%) and camphor (13.82%) (Sivropoulou et al., 1997). Djenane et al. (2011) reported that the major components of an essential oil well reflect its antimicrobial effects. Furthermore, the antimicrobial activity of an essential oil component should be related to the lipophilic character of the hydrocarbon skeleton and the hydrophilic character of the functional groups (Nowak et al., 2012). Thus, the following activity ranks of essential oil components were determined: phenols > aldehydes > ketones > alcohols > ethers > hydrocarbons (Kalemba and Kunicka, 2003).

Accordingly, since *A. roxburghiana* essential oil has higher relative abundance of the two ketones *cis*-thujone and camphor, we could have expected antimicrobial activity to be higher than that of *E. fruticosa,* of which the main component is the ether 1,8-cineole. Instead, our results support the hypothesis that the interactions between components should be taken into consideration in determining the antimicrobial activity of an essential oil, and synergistic effects should be observed (Burt, 2004). The whole essential oil may have greater antimicrobial activity than a mixture of its major components (Gill et al., 2002). In this study, the essential oils of *A. roxburghiana* and *E. fruticosa* showed 43 and 39 components, respectively, making it difficult to determine the possible synergistic effects between them.

The sensitivity of the bacteria to the two oils depended on the bacterial species. The results showed that *S. aureus* ATCC12600 was most sensitive among the Gram-positive bacteria, while *E. coli* ATCC11775 was most sensitive among the Gram-negative bacteria. As a general trend, *A. roxburghiana* oil was less effective than *E. fruticosa* oil, with MIC values ranging from 62.5 to 125 mg mL^{-1} and from 2.0 to 62.5 mg mL^{-1}, respectively. Although no specific references are available for the microbiological activities of *A. roxburghiana* and *E. fruticosa* essential oils, the present data showed their interesting biological activities, which are comparable to those reported in the literature for plants belonging to the same genera from different countries (Amri et al., 2013; Phetsang et al., 2017; Thanaseelungkoon et al., 2018; Aati et al., 2020).

The essential oil extracted from *E. fruticosa* in particular showed good antimicrobial activity in comparison to some more widely known plant species. For example, *Citrus* L. species (Rutaceae) *C. limon* (L.) Osbeck (lemon) and *C. sinensis* (L.) Osbeck (sweet orange) have shown 23 and 14 mm inhibition zones in a disc diffusion assay against *S. aureus*, respectively (Fisher and Phillips, 2006). For *Cinnamomum verum* J.Presl (Lauraceae), an inhibition zone of 24.7 mm against *E. coli* was reported (Mith et al., 2014). *Thymus vulgaris* L. (Lamiaceae) essential oil revealed antimicrobial activity against *E. coli* with an inhibition zone of 47 mm and MIC of 2.9 mg·mL^{-1} (Teixeira et al., 2013). These results were higher than that of *E. fruticosa* described in this study, but it has to be taken into consideration that they were obtained in different experimental conditions, and the comparison is purely descriptive.

3.3. Aphicidal activity

In order to study the influence of the essential oils on *M. persicae,* the aphid nymph mortality and the adult aphid fecundity were evaluated in comparison with azadirachtin, which has efficacy against different aphid species (*Aphis glycines*, *Aphis gossypii* and *M. persicae*) (Mordue and Blackwell, 1993; Pavela et al., 2004; Kraiss and Cullen, 2008; Shannag et al., 2014). The first mortality of nymphs occurred between 2 and 3

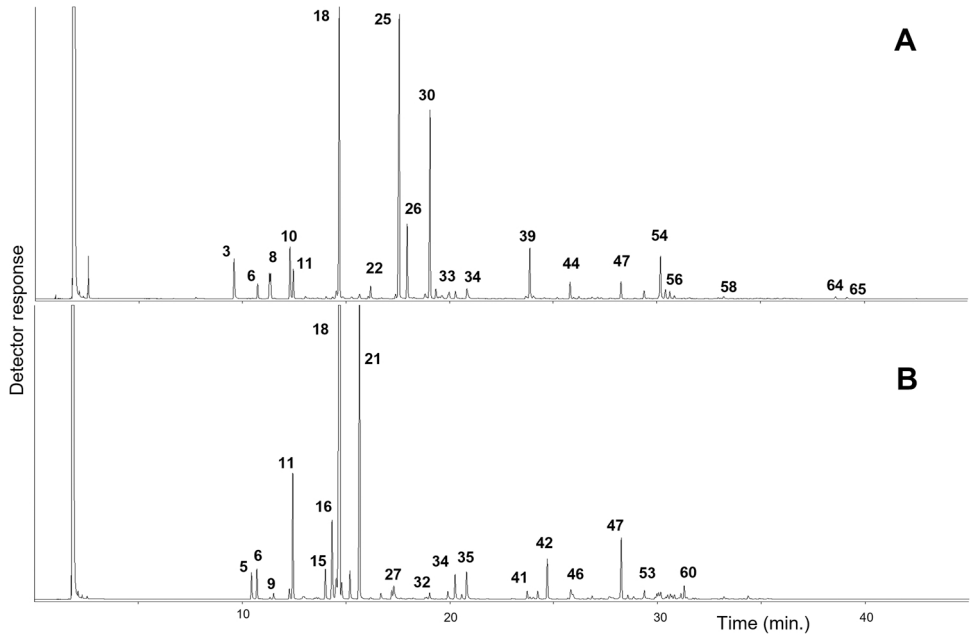

Fig. 1. GC trace of the essential oils from (A) *A. roxburghiana* ad (B) *E. fruticosa*. For peak identification, see Table 1.

Table 2
Mean radius of inhibition zones (mm) of the essential oils (500 mg·mL^{-1}) against pathogenic bacteria using the disc diffusion method.

Treatment	Bacterial strains					
	S. aureus ATCC12600	*L. innocua* LIN11	*E. faecium* ATCC19433	*Salmonella* sp. SAN39	*E. coli* ATCC11775	*P. fluorescens* ATCC13525
A. roxburghiana	10.5 ± 1.0 B	0.0 ± 0.0 C	0.0 ± 0.0 b	0.0 ± 0.0 b	10.0 ± 0.8 B	0.0 ± 0.0 C
E. fruticosa	27.0 ± 1.2 A	10.5 ± 1.0 B	12.5 ± 1.9 a	21.5 ± 6.4 a	12.0 ± 1.4 AB	11.5 ± 1.3 B
Ampicillin*	24.0 ± 2.3 A	23.5 ± 1.3 A	12.0 ± 2.3 a	–	–	–
Gentamicin*	–	–	–	19.0 ± 2.6 a	14.0 ± 0.8 A	22.0 ± 2.3 A

Mean values (N = 4) ± SD followed by different letters are significantly different at $p < 0.05$ (low letters) and $p < 0.01$ (capital letters) according to Games-Howell Test. *positive control (100 μg mL^{-1}).

Table 3
Minimum inhibitory concentration (MIC) values of essential oils against pathogenic bacteria (mean ± standard deviation; N = 2).

Species	MIC (mg·mL^{-1})					
	Bacterial strains					
	S. aureus ATCC12600	*L. innocua* LIN11	*E. faecium* ATCC19433	*Salmonella spp.* SAN39	*E. coli* ATCC11775	*P. fluorescens* ATCC13525
A. roxburghiana	62.5 ± 0.0	62.5 ± 0.0	62.5 ± 0.0	125.0 ± 0.0	62.5 ± 0.0	125.0 ± 0.0
E. fruticosa	2.0 ± 0.0	31.3 ± 0.0	15.6 ± 0.0	62.5 ± 0.0	7.8 ± 0.0	15.6 ± 0.0

days after introduction to plants treated with azadirachtin and between 3 and 6 days for plants treated with both the essential oils. Nymph mortality on plants treated with azaridachtin and both essential oils was higher with respect to the water control except in the case of Test 1, in which only azadiracthin showed different results from water. No difference occurred between the *E. fruticosa* and *A. roxburghiana* oils for each of the concentrations and aphid strains tested (Fig. 2). Similarly, both azadirachtin and the two essential oils reduced the adult aphid fecundity compared to water, and the effectiveness of both oils was not different from that of azadirachtin (Fig. 3).

Our experiments confirmed the previous results obtained with azadirachtin on *M. persicae* and also showed that both essential oils have similar effects on *M. persicae* aphids. The oils increased aphid nymph mortality and reduced adult fecundity similarly to azadirachtin. Interestingly, the oils were highly effective on both *M. persicae* strains, suggesting that they could be successfully applied in the field to control aphids in the presence of strains with high fitness and fecundity. However, the high relative amount of thujones of *A. roxburghiana* essential oil reported in this study may represent a limitation to its possible utilization as an insecticide for edible crops due to the well-known toxicity of thujones for human consumption (Németh and Nguyen, 2020). Further studies are necessary for this issue to assess the possible toxicity of the oils for humans, as well as to evaluate their residuals and the edibility of products obtained from treated crops.

4. Conclusions

Both *A. roxburghiana* Besser and *E. fruticosa* Rehder were revealed to be suitable species for cultivation at the sites described in this study, where predictable yields of aerial parts and essential oil showed promise in view of their cultivation. In particular, the essential oil content of the two species was comparable to or even higher than those obtained from

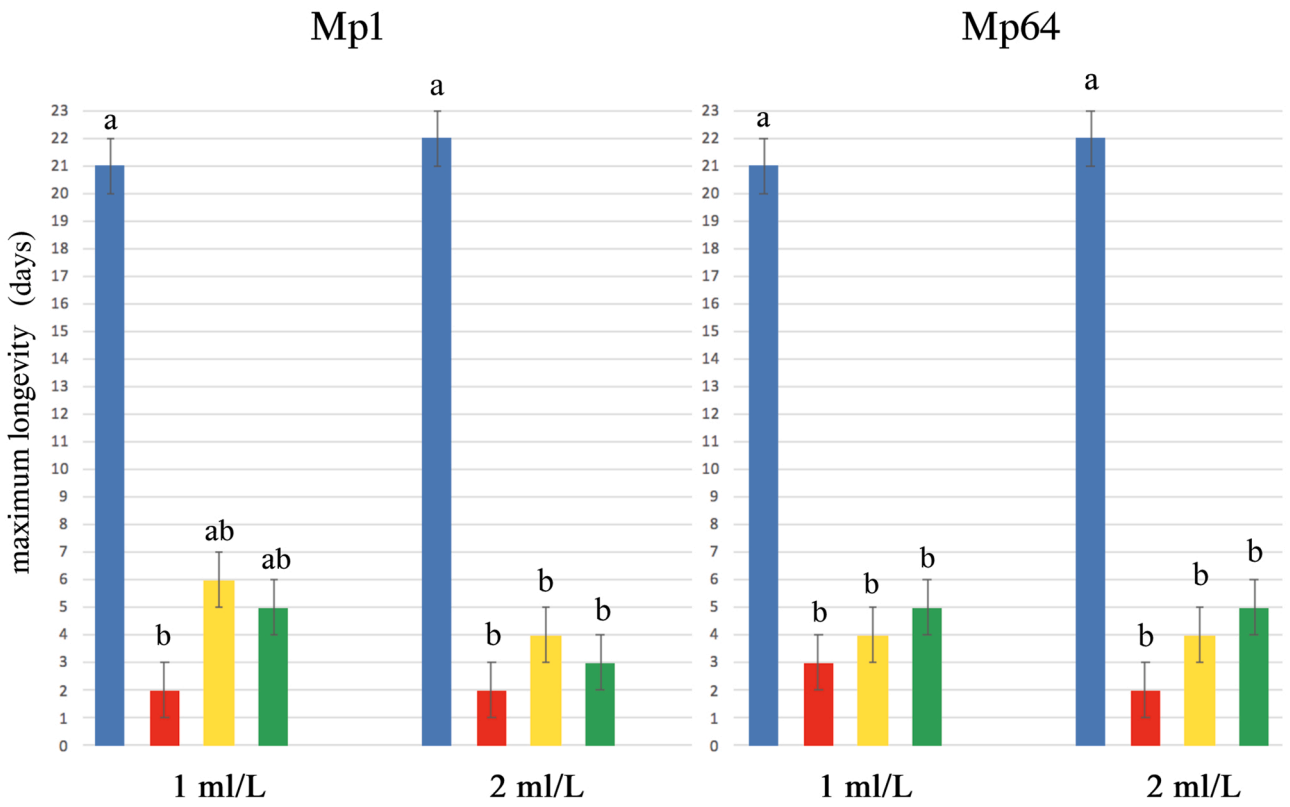

Fig. 2. Effects of water (blue), azadirachtin (red), and essential oils of *A. roxburghiana* (yellow) and *E. fruticosa* (green) on nymph mortality. Mortality was evaluated as maximum longevity of single nymph of *M. persicae* strains 1 (Mp1) and 64 (Mp64) in test 1 (essential oils at 1 mL·L^{-1}) and test 2 (essential oils at 2 mL·L^{-1}) on treated leaves. Results shown as mean ± SD of five independent determinations.

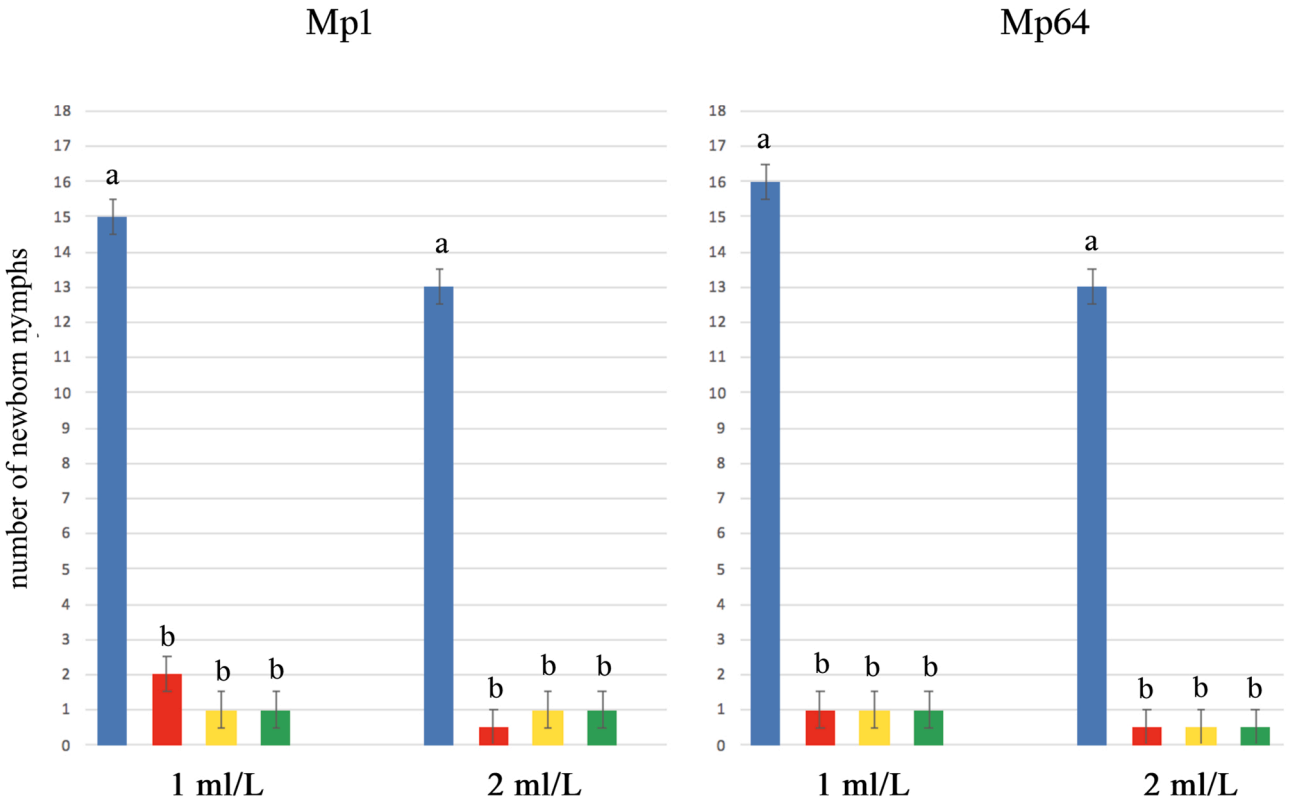

Fig. 3. Effects of water (blue), azadirachtin (red), and essential oils of *A. roxburghiana* (yellow) and *E. fruticosa* (green) evaluated as average total newborns for each female aphid of *M. persicae* strains 1 (Mp1) and 64 (Mp64) in test 1 (essential oils at 1 mL·L^{-1}) and test 2 (essential oils at 2 mL·L^{-1}). Results shown as mean ± SD of five independent determinations.

plants collected in their natural origins. The essential oil composition of *A. roxburghiana* found in this study confirmed previous reports, while that of *E. fruticosa* was more complete but different from reports by other studies. This indicates a possible influence of environmental factors characterizing the site of growth.

At the concentrations examined in this study, the essential oil of *E. fruticosa* revealed high in vitro antimicrobial activity against pathogenic Gram-positive and Gram-negative bacteria. The high nymph mortality rate and reduction of adult fecundity of aphids showed that the oils have prospects as a natural remedy for the control of the pest insect *M. persicae*. Considering these results, *A. roxburghiana* and *E. fruticosa* represent innovative crops as sources of natural extracts to be used in the agro-industry and improving sustainability. However, further studies are needed to assess the effect on other pests, the effect on the growth of staple and cash crops, the safety of their use on edible crops, and the possible toxicity of residuals for human consumption.

Funding

This research was partially supported by ROP ERDF 2014–2020 Lombardy – Innovation and Competitiveness, and partially by the Italian Ministry of Agricultural, Food and Forestry Policies under the project, 'Implementation of the FAO International Treaty on Plant Genetic Resources for Food and Agriculture', which is aimed at research and experimentation supporting the collection, characterization, and evaluation of plant genetic resources.

CRediT authorship contribution statement

Pietro Fusani: Conceptualization, Methodology, Investigation, Writing - Original Draft; **Domenico Ronga**: Investigation, Writing - Review & Editing; **Domenico Carminati**: Investigation, Writing - Review & Editing; **Mauro Mandrioli**: Resources, Writing - Review & Editing; **Gian Carlo Manicardi**: Resources; **Sergio Giannì**: Investigation; **Aldo Tava**: Conceptualization, Methodology, Investigation, Writing - Review & Editing;.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

Acknowledgments

The authors thank Prof. Kazumi Fujikawa of the Kochi Prefectural Makino Botanical Garden, Kochi, Japan, for the identification of *Artemisia roxburghiana* Besser plants.

P. Fusani wishes to thank Dr. Alessandro Bezzi, formerly of ISAFA, Istituto Sperimentale per l'Assestamento Forestale e l'Alpicoltura of Trento, for providing useful information about the origin of the plants studied.

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