

Seed Priming of *Trifolium repens* L. Improved Germination and Early Seedling Growth on Heavy Metal-Contaminated Soil

Laurence Galhaut · Alexis de Lespinay ·
David J. Walker · Maria Pilar Bernal ·
Enrique Correal · Stanley Lutts

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Abstract Seed priming effects on *Trifolium repens* were analysed both in Petri dishes and in two soils (one unpolluted soil and a soil polluted with Cd and Zn). Priming treatments were performed with gibberellic acid 0.1 mM at 22 °C during 12 h or with polyethylene glycol (−6.7 MPa) at 10 °C during 72 h. Both priming treatments increased the germination speed and the final germination percentages in the presence of 100 µM CdCl₂ or 1 mM ZnSO₄. Flow cytometry analysis demonstrated that the positive effect of priming was not related with any advancement of the cell cycle in embryos. Seed imbibition occurred faster for primed seeds than for control seeds. X-ray and electronic microscopy analysis suggested that circular

depressions on the seed coat, in addition to tissue detachments inside the seed, could be linked to the higher rate of imbibition. Priming treatments had no significant impact on the behaviour of seedlings cultivated on non-polluted soil while they improved seedling emergence and growth on polluted soil. The two priming treatments reduced Zn accumulation. Priming with gibberellic acid increased Cd accumulation by young seedlings while priming with polyethylene glycol reduced it. Priming improved the light phase of photosynthesis and strengthened the antioxidant system of stressed seedlings. Optimal priming treatment may thus be recommended as efficient tools to facilitate revegetation of former mining area.

Laurence Galhaut and Alexis de Lespinay contributed equally to this work.

L. Galhaut · A. de Lespinay · S. Lutts (✉)
Groupe de Recherche en Physiologie végétale (GRPV), Earth and Life Institute—Agronomy (ELI-A), Université catholique de Louvain (UCL), Croix du Sud 5 (Bte 7.07.13), Louvain-la-Neuve 1348, Belgium
e-mail: stanley.lutts@uclouvain.be

D. J. Walker · E. Correal
Departamento de Recursos Naturales, Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario (IMIDA), Estación Sericícola, Calle Mayor, s/n, La Alberca 30150, Spain

M. P. Bernal
Department of Soil and Water Conservation and Organic Waste Management, Centro de Edafología y Biología Aplicada del Segura, CEBAS-CSIC, Apartado 164, 30100 Espinardo, Murcia, Spain

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Abbreviations

PEG Polyethylene glycol
d Day
h Hour
GA₃ Gibberellic acid

1 Introduction

Restoration of industrial sites and degraded landscapes is nowadays a major conservation focus. To rehabilitate these sites, stand establishment of vegetation is essential (Clemente et al. 2004; Ye et al. 2000). Revegetation

improves soil stabilisation and limits erosion, but also protects habitats and the natural beauty of the site. Revegetation has thus both a phytostabilisation and a biodiversity-enhancing effect. However, revegetation could be difficult because of the poor physical and chemical properties of the target soils, which reduce seed germination and seedling emergence (Murungu et al. 2003; Zhang et al. 2001). High heavy metal concentrations, as well as poor fertility linked to very low levels of organic matter, are common characteristics of soils following mining activities and constitute the major constraints inhibiting plant establishment (Bernal et al. 2007; Claassen and Hogan 2002).

In order to phytostabilise disturbed areas, a mix of various plant species is commonly used. Among these, white clover (*Trifolium repens*) is a common species used in revegetation strategies (Bidar et al. 2007; Smith et al. 2006). White clover is indeed adapted to a large set of environmental conditions and contributes to soil improvement by its nitrogen fixing ability. It may thus assume a positive role in phytoremediation strategy for both heavy metals (Lopareva-Pohu et al. 2011) and polycyclic aromatic hydrocarbon-contaminated soils (Meng et al. 2011). Heavy metals such as Cd or Zn may, however, impair seed germination in this species which justifies the use of high seeding densities during revegetation processes (de Lespinay 2009). Efficient seed germination and early seedling growth are essential for further plant establishment. Hence, any strategy improving seed germination on these difficult sites may help to increase the efficiency of the rehabilitation process and reduce the costs of revegetation, in particular through a reduction of seed densities.

Seed priming is a technique consisting of soaking seeds in appropriate solutions followed by dehydration prior to final sowing. Such pre-treated seeds often exhibit an improved germination behaviour and also a better seedling emergence and growth (Jisha et al. 2013; Parera and Cantliffe 1994). Seed priming has been shown promising for hastening seed germination under difficult environmental conditions (Bradford 1986; Frett and Pill 1995; Mihoub et al. 2005). To achieve the maximum economical and technical potential of priming, the treated seeds should be dried to an acceptable moisture content level for long-term storage and/or transport after the priming treatment. Most results until now were obtained with plants used for agronomical or horticultural purposes (Haigh et al. 1986; Harris et al. 1999; Murungu et al. 2003), and

priming data concerning plant species used for revegetation application remain scarce. Moreover, the ways in which seed priming interacts with soil conditions, such as heavy metal-contaminated soils, are rarely considered and poorly understood.

Despite numerous studies devoted to the application of seed priming, the underlying physiological and biochemical bases of germination improvement also remain largely unknown. Different hypotheses, such as osmotic adjustment (Bradford 1986), specific protein synthesis (Davison and Bray 1991; Gallardo et al. 2001) and hastening of reserves mobilisation (Farooq et al. 2006), have been proposed. In mature seeds, embryos usually contain cells that are at different stages of the cell cycle, while synchronisation of root tip cell is a prerequisite to allow further morphological evolution leading to radicle protrusion. DNA replication during the priming treatment and synchronisation of the cell cycle at the G_2 phase was thus previously reported in some plant species (Bino et al. 1992; de Castro et al. 2000; Lanteri et al. 2000; Redfearn and Osborne 1997). This, however, does not appear as a general rule, and efficient priming treatments do not always involve an advancement of the cell cycle (Gurusinge et al. 1999; Varier et al. 2010). Faster water absorption has also been reported in primed comparatively to unprimed seeds (Jisha et al. 2013), but to the best of our knowledge, water status in relation to ultrastructural properties of primed seeds was only marginally considered until now (Nagarajan et al. 2005). Some authors reported that seed priming may have an impact on seedling nutrition in response to P or Zn deficiencies (Ajouri et al. 2004) or in response to salt stress (Cayuela et al. 1996), but data concerning seedlings growing on heavy metals polluted soils are crucially lacking.

It was previously demonstrated that osmopriming improved germination of white clover seeds in the presence of Cd and that this positive effect requires both gene expression and protein synthesis (de Lespinay et al. 2010). This study also demonstrated that the maintenance of amylase activities and hastening of water absorption are directly related to the positive impact of priming in this species. However, ultrastructural modification of primed seeds, impact of priming on cell cycle and mineral nutrition of young seedlings issued from primed seeds were not considered until now. The objectives of the present study are therefore (i) to analyse the impact of priming treatments on seed water uptake in relation to seed structure, (ii) to study cell cycle

progression in mature embryos of primed versus unprimed seeds and (iii) to analyse the effect of seed priming on seedling emergence, growth and mineral nutrition in soil containing high concentration of heavy metals.

Former mining and industrial areas are frequently contaminated by several heavy metals. The present work thus considers a typical non-polluted agricultural soil used as control and a highly polluted soil issued from a mining area of eastern Belgium and already characterized for its high level of contamination in several pollutants (Lambrechts et al. 2011a).

2 Materials and Methods

2.1 Plant Material and Priming Treatments

Seeds of white clover (*T. repens*, cv. Huia) were provided by Advanta Co. (Kapelle, the Netherlands). Seeds were preliminary selected under binocular magnifying glass to remove hard dark seeds (less than 8 % in the considered seed lot). Only heart-shaped brown seeds (size 0.9–1.2 mm; 639 mg/1,000 seeds) were used for subsequent priming treatments and germination tests. Seeds were primed in 0.1 mM gibberellic acid (GA₃) or –6.7 MPa polyethylene glycol (PEG) 8000 solutions at 10 and 22 °C, respectively. Priming treatments were applied during 6, 12, 18 and 24 h in the presence of GA₃ and during 24, 48 and 72 h in the presence of PEG. The concentrations of GA₃ and PEG, temperature and treatment duration were determined from preliminary experiments (de Lespinay 2009).

Seed priming was carried out in the dark on filter circle paper (diameter 18.5 cm, VWR International) in Petri dishes, moistened with the above-mentioned solutions. After priming, seeds were rinsed for 3 min with water, briefly blotted on filter paper and dried under laminar flux at room temperature for 24 h until they reach their initial moisture. The primed and untreated (control) dry seeds were stored at room temperature during a maximum period of 24 h prior to sowing or analysis. The best results obtained for each priming treatment were selected for the subsequent analysis.

2.2 Germination Test

Germination assays were carried out in 9-cm-diameter Petri dishes. Four replicates of 50 seeds were considered

per treatment. The seeds were incubated on filter paper (8.5 cm diameter, Whatman Grade 1, Schleicher & Schuell, England) saturated with 4 ml of half-strength Hoagland solution in order to fulfil mineral requirement for optimal seed germination. The nutrient solution contained the following chemicals (in mM): 2.0 KNO₃, 1.7 Ca(NO₃)₂, 1.0 KH₂PO₄, 0.5 NH₄NO₃ and 0.5 MgSO₄ and (in μM) 17.8 Na₂SO₄, 11.3 H₃BO₃, 1.6 MnSO₄, 1 ZnSO₄, 0.3 CuSO₄, 0.03 (NH₄)₆Mo₇O₂₄ and 14.5 Fe-EDDHA. Additional germination test was performed with nutrient solution added with 100 μM CdCl₂ or 1 mM ZnSO₄. The use of Visual MINTEQ software (v2.3.0) confirmed that more than 98 and 96 % of Cd and Zn, respectively, remained in their free divalent ionic form. Petri dishes were placed in a controlled-temperature room (Economic Delux ECD01E, Snijders) under optimal temperature conditions (20±0.5 °C). Germination was monitored daily over a period of 8 days, and seeds were considered as germinated when radicle protrusion was ≥2 mm. Seeds were germinated under complete darkness for *T. repens*, according to ISTA procedure (ISTA 2008). Results were expressed as percent germination (*G*) and as days to reach 50 % germination (T50) according to $T50 = t_i + \frac{((N+1)/2 - n_i)}{n_j - n_i} \times (t_j - t_i)$ where *N* is the final number of seeds germinating and *n_i* and *n_j* are the total number of seeds germinated at times *t_i* and *t_j*, where $n_i < (N+1)/2 < n_j$.

2.3 Hydration Kinetics After Priming

For a quantitative analysis of imbibition rates, 500 mg of seeds was weighed and then placed in Petri dishes, on filter paper fully imbibed with Hoagland solution. At fixed times after the start of imbibition, seeds were removed from the Petri dishes, blotted, weighed and then returned to the filter paper, always fully imbibed with the solution. Imbibition was measured until the seeds reached a constant maximum weight. Each measurement was performed in triplicate. The increase of water content was analysed by fitting the experimental data with exponential and sigmoid functions using SigmaPlot (de Lespinay et al. 2010).

2.4 Microscopy and X-ray Photographs

Control and primed seeds were cut using a razor blade, and cross, longitudinal, transversal and micropylar region sections were performed. Just after cutting, fresh samples (cut and uncut entire seeds) were mounted on

sticky tape affixed to aluminium stubs. Mounted specimens were coated with gold in a sputter coater (Denton Vacuum Desk II) and examined using a scanning electron microscope (Jeol JMS-5410) operating at 20 kV.

Two-dimensional X-ray (2D X-ray) inspection was realised with a vertical X-ray beam, imaging through a horizontal sample tray scanned in the X - and Y -axes (Rodenstock lens, X-TEK leading X-ray technology, Arcoss Company). The X-ray source was a 100XiR vacuum air-cooled, open-tube microfocuss, with a tungsten target. The X-ray conditions for seed images were 60 kV and 150 μ A, with an exposure time of 2 s.

2.5 Flow Cytometry

Control and primed seeds were first dissected in order to isolate the radicle tip of *T. repens*. For isolation of radicle tip tissue, 1 mm of the distal part of the radicle was used. Seed tissues were then chopped for 30–60 s in a plastic Petri dish containing 0.2 ml extraction buffer (Partec CyStain PI Absolute P Nuclei Extraction Buffer; Partec GMBH, Münster, Germany), polyvinylpyrrolidone-10 (5 %, w/v), dithiothreitol (10 mM) and ascorbic acid (12 mM). The resulting extract was diluted with 0.2 ml of extraction buffer and then passed through a 30- μ m-pore filter into a 3.5 ml plastic tube. To this filtrate was added 1.6 ml of Partec CyStain PI Absolute P Staining Buffer, containing propidium iodide (PI) and RNase to give final concentrations of 50 and 17.5 μ g/ml, respectively. All stages of extraction and staining were performed at 2–5 °C. For each sample, tissues of 10 to 30 seeds were used, and determinations were made at least in triplicate for each treatment. Samples were analysed with a Partec PA II flow cytometer, employing a 20-mW argon ion laser light source (488 nm wavelength, model PS9600, LG-Laser Technologies GmbH, Kleinostheim, Germany) with an RG 590 long-pass filter. For each sample, a minimum of 5,000 nuclei were analysed. The distribution of embryo and radicle tip cells in the three phases of the interphase part of the cell cycle (G_0/G_1 , S and G_2) was determined for each analysis, using the Flowmax software of the cytometer, to represent G_0/G_1 and G_2 by a Gaussian function and S by a second degree polynomial broadened with a Gaussian function.

2.6 Soil Selection and Characterisation

Two soils were selected for the present study: (i) The first soil was a non-polluted agricultural soil originating from

the area of Corbais (50° 38' 9" N, 4° 39' 17" E; Belgium), (ii) the second soil was issued from a contaminated area in the vicinity of a fertilizer industry located in Prayon (50° 35' 10" N, 4° 40' 30" E; Belgium). Since the beginning of the eighteenth century and until 1974, the site was contaminated by atmospheric fallouts of different heavy metals. It presents sparse vegetation consisting mainly in species adapted to high metal concentration (*Viola calaminaria*, *Agrostis capillaries*, *Armeria maritime*, *Noccaea caerulea*), but the vegetation cover is not complete and several plots did not present vegetation and are thus prone to erosion process.

Soils were collected from the top 20 cm in an area of around 10 m², air-dried for 6 days and sieved to <2 mm for analysis. Soil electrical conductivity (EC) and pH-H₂O were determined using glass electrodes (WTW Multi 350i) in a 1:5 soil/water paste. Total element concentration was determined according to Page et al. (1982) with inductive-coupled plasma atomic emission spectrometry (ICP-AES; Thermo Jarrell Ash Iris Advantage). Total N (N_T) and organic C (C_{org}) were determined according to Kjeldahl and Walkley–Black methods (Page et al. 1982). Heavy metal concentrations were obtained by dissolving 0.5 g of soil in an open Teflon crucible with HNO₃ 65 % and HF 40 %. The mixture was gently heated on a hot plate until complete dryness. The residue was redissolved with HClO₄ 70 %, and the mixture was heated again until complete dryness. The residue was redissolved with aqua regia (HCl 37 % and HNO₃ 65 %) and filtered (Whatman 41). Finally, the solution was diluted to 50 ml with deionised water and analysed with ICP-AES. Results of soil analysis are shown in Table 1. Following these results, the study focused on Cd and Zn, which presented high concentrations by far exceeding maximal values for unpolluted substrates (Cd 1 mg kg⁻¹, Zn 150 mg kg⁻¹; Council of the European Communities 1986). Lead was also higher than maximal values, but the bioavailability of this element determined by 0.01 M CaCl₂ extraction was very low and it did not directly constitute a serious threat for young seedling growth (detailed data not shown). This soil was acid and presented higher Cu concentration than the soil of Corbais, even if not exceeding maximal values for unpolluted substrates.

2.7 Seedling Emergence and Growth

The soils were placed in pots (8.5 cm diameter, 14 cm high) each containing 30 seeds. For each treatment

Table 1 Characteristics of the soils from Corbais (non-polluted soil) and the soil from Prayon (polluted soil)

Parameter	Corbais	Prayon
Texture	Silty clay loam	Silty clay loam
EC (dS m ⁻¹)	0.36	0.49
CaCO ₃ (%)	23.7	12.5
OM (%)	3.12	2.68
Organic-C (g kg ⁻¹)	15.6	9.2
Total-N (g kg ⁻¹)	1.9	1.3
Available-K (g kg ⁻¹)	0.26	0.09
Available-P (g kg ⁻¹)	0.20	0.06
pH	7.5	6.1
Cd (mg kg⁻¹)	0.1	64.8
Cu (mg kg ⁻¹)	10	86
Fe (g kg ⁻¹)	14.7	25.2
Mn (g kg ⁻¹)	0.34	0.69
Ni (mg kg ⁻¹)	11	21
Pb (mg kg⁻¹)	20	1,924
Zn (mg kg⁻¹)	69	5,223

Methods used for soil analysis are detailed in “Materials and Methods”. Each value is the mean of four replicates. Values exceeding legal norms of contamination are indicated in bold

(priming×soil), 12 pots were used and were watered twice a week to field capacity. The experiment was run in a growth chamber in Louvain-la-Neuve (Belgium) with a temperature of 24±0.5 °C (day) and 14±0.5 °C (night), and the relative humidity was 65±5 %, with a 12-h photoperiod (250 μmol m⁻² s⁻¹ PAR). Seedling emergence was monitored over a period of 17 days, until it reached a plateau, using cotyledons apparition as criteria. Seedling height and total leaf number were measured each week until harvest (i.e. 32 days after sowing) on six pots per treatment. Shoots of *T. repens* were cut just above the soil surface and rinsed with deionised water. Fresh weight was then measured, as well as dry weight after 72 h at 70 °C.

2.8 Plant Mineral Analysis, Malondialdehyde Concentration and Chlorophyll Fluorescence

Plant shoots were digested in 35 % HNO₃ and evaporated to dryness on a sand bath at 80 °C. Minerals were then dissolved in 0.1 N HCl, and cation concentrations were determined by plasma emission spectrometry ICP-AES (Thermo Jarrell Ash Iris Advantage). To estimate

stress levels encountered by the cultivated plants, malondialdehyde and chlorophyll fluorescence were considered on four plants per treatment. Malondialdehyde (MDA), a by-product of lipid biomembrane peroxidation resulting from oxidative stress, was quantified in leaf tissue according to Heath and Packer (1968). Chlorophyll fluorescence-related parameters were measured by the Fluorescence Monitoring System II (Hansatech Instruments) after dark adaption for 30 min on the last unfolded leaf. A first saturating pulse (18,000 μmol m⁻² s⁻¹) was sent to the leaf. A constant intensity of actinic light (600 μmol m⁻² s⁻¹) was thereafter sent for 3 min on the leaf, and a second saturating pulse of 18,000 μmol m⁻² s⁻¹ was subsequently applied. PS II efficiency (Φ_{PSII}), non-photochemical quenching (NPQ) and photochemical quenching (q_p) were estimated according to Maxwell and Johnson (2000).

2.9 Statistical Analysis

The experiment was repeated three times. Shapiro–Wilk and Bartlett/Levene tests were performed to check normality and variance equality of the data, respectively. Data were subjected to an analysis of variance using SAS software (SAS System for Windows, version 9.1). The statistical significance of the results was analysed by the Student–Newman–Keuls (SNK) test at the 5 % level. Percentage values were arc-sin-transformed before analysis.

3 Results

3.1 Effect of Priming on Germination

Priming treatment had no impact on the final germination percentage of seeds germinating under control conditions (Table 2), except for long-term priming treatment with GA₃ which significantly reduced final germination percentage. Although Cd and Zn reduced germination of unprimed seeds, most priming treatments significantly increased *G* values in the presence of heavy metals comparatively to unprimed seeds.

Cadmium delayed the germination of unprimed seeds while Zn had no impact on T50 values. Priming with GA₃ during 6 or 12 h reduced T50 for seeds germinated in the absence or in the presence of heavy metals. As far as PEG priming was concerned, only the

Table 2 Impact of priming treatments on final germination percentages (G ; in %) and time to reach 50 % germination (T_{50}) in seeds of *Trifolium repens*

Priming	Duration (h)	t° ($^{\circ}\text{C}$)	G (in %)			T_{50} (days)		
			Control	Cd	Zn	Control	Cd	Zn
Unprimed			93.0 a	58.2 a	70.6 a	2.64 a	3.22 a	2.73 a
GA_3	6	10	92.6 a	68.3 b	91.3 c	1.47 b	2.01 b	1.62 c
	12		91.3 a	76.9 c	92.4 c	1.40 b	1.93 c	1.55 c
	18		92.6 a	71.4 b	89.5 bc	2.36 a	2.24 b	2.84 a
	24		60.1 b	64.5 ab	86.3 b	2.51 a	2.97 a	2.32 b
PEG	24	22	90.2 a	71.5 b	90.4 c	2.83 a	3.46 a	3.07 a
	48		92.6 a	75.4 bc	92.1 c	2.34 a	2.87 ab	2.75 a
	72		94.8 a	84.5 d	93.2 c	1.61 b	1.73 c	2.01 b

Seeds were unprimed or primed with 0.1 mM GA_3 or PEG -6.7 MPa for various durations. Germination was performed in Hoagland nutrient solution (control) or in solution containing 100 μM CdCl_2 or 1 mM ZnSO_4 . Each value is the mean of four replicates. For a given column, values followed by different letters are significantly different based on $\text{SNK}_{0.05}$

longest priming treatment significantly reduced T_{50} . Priming treatments with GA_3 during 12 h and PEG during 72 h were selected for further experiments.

3.2 Priming Effect on Hydration, Seed Structure and Cell Cycle

Water uptake occurred faster in primed seeds than in control seeds (Fig. 1). Water uptake of PEG pre-treated seeds occurred faster than for GA_3 pre-treated ones, and seed water content was similar for both primed and

control seeds at the end of the experiment. Control seeds displayed a sigmoid-shaped curve and primed seeds an exponential-shaped curve (Fig. 1).

The scanning electronic microscopy and X-ray analysis results are presented in Figs. 2 and 3. Unlike control seeds, PEG- and GA_3 -primed seeds revealed the presence of seed coat tears (Fig. 2 B), as well as circular depressions of 2–5 μm diameter (Fig. 2 F) on the seed coat surface while unprimed seeds remained smooth (Fig. 2 A, E). Electronic microscopy photographs were also performed at the micropyle and hilum region (Fig. 2

Fig. 1 Time course analyses of water uptake during the first hours of germination for primed and unprimed (control) seeds of *Trifolium repens*. Seeds were primed with 0.1 mM GA_3 during 12 h or with PEG -6.7 MPa during 72 h. Each value is the mean of three replicates of 0.5 g of seeds, and vertical bars represent standard errors

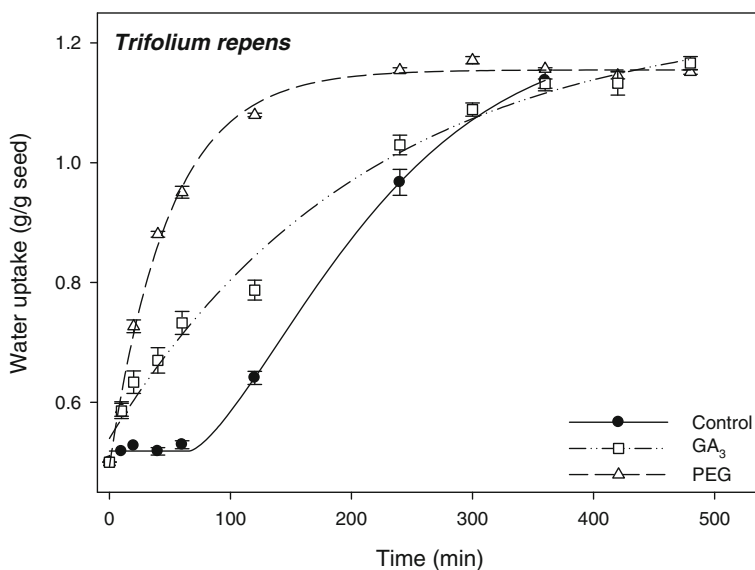
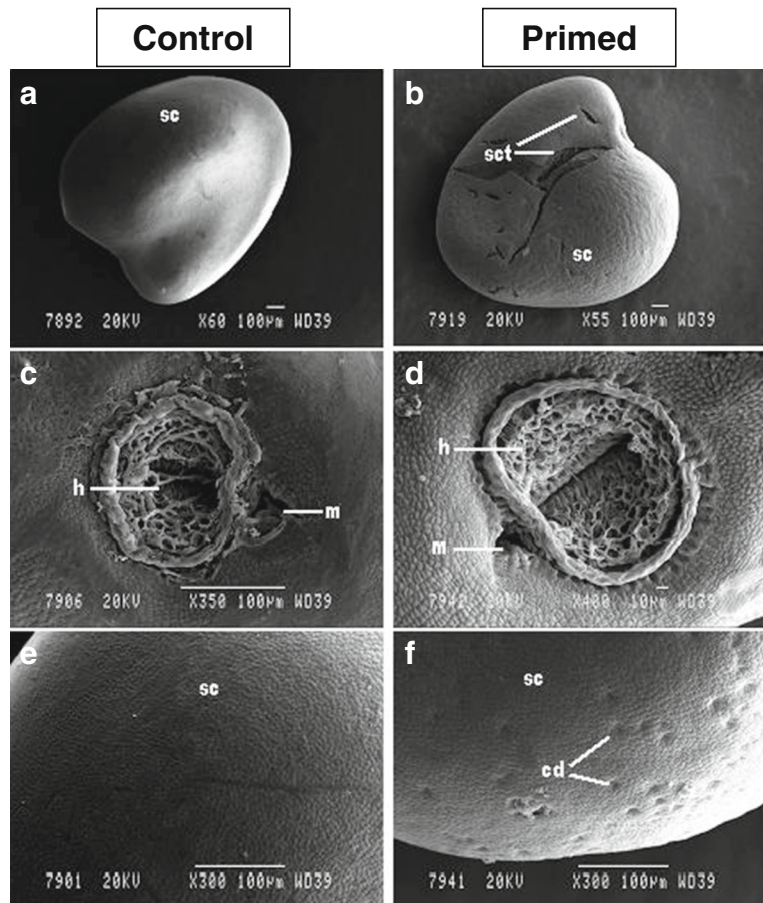


Fig. 2 Scanning electron micrographs of *Trifolium repens* seeds showing the entire seed (A, B), the hilum and micropyle region (C, D) and a close-up of the seed coat surface (E, F) of control unprimed seeds (A, C, E) and seeds that were primed in GA₃ (B, D, F). Primed seeds show a modified seed coat and the presence of circular micro-depressions in the seed coat surface (*cd* circular depression, *h* hilum, *m* micropyle, *sc* seed coat, *sct* seed coat tears). Magnifications and scales are indicated at the bottom of each micrograph



C, D) which, being a major water-entrance zone, was supposed to be enlarged after priming. However, no particular modification of this region was observed after the priming treatments. In order to get a clear view from inside the seed, X-ray photographs were taken. Unlike control seeds, most PEG-primed seeds showed a tissue detachment between the cotyledons and radicle, creating a free space between these organs (Fig. 3).

Nuclei percentage in the G_0/G_1 , S and G_2 stages of the cell cycle in radical tips for control and primed seeds remained identical, whatever the treatment (Table 3). A similar observation was made for the $G_2/(G_0/G_1)$ ratios. About 53 % of the radicle tip cells remained at the G_0/G_1 stage (Table 3). Although the settings of our flow cytometer would have allowed us to detect 8C or 16C signals, these were not noticed whatever the treatment. Differences in the cell cycle stage were observed among *T. repens* embryo tissues of unprimed control seeds (Table 4). The major difference was observed when

comparing cotyledons and radicle tissues. Cotyledons had 88 % of cells at G_0/G_1 compared to 58 % for radicles and radicle caps and 54 % for radicle tips and radicle meristems, there being a higher proportion of actively-dividing cells in the radicle tip (Table 4).

3.3 Seedling Emergence and Growth

As shown in Fig. 4, priming treatment did not confer any advantage to seedlings emergence in the non-polluted soil Corbais, and maximal seedling emergence was observed already after 13 days. In contrast, in the polluted soil Prayon, seedling emergence of unprimed seeds remained low and did not exceed 40 % at the end of the treatment. Seedlings issued from primed seeds exhibited a higher emergence than unprimed seeds, especially for PEG-treated seeds. Both priming treatments lead to a higher seedling emergence at the end of the experiment (ca. 20 %) comparatively to seedlings issued from unprimed seeds. Seedlings cultivated on

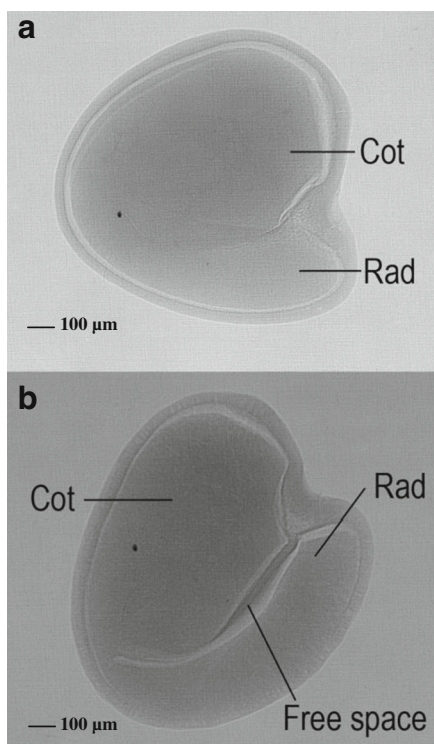


Fig. 3 X-ray radiographs of a *Trifolium repens* seed without priming (a) and a seed that was primed in PEG (b), showing the internal morphology. Radicle tip (*Rad*), cotyledon (*Cot*) and free space, at $\times 45$ magnification

polluted soil (Prayon) had a lower height and number of leaves than those cultivated on Corbais, but priming treatment had no significant influence on this respect (data not shown). Similarly, priming had no impact on the shoot dry weight from seedlings maintained on the Corbais soil (Table 5) but significantly improved the final dry weight of seedlings grown on the Prayon polluted soil. The polluted soil also reduced the shoot

water content of seedlings issued from unprimed seeds and, to a lower extent, from PEG-primed seeds, but priming with GA_3 significantly reduced the deleterious impact of pollution on this parameter.

3.4 Plant Mineral Analysis, Chlorophyll Fluorescence and Malondialdehyde Concentration

Priming treatments had no impact on Zn concentrations of plants maintained on the non-polluted soil Corbais (Fig. 5). Both priming treatments reduced Zn accumulation in shoots of plants cultivated in Prayon soils comparatively to seedlings issued from unprimed seeds. While Cd was not detected in seedlings cultivated on Corbais soil, the two priming treatments had a dual impact on the seedling maintained on Prayon substrate: GA_3 obviously increased Cd accumulation comparatively to seedlings issued from unprimed seeds while priming with PEG reduced it. In contrast, priming had no impact on Pb accumulation. Similarly, priming had no impact on P concentration in seedlings cultivated in Corbais soil, but it significantly improved P nutrition in seedlings cultivated on Prayon.

Seedlings issued from unprimed seeds and cultivated on Prayon exhibited lower values for maximal efficiency of PSII photochemistry in the dark-adapted state (F_v/F_m), Φ_{PSII} and q_p (Table 6), as well as higher values of NPQ comparatively to those maintained on the Corbais soil. Seed priming reduced the deleterious impact of polluted soil on chlorophyll fluorescence-related parameters, PEG priming being the most efficient, especially for NPQ.

All seedlings cultivated on Corbais soil accumulated similar amounts of MDA (Fig. 6). Lipid peroxidation strongly increased in response to polluted soil Prayon

Table 3 Percentage of nuclei at the G_0/G_1 , S and G_2 stages of the cell cycle and the $G_2/(G_0/G_1)$ ratio in primed and unprimed seeds, as determined by flow cytometry

Priming	Cell cycle			
	G_0/G_1 (%)	S (%)	G_2 (%)	$G_2/(G_0/G_1)$ ratio
Control	52.9 (1.3) a	9.2 (1.7) a	37.9 (2.4) a	0.721 (0.060) a
PEG	52.9 (2.4) a	6.9 (1.8) a	40.1 (0.9) a	0.766 (0.052) a
GA_3	52.9 (2.3) a	10.0 (1.3) a	37.1 (1.6) a	0.713 (0.062) a

The value in parentheses corresponds to the standard error for six determinations, each consisting of 20 radicle tips of *Trifolium repens*. For a given column, values followed by different letters are significantly different based on SNK_{0.05}

Table 4 Average percentage of nuclei at the G_0/G_1 , S and G_2 stages of the cell cycle and the $G_2/(G_0/G_1)$ ratio, as determined by flow cytometry in different tissues of 20 control (non-primed) seeds of *Trifolium repens*

Seed tissue	Cell cycle			
	G_0/G_1 (%)	S (%)	G_2 (%)	$G_2/(G_0/G_1)$ ratio
Cotyledons	87.6 (1.0) a	4.1 (0.9) a	8.4 (0.8) a	0.096 (0.010) a
Radicle ^a	57.6 (0.5) b	10.4 (1.1) c	32.0 (1.3) b	0.559 (0.025) b
Radicle cap ^b	57.4 (1.7) b	8.8 (1.4) b	33.9 (0.9) b	0.594 (0.032) b
Radicle tip	52.9 (1.3) b	9.2 (1.7) bc	37.9 (2.4) b	0.721 (0.060) c
Radicle meristem ^c	53.9 (3.3) b	8.5 (2.4) b	37.6 (3.6) b	0.709 (0.105) c

The values in parentheses correspond to the standard error ($n=3$). For a given column, values followed by different letters are significantly different based on $SNK_{0.05}$

^a“Radicle” represents the entire radicle (1.5–3 mm long)

^b“Radicle cap” represents the first 0.3 mm of the radicle tip

^c“Radicle meristem” is the zone equivalent to the radicle tip minus the radicle cap

but was significantly lower in seedlings issued from primed seeds than from unprimed ones.

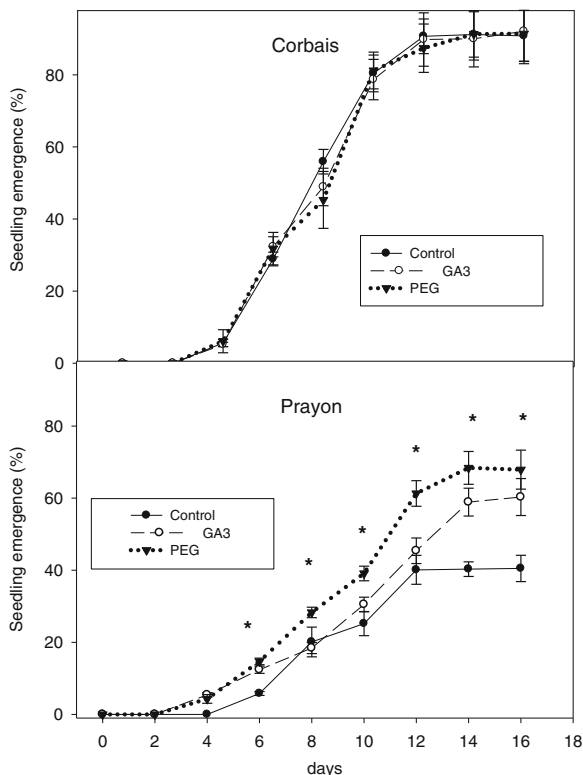


Fig. 4 Cumulative seedling emergence of *Trifolium repens* for unprimed (control) and primed seeds on the Corbais and Prayon soils. Each value is the mean of six replicates, and vertical bars represent standard errors. Asterisks indicate significant effect of priming relative to control plants ($P < 0.05$)

4 Discussion

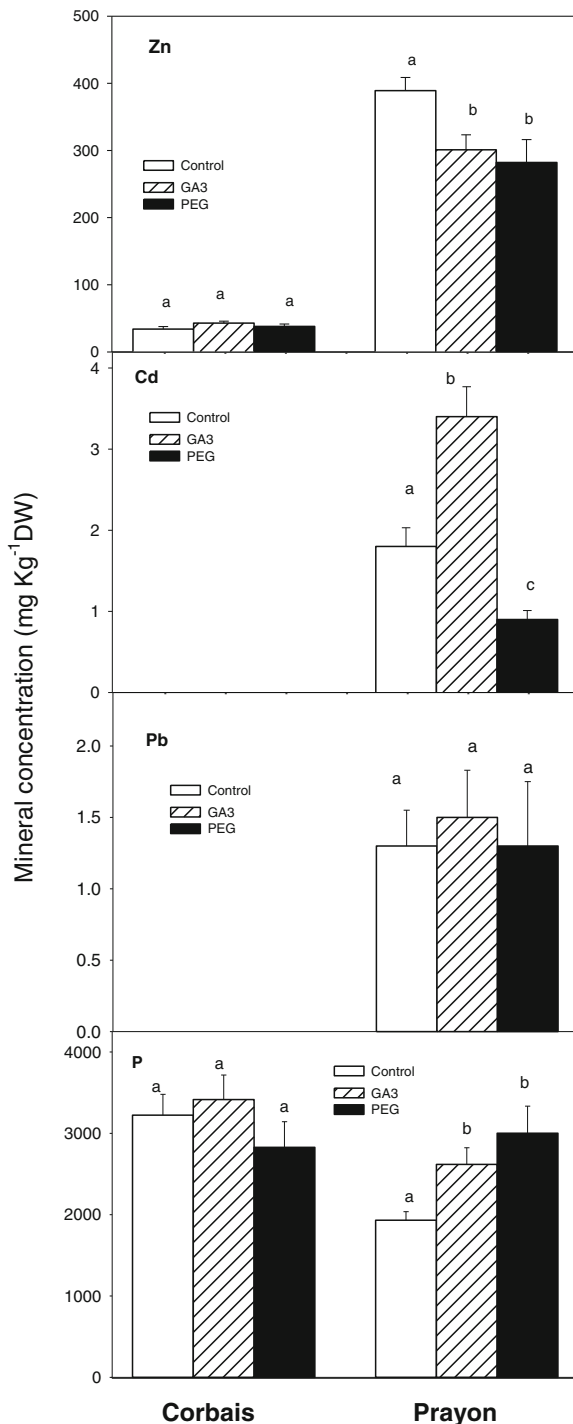
4.1 Cell Cycle Activation Is Not Required for Priming Effect in White Clover

Some authors reported that improvement of germination in different plant species could be linked to a priming-induced synchronisation of embryo cells at the G_2 phase of the cell cycle in relation to DNA replication (Ashraf and Bray 1993; Bino et al. 1992; de Castro et al. 2000; Redfearn and Osborne 1997). Some data also suggest that cell division may be found prior to radical protrusion (de Castro et al. 2000; Masubelele et al. 2005).

Table 5 Impact of seed priming treatments on the final dry weight and shoot water content of seedling of *Trifolium repens* maintained for 30 days on soil from Corbais or soil from Prayon contaminated with heavy metals

Soil	Priming treatment	Dry weight (mg)	Water content (%)
Corbais	Unprimed	13.5±0.7 a	86.5±0.6 a
	GA ₃	12.9±1.4 a	84.9±1.0 a
	PEG	13.9±1.2 a	85.3±1.2 a
Prayon	Unprimed	5.1±0.3 c	74.3±1.4 b
	GA ₃	9.8±0.2 ab	85.7±0.5 a
	PEG	7.4±0.5 b	78.2±0.3 b

Seeds were primed with 0.1 mM GA₃ during 12 h or PEG –6.7 MPa during 72 h. Each value is the mean of 10 replicates±SE. Values followed by the same letter are not significantly different according to $SNK_{0.05}$



In the present case, flow cytometry analyses obviously showed that germination improvement via priming was not related with DNA replication or any advancement of the cell cycle. Although the $G_2/(G_0/G_1)$ ratio differed according to the considered organ at the

Fig. 5 Mineral concentrations in shoot dry matter (DM) for *Trifolium repens* plants grown in pots and harvested at day 32 after sowing on non-polluted Corbais or heavy metal-polluted Prayon soils. Seeds were primed either with 0.1 mM GA₃ (12 h at 10 °C) or with PEG -6.7 MPa (72 h at 22 °C) before sowing and compared with unprimed (control) seeds. Each value is the mean of 10 replicates, and vertical bars represent standard errors. Bars with same letter are not significantly different according to SNK_{0.05}

embryo level (Table 4), it has to be noted that priming treatments had no significant impact on the cell cycle (Table 3). These data support the view of Gurusinge et al. (1999), who reported that DNA synthesis during seed priming is not essential for germination advancement. DNA replication during seed priming seems to be a species-dependent process in relation to endogenous ABA levels and/or sensitivity (Gendreau et al. 2008). In some cases, positive priming effects are related to DNA repair rather than DNA replication (Jisha et al. 2013; Varier et al. 2010). Some differences may even be recorded between cultivars from the same species, as recently demonstrated through the use of hydroxyurea (inhibitor of DNA synthesis) and cytochalasine-D (inhibitor of actin polymerisation) in two distinct cultivars of rapeseed (Pace et al. 2012). Hence, although beneficial effect of priming in white clover requires gene expression and protein synthesis, it does not require cell cycle activation in this species. Özbingöl et al. (1999) demonstrated that temperature plays a key role on the activation of the cell cycle during priming: it the present work, however, priming treatments were performed at 22 °C (with PEG) and at 10 °C (with GA₃), and in both cases, no impact of priming on cell cycle was detected in radicles.

4.2 Priming Treatments Improved Water Uptake

Our data suggest that improvement of germination in primed seeds could be linked to mechanical and physical effects of pre-treatment. Indeed, in all cases, primed seeds exhibited a faster imbibition in comparison with non-primed ones (Fig. 1), although pre-treated seeds were dried after priming to reach the same water content as non-primed seeds. It has already been reported that priming improvement of germination could be directly related to the modification of seed water relations (Bradford 1986; Nagarajan et al. 2005). Although water is known to enter the seed through the hilum and micropyle or through the strophiole in leguminous species

Table 6 Impact of seed priming treatment on chlorophyll fluorescence-related parameters recorded on seedlings of *Trifolium repens* maintained for 32 days on unpolluted soils (Corbais) or heavy metal-polluted soil (Prayon)

Soil	Priming treatment	F_v/F_m	Φ_{PSII}	q_P	NPQ
Corbais	Unprimed	0.83±0.01 a	0.80±0.01 a	1.01±0.04 a	0.21±0.01 a
	GA ₃	0.79±0.03 a	0.83±0.02 a	0.94±0.05 a	0.32±0.02 b
	PEG	0.80±0.02 a	0.80±0.04 a	1.00±0.03 a	0.24±0.00 a
Prayon	Unprimed	0.65±0.02 c	0.70±0.02 b	0.81±0.03 b	0.61±0.05 d
	GA ₃	0.72±0.02 b	0.71±0.01 b	0.94±0.01 a	0.48±0.03 c
	PEG	0.75±0.01 ab	0.78±0.03 ab	1.02±0.01 a	0.33±0.03 b

Seeds were primed with 0.1 mM GA₃ at 10 °C during 12 h or with PEG -6.7 MPa at 22 °C during 72 h. Each value is the mean of 10 replicates±SE. Means followed by the same letters are not significantly different according to SNK_{0.05}

(Garczarska et al. 2007; Martens et al. 1995; Pietrzak et al. 2002), we did not observe any particular modification of these structures after priming, despite a faster seed hydration (Fig. 2). SEM analysis clearly showed that primed seeds of *T. repens* exhibited seed coat tears and circular depressions (Fig. 2 F) that can favour seed imbibition. X-ray photographs also showed tissue detachment leading to free space, especially between the cotyledons and radicle (Fig. 3), which was never observed for control unprimed seeds. Our study therefore suggests that voids created inside the seed as a result of priming make water flow easier, thus contributing to tissue hydration. These observations support the view of Nagarajan et al. (2005) who hypothesised that better

performance of primed tomato seeds may be attributed to modifications of seed water-binding properties during imbibition. Similarly, it was demonstrated that PEG-primed seeds of *Lesquerella fendleri* displayed a higher ability to germinate under low water availability (Windauer et al. 2007). In a similar way, it was demonstrated in soybean that the cuticle of a permeable seed coat was mechanically weakened and developed small cracks through which water could pass (Ma et al. 2004). Beside structural changes, priming-induced modification in aquaporin gene expression may also be involved in the faster imbibitions of primed seeds (Chen et al. 2013). Thus, if germination in *T. repens* does not require cell division but only cell elongation of the root tip cell, it is conceivable that facilitation of water uptake should lead to a faster pressure potential-driven cell expansion, as suggested by the recorded decrease in T50.

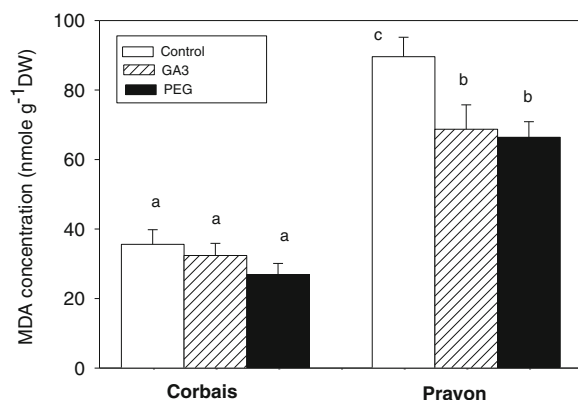


Fig. 6 Shoot malondialdehyde concentration in *Trifolium repens* plants grown in pots and harvested at day 32 after sowing on non-polluted Corbais or heavy metal-polluted Prayon soils. Seeds were primed either with 0.1 mM GA₃ (12 h at 10 °C) or with PEG -6.7 MPa (72 h at 22 °C) before sowing and compared with unprimed (control) seeds. Each value is the mean of 10 replicates, and vertical bars represent standard errors. Bars with same letter are not significantly different according to SNK_{0.05}

4.3 Priming Treatments Improved Germination and Seedling Performances on a Heavy Metal-Polluted Substrate

Priming treatments were shown to improve seed germination but also seedling emergence (Haigh et al. 1986), even under difficult environmental conditions such as abiotic stresses (Bradford 1986; Mihoub et al. 2005). According to Danneberger et al. (1992), differences between primed and unprimed material are more obvious under less favourable germination conditions than under optimal conditions. Our data agree with this view since no differences were recorded between seedlings issued from primed and unprimed seeds on Corbais soil: on this agricultural substrate with optimal level of available nutrients, priming indeed did not confer any advantage. However, the situation appeared quite

different for seedlings maintained on the highly contaminated Prayon substrate: in this case, both priming treatments significantly improved seedling emergence, plant growth and photosynthesis-related parameters and reduced oxidative damages comparatively to seedlings issued from unprimed seeds.

For unprimed seeds, the final percentage of seedling emergence (Fig. 4) was lower than germination rate recorded in the presence of high Cd or Zn doses (Table 2). This suggests that early seedling growth, taking place between germination *sensu stricto* and emergence, might be inhibited by heavy metals. It has, however, to be mentioned that Cd, Zn and Pb are simultaneously present in the Prayon substrate while the impact of heavy metal during germination was tested separately for Cd and Zn. It is still not easy to determine if several heavy metals present in the soil interact in a strictly additive way on the seedling behaviour. Copper is also present in Prayon soil: this essential element may become toxic at high concentration, and it produces numerous effects on germination, seedling growth and on photosynthesis (Mihoub et al. 2005). From a physiological point of view, the situation is rather complex and depends on numerous factors. Metal bioavailability is a crucial parameter influencing metal absorption (Lambrechts et al. 2011b). Bioavailable soluble metals are then transported to the seeds during the imbibition step, which is a passive process preceding metabolic reactivation of embryonic tissues. Such a passive process occurs mainly through the apoplasm: the subsequent distribution of heavy metals at this early developmental stage remains poorly studied, although their impact may differ according to the proportion of toxic elements reaching the symplasm, especially in the embryo. When radicle has protruded and functional seminal roots start to develop shortly after emergence, transpirational stream is induced and contributes to heavy metal translocation to the aerial parts of the young seedling. Jeliaskova et al. (2003) considered that the rate of seedling emergence on heavy metal-contaminated environment is a less reliable criterion than seedling growth for assessing metal tolerance in plants. Young seedling stage is indeed sensitive to ionic stress, which could be, at least partly, related to an incomplete endoderm development allowing important by-pass fluxes (Lefèvre et al. 2009). Conversely, heavy metal may compromise P nutrition (Bernal et al. 2007), and our work shows that both PEG and GA₃ priming contribute to maintain P uptake in young seedlings.

It is noteworthy that both priming treatments improved seedling emergence and seedling growth on polluted soils but that they did not impact plant height or leaf number, therefore suggesting that priming did not accelerate plant development. The main difference between the two tested priming treatments was related to Cd nutrition on the Prayon soil: GA₃ indeed increased Cd concentration while PEG treatment reduced it (Fig. 5). Gallardo et al. (2001) and Yacoubi et al. (2011) clearly demonstrated that priming induces the expression of a large set of genes and appearance of several proteins. Cadmium, which is a non-essential element, penetrates in plant cells through non-selective Ca channels, and it may therefore be hypothesized that GA₃ on the one hand and PEG on the other hand had an opposite effect on these targets during priming. Bush (1996) already demonstrated that GA₃ increases cytosolic calcium in aleurone cells, suggesting an improved activity and/or density of the transporters. Liu et al. (2010), however, demonstrated that PEG may also increase Ca²⁺ cytosolic concentration in roots through hyperpolarization-activated calcium permeable channels. Hence, the contrasting effect on GA₃ and PEG on shoot Cd concentration still needs to be explained. Since seedlings issued from GA₃- and PEG-primed seeds exhibited similar dry weight on Prayon soil (Table 5), it may be concluded that one type of priming may improve tolerance towards an accumulated phytotoxic elements while other types of priming treatments may induce an opposite strategy of avoidance resulting in a significant decrease in this element accumulation. Moreover, what is valid for one element is not necessarily valid for another since GA₃ and PEG triggered similar reduction in Zn accumulation by young plants.

Improved growth of white clover seedling as a consequence of priming may be directly correlated to improved photosynthetic properties and ability to cope with heavy metal-induced oxidative stress. Indeed, polluted soil strongly reduced the F_v/F_m values, thus suggesting a structural and functional perturbation of PSII while GA₃ and PEG priming obviously mitigated the deleterious impact of stress. Photochemical quenching increased in response to priming treatments, suggesting that the obtained seedling was able to trigger protective mechanisms allowing the maintenance of the electron transfer rate. Conversely, the stress-induced increase in NPQ, which might be considered as a strategy to avoid over-energization of photosynthetic apparatus, was less required in seedlings issued from primed seeds, as a

consequence of an improved ability to efficiently use the incident light energy.

Heavy metals also induce reactive oxygen species leading to oxidative stress in plant tissues. Reactive oxygen species are able to react with DNA, proteins and lipids and induce a wide range of structural and metabolic disorder. Kibinza et al. (2011) mentioned that priming treatment induces catalase synthesis by activating expression and translation of the H₂O₂-scavenging enzyme in sunflower. Similarly, Chen and Arora (2011) reported that osmopriming may strengthen the antioxidant system in *Spinacea oleracea*. Our data clearly demonstrated that MDA, a by-product of membrane lipids peroxidation, is drastically reduced in seedlings issued from primed seeds, thus suggesting that they were able to cope more efficiently than unprimed seedlings with secondary oxidative stress.

Taken together, our data suggest that optimal priming treatments may, in some cases, improve both germination and early seedling growth of white clover on heavy metal-polluted substrates. It should, however, be kept in mind that the recorded impact of priming treatment is valid only for a plant material–soil combination. Similar priming treatments than those used in the present study did not afford any advantage on white clover seedling maintained on other polluted substrates from the south-east of Spain (de Lespinay 2009). Therefore, a better comprehension of the molecular and biophysical mechanisms involved in priming treatments is still required.

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