Response of Perennial Woody Plants to Seed Treatment by Electromagnetic Field and Low-Temperature Plasma

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Radiofrequency (5.28 MHz) electromagnetic radiation and low-temperature plasma were applied as short-term (2–15 min) seed treatments to two perennial woody plant species, including Smirnov's rhododendron (*Rhododendron smirnowii* Trautv.) and black mulberry (*Morus nigra* L.). Potential effects were evaluated using germination indices and morphometry. The results suggest that treatment with electromagnetic field stimulated germination of freshly harvested *R. smirnowii* seeds (increased germination percentage up to 70%), but reduced germination of fresh *M. nigra* seeds (by 24%). Treatment with low-temperature plasma negatively affected germination for *R. smirnowii*, and positively for *M. nigra*. The treatment-induced changes in germination depended on seed dormancy state. Longer-term observations revealed that the effects persisted for more than a year; however, even negative effects on germination came out as positive effects on plant morphometric traits over time. Treatments characterized as distressful based on changes in germination and seedling length increased growth of *R. smirnowii* after 13 months. Specific changes included stem and root branching, as well as increased leaf count and surface area. These findings imply that longer-term patterns of response to seed stressors may be complex, and therefore, commonly used stressor-effects estimates, such as germination rate or seedling morphology, may be insufficient for qualifying stress response. Bioelectromagnetics. © 2016 Wiley Periodicals, Inc.

Keywords: germination; *Morus nigra*; plant development; pre-sowing seed treatment; *Rhododendron smirnowii*

INTRODUCTION

A diversity of dormancy-breaking agents can be used for pre-sowing seed processing to increase seed germination and/or crop yield [Graeber et al., 2012]. Physical treatments are considered to be more ecologically friendly than chemical treatments [Aladjadjiyan, 2012]. Improved seed performance (e.g., increase in germination by up to 20% in most cases) can be achieved for a large variety of annual plants after short-duration treatment of seeds with an electrostatic or pulsed electromagnetic field (EMF) (reviewed in Maffei [2014]; Pietrusziewski and Martinez [2015]), or low-temperature non-equilibrium plasma (or cold plasma, CP) (reviewed in Randeniya and de Groot [2015]). These stressors are a prospective inexpensive tool for inducing systemic eustress response in plant seeds: short exposure to EMF or CP leads to faster germination and seedling development followed by positive long-term effects on plant metabolism, biomass production, fruit ripening, nutritional quality [Racuciu et al., 2006; Bhardwaj et al., 2012], and disease resistance [Filatova et al., 2014; Jiang et al., 2014]. The majority of such studies have been performed on annual plants. Only a few reports are available on the response of perennial

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species to short-term seed treatment with EMF [Chao and Walker, 1967; Aladjadjiyan, 2003a,b], and only one paper has been published on CP effects [Puac et al., 2005].

The influence of EMF on biological objects has been examined mainly in the low-frequency (50–100 Hz) or microwave (300 MHz–300 GHz) regions [Maffei, 2014; Pietrusziewski and Martinez, 2015]. Effects of radiofrequency (RF) EMF are investigated less, although emissions from transmitting antennas, radar systems, and modern electronic equipment can increase the amount of plant exposure to such radiation in their environment. The aim of this study was to evaluate the effects of short-term seed treatment by RF (5.28 MHz) EMF and CP on two perennial woody angiosperms from different phylogenetic groups: Smirnov's rhododendron (*Rhododendron smirnowii* Trautv., order *Ericales*, family *Ericaceae*) and black mulberry (*Morus nigra* L., order *Rosales*, family *Moraceae*). *R. smirnowii* is a slow-growing decorative shrub. *M. nigra* is a fast-growing deciduous tree with edible fruit. These plants are not traditional agricultural plants, but both are important for decorative gardening. Furthermore, *M. nigra* is similar to the more popular *Morus alba* and has potential for medicinal use.

We chose perennial species for investigation with the intention to perform longer-term observations. These are important for several reasons: (i) evaluation of the early effects on plant development; (ii) estimation of the sustainability of the observed effects; and (iii) evaluation of the impact of treatments on aesthetic traits, quantities of pharmaceutically important secondary metabolites, and resistance to common diseases. Both *R. smirnowii* and *M. nigra* belong to species characterized by a physiological seed dormancy [Bewley et al., 2006; Finch-Savage and Leubner-Metzger, 2006; Linkies and Leubner-Metzger, 2012]. Such seeds become competent for germination after dormancy or after-ripening phase, and their germination is very different in dependence of the dormancy state. Aiming to estimate the impact of dormancy state on the effects of seed treatments with EMF and CP, we have also compared how these physical stressors change germination of freshly harvested and after-ripened seeds.

MATERIALS AND METHODS

Plant Material

Seeds of *R. smirnowii* were collected from Kaunas Botanical Garden, Vytautas Magnus University (Kaunas, Lithuania) in September 2013. Seeds were carefully cleaned under a stereomicroscope Motic SMZ-171 (Motic Deutschland, Wetzlar, Germany). The weight of 1000 cleaned *R. smirnowii* seeds was 143 ± 10 mg. Seeds of *M. nigra* were collected in Dubrava Arboretum in July 2013; the weight of 1000 cleaned *M. nigra* seeds was 1020 ± 53 mg. Some of the collected seeds were used for the in vitro germination test experiments in the same year (fresh seeds); others were stored for 6 (*R. smirnowii*) or 8 (*M. nigra*) months under dry conditions in the dark at 5 °C (*R. smirnowii*) or 10 °C (*M. nigra*), and used for in vitro experiments in April 2014 (after-ripened seeds). Seeds were visually examined for quality, and 200 or 300 seeds were packed into small plastic bags and taken to the B. I. Stepanov Institute of Physics of the National Academy of Sciences of Belarus (Minsk, Belarus) for treatment with physical stressors.

Seed Treatment With EMF and CP

The conditions for seed treatment were those reported as the most efficient for numerous annual plant species [Azharonok et al., 2009; Filatova et al., 2014]. The scheme of the experimental setup for seed treatment by RF EMF and CP at the B. I. Stepanov Institute of Physics, National Academy of Sciences of Belarus is shown in Figure 1. For seed treatment with RF EMF, plastic bags with seeds were placed in a container (Fig. 1, 1) which was mounted in the center of the water-cooled inductor (Fig. 1, 2), 90 mm long and 80 mm in diameter. The inductor was powered by a commercial RF (5.28 MHz) generator produced by VNIITVCH, Saint Petersburg, Russia (Fig. 1, 3). CP treatment was carried out in a planar geometry reactor consisting of two plane-parallel water-cooled (120 mm diameter) copper electrodes (Fig. 1, 4 and 5) and placed in a stainless steel vacuum chamber (Fig. 1, 6) with the inner volume of 0.053 m^3. The

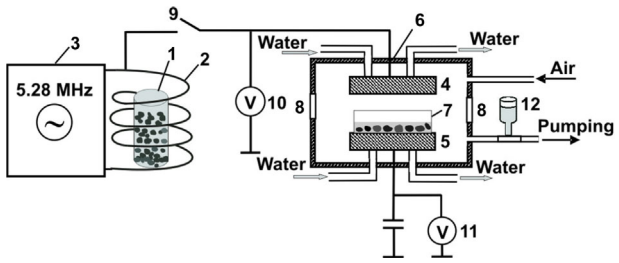

Fig. 1. Scheme of experimental setup used for seed treatment with vacuum, CP, and EMF: (**1**) dielectric container with seeds, (**2**) inductor, (**3**) RF generator, (**4**) powered electrode, (**5**) grounded electrode, (**6**) vacuum chamber, (**7**) Petri dish with seeds, (**8**) window, (**10, 11**) voltmeters, (**12**) thermistor vacuum gauge.

distance between electrodes was 20 mm. The RF power supply was connected to the plasma reactor through a switch (Fig. 1, 9).

Plastic bags with seeds were placed in the center of the water-cooled inductor and exposed to the EMF at atmospheric pressure and room temperature for 5, 10, and 15 min (these treatments are further abbreviated as EMF5, EMF10, and EMF15, respectively). The root mean square values of magnetic H and electric E components of EMF strength in the inductor were 590 A/m ($B \approx 0.74$ mT) and 12.7 kV/m, respectively, as determined using a high frequency field strength meter PZ-15 (SDB RME, Saint Petersburg, Russia) with an accuracy of 10%. Since solenoid dimensions are much higher than the sample size (10×4 mm^2), the magnetic field inhomogeneity within the tested sample was negligible. Seed heating during EMF exposure was controlled using a chromel–alumel thermocouple (IFS NAS, Minsk, Belarus) connected to millivoltmeter M2018 (BVS, Saint Petersburg, Russia). The measurements were performed immediately after turning off EMF. Exposure to EMF did not cause seed heating under the experimental conditions used.

Plasma treatment was performed in air at a pressure of 60 Pa measured by a thermistor vacuum gauge (Fig. 1, 12) with an accuracy of 20%. A capacitively coupled RF (5.28 MHz) discharge was operated at a specific power density of 0.68 W/cm^2. The RF voltage and the discharge current were determined from the readings of voltmeters (Fig. 1, 10 and 11). Under the experimental conditions, the gas temperature did not exceed 37 °C [Filatova et al., 2014]. Seeds were evenly dispersed on the inner surface of an open, sterile Petri dish that was placed on the grounded electrode. In every experiment, before plasma ignition between the electrodes, a pressure of 60 Pa (partial vacuum) was achieved by pumping air from the chamber for 7 min. Therefore, a 7 min "vacuum" treatment was used as an additional control in the CP experiments. The duration of further CP exposure was 2, 5, or 7 min (these treatments are further abbreviated as CP2, CP5, and CP7, respectively). Seed treatments for all experimental conditions and plant species were replicated three times.

Seed Surface Analysis, Germination Tests, Morphometric Estimation of Seedling Growth

The workflow scheme of all performed experiments is presented in Figure 2. The effectiveness of pre-sowing RF EMF and CP seed treatments was examined by seed germination tests in vitro. The tests were started 7 days after treating the seeds with physical stressors. Before germination tests, seed surface was analyzed using a scanning electron microscope (SEM) (Hitachi TM3000, Hitachi High-Technologies, Tokyo, Japan).

The untreated (control) seeds and seeds exposed to EMF, vacuum, and CP were evenly distributed on two layers of humid filter paper in 90 mm diameter plastic Petri dishes (three replicates of 50 seeds each) and watered with 3 ml distilled water. Petri dishes with seeds were placed in a climatic chamber (Pol-Eko-Aparatura KK 750, Pol-Eko-Aparatura, 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: 25 °C

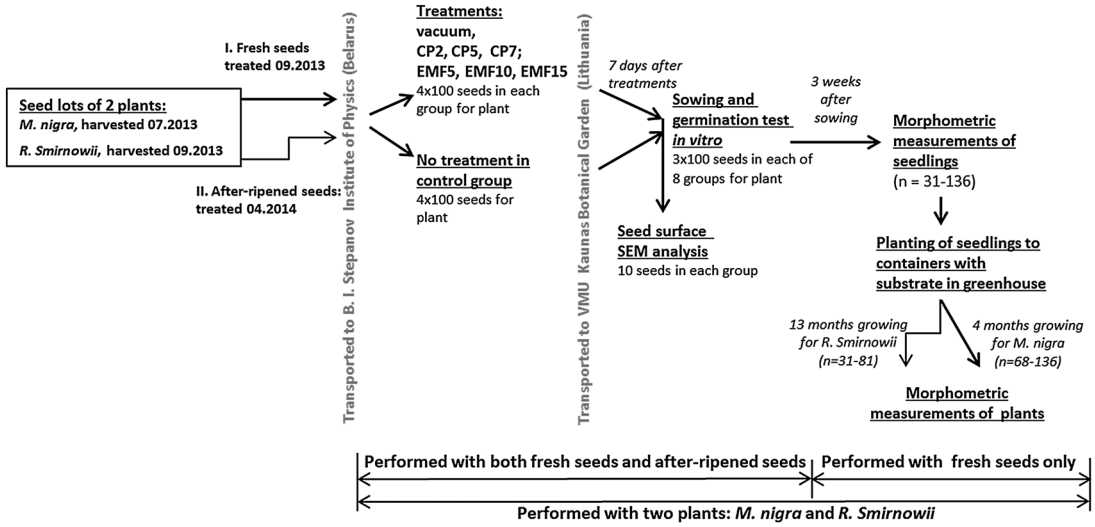

Fig. 2. Workflow scheme of performed experimental research.

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.

The effects of stressors on germination were estimated by the induced changes in parameters of germination kinetics, derived using application of Richards' function [Richards, 1959] for the analysis of germinating seed population [Hara, 1999]: Vi, the final germination percentage indicating seed viability, Me, the median germination time ($t_{50\%}$) indicating the germination halftime of a seed lot or germination rate, and Qu, 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 $\pm$ Qu).

After morphometric measurements, seedlings were carefully transferred from Petri dishes to plastic growth containers filled with soil and taken to the greenhouse. A neutral peat substrate was used for *M. nigra*, and an acidic peat substrate (pH 3.5–4.5) was used for *R. smirnowii*. For *M. nigra*, four seedlings were planted per $10 \times 10 \times 8$ cm container; up to 50 seedlings of *R. smirnowii* were planted in each $60 \times 40 \times 20$ cm container. Care was taken to maintain the same conditions of light, temperature (varied between 21 °C and 25 °C), and moisture (55–65%) for all experimental groups and replicates. The numbers of leaves and branches, leaf surface area, and seedling height were periodically measured.

Statistical Analysis

Statistical analysis of the results was performed using Statistica 10 software (issued by IBM Lietuva, Vilnius, Lithuania to Vytautas Magnus University). The main purpose of the analysis was to compare parameters in pairs—control and affected group (or in pairs 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 for comparing different conditions of affected groups. The dispersion homogeneity tests showed that dispersions of measured parameters in control and affected groups were unequal although all data sequences had normal (Gaussian) distribution. Results were considered statistically significant at $P < 0.05$. The number of measured plants in the control and treatment groups varied from 31 to 136.

RESULTS

Seeds of *R. smirnowii* and *M. nigra* belong to separate phylogenetic groups, characterized by different internal seed structure [Finch-Savage and Leubner-Metzger, 2006]. Shape and size of their seeds is also very different (Fig. 3).

Distinctive structural patterns of the seed-coat surfaces were revealed by SEM at 800× magnification (Fig. 4A and D). Since structural damage induced in the seed-coat surface is an important factor facilitating water penetration, SEM images were used to examine seed surfaces after treatment by physical stressors (Fig. 4B, C, E, F). Differences from the control group were not observed for the seed groups treated by vacuum and EMF (data not shown). However, CP treatment caused obvious etching of the seed surface for both plant species, and the damage increased with the duration of the treatment (Fig. 4), as was reported by other authors [e.g., Stolarik et al., 2015]. This indicates that direct interaction of active CP particles with seed surface during treatment produced more aggressive conditions in comparison to EMF treatment. An etched surface was observed in only half of the seeds. Careful scanning of both sides of each seed (seeds were carefully turned using a pincette to scan the opposite side) revealed that only one side of each seed was affected by CP etching. Presumably, the side facing the bottom of the Petri dish remained intact, and only the side directed toward treatment with CP was damaged.

Results obtained in laboratory tests for fresh and after-ripened seed germination are presented as germination curves in Figures 5 and 6. To quantify changes in the kinetics of germination, induced by seed treatments, the data points of each experimental replicate were fitted to Richards plots [Hara, 1999]. From the resulting 93 plots, the indices of germination kinetics

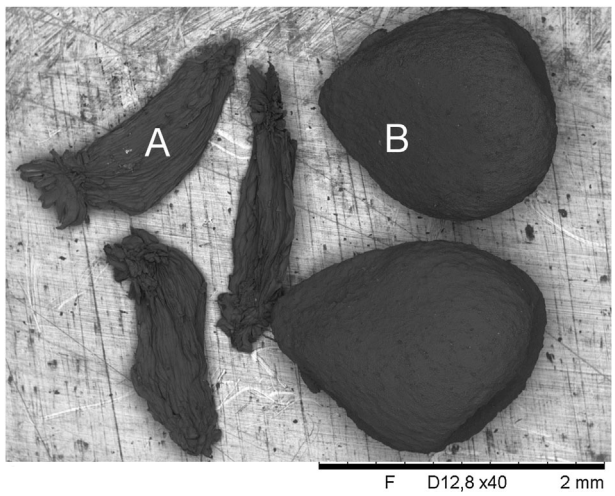

Fig. 3. SEM images of *R. smirnowii* (**A**) and *M. nigra* (**B**) seeds. Magnification: 40×.

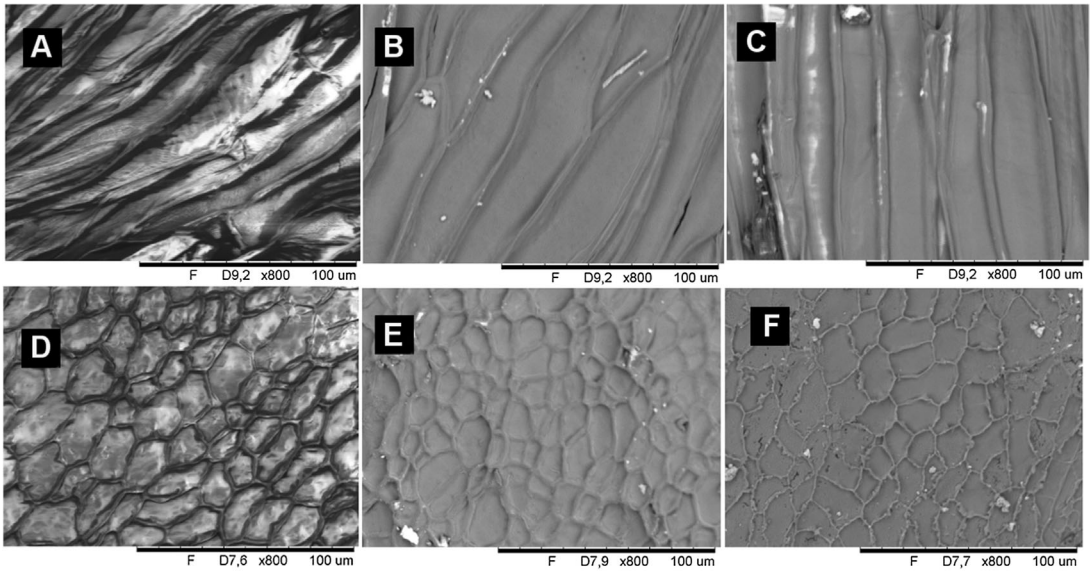

Fig. 4. SEM images of *R. smirnowii* (**A–C**) and *M. nigra* (**D–F**) seed surfaces. Surfaces in control (**A**, **D**) and experimental groups after seed treatment by CP5 (**B**, **E**) and CP7 (**C**, **F**). Magnification: 800×.

Vi, Me, and Qu were obtained (Tables 1 and 2). Selected examples of eight such plots are presented in Figure 7. Germination results fitted Richards plots very well—the measure of the goodness of fit Er [Hara, 1999] varied from 0.33 ± 0.09 (CP5) to 1.84 ± 0.36 (vacuum) for after-ripened seeds, and from 0.81 ± 0.03 (CP7) to 3.54 ± 0.06 (EMF10) for fresh seeds in *R. smirnowii*; in *M. nigra* from 1.08 ± 0.07 (CP2) to 2.24 ± 0.41 (CP5) for after-ripened seeds and from 1.44 ± 0.46 (EMF10) to 5.57 ± 0.76 (vacuum) for fresh seeds.

In the control, the final germination percentage or viability of *R. smirnowii* was much lower for fresh than for after-ripened seeds (Table 1, $P < 0.05$); the opposite was observed for *M. nigra* (Table 2, $P < 0.001$). However, for both species after-ripening decreased median germination time (Me) by ~2 days (Tables 1 and 2, for both $P < 0.001$) indicating substantial increase in the germination rate. Decrease in quartile deviation, Qu by 38% for *R. smirnowii*, and by 70% for *M. nigra* (Tables 1 and 2, for both $P < 0.0001$), shows smaller dispersion of germination time in after-ripened seed lots in comparison to their freshly harvested counterparts. Faster and more uniform germination of control seeds indicated that after-ripening during seed storage for 6 and 8 months had

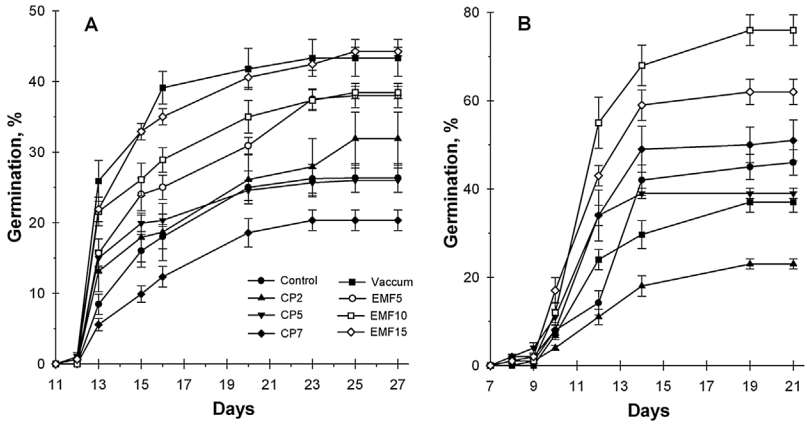

Fig. 5. *R. smirnowii* germination dynamics in vitro. (**A**) Fresh seeds, (**B**) after-ripened seeds. Percentage of germinated seeds is shown on ordinate axis, days after sowing–on abscissa axis. Points represent mean values of three replicates ± standard error of mean.

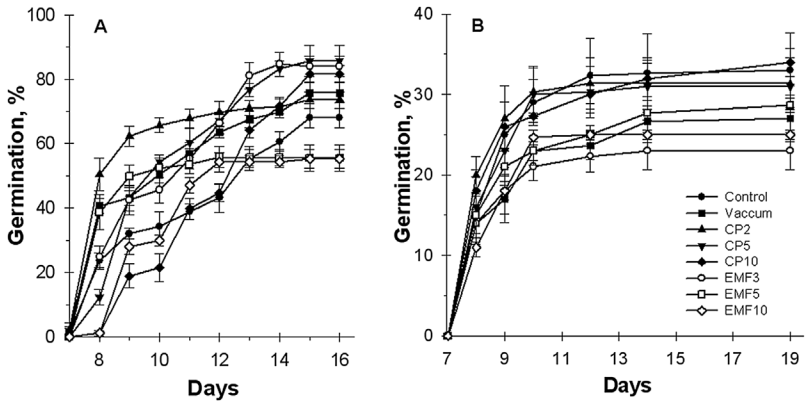

Fig. 6. *M. nigra* germination dynamics in vitro. (**A**) Fresh seeds, (**B**) after-ripened seeds. Percentage of germinated seeds is shown on ordinate axis, days after sowing—on abscissa axis. Points represent mean values of three replicates ± standard error of mean.

released dormancy both for *R. smirnowii* and *M. nigra* seeds.

There was considerable variation in the effects of EMF, CP, and vacuum treatments on seed germination dynamics (Figs. 5 and 6). Curves representing the percentage of germinated seeds after different treatments intersected in some cases. The most positive response in germination of fresh *R. smirnowii* seeds, clearly observable from the first day of radicle appearance, was induced by vacuum and EMF treatments, and longer-duration EMF treatment was more favorable (Fig. 5A, Table 1). Vacuum and EMF15 treatment increased the final germination percentage by more than 65% (from 26% to 43% and 44.7%), and EMF5 treatment increased Vi by 46%. Shorter treatment by CP (CP2 and CP5) did not change Vi of fresh *R. smirnowii* seeds (Table 1), but moderately negative effect (decreased Vi, increased Me and Qu) was observed in CP7 group. In comparison to vacuum treatment, CP had negative effect on germination; all CP treatments caused decrease in Vi ($P < 0.05$) (having a negative effect on seed viability), and CP2 and CP7 increased Me (decreasing rate of germination) and decreased Qu ($P < 0.05$) (decreasing uniformity of germination) (Table 1).

The pattern of response of after-ripened *R. smirnowii* seeds to stressors was similar only by the preference for EMF treatment (Fig. 5B); in EMF10 group Vi increased by 65%, but EMF15 treatment was less effective (+35%). In contrast to fresh *R. smirnowii* seeds (Fig. 5A), the response of after-ripened seeds to vacuum treatment was negative (−20%). CP treatment had either no significant effect (CP7) or up to 50% decreased seed viability (Vi) for CP2 treatment. However, all seed treatments, except CP2, resulted in a slightly increased germination rate (decreased Me by more than 1 day). In contrast to fresh seeds, germination dispersion indices Qu for

TABLE 1. *R. smirnowii* Germination Indices Calculated From Richards Plots

	Fresh seeds			After-ripened seeds		
Treatment	Vi	Me	Qu	Vi	Me	Qu
Control	26.0 ± 2.3	14.58 ± 0.18	1.38 ± 0.03	46.0 ± 2.9	12.49 ± 0.11	0.86 ± 0.08
Vacuum	43.0 ± 2.3*	12.81 ± 0.04**	0.35 ± 0.02**	37.0 ± 2.3*	11.48 ± 0.16*	0.89 ± 0.16
CP2	32.7 ± 3.8	14.65 ± 0.39	1.87 ± 0.20*	23.0 ± 1.2**	12.20 ± 0.35	1.24 ± 0.20
CP5	26.0 ± 1.7	12.82 ± 0.02**	0.32 ± 0.00**	39.0 ± 1.2*	10.66 ± 0.16**	0.78 ± 0.02
CP7	20.0 ± 1.2*	15.18 ± 0.16*	1.61 ± 0.06*	51.0 ± 3.5	11.44 ± 0.22*	0.89 ± 0.06
EMF5	38.0 ± 1.7*	14.23 ± 0.25	1.47 ± 0.09	–	–	–
EMF10	38.3 ± 1.2*	12.95 ± 0.13**	0.54 ± 0.19**	76.0 ± 3.4*	11.27 ± 0.15*	0.82 ± 0.02
EMF15	44.7 ± 0.9**	13.38 ± 0.24*	0.95 ± 0.22	62.0 ± 2.9*	11.10 ± 0.12**	0.81 ± 0.01

Vi, final germination percentage; Me, median germination time ($t_{50\%}$); Qu, quartile deviation.
Mean values ± standard error of mean of indices are presented.
Seed treatments for all experimental conditions were replicated three times (3 × 50 seeds, $n = 50$ for one replicate).
*Significantly different from control group ($P < 0.05$).
**Significantly different from control group ($P < 0.01$).

TABLE 2. *M. nigra* Germination Indices Calculated From Richards Plots

	Fresh seeds			After-ripened seeds		
Treatment	Vi	Me	Qu	Vi	Me	Qu
Control	70.0 ± 3.0	10.30 ± 0.10	2.12 ± 0.08	34.0 ± 2.9	8.22 ± 0.05	0.63 ± 0.02
Vacuum	75.7 ± 3.2	$8.45 \pm 0.15^{**}$	$1.26 \pm 0.03^{**}$	$27.3 \pm 2.6^{*}$	8.30 ± 0.09	$0.83 \pm 0.02^{**}$
CP2	$76.7 \pm 0.9^{**}$	$7.73 \pm 0.33^{**}$	$0.34 \pm 0.08^{**}$	30.3 ± 1.5	$7.85 \pm 0.01^{**}$	$0.35 \pm 0.07^{*}$
CP5	$86.0 \pm 2.3^{**}$	$9.29 \pm 0.11^{*}$	$1.20 \pm 0.14^{*}$	31.0 ± 2.9	8.15 ± 0.20	0.52 ± 0.10
CP7	$82.3 \pm 2.0^{**}$	$11.6 \pm 0.02^{**}$	$1.55 \pm 0.04^{*}$	34.0 ± 1.7	$8.02 \pm 0.04^{*}$	0.59 ± 0.11
EMF5	$84.3 \pm 3.2^{**}$	$9.78 \pm 0.20^{*}$	$1.66 \pm 0.10^{*}$	$23.0 \pm 2.3^{*}$	$7.94 \pm 0.05^{*}$	0.58 ± 0.10
EMF10	$53.0 \pm 2.9^{**}$	$7.83 \pm 0.07^{**}$	$0.32 \pm 0.11^{**}$	29.0 ± 1.2	8.15 ± 0.21	0.65 ± 0.19
EMF15	$53.3 \pm 2.0^{**}$	$9.53 \pm 0.08^{*}$	$0.90 \pm 0.33^{**}$	$26.3 \pm 1.2^{*}$	$8.04 \pm 0.03^{*}$	$0.22 \pm 0.02^{**}$

Vi, final germination percentage; Me, median germination time ($t_{50\%}$); Qu, quartile deviation.
Mean values $\pm$ standard error of mean of indices are presented.
Seed treatments for all experimental conditions were replicated three times (3×50 seeds, $n = 50$ for one replicate).
*Significantly different from control group ($P < 0.05$).
**Significantly different from control group ($P < 0.01$).

after-ripened *R. smirnowii* seed lots were not affected by seed treatments (Table 1). The germination of fresh *M. nigra* seeds for the initial days of radicle protrusion was characterized by larger intergroup variation than that of after-ripened seeds (Fig. 6), as was true for *R. smirnowii* (Fig. 5). Similarly, fresh seeds responded more positively to stressor treatment as well (Table 2). In contrast to *R. smirnowii* (Table 1), vacuum had no

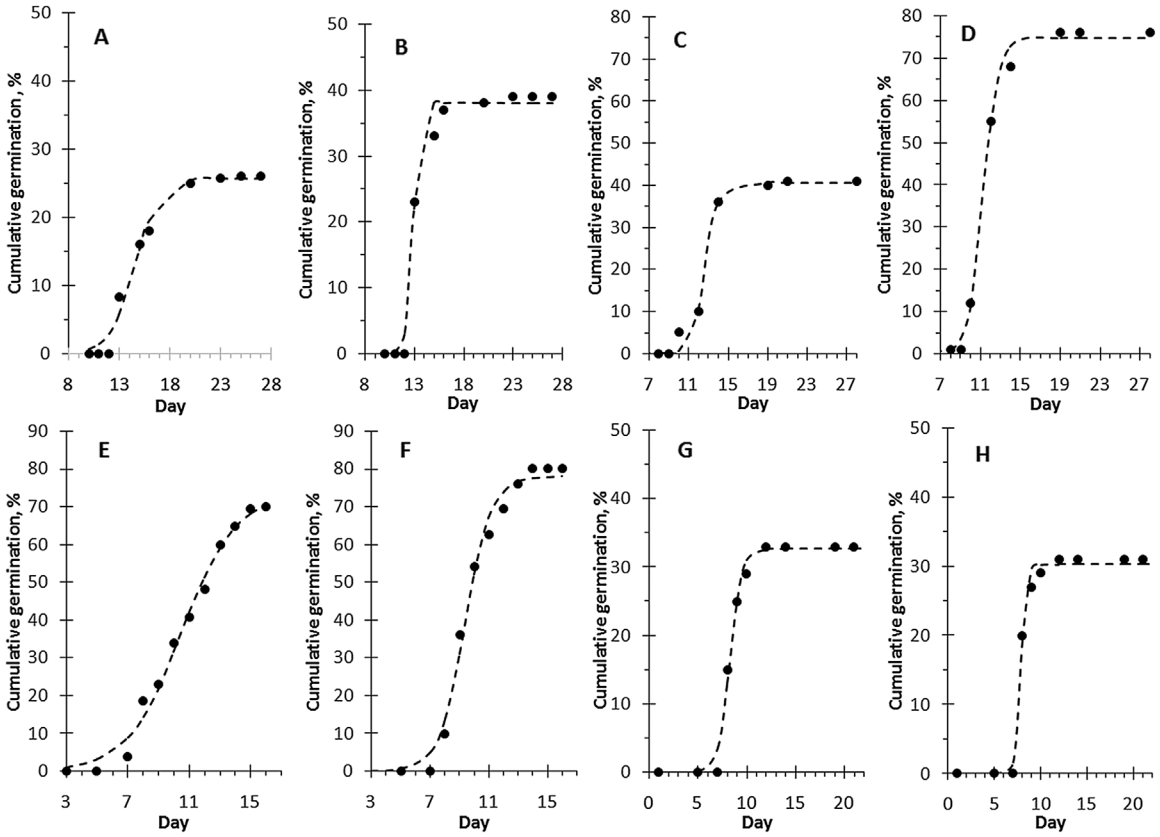

Fig. 7. Examples of germination curves calculated using Richards fitting function. Upper panel represents Richards plots for germination of *R. smirnowii*: (**A**) fresh seeds, control, (**B**) fresh seeds, EMF10, (**C**) after-ripened seeds, control, (**D**) after-ripened seeds, EMF10. Lower panel represents Richard plots for germination of *M. nigra*: (**E**) fresh seeds, control, (**F**) fresh seeds, CP5, (**G**) after-ripened seeds, control, (**H**) after-ripened seeds, CP2.

effect on Vi and positive response in *M. nigra* germination was caused by all CP treatments and by EMF5 (up to 23% increase in germination percentage in CP5 group). Negative effects in the final germination percentage of *M. nigra* were observed for longer-duration EMF treatment (10 and 15 min), in contrast to positive effects of EMF on fresh seeds of *R. smirnowii* (Fig. 5A). The only positive EMF effect on *M. nigra* germination percentage Vi in fresh seeds was observed in EMF5 group (Table 2). All treatments increased the uniformity of germination (decreased Qu), and the rate of germination—Me decreased in all groups except CP7, the most effective being the CP2 treatment that caused decrease in the germination halftime of a seed lot by 2.6 days.

Compared to fresh seeds (Fig. 6A), after-ripened *M. nigra* seeds responded differently to treatments (Fig. 6B); after-ripening reduced sensitivity to CP treatment and yielded a negative response to vacuum and EMF treatment. The change from a eustressful to a distressful response caused by seed storage was particularly striking for the short-duration (5 min) EMF treatment. Effects of treatments on Me and Qu were much smaller or absent in comparison to fresh seeds (Table 2); the largest decrease in the germination halftime Me was also caused by CP2, but only for 0.4 days. Vacuum, CP5, and EMF10 treatments did not affect germination rate in after-ripened *M. nigra* seeds, although the same treatments increased germination rate in fresh seeds.

Seed germination indices and seedling length are commonly used as the main criteria for estimating pre-sowing treatment efficiency [Racuciu et al., 2006; Sera et al., 2010; Bormashenko et al., 2012]. We also performed morphometric studies on the seedlings. Seedling length (total length of sprouts and roots) in 3 weeks after appearance of the first radicle is shown in Table 3. Data for fresh seeds only are presented, since effects of treatments on fresh seeds are more pronounced (Figs. 5A and 6A). The effects of stressors on seed germination are maintained for seedling length at least to some extent (Table 3). Vacuum treatment had the strongest positive effect (+24%) on seedling length in *R. smirnowii*, and seedlings in the EMF10 and EMF15 groups were significantly (by 16% and 11%, respectively) longer than those of the control. Again, in CP7 group inhibition of germination was followed by reduced seedling growth (by 14%). Although seedling length did not differ from the control after CP2 treatment, it significantly decreased (by 18%) compared to the vacuum-treated group. However, the effect of seed treatment on seedling length (Table 3) did not strongly correlate with the effects on germination for *M. nigra* (Fig. 6A). Seedling length was different from the control only in CP2 and EMF5 groups, while seedling growth was slightly inhibited despite positive effects on germination dynamics.

TABLE 3. Average Length (mm) of *R. smirnowii* and *M. nigra* Seedlings Obtained In Vitro 3 Weeks After Appearance of First Radicle

Treatment	*R. smirnowii*	*M. nigra*
Control	7.0 ± 0.4	34.7 ± 1.0
Vacuum	$8.7 \pm 0.3^{*}$	35.1 ± 1.2
CP2	$7.1 \pm 0.2^{**}$	$34.5 \pm 1{,}1$
CP5	8.4 ± 05	33.0 ± 0.9
CP7	$6.0 \pm 0.3^{*,**}$	$30.8 \pm 0.9^{*,**}$
EMF5	7.1 ± 0.3	34.0 ± 0.8
EMF10	$8.1 \pm 0.3^{*}$	35.2 ± 1.4
EMF15	$7.8 \pm 0.3^{*}$	$30.0 \pm 1.0^{*}$

Mean values ± standard error of mean of total seedling length (sprouts and roots) are presented.
Number of plants in experimental groups ranged from 31 to 85 (*R. smirnowii*) and from 68 to 136 (*M. nigra*).
*Significantly different from control group ($P < 0.05$).
**Significantly different from group treated by vacuum ($P < 0.05$).

After measurement of seedling length, seedlings were removed from Petri dishes and planted in containers with soil in the greenhouse. After 4 months, the morphology of *M. nigra* was examined to evaluate early plant development (Table 4). The height of above-ground plant tissues was measured in contrast to the total seedling length measured in vitro (Table 3). Only the vacuum treatment increased plant height slightly (by 12%). After CP treatment, plants were shorter than those in the vacuum group, but only CP2 group differed from controls (−18%); EMF5 and EMF15 treatment resulted in slightly reduced (7–8%) height. Some treatments (EMF5, EMF15, and CP2) slightly, but significantly, increased the number of leaves per plant, and stronger effects on leaf surface area (up to 41% by EMF10) were observed. However, some treatments that led to the increased leaf number had no effect on leaf surface area (CP2), and vice versa (CP5 or EMF10). Therefore, an unexpected result was obtained for total mean leaf surface area per plant: there were higher values for this estimate of leaf biomass accumulation for the CP7, EMF10, and EMF15 groups (Table 4). The final effect on germination (Fig. 6A) was the most negative in EMF10 and EMF15 groups. This reveals that plant response to stressors over longer time scales may not maintain the same trend.

R. smirnowii plants were tiny and developed fewer leaves after 4 months in comparison to *M. nigra*; therefore, similar measurements on *R. smirnowii* plants were performed after 13 months growing in the

TABLE 4. Morphometric Parameters of Plants in *M. nigra* Experimental Groups After 4 Months

Treatment	h (mm)	n_l	s_{leaf} (cm^2)	s_{plant} (cm^2)
Control	26.0 ± 0.8	2.68 ± 0.12	0.42 ± 0.02	1.13
Vacuum	$29.3 \pm 0.8^{*}$	2.73 ± 0.24	0.46 ± 0.03	1.26
CP2	$21.2 \pm 1.3^{*,**}$	$3.17 \pm 0.22^{*}$	0.42 ± 0.02	1.33
CP5	$23.8 \pm 1.3^{**}$	2.67 ± 0.25	$0.47 \pm 0.02^{*}$	1.25
CP7	$25.5 \pm 1.4^{**}$	2.68 ± 0.20	$0.56 \pm 0.03^{*,**}$	1.5
EMF5	$23.8 \pm 0.5^{*}$	$2.86 \pm 0.10^{*}$	0.44 ± 0.03	1.25
EMF10	24.8 ± 2.5	2.71 ± 0.32	$0.59 \pm 0.04^{*}$	1.60
EMF15	$24.2 \pm 0.8^{*}$	$2.94 \pm 0.21^{*}$	$0.58 \pm 0.03^{*}$	1.70

h, mean aboveground height of plant; n_l, mean number of leaves per plant; s_{leaf}, mean surface area per leaf; s_{plant}, total leaf surface area per plant.
Total leaf area per plant was calculated by multiplying mean surface area per leaf by mean number of leaves.
Mean values ± standard error of mean are presented.
Number of plants in experimental groups ranged from 68 to 136.
*Significantly different from control group ($P < 0.05$).
**Significantly different from group treated by vacuum ($P < 0.05$).

greenhouse (Table 5). The effects of short-duration (2–15 min) seed treatment persisted for more than 1 year. Comparison of seedling length (Table 3) and plant height (Table 5) shows that longer-term effects of CP treatment on plant morphology did not follow the initial trend: the positive effect of vacuum treatment became insignificant, a negative effect of CP5 was revealed, and—in complete contrast to seedlings (Table 3)—CP7-group plants showed the most rapid growth. Moreover, the latter group demonstrated unusual branching characteristics (Table 6)

TABLE 5. Morphometric Parameters of Plants in *R. smirnowii* Experimental Groups After 13 Months

Treatment	h (mm)	n_l	s_{leaf} (cm^2)	s_{plant} (cm^2)
Control	9.7 ± 0.4	7.8 ± 0.3	0.15 ± 0.01	1.2
Vacuum	10.6 ± 0.4	$9.8 \pm 0.4^{*}$	$0.52 \pm 0.05^{*}$	5.1
CP2	10.9 ± 0.5	$10.3 \pm 0.3^{*}$	$1.00 \pm 0.08^{*,**}$	10.3
CP5	$8.0 \pm 0.4^{*,**}$	$8.6 \pm 0.3^{**}$	$0.47 \pm 0.04^{*}$	4.0
CP7	$16.4 \pm 0.8^{*,**}$	$15.0 \pm 1.1^{*,**}$	$1.26 \pm 0.10^{*,**}$	19.0
EMF5	10.1 ± 0.3	$9.8 \pm 0.3^{*}$	$0.68 \pm 0.06^{*}$	6.7
EMF10	9.6 ± 0.4	$9.8 \pm 0.4^{*}$	$0.54 \pm 0.05^{*}$	5.3
EMF15	$11.0 \pm 0.4^{*}$	$10.9 \pm 0.5^{*}$	$0.73 \pm 0.07^{*}$	8.0

h, mean aboveground height of plant; n_l, mean number of leaves per plant; s_{leaf}, mean surface area per leaf; s_{plant}, total leaf surface area per plant.
Total leaf area per plant was calculated by multiplying mean surface area per leaf by mean number of leaves.
Mean values ± standard error of mean are presented.
Number of plants in experimental groups ranged from 31 to 81.
*Significantly different from control group ($P < 0.05$).
**Significantly different from the group treated by vacuum ($P < 0.05$).

TABLE 6. Percentage (%) of *R. smirnowii* Plants With Various Numbers of Stems (n_s) in Experimental Groups After 13 Months

	n_s			
Treatment	1	2	3	4
Control	100	0	0	0
Vacuum	96.3	3.7	0	0
CP2	97.4	2.6	0	0
CP5	94.9	5.1	0	0
CP7	67.6	24.3	5.4	2.7
EMF5	100	0	0	0
EMF10	96.6	3.4	0	0
EMF15	95.0	5.0	0	0

Number of plants in experimental groups ranged from 31 to 81.

with many more leaves per plant and larger total leaf surface area (Table 5).

Different members of the genus *Rhododendron* display sympodial branching that is often pubescent on first-year stems. Normal branching starts in the second or later years of development. Consistent with this characteristic, plants in the control and EMF5 groups developed a single stem in the first year. However, plants in other experimental groups showed various degrees of early branching induced by seed treatments (Table 6). The CP7 treatment was most effective in causing branching; more than 30% of plants developed more than one stem, and (unique among the groups) two plants had three stems, and one plant had four stems. It was not possible to distinguish the main stem among several branches, since branching started from the stem collar, and all stems appeared equivalent. Shorter-duration CP treatments also induced branching, but to a smaller extent. This finding could explain the large differences in the numbers of leaves and in leaf surface area among the treatments; the degree of branching correlated well with differences in numbers of leaves and total leaf surface area (Table 5). Vacuum and longer-duration EMF treatments also led to the early appearance of two stems in some plants. Exposure of seed potatoes to a static EMF (20–80 mT for 1 h) was shown to increase the number of stems [Marks and Szecowka, 2010]. Our results indicate that RF EMF and CP can induce similar changes in the development of rhododendrons; however, no branching effects were noticed in *M. nigra*. Inducing branching in decorative shrubs by CP treatment can be an important tool for increasing their aesthetic value. Therefore, further long-term observations should be performed to determine whether plants will flower earlier or will form more impressive crowns as a result of larger numbers of stems and leaves. Compared to controls, the

number of leaves per plant in all treatments, except CP5, was significantly larger ($P < 0.05$ in CP2, EMF5, and EMF15 groups) for *M. nigra* and *R. smirnowii* ($P < 0.05$), and the mean surface area per leaf increased from more than threefold (CP5 and vacuum treatment) to more than eightfold (CP7) for *R. smirnowii*. The differences in the mean total leaf surface area per plant for *R. smirnowii* were even larger, from 3.4-fold (CP5) to almost 16-fold (CP7).

R. smirnowii plants with the best growth from CP7 group were characterized by the lowest germination rates (Fig. 5A), and their seedling length did not differ from that of the control after 3 weeks (Table 3). Moreover, after more than 1 year, rhododendrons of all treatment groups had better growth parameters than control plants (Table 5). Although *M. nigra* did not show such differences in growth among treatment groups (Table 4), the total leaf area per plant also exceeded the control in all groups, and the largest difference was in EMF15 group (1.5-fold larger than the control), which had the lowest seed germination (Fig. 6A).

In order to better understand the reasons for these large differences in plant growth, we examined the roots of several *R. smirnowii* plants of mean height in some experimental groups. Examples of representative specimens from the control, vacuum, CP2, and EMF15 groups are shown in Figure 8 (statistical quantification was not performed because we aimed to preserve plants for longer-term observation and biochemical study).

The control plant had one main root with a few weak lateral branches, and all plants from the treatment groups had more developed root systems with numerous, large lateral branches. Roots of the CP7-group plant had the most developed branches, suggesting that this group would obtain nutrients more effectively than plants in the control and other experimental groups. The supply of nutrients is critical for plant development and growth; therefore, this finding may help to explain some of the differences in biomass indices, since representative plants from the vacuum, CP7, and EMF15 groups weighed almost five-, eight-, and threefold more, respectively, than the representative control plant (see Fig. 8 legend).

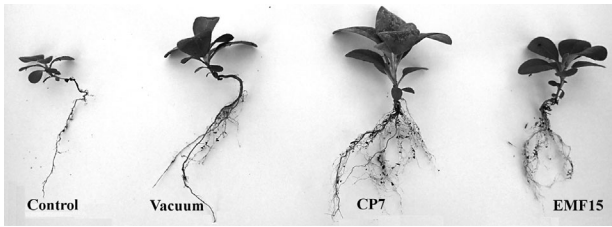

Fig. 8. Plant size and root system of *R. smirnowii*. Plants represent the control and experimental groups of seeds treated by vacuum, CP7, and EMF15. Height of plants selected for picture was close to mean height of corresponding experimental group (Table 5). Plants weighed 59, 267, 463, and 165 mg, respectively.

Considerable effects of similar seed treatments on growth of four ornamental tree species of family *Fabaceae* were reported previously [Aladjadjiyan, 2003a,b]: pre-sowing seed treatment for 10 min by the permanent 0.15 T magnetic field has doubled the germination percentage of *Robinia pseudoacacia*, and in 14 days after sowing fresh weight of shoots increased by more than threefold; the same treatment for 30 min resulted in more than fourfold increase in germination percentage of *Laburnum anagiroides*, and more than twofold increase in fresh weight of shoots. Other studies on the response of perennial plant seeds to EMF and CP treatment were focused only on germination effects, while the growth of the obtained seedlings was not investigated [Chao and Walker, 1967; Puac et al., 2005].

DISCUSSION

To summarize our findings, the effects of short-duration pre-sowing seed treatment by CP and EMF were dependent on plant species. Seeds of *R. smirnowii* were positively affected by EMF and negatively by CP, whereas germination of *M. nigra* seeds was inhibited by EMF and stimulated by CP. Comparison of our results obtained with two modes of treatment (CP vs. EMF) on germination of two plant species can be helpful for estimating the importance of effects on seed coat surface for germination outcomes. Etching of the seed coat surface only by CP treatment was observed for both species (Fig. 3), but germination of *M. nigra* was stimulated and that of *R. smirnowii* was suppressed by CP. Moreover, vacuum and EMF treatments exerted strong positive effects on seed germination in some cases, although these treatments did not change seed coat structure (at least to the extent visible by SEM). Therefore, we cannot associate positive or negative responses with induced SEM-visible changes in the structure of seed surface. Many recent studies intend to explain stimulating CP effects on germination mostly by the induced changes in seed coat surface leading to increased hydrophilization of seed tissues [Bormashenko et al., 2012, 2015; Ling et al., 2014; Stolarik et al., 2015]. Such explanation can be indeed satisfactory for plants of families (*Fabaceae*, *Poaceae*, etc.) used in these studies, because these plants are characterized by physical dormancy which is determined by one or more water

impermeable palisade cell layers in the seed coat [Baskin and Baskin, 2004].

However, the most prevalent kind of seed dormancy in gymnosperms and all major clades of angiosperms is physiological dormancy [Baskin and Baskin, 2004]. Germination of such seeds is dependent on the inner seed processes (not restricted by the seed coat), and they require a period of dry after-ripening or application of dormancy-breaking agents (hormones, scarification, stratification, etc.) for germination. Perennials used in our study, *R. smirnowii* and *M. nigra*, represent a large part of plants by the germination traits, since both are characterized by the physiological seed dormancy. Our results show that effects of EMF, CP, and vacuum treatments differed for freshly harvested and after-ripened seeds in both species. Within-species effects sometimes differed strongly or were opposite for different seed dormancy state (Figs. 5 and 6). Fresh seeds of *R. smirnowii* and *M. nigra* were more responsive and responded more positively to treatment by CP and EMF than after-ripened seeds. These results provide evidence that vacuum, CP, and EMF treatments can be considered as factors releasing physiological dormancy. The ability of CP to affect breaking dormancy in Lamb quarters (*Chenopodium album* agg.) seeds was investigated previously [Sera et al., 2009]. The authors estimated CP effects in seeds stored for 6 and 7 months, but did not obtain differences in Vi and Me; only Qu values differed for four durations of CP treatment. It is doubtful that only 1 month difference in after-ripening time could be sufficient for appreciable difference in the dormancy state between compared lots of Lamb quarters seeds [Sera et al., 2009]. We compared freshly harvested and after-ripened seeds (stored for 6 or 8 months after harvest), and the effects of vacuum, CP, and EMF on indices of germination kinetics (Tables 1 and 2) for both studied species obviously differed between dormant and after-ripened seeds lots.

However, in some cases applied seed treatments negatively affected germination. Further investigations are needed for understanding molecular reasons for species dependent preferences in seed germination response to treatment with various physical stressors and their different doses. Molecular mechanisms that decrease seed dormancy during ripening are not entirely understood [Shu et al., 2016], and it is likely that physical stressors exert their effects on germination by interfering with those mechanisms. Recent studies provide evidence that during storage, biochemical changes continue to occur in seeds at various levels, including hormonal balance [Graeber et al., 2012, Shu et al., 2016], gene expression [Liu et al., 2012], oxidative processes [Cadman et al., 2006], mRNA content, and protein translation [Bahin et al., 2011; El-Maarouf-Bouteau et al., 2013]. All of these processes can lead to systematic changes in molecular signal transduction machinery that result in different responses to stress in fresh and after-ripened seeds, as was observed in our experiments. For the same reasons, the stress response is also likely to differ for desiccation-tolerant seeds stored for different duration. However, information on the year of seed harvest is often lacking in published reports; this uncertainty can help to explain contradictory results. The finding that fresh seeds respond more strongly and positively to stressors than do after-ripened seeds may be important for the development of effective treatment technologies and should be taken into account in similar studies, in particular for physiologically dormant seeds.

Longer-term observations of seedling development after pre-sowing seed treatment by stressors have revealed that, regardless whether the germination response is positive or negative, plants grown from treated seeds performed better than control plants in all experimental groups (developed more leaves and had greater total leaf surface area). Moreover, the most negative stressor effects on seed germination (Figs. 5A and 6A) were followed by the most rapid leaf growth during later stages of plant development. This phenomenon occurred in both plant species (Tables 2 and 3), but was particularly pronounced for *R. smirnowii*, possibly due to stress-induced stem branching (Table 6). These findings raise doubts about the validity of conclusions on positive (eustress) or negative (distress) effects of seed stressors, when based only on germination test results, or measurements of seedling length in early stages of growth. For example, in the recent study of CP effects on wheat seed germination, growth, and yield, the authors selected only the best germinating experimental group for longer duration field studies [Jiafeng et al., 2014]. However, our results demonstrate that such logical extrapolation is not always valid since even treatment groups with inhibited germination may perform better and could possibly bring higher biomass yields.

It is not easy to explain the large differences in plant development from control seeds and seeds treated by physical stressors for only 2–15 min. Numerous explanations of mechanisms behind the interaction of plant seeds with magnetic fields and CP have been suggested, including modulation of water properties, induced changes in seed coat or membrane structure, improvement in water uptake, Ca^{2+} transport, and activation of metabolic enzymes that could

increase amount of photosynthetic pigments and stimulate protein biosynthesis in leaves [Galland and Pazur, 2005; Maffei, 2014; Randeniya and de Groot, 2015]. However, such explanations are insufficient, and many authors point to the lack of systematic knowledge about the physiological and molecular basis of such effects [Galland and Pazur, 2005; Poinapen et al., 2013; Maffei, 2014]. The mechanisms behind the balance in hormetic stress responses have to be better understood for the development of more reliable technologies for pre-sowing seed treatments.

CONCLUSIONS

The effects of pre-sowing seed treatment of perennial woody plants by RF EMF and CP are species-dependent: germination of *R. smirnowii* seeds was positively affected by EMF and negatively by CP, in contrast to *M. nigra* seed germination, which was stimulated by CP and inhibited by EMF. Etching of seed coat surface by CP, but not vacuum and EMF treatment, was observed for both plant species. However, these changes cannot be directly associated with improved germination, since CP treatment stimulated germination of *M. nigra*, and inhibited germination of *R. smirnowii* seeds. For both tested plant species, stressor- and dose-dependent responses in germination were dependent on seed dormancy state: freshly harvested seeds of both species responded to stressors more strongly and positively than after-ripened seeds. Longer-term observations revealed that plants grown from treated seeds of all experimental groups performed better (developed more leaves and had greater total leaf surface area) than the control plants. Furthermore, plants from the experimental groups where inhibition of germination was the strongest (CP7 for *R. smirnowii* and EMF15 for *M. nigra*) showed the best growth in later stages of development. Treatments by vacuum, CP, and EMF induced early branching of stems and roots in *R. smirnowii*. Obtained results suggest that commonly used estimates of stressor effects, such as germination rate or seedling morphology, are not sufficient to define the stress response, at least for perennials.

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