Soil scarification favors natural regeneration of Pinus pinea in Lebanon forests: Evidences from field and laboratory experiments

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Authors: Joseph Nakhoul[1], Mathieu Santonja[2], Catherine Fernandez[2], Stéphane Greff[2], Anne Bousquet-Mélou[2], Sylvie Dupouyet[2], Nabil Nemer[3], Salim Kattar[4], Jihad Abboud[4], Bernard Prévosto[1]

Addresses:

1. INRAE, Aix Marseille Univ, UR RECOVER, 3275 route de Cézanne CS 40061, F-13182 Aix-en-Provence cedex 5, France.
2. Aix Marseille Univ, Avignon Université, CNRS, IRD, IMBE, Marseille, France.
3. Holy Spirit University of Kaslik – USEK, Kaslik, Jounieh, Lebanon
4. Faculty of Agronomy, Lebanese University, Dekwaneh, Lebanon.

Abstract

Pinus pinea is an important species for ecological and economic reasons in the Mediterranean area and especially in Lebanon. However, conditions of its natural regeneration have received little attention. Our study aimed to test the influence of soil disturbances, predation and autotoxicity on pine regeneration. A seed sowing field experiment was conducted in a mature stand in which two types of soil disturbances were tested (soil scarification and litter burning) in presence or absence of predation. In addition, a laboratory experiment evaluating the influence of litter (present, absent or burned) and green needle leachates (i.e. in order to mimic potential autotoxicity) on seed germination and seedling growth was conducted. Results showed a strong effect of soil disturbance, in particular soil scarification which promoted seedling emergence and early survival, whereas the role of predation was low. Forty to sixty days after seed installation, seedling density peaked at 14.9 ± 0.4 seedling.m^{-2} in the scarification treatment, 13.1 ± 0.4 in the burning treatment and 8.4 ± 0.4 in the control treatment. However, an unexpected high mortality rate was recorded at the end of the field experiment in all treatments, as after seven months seedling density dropped to 0.2 ± 0.02. In the laboratory experiment, we emphasized an autotoxic effect of green needle aqueous extract on seed germination and seedling growth in the presence of litter. This allelopathic effect could be potentially linked to the presence of quinic acid which was measured as the major metabolite detected in needle aqueous extracts. In conclusion, we recommend soil preparation treatments to favor seedling emergence in combination with thinning to achieve seedling development on the long term.

Key words*: Soil disturbances, allelopathic effect, seedling emergence, seedling survival, germination, litter burning.*

1. Introduction

Natural regeneration of a plant species is the renewal of a stand by its own seeds in a natural way without intervention of any artificial agent (Ford-Robertson, 1971). This process includes seed production and dispersal, seed germination, establishment and seedling growth (Madsen and Larsen, 1997). Germination, which plays a major role in the success of natural regeneration since it controls seedling emergence (Baskin and Baskin, 2001), is a physiological process that allows the transition of latent life of a seed to seedling development and represents a very vulnerable and sensitive phase (Harper, 1977). In the broadest sense, germination often refers to two main stages. The first stage includes the physiological events occurring in imbibed seeds which led to the emergence of a radicle (germination *sensu stricto*) while the second stage refers to the emergence of the aerial part (emergence). It is in fact an irreversible process (Bewley and Black, 1994) and therefore any spatiotemporal disturbance will lead to death with cascading negative impact on population recruitment (Harper, 1977; Silvertown and Charlesworth, 2001). Also, it is considered as crucial as high mortality rates that happen during it are linked to competition by the ground vegetation and/or predation (Harper, 1977; Nambiar and Sands, 1993; Castro *et al.*, 2002). A major ecological filter to overcome is a successful seedling establishment which is very connected to events happening in early life stages of plants (Suding and Goldberg, 1999; Burt-Smith and Tilman, 2003) and dependent to both climatic and soil characteristics (Rojo and Montero, 1996; Manso *et al*., 2009). In addition, seed germination and seedling establishment are under control of various environmental factors, including essentially air temperature, soil humidity and light access (Mayer and Poljakoff-Mayber, 1989). Thus, the success of regeneration including all these phases depends on various biotic and abiotic factors.

Several studies investigating the influence of plant-plant interactions on natural regeneration of pines have led to contrasted results. Both forest herbaceous and shrub layers as

well tree canopy can affect pine regeneration. For instance, an increase in shrub cover was found detrimental to the natural regeneration of *Pinus radiata* (O'Brien *et al.*, 2007). By contrast, a study conducted on *Pinus sylvestris* showed an increasing negative effect of competition between pine seedlings with increasing light intensities (Pardos *et al.*, 2007). Tíscar and Linares (2011) also reported that an intermediate canopy cover of *Pinus nigra* (40-60%) could assist pine seedling establishment. Interestingly, Prévosto *et al.* (2015) reported a negative effect of shrub on *Pinus halepensis* seedling emergence but, in the opposite, a positive effect on seedling survival and growth. Litter on the forest floor can also play an important role in tree regeneration (Everham *et al*., 1996; Sayer, 2006; Baker and Murray, 2010). However, the role of litter is complex as it can act as a mechanical and physiological barrier to seedling establishment (e.g. litter thickness; Baker and Murray, 2010) but, in the opposite, sometimes can play a positive role (e.g. limiting soil evaporation; Sayer, 2006). Management actions tend to remove this barrier with operations such as prescribed burning or scarification. Prescribed burning modifies soil properties although physical and biological properties are more affected than chemical properties (see Alcañiz *et al.*, 2018 for a review). Litter is turned into ashes which is usually detrimental to germination and early survival of pine seedlings (e.g. Reyes and Casal, 2004; Sagra *et al*., 2018). In contrast, soil scarification is often found beneficial to seedling establishment after seed fall due to a better contact seed-soil, improvement of nutrition conditions, and limitation of the competition by the ground vegetation (e.g. Harrington and Edwards, 1999; Karlsson and Orlander, 2000; Nadelhoffer *et al*., 2000; Prévosto *et al*. 2012).

Plant-plant interactions such as competition for resources, facilitation, and allelopathy are one of the biotic factors which could influence regeneration (Callaway and Walker, 1997). A strong attention has been paid during the last decades to allelopathy, demonstrating the key implication of plant-plant chemical interaction as a driver of plant community structure and ecosystem functioning (Wardle *et al.*, 1998; Inderjit *et al.*, 2011; Meiners *et al.*, 2014). Seed

germination and seedling performance are the main life stages usually affected by allelochemicals (i.e. phenolics or terpenoids), and frequent negative allelopathic effects are inhibition of seed germination (Herranz *et al.*, 2006; Fernandez *et al.*, 2013), delay of seed germination (Fernandez *et al.*, 2013; Hashoum *et al.*, 2017) and inhibition of seedling growth (Santonja *et al.*, 2018; Gavinet *et al.*, 2019). Several plant physiological processes such as photosynthesis, nutrient uptake, cell division or elongation can be influenced by the allelochemicals released (Chou, 1999; Inderjit and Duke, 2003). In addition to direct effects on target plant species, allelochemicals released can also inhibit seed germination and seedling establishment by affecting root symbionts and site quality through interference with decomposition, mineralization and humification processes (Kuiters, 1990; Kainulainen *et al.*, 2003). These allelopathic effects have been considered among causes of regeneration failure in conifers forest (Bong-Seop, 1992; Mallik, 2003; Fernandez *et al.*, 2008; Monnier *et al.*, 2011). Fernandez *et al.* (2008) showed that *P. halepensis* needle and root aqueous extracts strongly inhibited seed germination and seedling growth of *P. halepensis*. Bong-Seop (1992) also demonstrated a negative effect of needle and root aqueous extract of *Pinus densiflora* on its seed germination.

Pinus pinea (stone pine) is a very important and typical species in the Mediterranean area because of its ecological and economical value, and one of the most valuable trees in the reforestation programs in particular in Lebanon (MoE, 2014; Haroutunian *et al.*, 2017). Natural regeneration of the ageing *P. pinea* forests is thus a crucial step not only for economic purposes but also to adapt the future forests to climate change by selecting the most adapted seedlings to environmental conditions (Lucas-Borja, 2014). However, natural regeneration of *P. pinea* has hardly been achieved although some studies have been conducted in Greece (Ganatsas *et al*., 2008), Spain (Calama and Montero, 2007; Barbeito *et al.*, 2008) and Tunisia (Adili *et al.*, 2013). Factors controlling natural regeneration of this species were less studied than for other pine

115 species and allelopathic effects were never investigated. Although challenging, natural
116 regeneration can be successfully enhanced by soil treatments which can remove the natural
117 barrier formed by the litter layer and favor the contact between seeds and soil (Jäärats *et al.*,
118 2012). This positive effect was observed particularly in temperate forests (Mattson and
119 Bergsten, 2003; Landhäusser, 2009) but also in Mediterranean ones (Prévosto *et al.*, 2012) and
120 was even recommended for *P. pinea* (Adili *et al.*, 2013).

121 In this context, the objective of the present study was to analyze the influence of soil
122 disturbance on two main processes of *P. pinea* regeneration: seedling germination (*sensu*
123 *stricto*) and emergence and, seedling growth and survival. In a first *in situ* experiment, we tested
124 if soil disturbances can affect emergence and early survival of *P. pinea* seedling in Lebanon
125 forests. Because predation is often of crucial importance in field conditions for the initial
126 establishment phase, its influence was also tested. We hypothesized that the soil treatment, by
127 removing the physical and chemical barrier of the litter, could favor these two processes. In a
128 second laboratory experiment, we tested the influence of the interaction between soil treatment
129 and natural needle leachate on *P. pinea* seed germination and seedling growth. We hypothesized
130 that *P. pinea* allelochemicals could exert an autotoxic effect on the early establishment phase
131 and that this effect could be modulated by soil conditions.

132

133 **2. Material and Methods**

134

135 ***2.1. Experiment 1: Response of Pinus pinea seed germination and early seedling survival to***
136 ***soil treatments and predation in Lebanon forest***

137

138 *2.1.1. Study site*

139 The study area was located in an 88-year-old *P. pinea* stand in Lebanon (33°57’2’’N -
140 35°38’9’’E) most probably a forest plantation like most of the pine stands in the area although
141 the status of the pine (native or planted) is debated. The site is at an altitude of 237 m a.s.l. on
142 a flat terrain with sandy soil that is typical for stone pine stands in Lebanon. The stand did not
143 experience any disturbance such as forest fire during the last 50 years. The tree layer was
144 composed by *P. pinea* strictly and a weakly developed understory layer of *Quercus calliprinos*
145 with a poor herbaceous layer dominated by *Cichorium intybus*.
146 The climate was Thermo-Mediterranean characterized by a hot and dry summer. The
147 mean annual rainfall was 964 mm and the mean annual temperature was 18.6 °C (mean values
148 over the period 2008-2018, meteorological station of Zouk Mosbeh (33°57'02.0"N;
149 35°38'08.6"E°)). It is important to note that during the 200 days of the first experimental year,
150 rainfall (280 mm) was below the amount registered in the second year (800 mm). A
151 representative pure pine stand was chosen on a flat area with a closed cover. Its main
152 dendrometric characteristics were a basal area of 48 $m^2.ha^{-1}$, a density of 625 individuals.ha^{-1},
153 an average DBH per tree of 31.0 cm ± 0.9 cm and mean height of 10.2 ± 0.2 m.
154
155 *2.1.2. Field experiment and measurements of the early survival*
156 Eighteen 1 × 1m plots were installed in the selected stand and randomly distributed
157 according to 3 soil treatments (control, litter burning, and soil scarification). Plots were
158 separated by a buffer zone (1 to 2 m depending on site facilities). No treatment was applied for
159 the control, *i.e.* the soil remained covered by a pine litter of approximately 3 cm thick. Litter
160 burning was done in autumn 24 h before seed installation and after cutting shrubs, removing
161 herbaceous layer while leaving litter in place. Fire was started using an ethanol solution to
162 produce a homogeneous burning; it was performed carefully and was fully controlled. Soil

scarification consisted in manually loosening forest floor and topsoil at an approximate depth of 20 cm with a multi-toothed soil tillage tool after removing the litter.

Although no study was conducted in this area on the importance of *P. pinea* post-dispersal seed predation, by rodents and birds, it can be important due to the size and nutritional value of the seeds (Manso *et al.*, 2014). To consider this effect, a protection treatment was provided by wire mesh cages (30 cm × 30 cm, 1 cm mesh size). Each plot was then divided in four 0.5 m × 0.5 m subplots (2 with protection, 2 without protection) resulting in a total of 72 subplots.

Pinus pinea seeds were purchased from a local nursery (Native Nurseries LLC – Ain Zhalta) and had a high germination rate (98%) with a mean seed weight of 0.9 g (information provided by the nursery). In each subplot, 25 seeds were gently driven into the floor in a systematic way (5 rows for 5 seeds each one spaced by 5 cm).

The experiment was repeated during two consecutive years. First year, we started in 15 December 2017 until 3rd of July 2018 and the second-year experiment began 11 November 2018 and finished in 8 June 2019. For the second year, the experiment was repeated using new undisturbed plots in the same stand in order to avoid any effect from the previous experiment.

Measurements were taken once per week (29 weeks in total). During the first year, only the total number of living seedlings in each subplot was recorded whereas during the second year the individual fate of each seed/seedling was recorded (emerged, alive or not). A seedling was considered emerged when the cotyledons were visible. Causes of death were difficult to assess: a large part of seedlings died obviously because of drought (brown and dried seedling) whereas, for some seedlings, the aerial part was removed without knowing exactly the cause of this phenomenon. Missing seedlings were also considered dead.

2.2. Experiment 2: Response of Pinus pinea seed germination and seedling growth to soil treatments and needle leachate

This experiment was conducted in laboratory in order to improve our mechanistic understanding on the effects of (i) soil management practices, (ii) *P. pinea* needle leachates, (iii) and their interactions on *P. pinea* seed germination and seedling growth.

2.2.1. Material collection

Seeds of *P. pinea* were provided by the National Forests Office (France), and then stored in a cold chamber at 4°C until the start of the experiment.

Soil and litter samples used as bioassay substrate were collected from a mature *P. pinea* forest located at Montclam (Southeast France). Needle litter of the current year was collected on the ground under the pine canopy, manually sorted and oven-dried at 40°C for 5 days. A part of the litter was kept while the other part was used for used for the burning experiment. After drying, 3 cm thick of litter was put in containers (29 cm × 23 cm) and burned using an inflamed cord previously soaked with ethanol and removed immediately after ignition. The ashes were collected while the rest of the litter was kept at room temperature until the start of the experiment. After needle litter removal, soil was collected until a 20 cm depth, air-dried, sieved to a mesh size of 2 mm, and then stored at room temperature until the start of the experiment.

Green needles of *P. pinea* used to mimic natural leachates were collected from a *P. pinea* forest located at Coudoux (Southeast France). Green needles from several individuals were collected and then stored at -20°C until the beginning of the experiment in order to prevent compound degradation.

2.2.2. Laboratory bioassay

We mimicked both the natural field soil conditions (control treatment with soil + needle litter) and the two soil management practices used in the field experiment (litter burning: soil + litter ash; soil scarification: soil without litter). We mimicked the effects of natural leachates using green needle aqueous extracts because water-soluble compounds have been shown to be most involved in allelopathy (Reigosa *et al*. 1999; Fernandez *et al*. 2016), and more specifically in autotoxicity process limiting plant species regeneration (Robles *et al*. 1999; Alias *et al*. 2006; Fernandez *et al*. 2008). These aqueous extracts were prepared by soaking 222 g (fresh weight) in 1000 mL of deionized water (10% dry weight, as plant material is 55% water) for 24 h at room temperature (20 ± 1°C) in darkness (Fernandez *et al.* 2013; Hashoum *et al*. 2017). After 24 h, extracts were filtered through #42 Whatman® paper filter, a diluted extract at 2.5% was prepared, and both extracts were stored at 4°C until use. Allelopathic bioassays are frequently used with these two concentrations (e.g. Fernandez et al. 2013; Gavinet et al. 2019) and, by consequence, we used both concentrations in order to compare our results to those of previous studies. While the bioassays performed with a 10% needle aqueous extract correspond to a high allelochemical concentrations, the bioassays performed with 2.5% aqueous extract could more realistically mimic the natural conditions (Fernandez et al. 2013).

Plastic microcosms (15 cm length × 8 cm width × 8 cm height) were first filled with 500.0 g DM of soil corresponding to a thickness of 4.5 cm allowing root development. For the substrate type mimicking natural field conditions (i.e. control treatment), 3.5 g DM of needle litter corresponding to a 3 cm litter layer was added. For the substrate type mimicking the litter burning treatment, 0.54 g of litter ash was added at the soil surface, corresponding to the ash obtained after burning 3.5 g of needle litter.

Each microcosm was sown with 10 *P. pinea* seeds that were previously soaked in water for 24 h at 3°C to start the imbibition process (Fernandez *et al*. 2008). All the microcosms were first watered with 250 mL of deionized water, and then watered with 100 mL of deionized water

(control) or green needle aqueous extracts (2.5% and 10%). Ten replicates were performed for each treatment (3 substrate types × 3 green needle aqueous extract concentrations) for a total of 90 microcosms. The bioassays were conducted under controlled conditions in climatic-controlled room (Panasonic, France) under optimal conditions favoring *P. pinea* seed germination and seedling growth (Agrimi and Ciancio; 1993). First, we considered a 12 h: 12 h light: dark photoperiod, a 50% air humidity, a 20°C: 16°C light: dark temperature, and sealed microcosms over the first week to favor seed germination. Second, we considered a 12 h: 12 h light: dark photoperiod, a 80% air humidity, a 22°C: 18°C light: dark temperature and open microcosms to favor seedling growth.

Seed germination was monitored every day and used to compute total germination rate and germination speed. Germination rate was calculated as [(number of germinated seeds) / (number of sown seeds)] × 100 (Hashoum *et al*., 2017; Santonja *et al*., 2019). Germination speed was calculated using the Kotowski velocity coefficient (Mazliak, 1982; Santonja *et al*., 2018) as: $Cv = 100\ (\Sigma Ni / \Sigma NiTi)$, where Ni is the number of seeds germinated at time i, and Ti is the number of days since the start of the experiment. The higher the velocity coefficient, the faster the germination. A seed was considered as germinated when the protruding radicle achieved the length of 1 mm beyond the seed coat (Fernandez *et al*., 2013; Gavinet *et al*., 2019). Regarding seedling growth, lengths and biomasses of root and shoot were measured for each individual at the same age, i.e. 11 days after germination. Length was measured at a 1 mm accuracy and dry biomass was obtained after oven-dried plant material at 40°C for 3 days. In addition, we calculated the root: shoot ratio for both seedling length and biomass. An increase of this ratio corresponds to an increasing resource allocation to the root growth rather to the shoot growth while, in the opposite, a decrease of this ratio corresponds to an increasing resource allocation to the shoot growth.

2.2.3. Chemical analysis of green needle aqueous extracts

Green needles aqueous extract at 10% DM used in bioassays was analyzed by liquid chromatography (UHPLC Dionex Ultimate 3000, Thermo Scientific®) coupled to a Photo Diode Array detector and a High-Resolution Mass Spectrometer equipped with an ESI source (QqToF Impact II, Bruker Daltonics®). A volume of 5 µL of the filtered extract (RC syringe filter 0.2 µm, Restek®) was injected on UHPLC. Separation occurs on an Acclaim RSLC C18 column (2.1 mm × 150 mm, 2.2 µm, Thermo Scientific®) with an elution rate of 0.5 mL min^{-1} at a constant temperature of 40°C. Chromatographic solvents were composed of A: H_2O, and B: acetonitrile with both 0.1% formic acid. The chromatographic program consisted of 5% B during 2 min followed by a linear gradient up to 50% B during 7 min and then 2 min in isocratic mode. The analysis was followed by an elution of 100% B during 2 min until a return to initial conditions for column re-equilibration during 3 min for a total runtime of 16 min. UV spectra were acquired at 254 nm and 340 nm and mass spectra were acquired in negative and positive modes from 50 to 1200 amu at 2 Hz with MS parameters as follows: capillary 2500 V, nebulizer 3.5 bar N_2, dry gas 12 L min^{-1}, dry temperature: 200°C. DDA-MS^2 spectrum at 40 eV was also acquired in order to give complementary information on the major detected metabolites. Spectrometer was calibrated with formate/acetate solution forming clusters on studied mass range to ensure mass accuracy. Raw formula (DataAnalysis version 4.3, Bruker Daltonics®) and MS^2 mass spectra were compared to online database (Guijas *et al*. 2018).

2.3. Statistical analyses

Statistical analyses were performed with the R software (version 3.3.1). Significance was evaluated in all cases at $P < 0.05$. When necessary, normality and homoscedasticity of the data were checked using Shapiro-Wilk and Levene tests, respectively.

For the field experiment (first and second year), the effects of time, soil treatment, protection and their interactions on the number of living seedlings were analyzed with a generalized linear mixed-effects model using a Poisson distribution to take into account that the fact that data were not-independent count data (function *glmer*, package "lme4"). Subplots and plots were considered as random factors. In addition, for the second year, the effects of time, soil treatment, protection and their interactions on seedling survival and emergence were tested using Cox proportional-hazard regression models. These models estimate seedling survival or emergence time according to the different factors and taking into account censored data (Cox 1972, package "Survival"). Comparisons of survival curves were achieved using multiple log-rank tests between pairs of treatments.

For the laboratory experiment, two-way ANOVAs, followed by Tukey HSD tests for post-hoc pairwise comparisons, were used to test the effects of substrate type (control, litter burning or soil scarification), green needle aqueous extract concentration (0, 2.5 or 10%), and their interactions on *P. pinea* seed germination (germination rate and velocity) and seedling growth (root and shoot length and biomass).

3. Results

3.1. Field Experiment

3.1.1 Number of living seedlings according to the treatments (year 1 and year 2)

Results of the generalized linear mixed models showed that both time, soil treatment and protection affected the number of living seedlings (Table 1). The significant time × soil treatment and time × protection interactions highlighted that the effects of soil treatment and protection varied across the course of the experiment. In addition, the absence of significant

soil treatment × protection interaction suggested that both factors independently affect the seedling number.

The number of seedlings increased simultaneously for soil scarification and litter burning treatments for both years to reach a peak after 40 days and then continuously decreased until the end of the experiment (Fig. 1a and c). The values were slightly higher for soil scarification than for litter burning but became similar after 160 days. In contrast, the peak of seedlings in the control treatment (i.e. in presence of an undisturbed litter layer) was less pronounced and delayed (50-60 days) compared to the two other soil treatments.

We also recorded a slightly higher number of seedlings in the treatment with protection than without it starting day 40 till the end of experiment (Fig. 1b and d). It was noticeable that at the end of the experiment mortality was extremely high: only 18 seedlings (all plots together) were alive at the end of the experiment of the first year and 14 for the second year.

3.1.2 Seedling emergence and survival (year 2)

Soil treatment strongly influenced both seedling emergence and survival whereas protection treatment only influenced seedling survival (Table 2).

The probability of non-emergence was clearly higher in the control treatment than in the soil scarification and litter burning treatments throughout the course of the experiment (Fig. 2).

Survival probability decreased regularly with time and was higher with than without protection against predator (Fig. 3a). The probability of survival was the highest in the soil scarification and litter burning treatments and the lowest in the control treatment (Fig. 3b). At the end of the experiment, the survival was close to 0.

3.2. Laboratory experiment

3.2.1. Seed germination and seedling growth

Seed germination rate was on average 94% across all treatments and was not affected by substrate type or green needle aqueous extract (Table 3). Germination velocity was 5% higher with soil scarification compared to the control treatment (i.e. soil + litter) while a 10% needle aqueous extract reduced germination velocity by 4% (Table 3; Fig. 4a and b).

Shoot length was 9% lower with both litter burning and soil scarification compared to the control treatment (Table 3; Fig. 4c and d). By contrast, root length was 8% higher with litter burning and soil scarification compared to the control treatment and decreased with increasing needle aqueous extract concentration (Table 3; Fig. 4e and f). Seedling total length was not affected by substrate type while a 10% needle aqueous extract reduced their growth by 15% (Table 3). The root: shoot length ratio was 18% higher with litter burning and soil scarification treatments compared to the control treatment (Table 3). A 10% needle aqueous extract reduced this ratio (Table 3), and this reduction was more marked with the litter burning and soil scarification treatments compared to the control treatment (substrate type × needle aqueous extract interaction, Table 3).

Substrate type and needle aqueous extract also interacted in their effects on both seedling shoot, root and total biomasses (Table 3). A 10% needle aqueous extract reduced by 16% root biomass in the control treatment while, in the opposite, a 2.5% needle aqueous extract increased by 12% root biomass with the soil scarification treatment (Fig. 5a). In a same way, shoot biomass was reduced by 12% at high extract concentration with the control treatment while, in the opposite, shoot biomass was 18% higher at both low and high extract concentrations with the soil scarification treatment (Fig. 5b). The interactive effect of substrate type and needle aqueous extract on seedling total biomass followed the same trend as reported for shoot biomass (Supplementary Fig. S1). Finally, soil scarification increased by 8% the root: shoot biomass ratio compared to the control treatment, while a 10% needle aqueous extract reduced this ratio by 12% (Table 3; Fig. 4g and h).

3.2.2. Chemical analysis of green needle aqueous extracts

The UV chromatogram at 254 nm revealed a major detected metabolite in the solution at 48 s with a *m/z* of 191.0566 (100% of the Total Ion Chromatogram) in negative mode (Supplementary Fig. S2) that corresponds to the likely formula $C_7H_{11}O_6$ ($[M-H]^-$, mass defect: -2.9 ppm). The single search of this *m/z* in database suggests a possible correspondence with quinic acid ($C_7H_{12}O_6$, Metlin ID: 3389, last accession 10/09/2019). The comparison of the experimental MS^2 spectra with the one experimentally recorded in Metlin database supports the proposed structure (Supplementary Table 1). No single ions were detected in positive mode for the same retention time. No major metabolites were detected at 340 nm.

4. Discussion

4.1. Germination and seedling emergence

In field study, the delayed peak of seedling number in control (i.e. litter presence) compared to the two soil treatments indicated that emergence velocity increased with soil disturbance and was maximal for soil scarification. This result agreed with the lab experiment where litter decelerated germination velocity. Seed germination and early seedling establishment are highly sensitive to the presence of litter (Facelli and Pickett, 1991) with generally increasing negative effect according to the increase of litter amount (Facelli and Pickett, 1991; Xiong and Nilsson, 1999). In fact, several studies have shown the detrimental influence of litter on seed germination (Sayer, 2006; Fernandez *et al*., 2008; del Cerro Barja *et al*., 2009; Lucas-Borja *et al.*, 2012; Asplund *et al*., 2017; Liu *et al*., 2017). Litter constitutes a physical barrier for seed germination (Sayer, 2006; Liu *et al*., 2017) that prevents germinated seeds from reaching light (López-Barrera and González-Espinosa, 2001) and limits radicle growth (Facelli and Picket, 1991). Moreover, upper litter layer can induce extremely hot and

dry conditions that can limit seed germination and seedling growth (Ellsworth *et al*., 2004). A chemical effect of litter cannot be excluded because the lab experiment clearly showed a negative effect of green needle aqueous extracts on seed germination velocity, which approach natural leachates in the field (see section 4.3 below). In addition, several previous studies clearly demonstrated allelopathic potentialities of both green needles and needle litter (e.g. Nektarios et al. 2005; Santonja et al. 2019) that can act simultaneously on target species under field conditions. Emergence was slightly but significantly lower in the litter burning treatment than in the soil scarification treatment (Fig. 2), a finding in line with the laboratory experiment showing a reduced germination velocity (although not significant) between these two treatments (Fig. 4a). Reyes and Casal (2004) found that the germination rate of four pine species (not including *P. pinea*) decreased with an increasing amount of ash in a laboratory experiment. Similarly, Sagra et al. (2018) reported that ash was a limiting factor on seed germination and seedling survival of *P. pinaster* in a field experiment. Such results could be explained by the alteration of soil properties after controlled burning in particular a reduced soil moisture and soil respiration and an increased soil temperature (Plaza-Álvarez *et al*., 2017).

Unexpectedly, we found no influence of protection against birds and rodents on emergence although post-dispersal predation is usually high in Mediterranean pine forests (e.g. Ordóñez and Retana, 2004; Lucas-Borja *et al*., 2008) but an influence of protection treatment was detected on seedling survival showing the presence of seedling predation. This lack of effect can be explained by the absence or a very low rate of predation by seed consumers in our field conditions. Jackals and hyenas, effective predators of rodents (Mondal *et al.*, 2012), are frequently present in Lebanon forests (Tohme *et al.*, 1975; Tohme and Tohme, 1983) and could limit the impact of predation. In addition, in our experiment seeds were not placed on the top but within the litter and were therefore less exposed to predation.

412 ***4.2. Seedling early survival***

413 A clear positive effect of the soil scarification and litter burning treatments were found
414 on early survival in the field experiment. Previous studies, conducted on different pine species,
415 have also shown the beneficial effect of soil scarification (Beland *et al*., 2000; Nilsson *et al*.,
416 2006). This treatment enhances water supply by soil decompaction and by expanding soil
417 volume reachable by the root system (Lincoln *et al.*, 2007). For instance, establishment of *P.*
418 *sylvestris* regeneration was positively affected by soil scarification done before a rich seed fall
419 occurs (Karlsson and Örlander, 2000).

420 After 200 days of field experiment, it was remarkable that almost all seedlings died.
421 Although, it was not possible to precisely determine the cause of death for each seedling, most
422 of them died because of drought. Drought impact was most likely reinforced by the low light
423 availability for this reputed light-demanding species since the field experiment was conducted
424 under a closed pine cover with a high pine density (625 individuals.ha^{-1}) and basal area (48
425 m^2.ha^{-1}). Using a modelling approach, Sagra *et al*. (2018) showed that survival of one-year-old
426 *P. pinea* seedling was the highest in moderate shade conditions (i.e. Global Site Factor of 0.5).
427 This was explained by the amelioration of the seedling water status which largely outweighed
428 the negative impact of shade on carbon assimilation. However, in shadier conditions like ours,
429 seedlings were shown to be much more vulnerable to drought even to a moderate water stress
430 (Jiménez *et al*., 2009). More generally, it is well known that in early life stages drought is
431 leading to high mortality of most of the Mediterranean woody species (e.g. Pardos *et al*., 2007;
432 Lucas-borja, 2014) including *P. pinea* (Gonçalves and Pommerening, 2011). Moreover, in a
433 study of the natural regeneration of *P. pinea* in Tunisia, Adili *et al*. (2013) noted the absence of
434 young pine seedlings (<1 year) in small gaps with low transmittance (<20%).

435 Predation has also been reported as a factor affecting natural regeneration of *P. pinea* in
436 Italy (Masetti and Mencuccini 1991) and in Central Spain (Manso *et al*., 2014). In our study,

predation played a slight but significant role on survival during the second year of the field experiment. *Apodemus sylvaticus* and *Mus musculus* are rodents of *Pinus nigra* observed in Spain (Lucas-borja *et al*., 2010) and also found in Lebanese forests. In addition, emerged seedlings are also very easily detectable by the predators (including birds and rodents) in the absence of ground vegetation as it was observed in other studies (e.g. Sagra *et al*., 2017). Predators activity is also largely influenced by climatic factors which could explain variation from year to year (Manso *et al*., 2014).

In addition, fungal infections may cause pine seedlings disease and death (Raitelaityt *et al*., 2016). Young stone pine seedlings are often attacked by *Melampsora pinitorqua* Rostr. leading to its rapid dryness and death (Moriondo, 1951).

4.3. Potential allelopathic effects on seed germination and seedling growth

A delay in seed germination may have important biological implications, particularly under a Mediterranean climate where early-emerging species could be more competitive for access to resources (Herranz *et al.*, 2006; Fernandez *et al.*, 2013). In addition, the successful establishment of a species in the Mediterranean region is largely dependent on a well-developed root system for efficient capture of resources (Lloret *et al*., 1999; Green *et al*., 2005), particularly when water uptake is a limiting factor. By consequence, as green needle aqueous extract showed negative effects on both seed germination velocity, root length, root: shoot ratio and root biomass in the present study, this chemically mediated interaction could strongly impair *P. pinea* regeneration. These findings are in line with other findings that reported autotoxic effects for other pine species such as *P. halepensis* (Fernandez *et* al., 2008) or *P. densiflora* (Bong-Seop, 1992).

Litter presence and needle aqueous extract reduced root length and root: shoot ratio while, in the opposite, shoot length was increased in presence of litter. Longer shoots and

462 reduced roots in presence of litter can be explained by a stronger hypocotyl elongation due to
463 stronger light interception by the thick litter at the expense of the root system (Facelli and
464 Pickett, 1991). The presence of litter attenuated the positive effect of needle aqueous extract on
465 root biomass observed in bare soil and enhanced the sensitivity of pine seedlings to needle
466 aqueous extracts. Thus, seed germination and early seedling growth were significantly affected
467 by *P. pinea* needle aqueous extract and this autotoxic effect was sometime amplified by litter.
468 Quinic acid was the major metabolite detected from *P. pinea* green needle aqueous
469 extract (Supplementary Table S1). Previous studies showed that quinic acid is constitutively
470 present in needles of several conifer species such as *P. sylvestris* (Luethy-Krause *et al*., 1990)
471 or *Picea abies* (Jensen, 1988). Other studies suggested that this compound may act as
472 allelochemical to inhibit both plant and microbial growth. Balah (2012), studying the
473 allelopathic potentialities of *Jasonia montana*, isolated the 3, 5-dicaffeoyl quinic acid and the
474 presence of this polyphenol in a pot experiment decreased *Convolvulus arvensis* biomass.
475 Chlorogenic acid, an ester of caffeic acid with quinic acid, is also recognized as a strong
476 allelochemical (Chou and Waller, 1980; Rice, 1984; Reigosa and Pazos-Malvido, 2007). For
477 example, Reigosa and Pazos-Malvido (2007) showed that chlorogenic acid exhibited strong
478 inhibitory effect on *Arabidopsis thaliana* root growth.
479
480 **5. Conclusion**
481 The present study demonstrated that litter has detrimental physical and "potential"
482 chemical effects on *P. pinea* regeneration. In addition, we also highlighted an autotoxic effect
483 of green needle aqueous extract on seed germination and seedling growth that was amplified
484 by needle litter presence. We highlighted that soil scarification can alleviate this negative
485 impact of litter and enhance seed germination and seedling growth and survival. This method
486 was proved effective and largely recommended in pine forest regeneration operations.

Moreover, controlled litter burning, which is by far a less commonly used in the Mediterranean region, seems also an appropriate method to regenerate *P. pinea*, as already shown for *P. halepensis* (Prévosto and Ripert, 2008). Despite this positive impact of soil treatments, seedlings mortality after several months was very high under a closed canopy. Amelioration of survival can be improved by increasing light availability (Jiao-jun *et al*., 2003) and encouraging results on *P. pinea* regeneration were reported after heavy thinning (Adili *et al*., 2013). In addition, the reduction of the pine cover could reduce the quantity and concentration of needle leachates which are found detrimental on seed germination and early root growth. Hence, an ideal management of *P. pinea* stands should include soil preparation treatments, to enhance the initial seedling installation and thinning to achieve regeneration development on a longer term.

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754 **Tables**

755

756 **Table 1.** Results of the generalized linear mixed models of the number of seedlings according
757 to time, protection and soil treatment. df = degrees of freedom. *F*-values and associated *P*-
758 values (** for $P < 0.01$ and *** for $P < 0.001$) are indicated.

759

		Year 1		Year 2	
	df	*F*	*P*	*F*	*P*
Time	1	895.4	***	2303.6	***
Protection	1	0.7		10.5	***
Soil treatment	2	12.0	**	39.8	***
Protection× Time	1	6.4	**	42.2	***
Soil treatment × Time	1	60.8	***	46.4	***
Soil treatment × Protection	2	0.8		0.1	

760

761

762 **Table 2.** Cox proportional-hazard regression models: influence of protection and soil treatments
763 on seedling emergence and survival. df = degrees of freedom. *F*-values and associated *P*-values
764 (*** for $P < 0.001$) are indicated.

765

	Emergence			Survival	
	df	χ^2	*P*	χ^2	*P*
Protection	1	1.47		23.14	***
Soil treatment	2	205.58	***	102.28	***
Soil × Protection	2	1.78		0.32	

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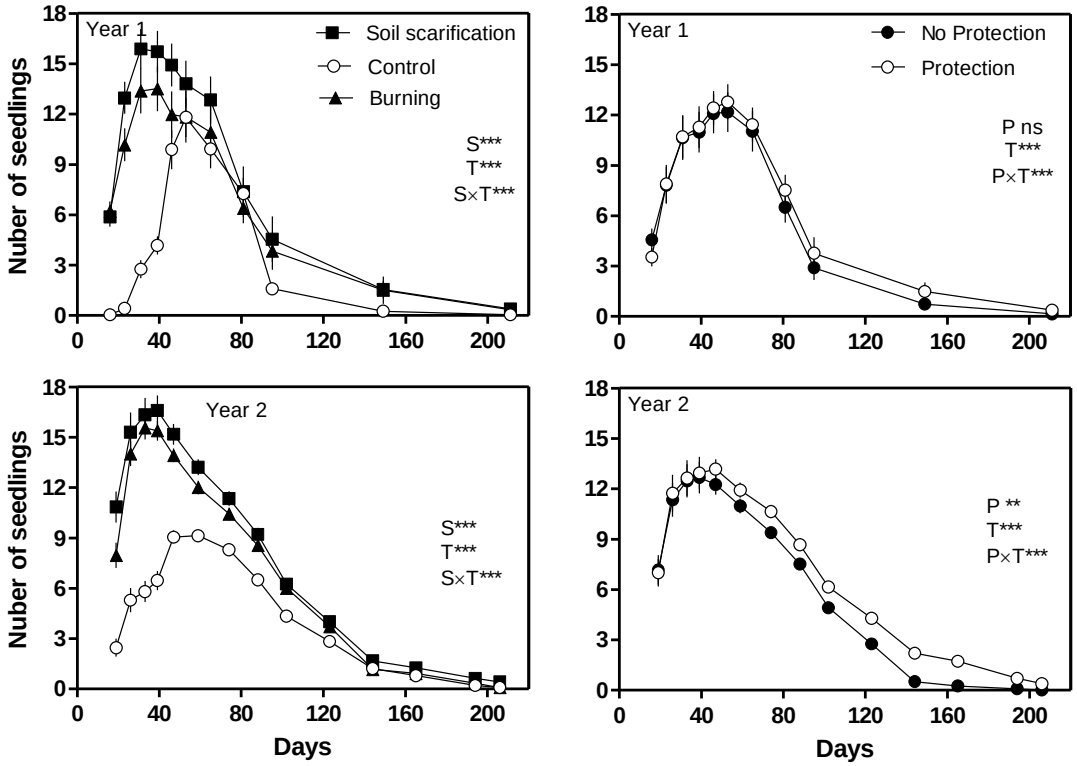
Table 3. Results of two-way ANOVAs testing for the effects of substrate type (control, litter burning or soil scarification), green needle aqueous extract concentration (0, 2.5 or 5%), and their interactions on *P. pinea* seed germination and seedling growth parameters. df = degrees of freedom. *F*-values and associated *P*-values (* for $P < 0.05$, ** for $P < 0.01$ and *** for $P < 0.001$) are indicated.

	Substrate type			Aqueous extract			Substrate × Extract		
	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Seed germination									
Germination rate	2	1.6		2	0.5		4	0.7	
Germination velocity	2	5.3	**	2	4.2	*	4	0.2	
Seedling length									
Shoot length	2	30.8	***	2	2.3		4	2.0	
Root length	2	8.1	***	2	65.0	***	4	2.2	
Total length	2	1.5		2	54.1	***	4	1.7	
Root: shoot length ratio	2	27.1	***	2	38.8	***	4	2.5	*
Seedling biomass									
Shoot biomass	2	0.2		2	0.7		4	2.7	*
Root biomass	2	4.9	**	2	19.8	***	4	3.1	*
Total biomass	2	0.2		2	1.2		4	3.0	*
Root: shoot biomass ratio	2	5.0	**	2	10.6	***	4	0.7	

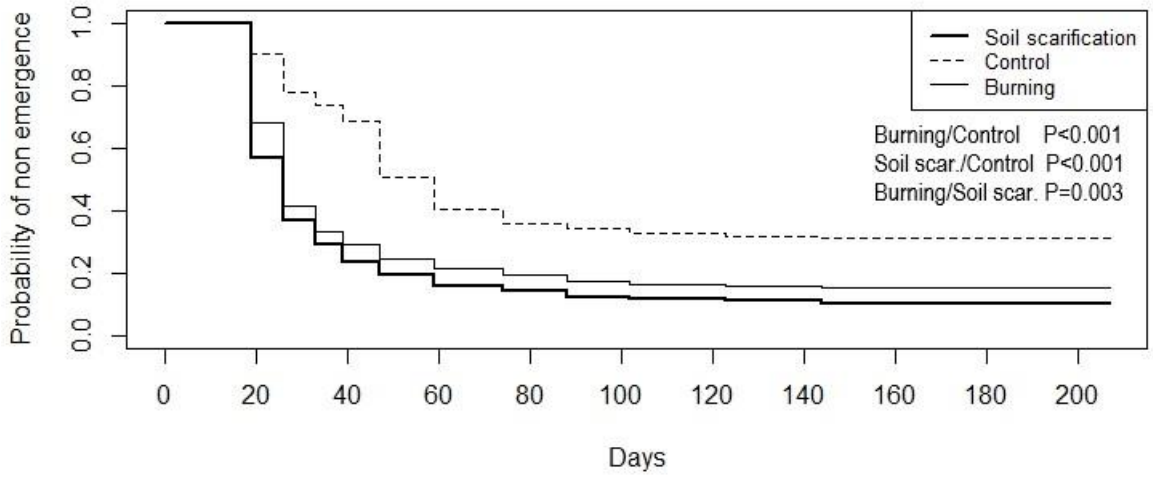
Figures

Fig. 1. Change of living seedling number with time (mean ± SE) according to soil treatment (a, c) and to protection (b, d) during the year 1 (a, b) and year 2 (c, d). S, T and P indicate results of generalized linear mixed models testing for the effects of soil treatment, time and protection on seedling number, respectively. *P*-values are indicated with the respective symbols ** for $P < 0.01$, *** for $P < 0.001$ and ns for non-significant effect. Days are the number of days since installation of the seeds.



786 **Fig. 2.** Change of the probability of non-emergence with time according to soil treatment.

787 Pairwise comparison between treatments are indicated.



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790 **Fig. 3.** Change of the probability of survival with time according to protection (a) and soil
791 treatment (b). Pairwise comparison between soil treatments are indicated.
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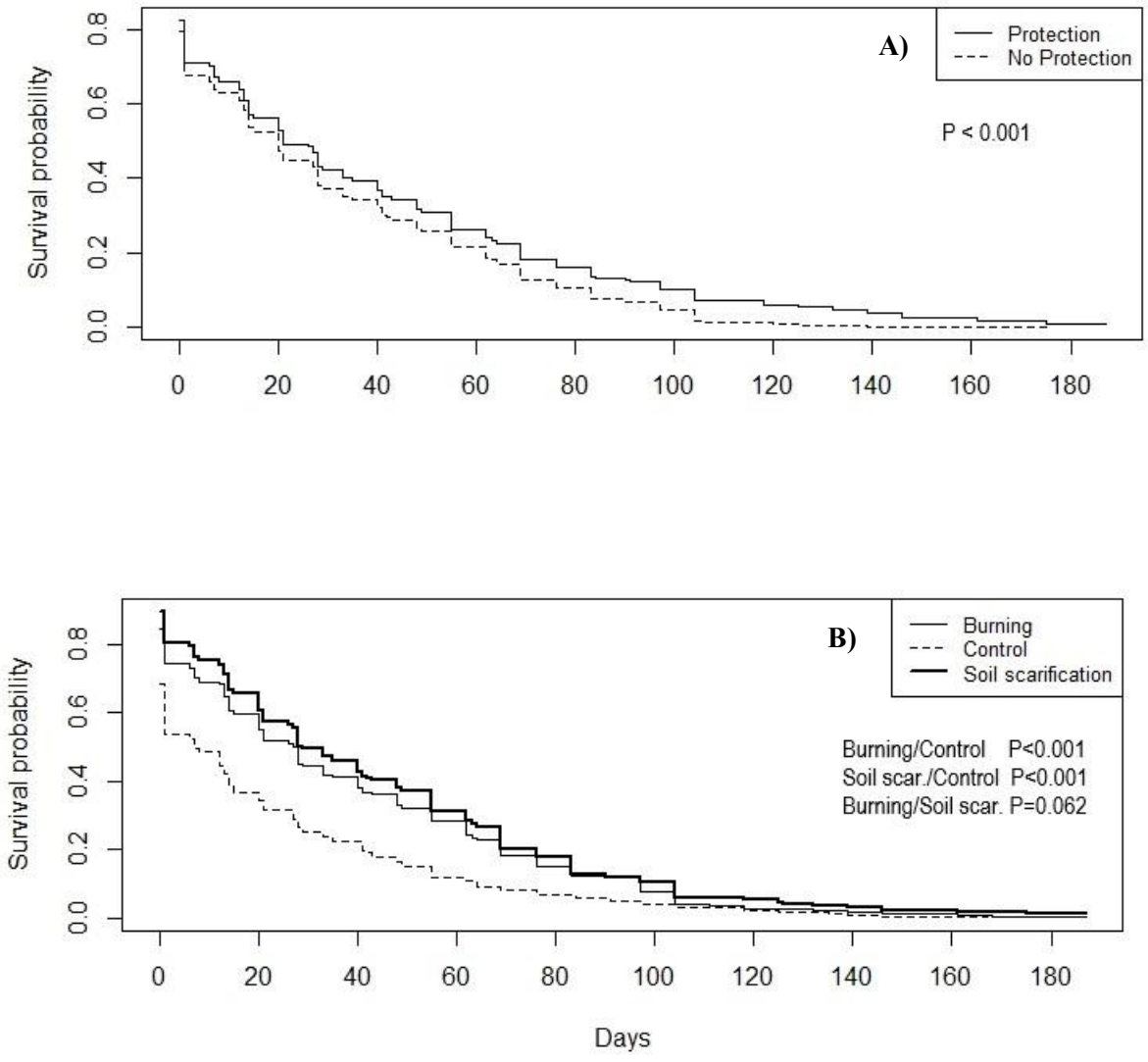


Fig. 4. Seed germination velocity (a, b), seedling shoot length (c, d), seedling root length (e, f), and root: shoot biomass ratio (g, h) according to substrate type (a, c, e, g) and green needle aqueous extract concentration (b, d, f, h). Values are mean ± SE; n = 30 for a and b; n = 267 to 286 for c, d, e, f, g and h. Different letters denote significant differences between treatments with b > a (post-hoc Tukey tests). LB = litter burning; SS = soil scarification.

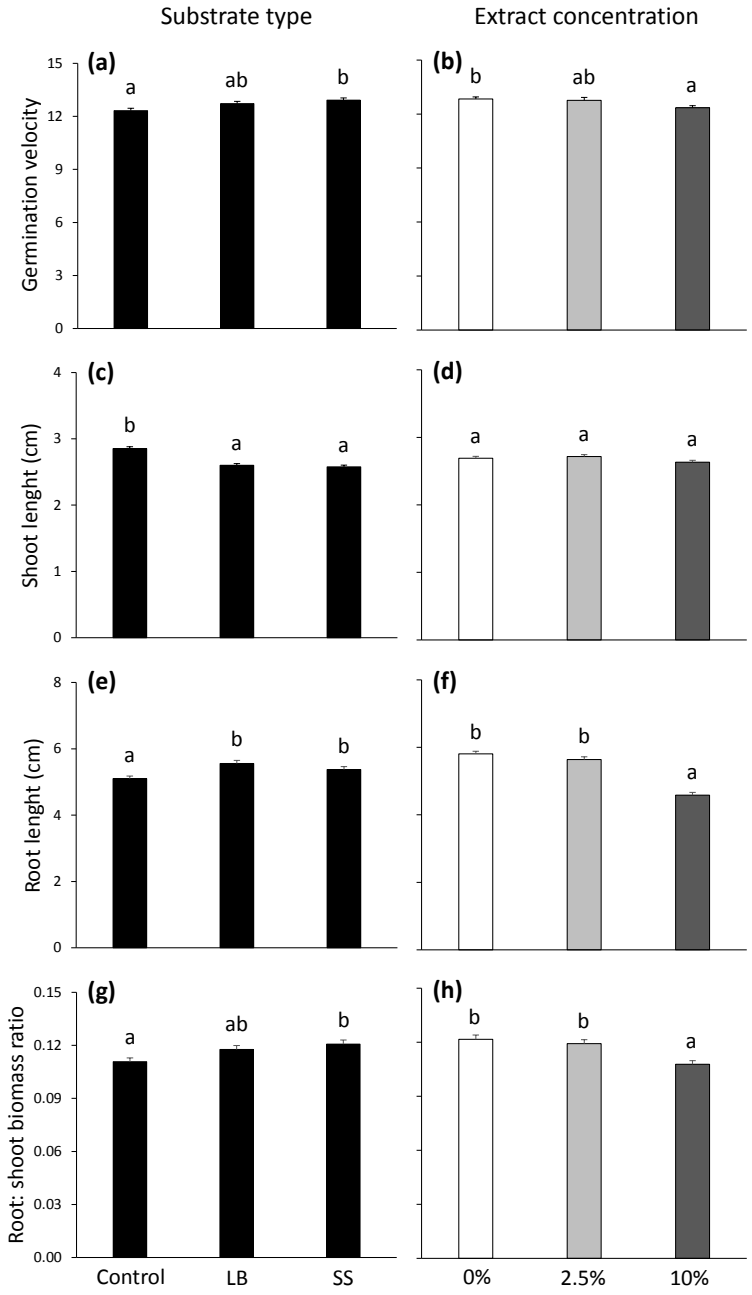
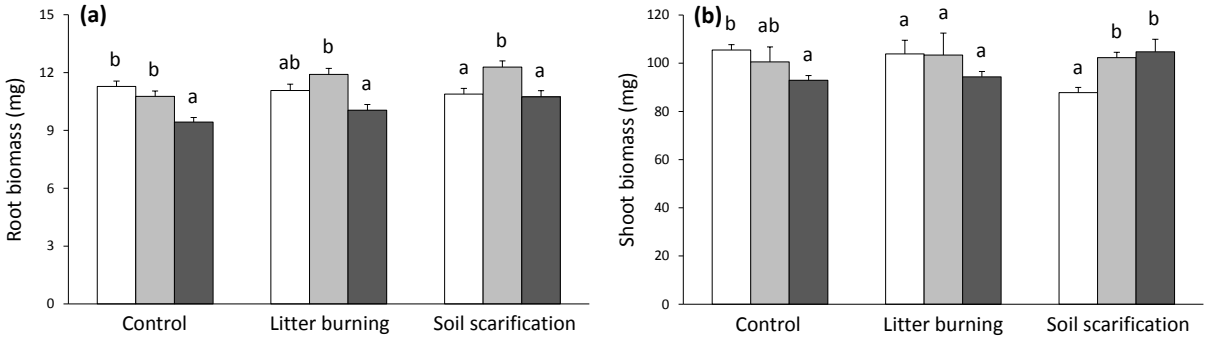


Fig. 5. Seedling root biomass (b) and shoot biomass (c) according to the substrate type × needle aqueous extract interaction (Table 1). Values are mean ± SE; n = 267 to 286. Different letters denote significant differences between extract concentrations according to the substrate type considered. White bar: control; light grey bar: 2.5% aqueous extract; dark grey bar: 10% aqueous extract.

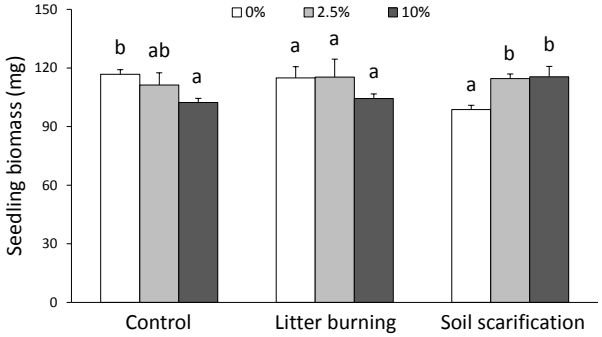


Supplementary Table and Figures

Supplementary Table S1. Comparison of the fragmentation pattern at 40eV of the major metabolite detected in the green needle UV chromatogram (47 s, *m/z* 191.0566 in negative mode) with the experimental MS^2 spectra at 40eV of quinic acid (Metlin database). I = fragment intensity in percentage of the Total Ion Chromatogram.

Experimental MS^2 fragments		Experimental MS^2 fragments of quinic acid	
m/z	I (%)	m/z	I (%)
44.9986	23.8	44.9982	47
55.0190	11.2	55.0191	25
57.0347	14.6	57.0349	21
58.0060	2.0	58.0055	1
59.0138	32.9	59.0142	51
60.0174	1.3	-	
67.0189	11.7	67.019	14
68.9983	1.2	-	
69.0345	13.3	69.0349	15
71.0137	12.3	71.0141	12
71.0501	1.0	-	
72.9930	5.0	-	
73.0295	4.5	73.0297	4
81.0345	9.9	81.0348	13
83.0139	1.4		
83.0500	3.6	83.0505	4
84.0216	2.4	84.0211	2
85.0293	100	85.0296	98
86.0326	6.9	-	
87.0085	19.9	87.0091	13
93.0343	60.7	93.0346	75
94.0376	6.1	-	
95.0135	1.2	-	
97.0291	2.0	97.029	2
99.0450	1.4	99.0457	<1
108.0215	12.8	108.021	16
109.0292	10.3	109.029	10
110.0324	1.0	-	

Supplementary Fig. S1. Seedling total biomass according to the substrate type × needle aqueous extract interaction (Table 1). Values are mean ± SE; n = 267 to 286. Different letters denote significant differences between extract concentrations according to the substrate type considered.



Supplementary Fig. S2. UV chromatogram (a) of the green needle aqueous extract (10% DM) analyzed by ultra-high performance liquid chromatography. Mass spectra (b) of the major metabolite detected in negative mode at 47 s. Fragmentation mass spectra at 40 eV of the major ion detected at 47 s.