Canadian Science Publishing | Botany

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Journal:	*Botany*
Manuscript ID	cjb-2018-0212.R1
Manuscript Type:	Article
Date Submitted by the Author:	09-Jan-2019
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Keyword:	essential oil, Invasive Alien Species, liposomes, Rosmarinus officinalis, seed germination
Is the invited manuscript for consideration in a Special Issue? :	Not applicable (regular submission)

Original research

Inhibitory effect of rosemary essential oil, loaded in liposomes, on seed germination of *Acacia saligna* (Labill.) Wendl., an invasive species in Mediterranean ecosystems

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Abstract

Acacia saligna (Labill.) Wendl. is native of south-western Australia, but has been planted extensively in many areas of the world, including the Mediterranean Region, becoming highly invasive especially in coastal habitats. The aim of this study was to test whether the indigenous *Rosmarinus officinalis* L. essential oil (EO), loaded in liposomes, can inhibit the seed germination of the invasive alien *A. saligna*. Variability in seed germination requirements and responses among populations were evaluated. Germination tests under light, constant temperatures and three concentrations of rosemary EO were carried out. Among the examined factors, only the EO amount and temperature had a highly significant effect on seed germination. The lowest EO quantity did not show differences as compared to the control, while the highest amount inhibited significantly seed germination of all populations at all the tested temperatures. No seed recovered the ability to germinate after the EO treatment with neither of the two washing methods. Our results allowed us to identify the minimum amount of rosemary EO capable of inhibiting the seed germination of the invasive *A. saligna.* These results could be useful for the control of this invasive alien species thus allow the conservation of indigenous Mediterranean plant species and habitats.

Keywords: essential oil, Invasive Alien Species, liposomes, *Rosmarinus officinalis*, seed germination.

Running title: Inhibition of *Acacia saligna* germination by rosemary essential oil.

Introduction

Invasive Alien Species (IAS) are considered, after habitat loss and fragmentation, as one of the greatest threats to the conservation of indigenous species and habitats worldwide (Crisóstomo et al. 2013). Moreover, these species cause also significant economic damage to natural ecosystems and to human health (DAISIE 2009; Strydom et al. 2012).

IAS are responsible for the reduction of native species abundance and diversity, change in nutrient cycling, water availability and fire regimes, altering both the structure and function of invaded ecosystems (Correia et al. 2014). Callaway and Aschehoug (2000) asserted that some IAS may be successful because they introduce novel mechanisms of interaction by releasing substances which interfere with primary and secondary physiological processes, e.g. ions and water uptake, enzyme functions, seed germination, seedling growth, photosynthesis and respiration of native plant species (Lorenzo et al. 2011).

The genus *Acacia* Mill. belongs to the Fabaceae (nom. alter. Leguminosae) family (Richardson et al. 2011) and includes some of the most important IAS in the world. About 23 species of this genus are major invaders in Mediterranean ecosystems (Werner et al. 2010). The effects of invasion by *Acacia* species on indigenous species and habitats have been widely documented (Le Maitre et al. 2011; Correia et al. 2014), particularly on species richness, community structure, soil bulk density, and water availability (Werner et al. 2010). Moreover, these species show several reproductive traits that may also contribute to their invasiveness, such as the ability to self-pollinate, vegetative reproduction and the production of a large amount of long-lived and highly viable seeds (Morris et al. 2011; Correia et al. 2014; Meloni et al. 2015).

Acacia saligna (Labill.) Wendl. is an evergreen phanerophyte, native of south-western Australia (Orchard and Wilson 2001) and is considered a neophyte for the Mediterranean Basin. This species grows in a wide range of ecological conditions, including semi-arid and arid Mediterranean climatic areas, especially on barren slopes, sand dunes (Midgely and Turnbull 2003), and abandoned lands

(Kutiel et al. 2004). Musil (1993) reported that *A. saligna* is tolerant to alkaline and saline soils, exhibiting greater tolerance to high concentrations of potassium, calcium and magnesium compared to several indigenous species (Fox 1995; Doran and Turnbull 1997).

One of the factors that may have a strong influence on *A. saligna* invasiveness is its ability to produce a large number of viable seeds (Strydom et al. 2012). *A. saligna* seeds may remain dormant but viable for more than 50 years, until the seed coat is sufficiently damaged to be permeable to water necessary for seed imbibition (Meloni et al. 2015). According to Holmes et al. 1987 and Holmes and Cowling (1997*a*, *b*) they maintain high viability (values of ca. 86-100%) in the soil seed bank even after several years.

In recent decade, this species has been widely used for stabilizing shifting sand dunes, rehabilitating sandy mining areas, protecting barren rocks along roadsides, and has been planted extensively in many parts of the world, becoming highly invasive in the Mediterranean Basin (Crisóstomo et al. 2013; Del Vecchio et al. 2013).

Essential oils (EOs) are produced more than 17,000 aromatic plant species belonging mostly to a few families, including the Myrtaceae, Lauraceae, Lamiaceae, and Asteraceae (Regnault-Roger et al. 2012). EOs contain compounds named Plant Secondary Metabolites (PSMs), such as terpenes and phenols and are known to have multiple ecological roles, notably defence against herbivores, pathogens and abiotic stresses (Iason et al. 2012). The production of these compounds is affected by the physical and chemical properties of the ecosystem where the plants grow, such as precipitation, temperature and edaphic features (Thompson 2005; Pirbalouti et al. 2015). PSMs may be present in all plant organs, and may be found in different concentrations in several parts of the plant. The concentration of these substances is the parameter that determines a positive influence (facilitation) and/or negative (competition) against other target organisms (Amiot et al. 2005; Iason et al. 2012). The beneficial or harmful influence of these compounds, may have an influence on

seed germination and seedling growth of other nearby plants, allelopathy or phytotoxicity (Rice 1984).

The Mediterranean biogeographic region is rich in aromatic plants, many of which belong to the Lamiaceae (Chizzola 2006), which is considered one of the largest and most distinctive families of flowering plants, with about 220 genera and almost 4000 species worldwide (Mitić et al. 2011). *Rosmarinus officinalis* L. (rosemary) has recently (2017) been placed in *Salvia* L. genus as *S. rosmarinus* (L.) Schleid. by Drew et al. (2017) and is known as *S. rosmarinus* Schleid. (Bartolucci et al. 2018) is one of the most important aromatic species of this family and it has been used since ancient times for medicinal and culinary uses (Dajic-Stevanovic et al. 2008). Rosemary is a Circum-Mediterranean evergreen indigenous nanophanerophyte, calcicolous and heliophilous, which grows in many substrata, preferring sandy, dry, calcareous and humus-poor soils. This species is characteristic of the Mediterranean maquis, garrigue and shrublands, and can be found from sea level up to 800 m a.s.l., and contains a quantity up to 1% of EO (Pintore et al. 2002; Bellumori et al. 2015).

Detailed studies of the effects of EO from several members of the Lamiaceae have shown that, depending on the type and concentration, EOs reduced or totally inhibited germination of a range of species (Angelini et al. 2003; Atak et al. 2016)

The aims of this research were to obtain preliminary results on the effect of rosemary EO on *A. saligna* seeds, in particular: 1) to define the potential phytotoxic effect of rosemary EO on seed germination, evaluating the minimum amount of rosemary EO able to inhibit seed germination, and 2) evaluate inter-population variability in the seed germination response in *A. saligna*.

In this study the EO was loaded in liposomes which are phospholipid nanovesicles able to limit the loss of volatile components and deliver them inside the seed potentiating the EO efficacy. Additionally, the EO incorporation in liposomes ensures its complete and homogenous dispersion in

the agar reducing its loss. To our knowledge, this is the first time that liposomes have been used as components of a germination substrate.

Material and methods

Plant material, essential oil and seed lot details

Flowering aerial parts of ten individuals of *R. officinalis* were collected in North Sardinia [Su Canale, Monti (OT; Italy), 40°50' N - 09°23' E]. Afterwards the plant material was air-dried in an oven (FP 115, BINDER) at 40°C with forced ventilation for 48 hours. The plant material was subjected to steam distillation to obtain the EO. The chemical composition of EO was analysed by using gas chromatography (GC-FID) and gas chromatography-mass spectrometry (GC-MS) according to Zuzarte et al. (2013).

A. saligna legumes (hereafter fruits) were collected in the period of natural dispersal (summer) in four naturalized Mediterranean populations (Table 1). Seeds were extracted from the fruits by hand and using laboratory tweezers. Prior to germination tests, seeds were stored under controlled conditions (20°C and 50% relative humidity) for two weeks.

Germination trials

Germination tests were performed at the Sardinian Germplasm Bank (BG-SAR) of the Hortus Botanicus Karalitanus (HBK) of the University of Cagliari**,** Sardinia (Italy). To detect the presence of water-impermeable integuments, i.e. the physical component of seed dormancy, seven different methods of both mechanical and chemical scarification were tested. In each method, four replicates of 25 seeds per Petri dish were used. Furthermore, other four replicates with 25 intact seeds per Petri dish were used as the control. Seeds were chipped with a scalpel (1), engraved with nail clippers (2), mechanically scarified with sandpaper no. 40 (3) and no. 80 (4), immersed in H_2SO_4 (96%) for 10 (5), 15 (6) and 20 (7) minutes (Azad et al. 2011). All seeds were sown under a laminar

flow hood (KB, Faster) in 90 mm diameter plastic Petri dish on 1% water agar substrate, which provided a solid, non-sterile medium for germination, independently from the treatment, and were incubated at a constant temperature of 15°C in a growth chamber (MLR-351, SANYO) in the light (12 h of irradiance per day). After sowing, each Petri dish was sealed with laboratory parafilm (parafilm M, Pechiney) to limit evaporation and prevent contamination spores and microorganisms from the outside.

The criterion for germination was visible radicle protrusion (≥ 2 mm). Seeds were scored daily, and germinated seeds discarded. When no additional germination occurred for two consecutive weeks, tests were stopped, and the viability of each remaining seed was checked by a cut test with a scalpel and subsequent observation of the endosperm under binocular microscope.

Several preliminary experimental tests were carried out to identify the more suitable formulation able to stably load the EO and easily blend with the germination substrate. In particular, during these preliminary experiments, soy lecithin, which forms liposomes, was identified as the best component for this aim.

The effect of soy lecithin (empty liposomes) on the seed germination of *A. saligna* was evaluated by testing four replicates with the addition of soy lecithin to the substrate and four replicates with only 1% agar. Seeds were incubated in a growth chamber at 15°C in the light (12 hours of irradiance per day). Meloni et al. (2015) showed that these conditions were optimal to achieve the highest germination for this species. For all the preliminary experiments, Asal1 seeds (Table 1) were used (due to their higher availability).

In each Petri dish used for the germination tests with the addition of EO, 50 ml of solution was used for the germination substrate. The substrate consisted of two layers: A (lower) and B (upper). Layer A contained 30 ml of solution, while the remainder 20 ml were used to create layer B on which seeds were sown. Layer A consisted of 1% agar. Layer B contained 1% agar, distilled water and liposomes containing 1.8 g of soy lecithin and EO (on the basis of the used amount). To ensure a

homogenous mixing of the EO and the agar substrate, the EO was loaded in liposomes which were prepared by sonicating the soy lecithin and the EO dispersed in water (Soniprep 150 Plus Sonicators, MSE) for 30 minutes, then immediately mixed with agar. It was not possible to prepare layer B without using EO loaded liposomes because the free EO added to the agar substrate precipitated.

We tested the effect of three different concentrations of *R. officinalis* EO (3.90, 7.81, 15.62 µl/50 ml of the substrate) loaded in liposomes containing the same amount of soy lecithin. Preliminary experiments showed that higher concentrations did not produce a solid substrate.

At the end of the experiments, to evaluate the reversibility of the EO effects, the ability of *A. saligna* seeds to recover their germination after the EO treatment was evaluated by washing seeds with Tween 80 PS ($C_{64}H_{124}O_{26}$, Polysorbate 80, Panreac) and sodium hypochlorite (NaClO) solutions. The Tween 80 washing lasted 10 minutes (the first five minutes with 200 ml/L Tween 80 in distilled water and the remaining five minutes with 100 ml/L). The sodium hypochlorite (860 mM) washing lasted 10 minutes (the first five minutes with 500 ml/L bleach in distilled water and the remaining five minutes with 250 ml/L). The response of *A. saligna* seeds from each population was evaluated on the basis of the seed availability, therefore we tested three temperatures (10, 15, 20°C) for the two Sardinian populations and two (15, 20°C) for the Sicilian ones, with photoperiod of 12/12 hours of irradiance per day.

Data analysis

Final germination percentages were calculated as the mean of four replicates (± SD). The rate of germination was estimated by using a modified Timson's index of germination velocity (TI) (Khan and Ungar 1984; Santo et al. 2015): $TI = \Sigma\, G/t$, where G is the percentage of seed germination at 2-day interval and t is the total germination period. Arcsine-transformed germination percentages and log10-transformed TI were analysed by ANOVA and consequent Fisher's Least Significant

Differences (LSD) *post hoc* test. Germination percentages of scarification pre-treatments were analysed by the non-parametric Kruskal-Wallis test, followed by a Mann-Whitney *U* test, due to the non-satisfaction of the ANOVA assumptions even after arcsine transformation. Data were graphed using Sigmaplot 11.0 (Systat Software Inc., London, UK), and all statistical analyses were carried out using the statistical software Statistica 7.0 for Windows (Software Statsoft Release 7).

Results

Phytochemical analysis

The rosemary EO was characterized by a total of 23 compounds, accounting for 99.8 % of the total composition. The chromatographic results, expressed as GC peak area percentages and calculated without any response factor correction, highlighted a very high percentage of oxygenated monoterpenes (51.8 %) and hydrocarbon monoterpenes (44.5 %), and a lower percentage of hydrocarbon sesquiterpenes (2.7 %) and other compounds (0.8 %). The main compounds were: α-Pinene (21.5 %), Bornyl acetate (16.8 %), Borneol (10.2 %), Camphor (9.7 %), Camphene (7.5 %), 1,8-Cineole (7.4 %), Verbenone (4.4 %), Limonene (4.3 %) and β-Pinene (3.0 %).

Effect of scarification on seed germination

Highly significant differences ($p < 0.001$) were detected among different scarification pre-treatments (Figure 1). In particular, the sandpaper no. 80, the scalpel and nail clippers were shown to be the best methods, with 100% of germinated seeds in the shortest time (TI > 8, for all). In contrast, the germination percentages obtained by chemical scarification were very low (< 50%) independently of the tested immersion time (TI of ca. 0.9).

As the fastest germination times were obtained by scarification using sandpaper no. 80, we used this scarification technique for the following germination tests using EO.

Effects of temperature and EO on seed germination

Preliminary experiments were carried out to evaluate the effect of the individual components in the culture medium. The addition of empty liposomes had no effect on germination, as the final percentages were statistically comparable ($p > 0.05$) to control (ca. > 95% for both). Both the EO concentration (Co) and temperature (Te) had highly significant effect ($p < 0.001$) on the final germination, while population (Po) did not affect the results ($p > 0.05$; Table 2).

Final germination of all the tested populations decreased with increasing EO amount (Figure 2). In particular, Asal1 showed inhibition of its seed germination at 10 and 20°C starting from 7.81 µl, while at 15°C the reduction in final germination occurred only starting from 15.62 µl. The highest tested EO 15.62 µl/Petri dish concentration caused an inhibition of germination of ca. 85% at 20°C and of ca. 95% at 10 and 15°C. For Asal2, no significant inhibition ($p > 0.05$) occurred up to 7.81 µl at all temperatures, while total inhibition was observed at 10°C under 15.62 µl. Asal3 seeds showed a decrease starting from 7.81 µl at both the two tested temperatures (15 and 20°C) and the 15.62 µl amount caused the inhibition of ca. 85% at 15°C and ca. 90% at 20°C. For Asal4 a significant decrease in seed germination was observed starting from 3.90 µl at 20°C, while starting from 7.81 µl at 15°C. The highest tested EO amount inhibited the final germination of ca. 73% at both the two tested temperatures. Overall, the suboptimal temperatures for this species (10 and 20°C) caused a highly significant effect ($p < 0.001$) of the EO on the seed germination and this pattern was also confirmed by the statistical analyses (interaction Co × Te in Table 2).

Discussion and Conclusions

This study highlights a qualitative and quantitative chemical composition of the rosemary EO that is the same as other oils used for the management of IAS and weeds, characterized by a phytotoxic effect on the seed germination of several species (Angelini et al. 2003; Atak et al. 2016). It is well known that the EOs are characterized by high concentrations of two or three compounds, compared

to the other components (which are present in trace) and generally these major compounds determine the biological proprieties of the EOs (Bakkali et al. 2008). Some monoterpenes present in the Rosemary EO, such as α-Pinene, Limonene, 1,8-Cineole, Borneol, Camphor, are known to inhibit seed germination in some species (De Martino et al. 2012). Based on its chemical composition, we hypothesised that EO would inhibit germination of *A. saligna*.

The use of empty liposomes in the culture medium did not influence the final germination of *A. saligna*. The incorporation of EO in liposomes limited the normal loss of EO volatile components thus delivering them into the seed and potentiating EO efficacy. This innovative method, used for the first time, might be used in further studies on species with slow germination because it preserves EO composition. Further studies might investigate the use of EO liposomes applied to the shoot of *A. saligna* plants, in the field.

Our study provides preliminary information about the phytotoxic effect of rosemary EO on *A. saligna* seed germination. In particular it showed that *A. saligna* seeds germinated when exposed to up to 15.62 µl of *R. officinalis* EO in the substrate, independently from the seed provenance. The interaction between temperature and EO presence in the substrate amplified the inhibitory effect of increasing EO amount. Overall, the inhibition of the seed germination was low, up to 7.81 µl, but the highest tested concentration showed that *A. saligna* seeds were highly sensitive to this EO, resulting in a potentially effective method for inhibiting germination in *Acacia saligna* that warrants further investigation.

The results obtained in the present study are similar to those of previous studies which showed the inhibition of the seed germination of several weeds (and cultivated plants associated to them) above a certain concentration of rosemary EO. In particular, as showed by Atak et al. (2016) for *Avena sterilis* L. seeds, concentrations of rosemary EO from 2 to 16 µl/Petri dish caused a decrease in germination from 61 to 12%, whilst at least 2 µl/Petri dish inhibited germination of *Sinapis arvensis*

L. by 89%. In our study, the germination inhibition of *A. saligna* seeds was more similar to that observed for *Avena sterilis*, showing that the rosemary EO effects are species-specific.

The present study showed that no *A. saligna* seed recovered the ability to germinate after EO exposure followed by either of the two washing methods. This indicates that the rosemary EO might cause irreversible damage to *A. saligna* seeds. Witzke et al. (2010) asserted that the phytotoxic effects of EOs on seed germination, seedling development and survival occur through several actions on different functional processes, such as: loss of membrane integrity, ion and solute transport through membranes, synthesis of molecules and macromolecules (e.g. membrane lipids, chlorophyll, proteins and nucleic acids) and cell division. Moreover, EOs may trigger changes in the mitochondrial adenosine triphosphate (ATP) production, which compromise the mitochondrial metabolism, in particular during seed germination and early seedling development (Botha et al. 1992; Neuburger et al. 1996). It was not possible to determine which physiological mechanism(s) caused the inhibition of germination of *A. saligna*.

The potential for using phytotoxicity in the management of weeds and invasive species has been well documented (Putman 1988; An et al. 1998). Moreover, germination inhibition by EOs applied to seeds of various crops, weeds and invasive alien species, has been reported in several studies (Nishimura et al. 1982; Azirak and Karaman 2008; Mutlu and Atici 2009; Gitsopoulus et al. 2013). Recently, EOs obtained from natural indigenous plants were widely tested in the control of weeds and IAS. The potential phytotoxic effect caused by the EOs of indigenous plant species might be a reliable means for the control and management of IAS and weeds.

On the basis of our encouraging results and the verified ability of the rosemary oil to inhibit the seed germination of our invasive study species, the EO of rosemary might be used to control the invasiveness of *A. saligna*. In conclusion, these results may be useful for the control against invasive alien species thus allowing the conservation of native plant species and the preservation of the Mediterranean habitats.

Further studies are in progress to evaluate the potential phytotoxic effect of *R. officinalis* EO on seedling development and survival of *A. saligna*, so that these treatments may be applied to reduce its establishment and seedling recruitment directly in natural habitats.

Acknowledgements

The authors wish to thank the Co.S.Me.Se and BG-SAR for financial support and the entire research group, in particular Lina Podda, Maria Letizia Manca and Carla Caddeo. A particular thank also to Anna Rita Succa for the linguistic revision of the manuscript.

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Tables

Table 1 - Population data and seed lot details.

Population code	Site	Island	Mean coordinates (UTM, WGS84)	Substrate	Distance from sea (m)	Mean elevation (m a.s.l.)	Mean seed mass (mg ± SD)
Asal1	Costa Rei - Muravera (CA)	SE Sardinia	41°14' N 09°24' E	Aeolian sands	10	3	17.94 ± 0.54
Asal2	La Maddalena (OT)	NE Sardinia	41°35' N 09°18' E	Granites	2	2	18.96 ± 1.04
Asal3	Arenella – Siracusa (SR)	SE Sicily	37° 00' N 15°16' E	Calcarenites	45	10	19.92 ± 0.82
Asal4	Punta del Pero - Siracusa (SR)	SE Sicily	37°02' N 15°17' E	Limestones	24	5-8	18.37 ± 0.67

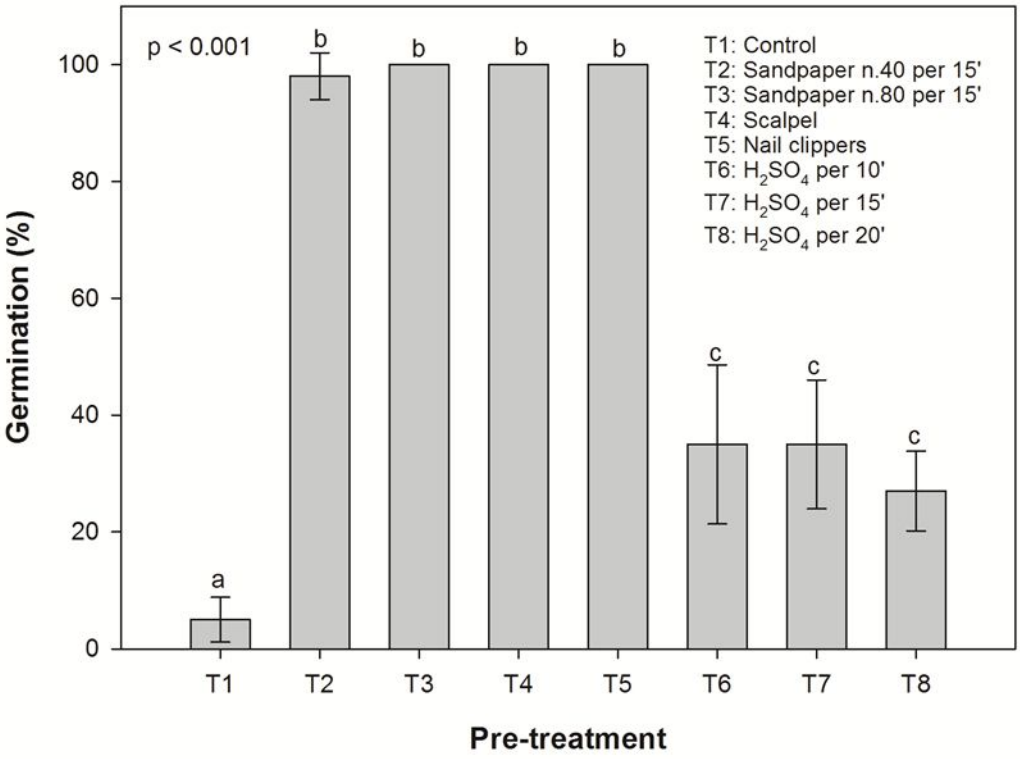
Table 2 - Effect of EO concentration (Co), temperature (Te), population (Po) and their interactions on the final germination of *Acacia saligna* seeds from the four investigated populations. *P* values were considered not significantly ($P > 0.05$, ns), significantly ($P < 0.05$, *) or highly significantly ($P < 0.001$, ***) different, by three-way ANOVA; SS = Sum of squares; DF = Degrees of freedom; MS = Mean square; *F* = Fisher variable; *P* = *p* value).

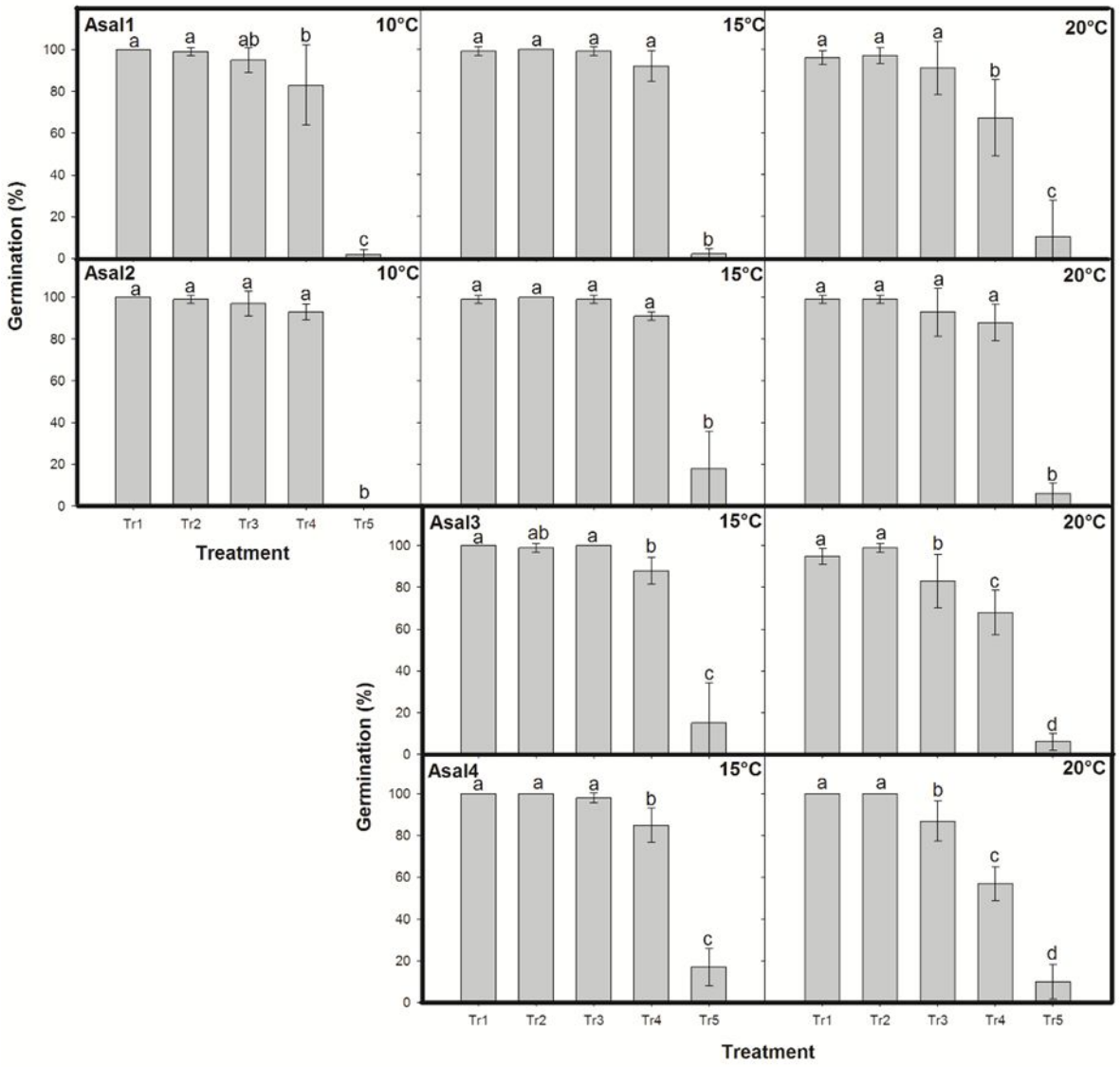
Effect	**SS**	**DF**	**MS**	***F***	***p***
EO concentration (Co)	181245.6	4	45311.4	702.14	***
Temperature (Te)	2250.0	1	2250.0	34.87	***
Population (Po)	448.8	3	149.6	2.32	ns
Co × Te	1760.0	4	440.0	6.82	***
Co × Po	1351.2	12	112.6	1.74	ns
Te × Po	214.8	3	71.6	1.11	ns
Co × Te × Po	1195.2	12	99.6	1.54	ns
Error	7744.0	120	64.5		

Figure captions

Figure 1 - Final germination of *Acacia saligna* seeds under different scarification pre-treatments. A Kruskal-Wallis test was carried out in order to identify differences among the tested pre-treatments. Values with different letters are significantly different at $p < 0.05$ (by Mann Whitney *U*-test).

Figure 2 - Effect of different concentrations of *Rosmarinus officinalis* essential oil (EO) on the seed germination of four *Acacia saligna* populations at constant temperatures (10, 15, 20 °C). Bars with the same letters are not significantly different at $p < 0.05$ (three-way ANOVA followed by Fisher's Least Significant Difference *post hoc* test). Data are the means (± SD) of four replicates. See Table 1 for the explanation of population codes; Tr1 = Control (only 1% agar); Tr2 = Soy Lecithin + Agar 1%; Tr3 = 3.90 µl EO; Tr4= 7.81 µl EO; Tr5 = 15.62 µl EO.


p < 0.001
b
b
b
b
a
c
c
c
T1: Control
T2: Sandpaper n.40 per 15'
T3: Sandpaper n.80 per 15'
T4: Scalpel
T5: Nail clippers
T6: H2SO4 per 10'
T7: H2SO4 per 15'
T8: H2SO4 per 20'
Germination (%)
0
20
40
60
80
100
T1
T2
T3
T4
T5
T6
T7
T8
Pre-treatment


Asal1
10°C
15°C
20°C
Asal2
10°C
15°C
20°C
Asal3
15°C
20°C
Asal4
15°C
20°C
Germination (%)
Treatment
Tr1
Tr2
Tr3
Tr4
Tr5