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FULL PAPER

PLASMA PROCESSES AND POLYMERS

Changes in Norway spruce germination and growth induced by pre-sowing seed treatment with cold plasma and electromagnetic field: Short-term versus long-term effects

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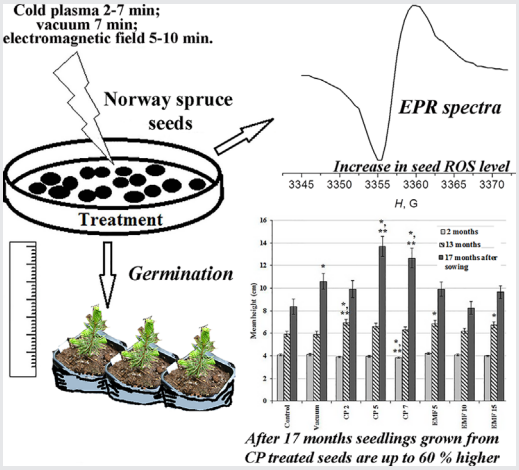
The effects of Norway spruce (*Picea abies*) seed treatment with cold plasma (CP), vacuum, and electromagnetic field (EMF) on germination and seedling growth are studied. CP treatments negatively affected germination 'in vitro', but for germination in cassettes CP (7 min) and EMF (5 min) significantly increased the germination yield. All treatments increase the germination rate in cassettes except CP (7 min) treatment having a negative effect. Seed treatment with CP and EMF increases the number of paramagnetic centers in dry seeds and modulate H_2O_2 production in germinating seeds. After the second vegetation season seedlings grown from CP (5 min) and CP (7 min) treated seeds, characterized by negative effects on either the germination rate or yield, have 50–60% larger height and 40–50% increased branching in comparison to the control seedlings.



KEYWORDS
cold plasma, electromagnetic field, EPR, germination, hydrogen peroxide, *Picea abies*

1 | INTRODUCTION

Forests and trees contribute to sustainable agriculture stabilizing the soil and the climate, regulating the drain, creating a habitat for pollinators, and natural enemies of agricultural pests. Woods and trees can also contribute to the food security for people serving as an important supplementary source of nutrition. Norway spruce (*Picea abies* (L.) Karst.) is one of the most important forest trees in Europe both due to economic and ecological aspects.[1] The highest forest coverage of Norway spruce is found in Sweden, Austria, and Czech Republic, where it covers more than 40% of total forest area.[2,3] In Lithuania it covers 23.4% of total forest lands and is considered as one of the most productive trees.[4] However, the Norway spruce is

subject to increasing concerns about forest decline – the promoted artificial spruce distribution makes European forestry vulnerable due to the high sensitivity of Norway spruce to climate change[5–8] and pollution.[9,10] In many countries, planting of Norway spruce seedlings is used in reforestation programs, and that underlies the need to develop novel technologies that could lead to improved seedling performance and resistance.

Plant seed treatment by electromagnetic fields (EMF) or cold plasma (CP) is recognized as an innovative tool for seed germination enhancement and early seedling growth (reviewed recently[11–15]). These effects are accompanied by long-term changes in plant metabolism, an increase in biomass production,[16–22] and disease resistance.[23–25] The majority of such studies have been performed on annual plants and reported moderate increase in germination (by up to 20% in most cases), whereas much stronger effects of EMF or CP on germination and plant development were demonstrated on perennials.[26–28] Only a few similar studies on effects of EMF or magnetic field have been performed on seeds of conifers to this date. Weak, sinusoidal magnetic field increased the length of Norway spruce seedlings slightly but had no clear effect on germination.[29] Electrostatic field improved germination, increased seedling height and root length during the middle and later stages of *Pinus tabuliformis* seedling development.[30] The most recent reviews outlining investigations on the CP effects on seeds do not refer to any studies performed on conifers.[11,12]

One of the aims of this study was to estimate the effects of pre-sowing treatment of Norway spruce seeds by radio-frequency CP and EMF treatments that were shown as effective for enhancing germination and seedling growth in other perennial woody plant species.[28] The effects on germination on early and longer term seedling development were assessed in order to evaluate the potential of applied seed treatments to acquire seedlings of Norway spruce with improved performance.

The molecular aspects of seed response to CP and EMF treatments are not yet clearly understood, therefore we intended to investigate the molecular events in the treated seeds elicited in response to CP and EMF treatments. A substantial increase in electron paramagnetic resonance (EPR) signal of dry pea seeds has been demonstrated after pre-sowing treatment with magnetic field,[31] as well as in the seeds of Scots pine (*Pinus silvestris*) and European larch (*Larix decidua*) after priming with polyethylene glycol or potassium salt.[32] In this study we present evidence that treatments of Norway spruce seeds with CP, vacuum and EMF also induce changes in seed EPR signal.

Numerous studies have demonstrated importance of reactive oxygen species (ROS) in the molecular processes that occur during seed imbibition and germination.[33–36] An array of enzymatic systems as well as several non-enzymatic processes can generate ROS in the membranes, outside or inside seed cells.[37,38] ROS accumulate in dry seeds during storage[39,40] and perform a regulatory and signalling function after the seeds get imbibed.[33,41] Hydrogen peroxide (H_2O_2) is recognized as one of the central molecules (see the most recent reviews[41,42]), the amount of which needs to be well-balanced for optimal germination.[41] The question of the possible involvement of biological ROS production in the effects exerted by CP and EMF treatments on seed germination has never been tackled before, therefore we hypothesized that such treatments can change inner ROS production in germinating seeds. Taking into account the key importance of H_2O_2 in activating germination events, in this study we estimated whether CP and EMF treatments can modulate the dynamics of H_2O_2 production in germinating Norway spruce seeds.

2 | EXPERIMENTAL SECTION

2.1 | Seed treatment by vacuum, CP, and EMF

Seeds of Norway spruce (Picea abies (L.) Karst.) harvested in 2014 were received from the Cone processing and seed storing fridge of the Dubrava Experimental-Training Forest Enterprise (Kaunas region). Seeds were visually checked for quality and packed into small plastic bags.

Seed treatments were carried out at the B. I. Stepanov Institute of Physics, NAS of Belarus (Minsk, Belarus). The selected conditions for seed treatment represented those reported as the most efficient for numerous annual[23] and several perennial[28] plant species. The equipment and conditions used for seed treatment was described earlier in more detail.[28]

Seed treatment with radio frequency EMF was carried out under the following experimental conditions: alternator frequency – 5.28 MHz; root-mean-square value of magnetic H and electric E components of EMF strength were equal respectively 590 A/m ($B \approx 0.74$ mT) and 12.7 kV/m; amplitude values $H^* = \sqrt{2}\bar{H}$ and $E^* = \sqrt{2}\bar{E}$ of 835 A/m ($B \approx 1$ mT) and 17.96 kV/m, respectively. Packed seeds were placed in plastic bags on the container at the center of the induction coil. Seed treatment was performed at atmospheric pressure and room temperature. Exposure to EMF did not cause seed heating under the experimental conditions used. Seed heating during EMF exposure was controlled using a chromel-alumel thermocouple connected to millivoltmeter M2018 (BVS, Saint Petersburg, Russia). The measurements were performed immediately after turning off EMF. EMF treatment of seeds lasted for 5, 10, and 15 min (these treatments are further abbreviated as EMF5, EMF10, and EMF15, respectively).

The planar geometry reactor for seed treatment by CP consisted of two plane-parallel, water-cooled copper

electrodes (120 mm diameter) placed in a stainless steel vacuum chamber. The low-pressure capacitively coupled radio frequency discharge in this reactor operated at 5.28 MHz and the specific power of 0.35 W/cm^3 was applied. Norway spruce seeds were processed with air plasma at a pressure of 60 Pa. Seeds were evenly dispersed on the surface of an open, sterile Petri dish and placed on the grounded electrode before pumping air from the chamber. In every CP experiment, before plasma ignition between the electrodes, a pressure of 60 Pa (partial vacuum) was achieved by pumping air from the chamber for approximately 7 min. Thus "vacuum" treatment was used as an additional control in the CP experiments. Further CP treatment lasted for 2, 5, or 7 min (these treatments are abbreviated as CP2, CP5, and CP7, respectively). Treatments for all experimental conditions were replicated three times. After treatment with CP and EMF seeds were stored in plastic bags at room temperature (20–22 °C) until the further investigation.

2.2 | Measurement of seed germination and seedling morphological parameters

Seed treatment by vacuum, CP, and EMF was performed in the middle of April, 2015. Germination tests were started 4 d after the treatment both 'in vitro' and in cassettes. For germination test 'in vitro' seeds were evenly distributed on two layers of filter paper in 13.5 mm diameter plastic Petri dishes (three replicates of 60 seeds each) and watered with 5 mL distilled water. Petri dishes with seeds were placed in a climatic chamber (Pol-Eko-Aparatura KK 750, Wodzisław Śląski, Poland) with automatic control of moisture (60%), light, and temperature. Alternating light and temperature regimes were maintained in the chamber (darkness: 14 °C for 8 h; light: 21 °C for 16 h). Seeds were provided additional water in a Petri dish, if necessary, to prevent drying. Germinated seeds (judged by the appearance of a visible 1-mm radicle) were counted daily until their number stopped increasing. For the germination tests in cassettes, the seeds were sown into seed-pots ($8 \times 8 \times 10$ cm) filled with peat substrate for conifers, placing the seeds in 0.5 cm depth from the surface of the substrate. Seed treatments were replicated three times for all experimental conditions (3×60 seeds, $n = 60$ for one replicate, including control seeds). The germinated seeds of Norway spruce were counted every second day as judged by the appearance of the top of green sprout from the surface of the substrate. For the first month, the containers with grown seedlings were kept in the green house of the Kaunas Botanical Garden, where the air temperature was maintained constant (20 °C).

The germination results of each experimental replicate were analyzed using the application of Richards' function[43] for the analysis of germinating seed population.[44] The indices of germination kinetics: V_i (%) – the final germination percentage indicating seed viability, M_e (days) – the median germination time ($t_{50\%}$) indicating the germination halftime of a seed lot or germination rate, Q_u (days) – the quartile deviation indicating the dispersion of germination time in a seed lot (half of seeds with an average growth time germinate in the range $Me \pm Q_u$), and the measure of the fit goodness E_r[44] were counted for control and treated groups.

After one month all the seedlings in cassettes were transported to a plant nursery. A special net was used to reduce insolation, with the aim to facilitate adaptation to field conditions, and care was taken to ensure uniform conditions for seedlings of all treated and control groups (lighting, irrigation, fertilizers for conifers). After one more month, the seedling height was measured and the number of branches was counted. Then the seedlings were carefully removed from plastic cassettes and planted directly into the soil in experimental plots. The morphological parameters of Norway spruce seedlings were measured: seedling height (2, 13, and 17 months after sowing), the number of needles per seedling (2 months after sowing), and the number of branches per seedling (13 and 17 months after sowing). The period between 13 and 17 months after sowing was the period of active vegetation (from late spring to early autumn, 2016).

2.3 | Measurements of EPR signal in dry seeds

Free radical signal intensities were measured by using EPR spectroscopy in samples of control seeds and seeds treated with CP and EMF. EPR spectra were recorded at 20 °C with Varian E112 EPR spectrometer (USA) operating at X-band frequencies (9.3 GHz) with 25 KHz field modulation.

Basic standard of Mn^{2+} in the MgO powder with the known number of spins was used for detection of EPR spectral parameters and the concentration of paramagnetic centers. The two-seed samples had good signal-to-noise ratios permitting accurate measurements of signal intensity. Therefore each sample consisted of two randomly selected seeds that were weighed and placed into quartz EPR tubes (4 mm inner diameter). EPR signals in dry seeds were measured 1 d after seed treatment with CP, vacuum and EMF. The EPR signal was registered in 8 different seed pares (8 repetitions x two seeds) for each experimental group, and their spectra were recorded three times each. The content of moisture in seed samples within the various experimental groups could differ slightly after CP, vacuum or EMF treatment. So, the standard material permanently fixed in the EPR cavity coaxially to the sample tube was used in all cases to take into account possible changes in the quality factor of the EPR resonator. Thus, both EPR spectra, those of Mn^{2+} and experimental sample, were simultaneously recorded under identical conditions and the ratio of their intensities was taken for further consideration. For obtaining more precise results the same quartz EPR tube was used for all

measurements. The sample tube was positioned exactly in the centrum of the cavity.

EPR spectrum was recorded as a single symmetric first derivative line for all experimental samples, and the following relation was used to determine a number N_1 of paramagnetic centers (NPCs) in the specimen:

$$\frac{N0}{N1} = \frac{I_0(\Delta H_0)^2}{I_1(\Delta H_1)^2},$$

where N_1, I_1, and ΔH_1 – NPCs, the peak to peak amplitude of the first derivative line intensity and the width parameter for line shape of specimen; N_0, I_0, and ΔH_0 – NPCs, the peak to peak amplitude of the first derivative line intensity and the width parameter for line shape of standard.

The NPCs value in each measurement was normalized for fresh weight m_1 of two seeds used for the sample:

$$N_1^n = \frac{N_1}{m_1}.$$

2.4 | Measurement of hydrogen peroxide release from the germinating seeds

Hydrogen peroxide production and release from seeds was measured using the method described by Leymarie et al.,[45] modified by replacing scopoletin with a more sensitive indicator – Ampliflu Red. Seed germination was initiated by placing the control seeds and the seeds exposed to CP, vacuum, and EMF on two layers of filter paper in 90 mm diameter plastic Petri dishes (105 seeds for each group) and watering with 3 ml of distilled water. The measurements of H_2O_2 production were performed for 2 d in seed samples taken from Petri dishes (kept in the dark thermostate at 25 °C), every 6 h with a 12 h interval at night. Five seeds of each experimental group were incubated in potassium phosphate buffer (20 mM, pH 7.4, 500 µl per 100 mg seeds) containing 5 µM Ampliflu Red (Sigma-Aldrich, St. Louis, USA) and 1 U/ml horseradish peroxidase (Sigma–Aldrich, St. Louis, USA) for 1 h at 25 °C shaking at 250 rpm in the dark. Such measurements were repeated in triplicate (3 samples × 5 seed) for each experimental point. The release of hydrogen peroxide in three samples of dry control seeds was measured in parallel. The fluorescence ($\lambda_{exc} = 535$ nm, $\lambda_{em} = 590$ nm) of 100 µl of incubation buffer from each vial was measured in a 96-well plate with a plate reader (Tecan Genios Pro, Tecan). H_2O_2 production was recalculated into molar H_2O_2 concentration using a linear calibration curve determined at every time point of measurements. Results were expressed as a mean ± standard error of three replicates, per 100 mg of the initial weight of dry seeds.

2.5 | Statistical analysis

Statistical analysis of the results was performed using Statistica 10 software. The purpose of the analysis was to compare parameters in pairs – control and affected group or vacuum and CP treatment group. Means of various parameters between the control and treatment groups were compared using Student's *t*-tests for independent samples, as there was no reason of comparing different conditions of affected groups. The differences were assumed as statistically significant at the level of $p \leq 0.05$.

3 | RESULTS AND DISCUSSION

3.1 | Changes in Norway spruce germination kinetics induced by seed treatment with CP and EMF

The effectiveness of radio-frequency EMF and CP seed treatments was examined by changes in seed germination kinetics (Fig. 1, Table 1). The germination tests in cassettes were performed because longer-term observations of the effects of pre-sowing seed treatment with stressors on perennial woody plant development were planned.

The results of germination tests revealed differences in kinetics of Norway spruce germination 'in vitro' (Fig. 1a) and in cassettes (Fig. 1b), as well as in the response to CP and EMF treatments. Germination 'in vitro' started earlier and germination percentage in most of the experimental groups (except CP5 and CP7) reached maximal value in 4 d from the onset of germination. All CP treatments had inhibiting effect on germination 'in vitro' and the extent of inhibition increased with the duration of treatment. The germination curves of EMF and vacuum treated seeds coincided with the control curve (Fig. 1a). The germination in cassettes was much slower in comparison to that 'in vitro', e.g., maximal germination percentage was reached in more than 10 d after the onset of germination (Fig. 1b). First sprouts of CP2 and EMF15 groups emerged from the substrate on the ninth day after sowing, and germination in the rest of the groups started in the two following days, although germination percentage in CP7 and CP5 groups was the lowest in the initial stages of germination (Fig. 1b). It is not easy to evaluate differences in the germination rate in the rising part of most curves, but it is obvious that the final germination percentage was the highest in EMF5 and CP7 groups. Inhibition of germination in cassettes was clearly pronounced only in CP5 group.

To quantify differences in the germination kinetics among experimental groups, germination data points of each experimental replicate were fitted to Richards plots,[43,44] as it has been described in more detail earlier.[28]

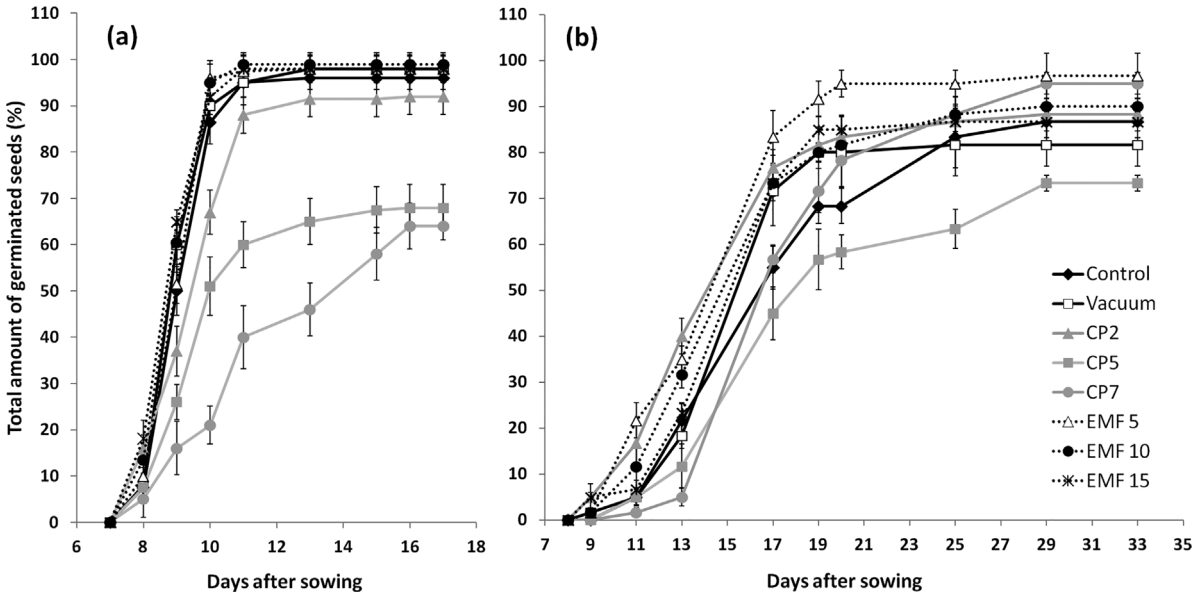


FIGURE 1 Germination dynamics of Norway spruce 'in vitro' (a) and in cassettes (b). The points represent mean values of three replicates ± standard error of mean. Seed treatments for all experimental conditions were replicated three times ($n = 60$ for one replicate)

The germination results of *Picea abies* fit the Richards plots well – the measure of the fit goodness E_r[44] varied from 0.63 ± 0.12% (vacuum) to 5.8 ± 0.36% (CP5). Values obtained for the germination kinetics indices: final germination percentage V_i, median germination time Me, and quartile deviation Q_u, are presented in Table 1. Seed germination percentage 'in vitro' was very high in control, vacuum and EMF treated groups (Table 1). Vacuum and EMF did not cause changes in other indices of germination kinetics. In contrast to vacuum, all CP treatments had clear negative effects on germination kinetics and the extent of this effect increased with the duration of treatment: the germination percentage, germination rate and uniformity was reduced (V_i decreased up to 33%, M_e increased by 1.7 d and Q_u increased 4.1 times in CP7 group in comparison to control).

The results of germination in cassettes were quite different in comparison to those obtained in 'in vitro' (Table 1). Pre-sowing seed treatment with CP7 and EMF5 had a significant positive effect on the final germination yield (V_i increased by 9.6 and 11.5% in comparison to control, respectively) in cassettes, whereas the effect of CP5 was negative (V_i decreased by 15.5%).

CP7 treatment significantly increased V_i (by 16.3%) and CP5 reduced V_i (by 10.3%) in comparison to the vacuum group. The majority of treatments accelerated the median germination rate of spruce seeds significantly – decreased M_e by more than 1 d was obtained in the vacuum, CP2,

TABLE 1 Norway spruce germination kinetics indices calculated from Richards plots

	Germination 'in vitro'			Germination in cassettes		
Treatment	**V_i [%]**	**M_e [days]**	**Q_u [days]**	**V_i [%]**	**M_e [days]**	**Q_u [days]**
Control	95.5 ± 2.1[a]	8.97 ± 0.02	0.38 ± 0.05	86.7 ± 2.0	15.87 ± 0.01	2.51 ± 0.05
Vacuum	97.7 ± 2.2	8.83 ± 0.12	0.41 ± 0.03	81.7 ± 4.6	14.54 ± 0.1*	1.38 ± 0.03*
CP2	91.6 ± 1.3*,**	9.3 ± 0.10*,**	0.77 ± 0.10*,**	88.3 ± 1.4**	13.39 ± 0.24*,**	1.73 ± 0.08*,**
CP5	67.7 ± 4.4*,**	9.4 ± 0.20*,**	0.71 ± 0.10*,**	73.3 ± 1.7*,**	15.97 ± 0.23**	2.28 ± 0.02*,**
CP7	64.0 ± 3.4*,**	10.7 ± 0.2*,**	1.56 ± 0.03*,**	95.0 ± 2.3*,**	16.52 ± 0.16*,**	1.84 ± 0.05*,**
EMF5	97.5 ± 2.5	8.95 ± 0.31	0.38 ± 0.04	96.7 ± 4.8*	13.81 ± 1.25*	1.93 ± 0.60*
EMF10	98.5 ± 1.4	8.82 ± 0.20	0.37 ± 0.03	90.0 ± 1.7	14.19 ± 0.20*	2.02 ± 0.04*
EMF15	98.3 ± 1.6	8.69 ± 0.35	0.45 ± 0.10	86.7 ± 3.5	14.48 ± 0.14*	1.61 ± 0.03*

[a]Mean values ± standard error of the indices are presented.
*Significantly different from the control group ($p \leq 0.05$).
**Significantly different from the vacuum group ($p \leq 0.05$).

EMF10 and EMF15 groups (the most effective was the CP2 treatment that reduced M_e by 2.5 d in comparison to control). However, CP7 treatment increased M_e by 0.7 d in comparison to control, thus the germination was slower; on the other hand, the germination percentage was higher in comparison to untreated seeds. All treatments increased the uniformity of spruce seed germination significantly (decreased Q_u), and the most effective in this respect was the vacuum treatment.

Our data (Table 1) show that kinetics of germination as well as the response of germination to seed treatment with CP and EMF can be strongly modulated by changing germination conditions. Seeds germinate much faster and germination percentage tends to be higher under 'in vitro' conditions. The negative effects of CP treatments on germination 'in vitro' were more obvious in comparison to germination in cassettes (although seeds in CP7 and CP5 groups also germinated very slowly in cassettes in the first 3 d after the germination onset, only CP5 treated seeds remained clearly inibited in the final stage of germination, while CP7 even increased final germination yield). Opposite, the stimulating effects of EMF treatments on germination were more obvious in cassettes (Fig. 1b, Table 1).

The difference between germination kinetics 'in vitro' and in cassettes may be explained by several reasons. Estimating the germination 'in vitro', the appeared radicle is counted when it reaches 1 mm length, whereas much longer sprout (>5 mm) appears from the substrate surface in the casette, therefore results of germination in cassettes reflect a later stage of germination. Numerous other factors can be responsible for the differences in germination kinetics and seedling appearence kinetics, such as slower penetration of water, reduced oxygen and light supply,[46] presence of various compounds in the soil and chemical interactions with soil microbiota may affect seed germination rate in the substrate – all these factors are absent when seeds germinate in the Petri dish.

3.2 | Changes in morphometric parameters of Norway spruce seedlings induced by seed treatment with CP and EMF

The morphometric parameters of Norway spruce seedlings were measured 2, 13, and 17 months after sowing, aiming to estimate the effects of pre-sowing seed treatment with stressors CP and EMF on plant development in the early stage, as well as on a longer time scale. There were no large differences among the experimental groups in the early stage of the development (2 months after sowing); the mean seedling height was slightly but statistically significantly smaller (by 5%) only in the CP7 group, in comparison to the control (Fig. 2).

However, the pattern of changes in plant height induced by treatment changed 13 months after sowing: CP2, EMF5, and EMF15 treatments significantly increased seedling height (by 17.0, 16.0, and 13.5%, respectively). Treatment-induced differences in seedling height increased progressively after 17 months, but seedlings in other groups (namely, vacuum, CP5, and CP7 treated groups) became remarkably higher in comparison to the control (by 26.8, 63.8, and 51.4%, respectively).

The number of needles per seedling in the early stage of seedling development (2 months after sowing) was most positively affected by EMF treatments – needle count was higher by 23, 19, and 17% in EMF5, EMF10, and EMF15 group in comparison to the control, respectively (Table 2).

The effects of vacuum and CP were smaller (CP2 and CP7) or absent (only in CP5 group). The mean number of branches per seedling after 13 months after sowing was higher (by 23%) only in seedlings from CP2 group. However, CP2 effect on branching was not statistically significant in the end of the vegetation season (17 months after sowing); instead the effects became apparent in other groups – seedlings in CP5, CP7, and vacuum groups had significantly larger number of branches (by 51, 40, and 28%, respectively) in comparison to the control (Table 2). EMF treatments were not effective in inducing branching of Norway spruce seedlings.

In summary, we report that although the parameters of the untreated Norway spruce seed germination are in the range reported as regular for good germination,[47] pre-sowing seed treatment with CP and EMF may have obvious positive effects on seed germination in cassettes: increasing the germination percentage (CP2, CP7, EMF5), decreasing germination time (vacuum, CP2, EMF10, EMF15) and increasing uniformity (all treatments). Only the CP7 treatment decreased germination rate slightly (by 4%), and this effect was related to the small negative effect on

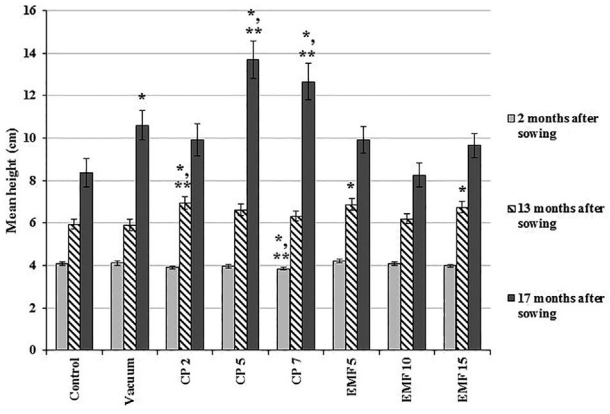


FIGURE 2 The height of Norway spruce seedlings. The results represent mean values ± standard error. The number of plants in the experimental groups ranged from 41 to 58. * – significantly different from the control group ($p \leq 0.05$), ** – significantly different from the vacuum group ($p \leq 0.05$)

TABLE 2 Mean number of needles and number of branches per Norway spruce seedling[a]

	Number of needles 2 months after sowing	Number of branches 13 months after sowing	Number of branches 17 months after sowing
Control	19.3 ± 0.6[b]	3.9 ± 0.2	4.7 ± 0.4
Vacuum	$22.1 \pm 0.6^{*}$	4.1 ± 0.3	$6.0 \pm 0.5^{*}$
CP2	$21.8 \pm 0.5^{*}$	$4.8 \pm 0.4^{*}$	5.5 ± 0.5
CP5	21.0 ± 0.7	4.6 ± 0.3	$7.1 \pm 0.5^{*}$
CP7	$20.3 \pm 0.5^{**}$	3.9 ± 0.2	$6.5 \pm 0.5^{*}$
EMF5	$23.7 \pm 0.5^{*}$	4.2 ± 0.2	5.1 ± 0.3
EMF10	$22.9 \pm 0.6^{*}$	3.7 ± 0.2	4.6 ± 0.3
EMF15	$22.6 \pm 0.5^{*}$	4.5 ± 0.3	5.5 ± 0.4

[a]The number of plants in the experimental groups ranged from 41 to 58.
[b]mean values ± standard error of the indices are presented.
*Significantly different from the control group ($p \leq 0.05$) .
**Significantly different from the vacuum group ($p < 0.05$).

seedling height (by 6%) 2 months after sowing (Fig. 2). Other treatments did not have an effect on the early seedling growth. However the situation has changed on a longer time scale (17 months after sowing), so that the seedlings in CP5 and CP7 groups, both characterized by negative effects on germination (Fig. 1, Table 1), were much higher (by 50–60%) and had more branches (40–50%) in comparison to the control seedlings. Although EMF treatments affected the uniformity of germination, germination percentage (EMF5), and germination rate (EMF10 and EMF15) positively, these treatments did not promote seedling growth or branching 17 months after sowing. A similar result was obtained in our recent study on other perennial woody plant species – *Morus nigra* and *Rhododendron Smirnowii*.[28] Seedlings of these perennials from the experimental groups where inhibition of germination was the strongest also showed the best growth in later stages of development.

3.3 | Changes in EPR signal of Norway spruce seeds induced by their treatment with CP and EMF

Aiming to gain more insight into the mechanism of sensing of CP and EMF by plant seeds we compared the levels of EPR signal in the control and treated Norway spruce seeds. There was a large intergroup variation in the number of paramagnetic centers (NPCs) during day one after seed treatment (data not shown), therefore we present the results obtained after 20 h when the signal stabilized (Fig. 3).

CP, vacuum and EMF treatments changed the NPCs in dry seeds but the extent of these changes depended on the duration of treatments. The largest increase (more than 30%) in seed NPCs was induced by CP7 and EMF10, while the effects of CP5 and EMF5 treatments were less pronounced (24 and 12%, respectively); values in CP2 and EMF15 groups did not differ from the control. NPCs was decreased by 19% after the vacuum treatment. We cannot provide an explanation for the latter effect. Taking into account that the vacuum group serves as an additional control for CP treatment, EPR signal in CP treated seeds was increasing progressively with the duration of treatment and is much larger (by 34, 53, 63% for CP2, CP5, and CP7 groups, respectively) as compared to the vacuum group than in EMF treated seeds (12, 32% for EMF5, and EMF10 groups, respectively) as compared to the control group.

Seed germination is a complex process that begins with water uptake and is associated with seed transition to a metabolically active state. Bearing in mind the role of H_2O_2 in activating germination events,[37,41,42] we examined whether seed treatment with CP and EMF can affect H_2O_2 production in germinating Norway spruce seeds.

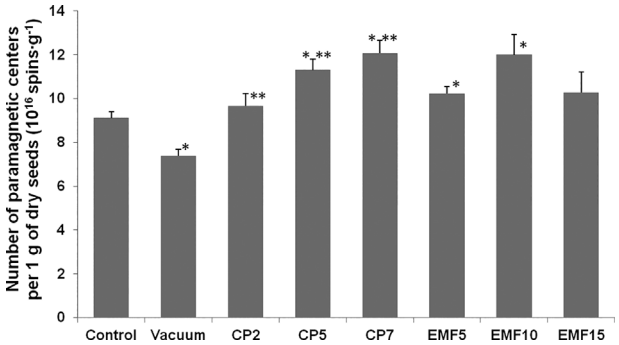


FIGURE 3 Number of measured paramagnetic centers in Norway spruce seeds 20 h after treatment with CP, vacuum and EMF. The points represent mean values of 8 replicates of seed pairs ± standard error. * – significantly different from the control group ($p \leq 0.05$), ** – significantly different from the vacuum group ($p \leq 0.05$)

H_2O_2 is a small and easily diffusible molecule that is released from seeds placed to a solution. The method for the estimation of seed H_2O_2 content used in this study was based on the reaction between the released H_2O_2 and the Ampliflu Red dye, i.e., the seeds were taken from the place of their germination in a Petri dish at indicated time points and incubated for 1 h in a buffer solution containing the dye. Therefore even "dry" seeds spent one hour in a solution and released the accumulated amount of H_2O_2. The obtained results show (Fig. 4) that H_2O_2 amount released from dry seeds remains at a constant level during 48 h of the experiment.

Under similar to 'in vitro' germination conditions used in this experiment (only differences were incubation in dark thermostat at 25 °C), Norway spruce seeds started to germinate on day four, thus we registered H_2O_2 production in the early stage of the seed response to imbibition. The release of H_2O_2 in all groups of the imbibed seeds displayed specific periodical patterns of changes in all groups of the germinating seeds. At different time points, the highest values of the released H_2O_2 amount exceeded the lowest values 2–4-fold, and twice (at 6 and 30 h) exceeded the level characteristic for dry seeds. Intermittent measurements did not allow exact estimation of the amplitudes and periodicity of these oscillatory changes.

However, throughout the 48 h of performed measurements, the level of H_2O_2 release from the three CP-treated groups of seeds was very similar and considerably lower than that from the three EMF-treated seed groups. The differences between CP and EMF data points in the majority of the time points were statistically significant ($p \leq 0.05$). For many time points, the values for CP groups were statistically significantly lower ($p \leq 0.05$), and the values for EMF groups – higher ($p \leq 0.05$) in comparison to the values of control seeds.

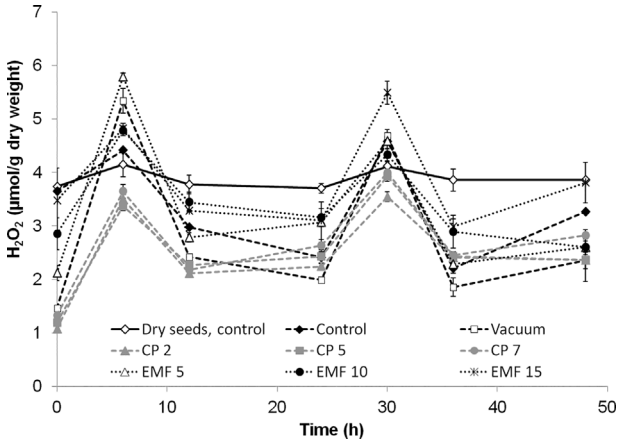


FIGURE 4 Time course of hydrogen peroxide production in dry and imbibed seeds of Norway spruce. The points represent mean values of three replicates ± standard error

To our knowledge, the oscillatory dynamics of H_2O_2 production and release from germinating seeds is reported here for the first time, although resembling trends were obtained for the *Arabidopsis thaliana* seeds when their H_2O_2 production was measured for 24 h after imbibition.[45] We have also observed similar waves of H_2O_2 release from germinating seeds for other conifer species – Scots pine (*Pinus silvestris*), but periodic dynamics was not characteristic for seeds of two tested crop species – radish (*Raphanus sativus*) and sunflower (*Helianthus annuus*) (data not shown).

The intracellular or extracellular sources of ROS production in germinating seeds are in general not well documented,[37,41,42] and they were not studied in Norway spruce seeds. Species dependent differences in the molecular nature or regulatory interactions of such sources may be responsible for the occurrence of oscillatory dynamics of H_2O_2 production in germinating seeds. The release of H_2O_2 from germinating seeds is determined on the balance between H_2O_2 producing processes and H_2O_2 elimination by the antioxidative system. It was found that expression of hundreds of genes both in seeds[48] and in plants is controlled by the circadian clock and undergo significant diurnal variations, e.g., roughly one-third of expressed *A. thaliana* genes is circadian regulated, including the genes involved in response to hormones, stress and ROS.[49,50] Such variations may be responsible also for the observed pattern of H_2O_2 release (Fig. 4).

Thus, this study provides the first experimental evidence that pre-sowing treatment with CP, vacuum and EMF affects NPCs in dry seeds and modulates production of H_2O_2 in germinating seeds of Norway spruce. It is assumed that EPR spectra of seeds generally consist of signals assigned to Fe(III), Mn(II), and relatively stable organic radicals – lipid peroxides,[40] melanin type pigments,[51] and semiquinones originating from the oxidized tannins,[52] which are the major pigments in the testa of conifers.[53] Numerous studies have reported that stable radicals are primarily located in the testa of dry seeds,[40,51,54] where they are formed from organic antioxidants that are responsible for UV protection[55] and have a radical scavenging function.[56,57] The high amounts of stable organic radicals in seeds can interfere with the estimation of stress-derived radicals. However, our results (Fig. 3) indicate that short treatments with CP and EMF can induce appreciable increase in EPR signal in dry seeds. The applied treatments can increase the number of stable radicals directly, affecting their organic non-radical counterparts, or non-directly – they may be produced by scavenging less stable free radicals or ROS, e.g., superoxide or hydroxyl radical can react with polyphenolic pigments and convert them to stable radicals. The first alternative is highly probable for CP treatment that involves bombardment of seed testa

with ionized particles and radicals. It was demonstrated using TOF-SIMS spectroscopy that mass peaks of O^-, CN^-, and CNO^- secondary ions was several times more intense in the lentil, bean and wheat seed surfaces treated with air plasma in comparison to non-treated seeds.[58] Authors conclude that cold air plasma treatment enriched the surface of seeds with oxygen containing functional groups and that resulted in the essential improvement in the wettability of seeds, and eventually stimulated their germination. We consider that chemical modifications of organic compounds on the seed surface by plasma may lead to formation of organic radicals seen by EPR measurements (Fig. 3). However, it is hard to explain the similar effect of EMF treatment in the same way. EMF10 treatment was also effective in increasing NPCs (Fig. 3) and that gives evidence in favour of non-direct reasons. Since ROS generation is regarded as a versatile mechanism of plant stress response,[59–61] we hypothesize that NPCs increase in EMF treated seeds may be caused by the induced ROS burst leading to the secondary conversion of ROS scavenging pigments to organic radicals in treated seed testa. The increased EPR signal was also reported in after pre-sowing treatment of pea seeds with magnetic field.[31] Both direct and indirect (acting as a secondary effect) reasons may cause increase in NPCs in the case of CP treatment. ROS generation after treatment with atmospheric argon CP was demonstrated in human lymphoma cells and extracellular milieau by using EPR-spin trapping.[62]

Thus, our data may serve as evidence that an increase in organic radical (and, likely, ROS) production is involved in the early stages of the response to CP and EMF treatments in dry seeds. The level of seed testa pigmentation is closely related to seed longevity, dormancy and germination traits,[63,64] because the same gene locus regulates the biosynthetic pathways of flavonoids and the abscisic acid (ABA) – a phytohormone inducing dormancy and inhibiting germination.[65] Seed treatment with CP and EMF induces changes in NPCs, most possibly modulating the redox state of the seed coat flavonoids. It remains to elucidate further whether these changes are translated into the effects on seed germination, since we could not find any clear correlations between the treatment-induced changes in the NPCs (Fig. 3) and the parameters of seed germination (Table 1). For example, similar NPCs changes in CP5, CP7, and EMF10 groups were associated with very different outcomes in germination kinetics.

Many reports have shown that the transition from a quiescent to a metabolically active germinating seed is associated with ROS generation. Production of H_2O_2 is a universal feature of the early imbibition period in seeds of numerous crops[37]. It is considered that H_2O_2 is an important player in a complex regulatory network of germination events controlled by the plant hormones ABA, gibberellic acid, ethylene, etc.[37,41,42] Lack of H_2O_2 leads to suppressed germination, however, the excess of H_2O_2 may cause delayed or inhibited germination due to oxidative damage.[37] Therefore changes induced in H_2O_2 production by seed treatment with vacuum, CP and EMF may be tightly related to the effects of these stressors on germination. The obtained results (Fig. 4) indicate that CP treatments decrease the level of H_2O_2 production in seeds of Norway spruce, and these effects correlate with negative effects of CP treatments on the germination 'in vitro' (Fig. 1a), and with CP5 and CP7 (but not CP2) treatments in the initial stages of germination in cassettes (Fig. 1b). We did not register H_2O_2 production in germinating seeds for the entire germination period (4 d), but there is some possibility that negative effects of all CP treatments on the germination 'in vitro' as well as negative effects of CP5 treatment on the final germination percentage or of CP7 – on germination time in cassettes (Table 1) can be explained by insufficient amounts of H_2O_2. In contrast, EMF treatments resulted in increased H_2O_2 production (Fig. 4) and positively affected germination indices, but due to better germination of the control seeds 'in vitro' stimulating EMF effects were pronounced only in cassettes (Table 1). Thus, our data provide the first evidence, that effects of CP and EMF on seed germination at least partially may be explained by the induced changes of H_2O_2 amount in germinating seeds. However, further studies are needed to elucidate these effects in more detail and to define their connection to hormonal changes in germinating seeds.

Another intriguing result of this study was that the treatment-induced pattern of changes in seedling growth varied with time so that seedlings grown from CP5 and CP7 treated seeds, characterized by negative effects on germination (Table 1) had considerably larger height and more branches in the end of the second vegetation season (17 months after sowing); and the opposite, EMF treatments that positively affected germination, had no effect on seedling growth on a longer time scale. The differences in the CP effects observed in early seedling development (germination and early growth) and on a longer time scale probably might be explained by the continuous seed stress response development, involving adaptive mechanisms that continue to operate in plants growing from treated seeds after completion of germination.

CP induced changes in the chemical and physical structure of seed surface,[58,66,67] that facilitate water penetration and may lead to increased EPR signal (Fig. 3). Probably, these effects also might be considered among the up-stream factors in stress signal perception by CP treated seeds, although in other studies stimulation of germination by CP was obtained under conditions not related to changes in SEM-visible seed surface.[68] In contrast to CP, EMF treatment does not cause seed surface etching[28] and hardly can induce chemical modifications, but our results show that EMF also induces increase in seed EPR signal (Fig. 3). That

may indicate that ROS production is a primary event leading to the secondary formation of stable organic radicals.

Our data show that plants developed mechanisms to respond very efficiently to short and rather moderate stress experienced in the seed (embrio) stage. Long-term observations show (this study and[28]) that negative effects on seed germination can be followed by strong positive effects on plant growth and development. The possible outcome of stress response is increase in plant fitness, performance and survival chances under unfavorable conditions. Possibly, more powerful "emergency" signal can work by selecting smaller number (decreased germination percentage) but stronger individuals capable to mobilise maximally their inner resources in the response to more extensive stress. The part of seed population does not germinate and does not compete for the resources with the remaining individuals that are able to switch on the maximal growth programme surpassing both plants in the control group and other groups with stimulated or unaffected germination.

4 | CONCLUSION

Pre-sowing treatment of Norway spruce (*Picea abies*) seeds with CP, vacuum, and EMF can effectively improve seed germination and seedling growth. Both kinetics of germination and the response of germination to seed treatment with CP and EMF was modulated by changing germination conditions. CP treatments negatively affected germination 'in vitro', but for germination in cassettes CP7 and EMF5 significantly increased (by 8–10%) the germination yield, so that only the effect of CP5 was negative. The majority of treatments accelerated the germination rate in cassettes, with CP2 treatment being the most effective, and only the CP7 treatment affected the germination rate negatively. Seed treatments with CP and EMF increase the number of paramagnetic centers in dry seeds, however changes in EPR signal do not correlate with changes in the germination kinetics. Our study provides the first evidence that H_2O_2 release from germinating seeds of Norway spruce displays periodic variations and the level of H_2O_2 release is modulated by the pre-sowing seed treatment with CP and EMF. We hypothesize, that effects of CP and EMF on seed germination at least partially may be explained by the induced changes of H_2O_2 amount in germinating seeds. Long-term observations revealed that the treatment-induced pattern of seedling growth changes varies with time. At the end of the second vegetation season (17 months from sowing) seedlings grown from CP5 and CP7 treated seeds, characterized by the negative effects on either germination rate or yield, had 50–60% larger height and 40–50% increased branching in comparison to the control seedlings. In contrast, the EMF treatments that positively affected germination had no effect on seedling growth on a longer time scale. The hypothesis of continuous seed stress response development is suggested to explain such findings.

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