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ORIGINAL ARTICLE

$CaCl_2$ treatment improves drought stress tolerance in barley (*Hordeum vulgare* L.)

Małgorzata Kaczmarek[1] · Olga Fedorowicz-Strońska[1] · Katarzyna Głowacka[1,4] · Agnieszka Waśkiewicz[3] · Jan Sadowski[2]

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Abstract Spring drought can adversely affect the productivity of barley (*Hordeum vulgare* L.) by reducing the yield. Because seed osmopriming can enhance crop productivity, we examined the potential of $CaCl_2$ treatment to improve drought tolerance in spring barley. Initially, we applied the priming procedure (5, 50, and 500 mM) to caryopses and assessed its effectiveness using a routine germination test, followed by measuring the level of divalent cations. Since drought adaptation is a complex phenomenon, we tested a comprehensive set of physiological parameters including (1) relative water content (RWC), (2) gas exchange parameters, and (3) photosynthetic pigments concentration in leaves of 3-week-old plants developed from the seeds subjected to osmopriming, followed by exposure to increasing water shortage. The plants were sampled at two selected time points, determined by soil moisture retention (pF = 3.6 and 4.2). The effect of $CaCl_2$ pretreatment was characterized in three distinct spring barley varieties, which differed in their response to drought stress (drought-tolerant Sebastian and Cam/B1/C1 and drought-susceptible Georgie), to assess potential interactions between osmopriming and genetically determined drought tolerance. Our results clearly demonstrate that $CaCl_2$ priming improves drought tolerance in stress-tolerant as well as drought-susceptible barley cultivars. Furthermore, we show that the beneficial effects of calcium preconditioning interact significantly with genetically determined drought tolerance.

Keywords Barley (*Hordeum vulgare* L.) · Drought tolerance · Osmopriming · Germination · Calcium chloride · Divalent ion content · Gas exchange parameters

Introduction

Drought is one of several environmental stresses that cause drastic changes in the growth, physiology, and metabolism of plants and as a result affect global grain production. There are various definitions of drought (Wilhite and Glantz 1985; Dracup et al. 1980), but with respect to agriculture and food production it can be defined as the situation where there is insufficient soil moisture to meet the needs of a particular crop at a particular time. Since 1980s, drought has become more frequent and intense (Food and Agriculture Organization of the United Nations [FAO (2012), http://www.fao.org/docrep/017/aq191e/aq191e.pdf]. It is most commonly associated with parts of the world such as Africa and Australia, but regional droughts in Europe are likely to become more severe and widespread (Olesen et al. 2011). Consequently, there is a pressing need for improvement in the adaptation of crop

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✉ Olga Fedorowicz-Strońska
ofed@igr.poznan.pl

1 Institute of Plant Genetics, Polish Academy of Sciences, Strzeszyńska 34, 60-479 Poznań, Poland

2 Department of Biotechnology, Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University, Umultowska 89, 61-614 Poznań, Poland

3 Department of Chemistry, Faculty of Wood Technology, Poznań University of Life Sciences, Wojska Polskiego 38/42, 60-637 Poznań, Poland

4 Present Address: Institute for Genomic Biology, University of Illinois, 1206W. Gregory Dr., Urbana, IL 61801, USA

species to drought stress so that high yield and quality can be maintained.

Seed priming is one of several approaches used to improve crop productivity (Bradford 1986). Priming refers to partial imbibition of seeds brought about, for example, by reducing the soaking time in water (hydropriming), using soaking solutions with low water potential (osmopriming or osmoconditioning), or by treating seeds with hormones (Chen and Arora 2013). Primed seeds initiate germination without radicle protrusion (Bradford 1986). The seed priming approaches listed above have been used to accelerate synchronized seed germination and the establishment of vigorous seedlings, and to stimulate vegetative growth and crop yield in many field crops including wheat (Iqbal and Ashraf 2007a), chickpea (Kaur et al. 2002) and cotton (Casenave and Toselli 2007). In addition, it has been reported that seedlings developing from primed seeds emerge faster, grow more vigorously, and perform better under diverse conditions such as drought and salinity stress (Harris et al. 2002; Farooq et al. 2009; Evangelina et al. 2011). However, the effectiveness of different priming agents is dependent on both the crop used and the stress conditions imposed (Iqbal and Ashraf 2005).

Seed osmopriming improves germination performance in various species such as *Brassica oleracea* (Soeda et al. 2005) and *Cucumis melo* (Farooq et al. 2007). Furthermore, in a range of field crops, including rice (Zheng et al. 2002; Basra et al. 2004; Farooq et al. 2006a), wheat (Nayyar et al. 1995) and sunflower (Kathiresan et al. 1984), osmopriming with calcium chloride solutions has proved effective in improving germination rate and stand establishment. Calcium ions have essential structural and signaling roles in all plants (Hirschi 2004) and are important for charge and/or osmotic balance (Leight et al. 1986). Ca^{2+} status influences plant productivity as well as tolerance to both biotic and abiotic stresses; thus, plants with calcium deficiencies are more susceptible to pathogens and osmotic stress (Bangerth 1979; Marchner 1995; Sanders et al. 1999; Knight 2000). Indeed, $CaCl_2$ has been shown to improve stress tolerance in many studies (Xiang et al. 2008; Jaleel et al. 2007; Upadhyaya et al. 2011; Ma et al. 2005; Xu et al. 2013).

Barley is one of the four main cereal crops globally, with 41% of worldwide production occurring in Europe, and is grown mainly for food and malting [FAO Report (2012), http://www.fao.org/docrep/017/aq191e/aq191e.pdf]. In western and central Europe, it is primarily grown as an annual crop of spring barley. Barley is highly appropriate for drought studies since, compared to winter cereals, such as winter wheat, it is extremely vulnerable to drought (Olesen et al. 2011). Drought can cause damage throughout the growing season, but for spring barley two time points are critical for crop yield: the spring, when the plants are at early vegetative stage, and the summer, when the fruit is developing and ripening (Arora et al. 1987; Petr 1987; Quiring and Papakryiakou 2003; Zimolka 2006; Trnka et al. 2007). Thus, drought in spring can kill seedlings, as their root systems are not fully developed, while drought in summer can reduce both the number of caryopses and their formation in the mature plants. Plant population reduction caused by spring drought cannot be compensated by increasing grain number in each ear as there is only one floret in each spikelet (HGCA (2016), The barley growth guide). Additionally, the number of spikes per plant is determined at an early developmental stage before the spike is completely formed (Houston et al. 2013; von Korff, Max Planck Research Report 2010). Drought may impair N uptake from fertilizers, which during early spring can further limit adequate ear number/m^2, an important yield component (HGCA (2016), The barley growth guide). Finally, drought leads not only to a decrease in yield, but also to a decrease in the variability of the yield response, as severe droughts impair spring barley production irrespective of soil conditions (Trnka et al. 2007).

The first line of defense against drought is the accumulation of osmolytes to reduce cell water loss and to maintain tissue turgor. Plants regulate water loss through precise control of stomatal closure. In addition, photosynthetic membranes are protected against reactive oxygen species (ROS), which are generated as a response to water potential decrease, by the production of carotenoids and decreased degradation process (Munne-Bosch and Alegre 2000). Prolonged drought causes the above defensive mechanisms to fail and consequently leads to impaired photosynthesis. As a result, plant growth and yield are further affected.

In this study, the potential benefit of $CaCl_2$ osmopriming on drought tolerance in spring barley was examined. Due to the complexity of drought adaptation, a comprehensive set of physiological parameters including (1) relative water content (RWC) and (2) gas exchange parameters were determined in plants developed from the seeds subjected to osmopriming followed by exposure to increasing water shortage. Given the relatively high diversity in drought vulnerability among the barley varieties, we might hypothesize that there are interactions between seed priming effects and genetically determined drought tolerance. Therefore, we also characterized the effects of $CaCl_2$ pretreatment on three distinct spring barley genotypes that respond differently to drought stress. We show that $CaCl_2$ osmopriming improves drought tolerance in stress-tolerant as well as drought-susceptible barley cultivars and that the beneficial effects of calcium preconditioning interact significantly with genetically determined drought tolerance.

Materials and methods

Plant material

Experiments were carried out on three spring barley (*Hordeum vulgare* L.) genotypes differing in the response to drought stress: the drought-tolerant varieties Sebastian and Cam/B1/C1 and drought-susceptible variety Georgie (seeds kindly provided by Prof. Andrzej Górny, Institute of Plant Genetics collection). Sebastian is a spring two-row malting type barley variety of Danish origin, widely cultivated in the Czech Republic (Nesvadba et al. 2008). Georgie is also a spring two-row cultivar of British origin released in 1975. It is a poor malting cultivar with tall straw, which is susceptible to mildew (Ellis et al. 1997). Cam/B1/C1 is a breeding line of *H. vulgare* originating from Syria, whose photosynthetic apparatus is well adapted to harsh environmental conditions (Górny 2001).

Seed pretreatment and osmopriming

The initial seed moisture content was 7.78, 8.77, and 8.95% (on a dry weight basis) for CamB1/C1, Georgie and Sebastian, respectively. The seeds were surface-treated with JOCKEY 201FS (BASF Crop Protection, Poland) for 5 min to avoid fungal invasion. The seeds were primed with three $CaCl_2$ (Sigma Aldrich) concentrations (5, 50, and 500 mM), alongside a water-treated control, for 24 h at 23 $\pm$ 2 °C with continuous shaking at 400 rpm. The ratio of seed weight to solution volume was 1:25 (g/ml). After osmopriming the seeds were given three surface washes with distilled water, allowed to slowly dry at RT to approximately 35.3–41.1% seed moisture content and then sown the next day.

Growth conditions

The plants were grown in a greenhouse in 1 dm^3 pots filled with a mixture of sand and soil (2:7 w/w; provided by the Institute of Soil Science and Plant Cultivation, Puławy, Poland). The following parameters were maintained: 23/14 °C day/night, 55% RH, and a photoperiod of 10 h during the growth stage. Initially, eight seeds were sown per pot and then thinned to five. Each parameter combination (genotype * seed treatment * drought vs. control * time point) was represented by five plants per pot. The entire experiment was performed with three replicates. The pots were watered and weighed every day and optimal soil moisture (8–12%), corresponding to a soil moisture retention (pF) between 2.4 and 3.0, was maintained. The pF curve was drawn for soil used in all experiments (kindly provided by Prof. Grzegorz Józefaciuk, The Bohdan Dobrzanski Institute of Agrophysics of Polish Academy of Sciences, Lublin, Poland) (Online Resource 3). It served to distinguish two stages of drought: mild at 3.6 pF, and severe (beyond permanent wilting point) at 4.2 pF. Three-week-old plants were exposed to drought stress by withstanding of water to ensure a water content corresponding to 3.6 or 4.2 pF over following 4 days.

Determination of ion content

Determination of ion content was performed on caryopses prepared as described above in 'Seed pretreatment and osmopriming' and on leaves of 3-week-old seedlings prepared as follows. The plants were grown in a growing chamber in 1 dm^3 pots filled with a mixture of sand and soil (2:7, w/w; provided by the Institute of Soil Science and Plant Cultivation, Puławy, Poland). The following parameters were maintained: 20/18 °C day/night, 55% RH and a photoperiod of 16 h during the growth stage. Initially, ten seeds were sown per pot and then thinned to five. Each parameter combination (genotype * seed treatment * drought vs. control * time point) was represented by five plants per pot. The entire experiment was performed with three replicates. The pots were watered and weighed every day to maintain optimal soil moisture (8–12%). Ca^{2+} ion content corresponding to pF $<$2.8 was determined to confirm Ca^{2+} uptake during osmopriming. Additionally, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} contents were measured to control for any adverse effects of osmopriming that altered ion balance in a non-specific fashion.

Ion content was determined as follows: approximately 0.5 g ($\sim$10 caryopses) was dried in an oven (Memmert) at 80 °C for 24 h, then crushed to powder in a homogenizer (TissueLyser, Qiagen) using grinding jars with stainless steel beads (Qiagen, cat. No. 69985, jars 10 ml, beads 20 mm). Leaf samples of approximately 0.5 g were flash frozen in liquid nitrogen and then ground in a mortar. Concentrations of ions were analyzed by flame atomic absorption spectrometry for Cu^{2+}, Fe^{2+}, Mn^{2+}, and Zn^{2+} and by atomic emission spectrometry for Ca^{2+} and Mg^{2+} (Agilent Technologies AA Duo-AA280FS/AA280Z spectrometer, Agilent Technologies, Mulgrave, Victoria, Australia). The Agilent spectrometer was equipped with one-elemental Varian hollow-cathode lamps (HCLs). Calibration curves were prepared before each analysis with four replicates per element concentration. All measurements were carried out in three independent experiments ($n = 30$).

Seed germination

Germination tests were performed on three replicates ($n = 150$) at 20 °C for 7 days with an optimal water regime as recommended by the International Seed Test Association (International Rules for Seed Testing Edition 2012, ISBN 978-3-906549-68-2). The number of germinated caryopses was evaluated daily. Caryopses were considered to have germinated when the radicle was 2 mm long. Seed germination parameters, such as mean length of incubation time (MLIT; Czabator 1962), percentage of maximum germination (amount of germinated seeds in relation to all seeds used to the test in a replicate), and percentage of normally developed seedlings (amount of normally developed seedlings in relation to all seeds used to the test in a replicate), were calculated.

Relative water content

Relative water content (RWC) was measured for the middle part of a fully developed second leaf of each plant assayed ($n = 7$) and was determined according to Barrs's formula (1968): RWC = [(FW − DW)/(TW − DW)] × 100%, where FW is fresh weight, DW is dry weight and TW is turgid weight. To measure TW, 3 cm long leaf sections were placed at 4 °C in the dark for 4 h in Eppendorf tubes containing distilled water to allow complete rehydration. Dry weight (DW) was measured after drying leaf sections in glass tubes for 24 h at 80 °C.

Gas exchange measurements

Gas exchange parameters were evaluated on plants developed from the seeds subjected to osmopriming with 50 mM $CaCl_2$ vs. H_2O (see "Seed pretreatment and osmopriming") and subjected to drought stress (see "Growth conditions") with three replicates ($n = 9$). Parameters were measured using an infrared gas analyzer (Ciras-2, PP Systems, Hitchin, UK) with a Parkinson leaf cuvette (PLC6; PP Systems, Hitchin, UK). In the leaf cuvette, the air temperature, air relative humidity, CO_2 concentration, and light were maintained at 23 °C, ambient humidity, 370 μmol CO_2 per mol of air, and 1000 μmol/m^{2}s, respectively. Gas exchange parameters included: photosynthetic rate (P_N; μmol CO_2/m^{2}s), transpiration rate (E; mmol H_2O/m^{2}s), stomatal conductance (G_S; mmol H_2O/m^{2}s), and intracellular CO_2 concentration (C_i; μmol CO_2/m^{2}s). Additionally, instantaneous water use efficiency (WUE_{inst}; μM CO_2/mM H_2O) as a ratio of P_N/E, and intrinsic water use efficiency (WUE_{intr}; μM CO_2/mM H_2O) as a ratio of P_N/G_S, were calculated. The measurements were performed on 25 × 7 mm areas of the middle parts of fully expanded third leaves.

Measurement of chlorophyll and carotenoid content

Chlorophyll and carotenoid content was measured in dimethyl sulfoxide (DMSO) (Merck) extracts. For extraction, 3 cm long sections were taken from the middle part of a second leaf of each plant assayed ($n = 7$) and incubated in 13 ml Falcon tubes with 5 ml DMSO at 60 °C for 4 h in dim light. All extracts were assayed in Shimadzu UV-1800 spectrophotometer (Shimadzu Scientific Instruments, Kyoto, Japan) for absorbance at 663, 652, 645, and 480 nm using 10 × 10 mm cuvette. Chlorophyll a and b content (mg g^{-1} fresh weight) were calculated from absorbance at 663 nm (A_{663}) and 645 nm (A_{645}), respectively, using the formula of Wellburn (1994):

Chl a = (12.19 A_{663}) − (3.45 A_{645}),

Chl b = (21.99 A_{645}) − (5.32 A_{663}).

Carotenoid concentrations (μmol g^{-1} fresh weight) were determined by the equation of Price and Hendry (1995):

$$\text{carotenoids} = [(A_{480} + 0.114 \times A_{663}) - (0.638 \times A_{645}) \times V/112.5 \times \text{gFW},$$

where V = volume of the sample (ml), A = absorbance and FW = fresh weight (g).

RWC, gas exchange parameters values, along with chlorophyll and carotenoid content, were presented as a drought susceptibility index: DSI (%) = (D/C) × 100%, where D and C represent the mean values of the parameters measured under drought stress and control conditions, respectively (Fisher and Maurer 1978; Rapacz et al., 2010).

Statistical analysis

Analysis of variance (ANOVA) was used to examine the differences between genotypes (CamB1/C1, Georgie, and Sebastian) and types of treatment (H_2O vs. $CaCl_2$ treatment) at each drought stage (mild vs. severe) with respect to drought-related traits (Gomez and Gomez 1984). Additionally, ANOVA was used to determine differences between genotypes for each treatment (three $CaCl_2$ concentrations and H_2O control) with respect to ion content and germination parameters. The differences between genotypes and treatments were estimated according to two-way ANOVA with Bonferroni's correction for multiple comparison at significance level $P < 0.05$ and $P < 0.01$ using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA; http://www.graphpad.com). The reported data are the mean ± standard error of the mean (SEM) of nine replicates, except for RWC and chlorophyll and carotenoid content measurements, which were performed on 7 and 15 replicates, respectively.

Results

Determination of optimal $CaCl_2$ concentration for osmoconditioning

Determination of divalent cation content

There was a positive correlation between Ca^{2+} content in caryopses and $CaCl_2$ concentration used for osmopriming

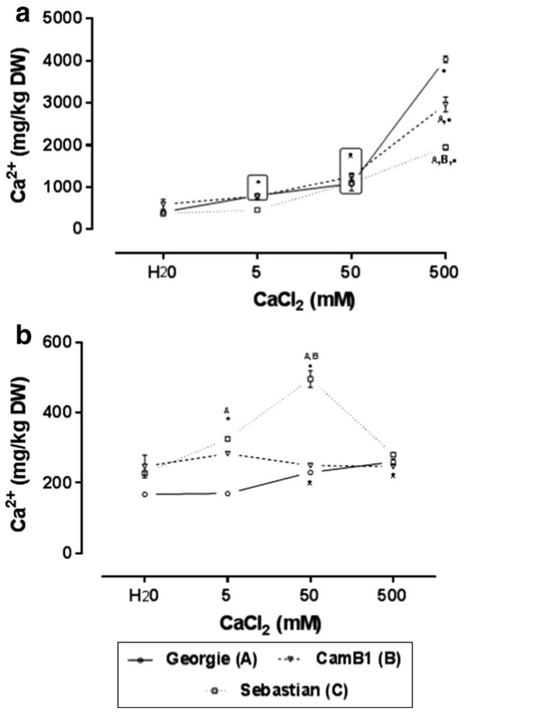

Fig. 1 Ca^{2+} ion content in barley caryopses (**a**) and leaves (**b**) relative to different $CaCl_2$ concentrations and H_2O-treated control. *Values* are mean ± SEM of three independent experiments ($n = 30$). *Letters* indicate genotype-dependent differences at $P \leq 0.05$. The remaining *symbols* designate the significance levels of the differences between control (H_2O) and $CaCl_2$-pretreated groups as $P \leq 0.05$ (*asterisk*) and $P \leq 0.01$ (*filled circle*), respectively

(Fig. 1). The prevalent elevation of Ca^{2+} content was recorded between treatments with 50 and 500 mM $CaCl_2$. A difference between the genotypes was noticeable only after priming with the highest concentration of $CaCl_2$. Measurement of other ion concentrations in barley caryopses, namely Fe^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, and Cu^{2+}, revealed differences between the varieties, but no statistically significant effects of seed pretreatment (Online Resource 1). All measurements were repeated on leaves of 3-week-old barley seedlings. A substantial elevation in Ca^{2+} content was observed only for the Sebastian genotype pretreated with 50 mM $CaCl_2$. Moreover, in this case, the increase in Ca^{2+} content was accompanied by simultaneous increases in Mg^{2+} and Zn^{2+} levels.

Germination test

The germination tests revealed marked differences among the untreated barley varieties, which constitute the reference groups for the assessment of preincubation with different doses of $CaCl_2$. Thus, Cam/B1/C1 clearly had the longest MLIT (Fig. 2a), while Georgie seeds germinated more efficiently (Fig. 2b) and with higher percentage of normal seedlings (Fig. 2c) than Sebastian and Cam/B1/C1. In general, a substantial improvement in all parameters was observed after pretreatment with 5 mM and 50 mM $CaCl_2$ (Fig. 2), with the most significant effect at the latter, higher concentration. By contrast, when the highest dose of $CaCl_2$ (i.e., 500 mM) was used, a reversal of this trend was observed, with a severe deterioration in germination parameters regardless of the barley cultivar used. The statistical significance of the effect of genotype and treatment on germination parameters was confirmed by two-way ANOVA for the whole experiment (Fig. 2).

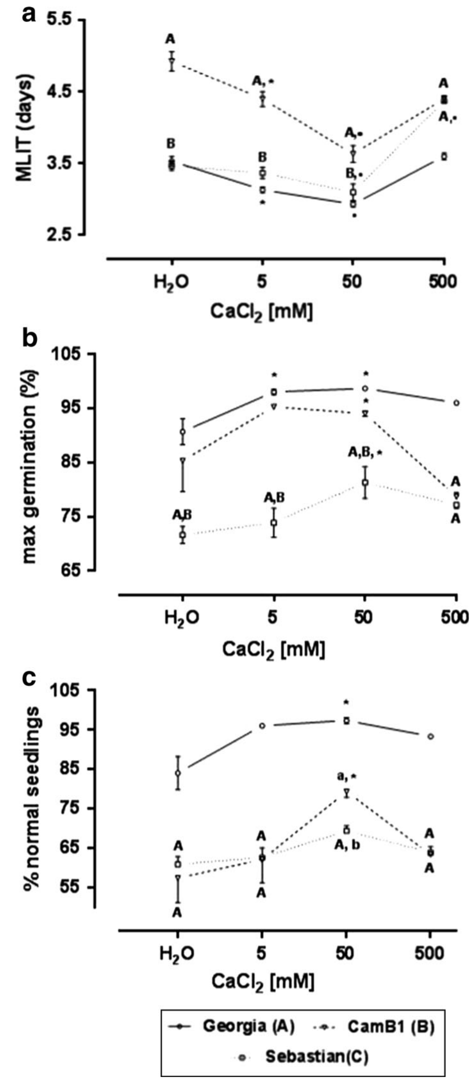

Fig. 2 Effect of $CaCl_2$ treatment vs. H_2O-treated control on: **a** mean length of incubation time in days; **b** maximum germination as percentage of germinated seeds; and **c** percentage of normally developed seedlings. *Values* are mean ± SEM of three replicates ($n = 150$). *Capital* and *small letters* indicate significant differences between genotypes within treatment type as $P \leq 0.01$, and $P \leq 0.05$, respectively. The remaining *symbols* designate the significance levels of differences between control (H_2O) and $CaCl_2$-pretreated groups at and $P \leq 0.01$ (*filled circle*) and $P \leq 0.05$ (*asterisk*), respectively

Based on the above results, osmoconditioning with 50 mM $CaCl_2$ was chosen for subsequent analysis. This concentration appears to be most beneficial with respect to germination parameters, regardless of genotype used (Fig. 2a–c). It was also the concentration that significantly raised Ca^{2+} content in seeds, independently of genotype (Fig. 1a). This decision was validated by assessment of, for example, the divalent cation content in the leaves of plants grown from pretreated seeds (Online Resource 2). We also took into account the effect of the $CaCl_2$ concentration on RWC values in plants subjected to progressive water deficit; osmoconditioning with 50 mM $CaCl_2$ gave the highest RWC (data not shown).

The effect of $CaCl_2$ osmopriming on drought-related traits during water restriction stress

Leaf RWC

Under conditions of cumulative water deficit, the DSI calculated for RWC in barley leaves reduced in response to drought intensity, but not in a linear fashion (Fig. 3a). A substantial drop in RWC was observed as a result of the transition to severe drought, but mild water stress induced only a limited change in this parameter. Of the three osmopriming pretreatments used, only 50 mM $CaCl_2$ resulted in a noticeable improvement in drought tolerance as measured by the RWC value and this effect was statistically significant for Sebastian and Georgie genotypes (Fig. 3a).

Gas exchange parameters

Drought gradually reduced all gas exchange parameters in all genotypes tested compared to the well-watered control (Fig. 3). The only exception was elevated intracellular CO_2 concentration recorded in $CaCl_2$ treated Sebastian genotype at mild drought stage (almost twice as high as the value recorded in control plants) (Fig. 3e).

A significant elevation of photosynthetic rate after priming was indicated for Sebastian and Cam/B1/C1 under severe drought conditions, while in contrast the Georgie genotype showed a substantial decrease on mild drought (Fig. 3b). However, after transition to severe water restriction, the difference in P_N value between $CaCl_2$-treated and control plants diminished.

A similar pattern of changes as an effect of osmoconditioning was observed for transpiration efficiency and stomatal conductance (Fig. 3c, d) with a substantial increase observed in Georgie and CamB1 in the former and Georgie and Sebastian in the latter. Under severe water deficit $CaCl_2$ treatment enhanced both parameters in Sebastian genotype.

Under mild drought conditions, osmoprimed CamB1 and Sebastian plants recorded increased levels of intracellular CO_2 concentration (Fig. 3e).

The patterns of change observed for WUE_{inst} and WUE_{intr} were very similar revealing substantial decrease of DSI values in $CaCl_2$-treated Sebastian plants for both stages of drought stress (Fig. 3f, g). Additionally, under mild drought conditions, the $CaCl_2$-treated CamB1 demonstrated reduced WUE_{inst} values than H_2O treated control plants.

Carotenoid and chlorophyll content

Drought decreased the content of chlorophyll (both a and b) and carotenoids for all genotypes and treatments used (Fig. 4a–c). Among H_2O-treated plants, the Sebastian genotype had the highest content of chlorophyll (both a and b) for both mild and severe drought, and of carotenoids for severe drought. Cam/B1/C1 had the lowest chlorophyll a and carotenoids content and Georgie had the lowest chlorophyll b content when subjected to severe drought. $CaCl_2$ treatment evoked a beneficial effect on all photosynthetic pigment contents in all tested genotypes, except for chlorophyll b content in the Cam/B1/C1 variety and chlorophyll a and b in Sebastian cultivar under mild drought.

Discussion

Determination of optimal $CaCl_2$ concentration for osmoconditioning

Primed seed germination

We observed significant reduction in percentage of maximum germination as an effect of $CaCl_2$ pretreatment that should allow roots to become established more rapidly during favorable sowing conditions (Kwon et al. 2009). We also demonstrated a distinct interaction between seed conditioning and barley genotype. To the best of our knowledge, this is the first report of such phenomena. The highest interaction value was observed for the MLIT parameter, which accounts for 10.75% of total variance. From this, we assume that, of all the tested barley varieties, the relatively drought-tolerant Sebastian, initially characterized as having the worst germination parameters, should benefit the most from $CaCl_2$ pretreatment of seeds.

Seed osmopriming with $CaCl_2$ are beneficial for other cereal crops including rice, in which emergence and seedling growth are enhanced (Farooq et al. 2006b). As a result, the concentration of K^+ and Ca^{2+} in both seeds and seedlings increases, while α-amylase activity is enhanced

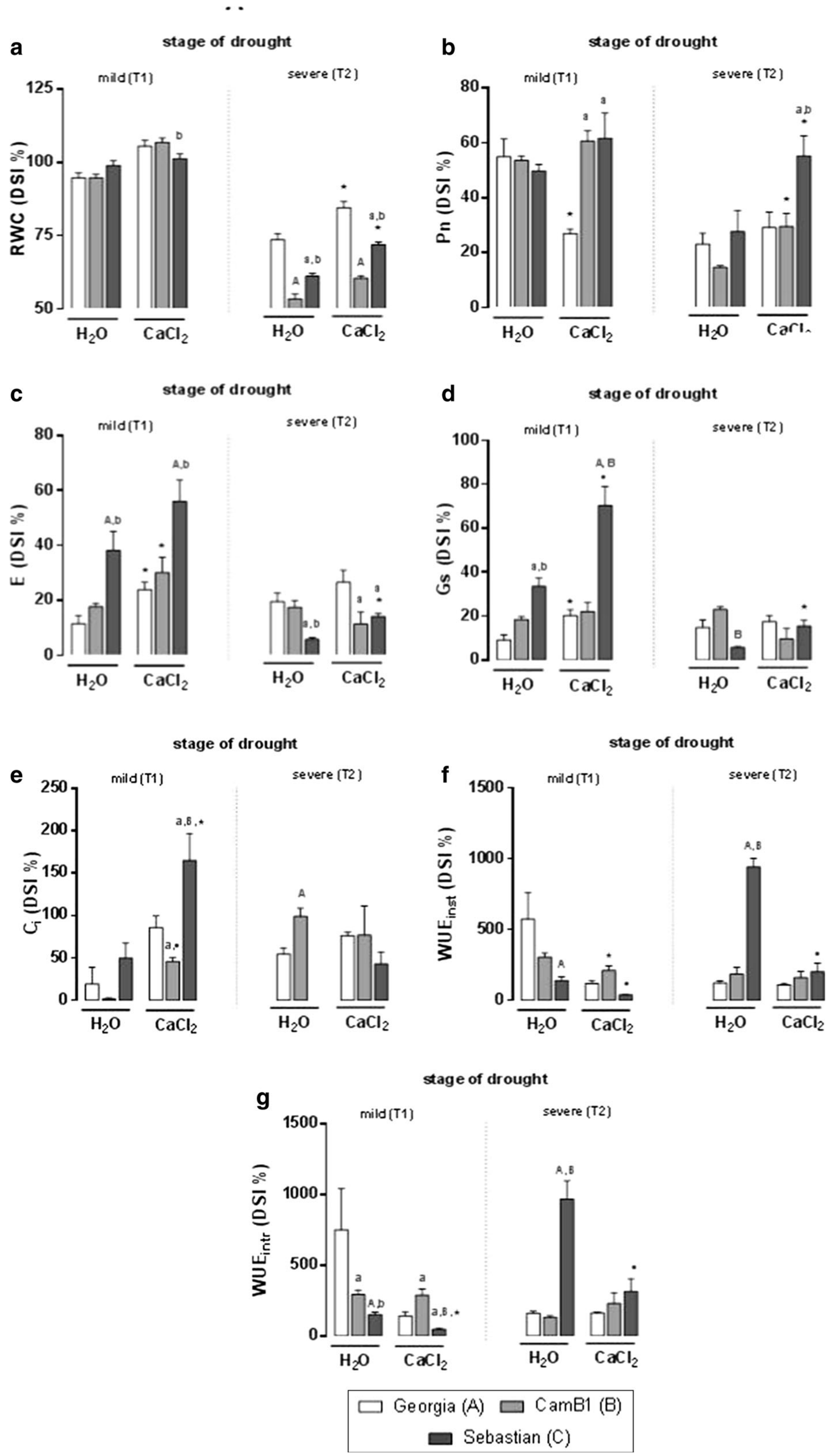

Fig. 3 Effect of 50 mM $CaCl_2$ on relative water content and gas exchange parameters in barley leaves of 3-week-old plants, developed from the seeds subjected to osmopriming, followed by exposure to increasing water shortage under two drought conditions, mild at pF = 3.6 and severe at pF = 4.2: **a** relative water content (RWC), **b** photosynthetic rate (P_N), **c** transpiration rate (*e*), **d** stomatal conductance (G_S), **e** intracellular CO_2 concentration (C_i), **f** instantaneous water use efficiency (WUE_{inst}), and **g** intrinsic water use efficiency (WUE_{intr}). *Vertical bars* are mean ± SD of $n = 9$. *Capital* and *small letters* indicate genotype-dependent differences for a given drought stage (mild or severe) at $P \leq 0.001$ or at $P \leq 0.05$, respectively. The remaining *symbols* designate the significance levels of differences between control (H_2O) and $CaCl_2$-pretreated groups for a given drought stage (mild or severe) at $P \leq 0.01$ (*filled circle*) and $P \leq 0.05$ (*asterisk*), respectively

and reducing sugar content enlarge (Farooq et al. 2006c; Jisha et al. 2013). The effects of $CaCl_2$ preconditioning in barley were described for the first time by Abdulrahmani et al. (2007), who reported, opposite to our findings, a failure in improving germination with $CaCl_2$. However, this disparity could be at least partially explained by the

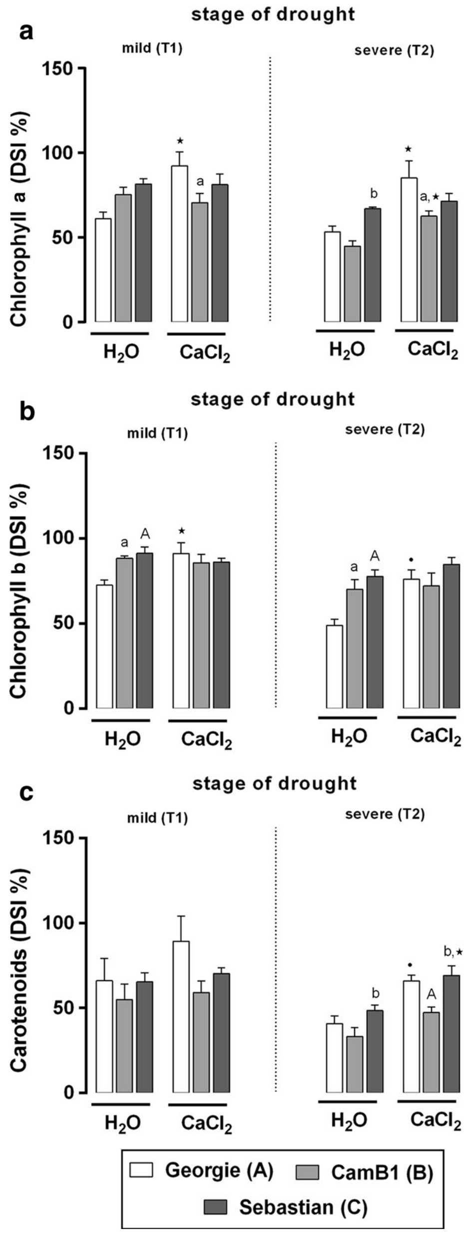

Fig. 4 Effect of 50 mM $CaCl_2$ on photosynthetic pigment content in barley leaves of 3-week-old plants, developed from the seeds subjected to osmopriming, followed by exposure to increasing water shortage under two drought conditions, mild at pF = 3.6 and severe at pF = 4.2: **a** chlorophyll a, **b** chlorophyll b, **c** carotenoids. *Vertical bars* are mean ± SEM of $n = 15$. *Capital* and *small letters* indicate genotype-dependent differences for a given drought stage (mild or severe) at $P \leq 0.001$ or at $P \leq 0.05$, respectively. The remaining *symbols* designate the significance levels of differences between control (H_2O) and $CaCl_2$-pretreated groups for a given drought stage (mild or severe) at $P \leq 0.01$ (*filled circle*) and $P \leq 0.05$ (*asterisk*), respectively

substantially different pretreatment conditions in our experiments, i.e., higher $CaCl_2$ concentration and a longer treatment time, along with genotype impact on $CaCl_2$ preconditioning efficiency. Nevertheless, our data have been definitively confirmed by several very recent reports (Jalilian et al. 2014; Esmaeili and Farahmanfar 2013; Tabatabaei 2013), in which a significant improvement in germination parameters using a similar seed preconditioning procedure also observed.

Divalent cation accumulation

We further characterized the preconditioning procedure by assaying the content of calcium and other divalent cations in pretreated barley seeds. We reported dose-dependent uptake of calcium by barley caryopses during preconditioning which has not been noted previously, along with significant differences in accumulated calcium between cultivars at the highest calcium dose used. Hence, these data may substantially add to the current understanding of mechanisms underlying the effects of calcium preconditioning.

Calcium plays a central role in many defense mechanisms that are induced by drought; these mechanisms can be either structural, for example, leading to increased cell wall integrity (Guimarães et al. 2011), or may elicit a signaling response (Xu et al. 2013). An increase in external Ca^{2+} could help to maintain an optimal K^+/Na^+ ratio in the cytosol by regulating K^+ transport across the plasma membrane (Iqbal and Ashraf 2007b; Laohavisit et al. 2013). Nevertheless, other divalent cations have similar effects (Shabala et al. 2005) and therefore we decided to verify whether $CaCl_2$ osmopriming could alter the content of Mg^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, and Fe^{2+}. Although each barley variety was characterized by a distinct pattern of ion content, no evidence for any pre-sowing related effects of divalent cations other than Ca^{2+} were observed.

Three weeks after the initial measurements in seeds, the assessment of divalent cations was repeated in leaves. Apart from a substantial elevation of Ca^{2+} content observed only in the Sebastian genotype pretreated with 50 mM $CaCl_2$, simultaneous increases in Mg^{2+} and Zn^{2+} were also reported. Both cations play a major role in calcium-related adaptation to drought. Mg^{2+}-ATPase is one of two systems involved in the active transport of Ca^{2+} across the plasma membranes of higher plants (Bonza et al. 2000) and Zn^{2+} acts as a cofactor for an enzymatic antioxidant defense mechanism (Chen and Arora 2013; Conrath 2006). Our study design did not allow us to clearly differentiate between osmotic and ionic effects of $CaCl_2$ preincubation. However, the apparent dose dependency of seed calcium content, as well as the sustained alteration in levels of some divalent ions (Ca^{2+}; Mg^{2+}; Zn^{2+}) in the leaves of plants developing from these seeds, suggests that the ionic effect predominates.

Effect of $CaCl_2$ treatment on barley drought stress tolerance

Treatment with 50 mM $CaCl_2$ had a significant impact on five and six out of ten physiological parameters evaluated under mild and severe drought conditions, respectively. During the initial stages of drought, $CaCl_2$ treatment exclusively influenced parameters relating to stomatal factors like G_S, E, C_i and both WUE_{inst} and WUE_{intr}. As drought progressed, $CaCl_2$ treatment modified P_N values along with the P_N derivatives, WUE_{inst} and WUE_{intr}. Moreover, photosynthetic pigment content was treatment-dependent at advanced drought time points, as revealed by ANOVA.

Preconditioning with $CaCl_2$ led to a genotype-dependent increase in RWC in Sebastian and Georgie cultivars exposed to severe drought. As both E and G_S values increased in $CaCl_2$-treated Georgie under severe drought conditions, stomatal factors responsible for higher tissue turgor can be excluded.

The highest chlorophyll and carotenoid contents after H_2O treatment at both drought stages were observed in the drought-tolerant Sebastian variety, reflecting results obtained by Guo et al. (2009). Nevertheless, drought-susceptible Georgie plants gained the most from $CaCl_2$ treatment, as they had the highest chl a content over the whole period of drought stress and during severe drought their chl b and carotenoid contents were almost equal to those of drought-tolerant Sebastian. Plants exposed to drought experience a decrease in photosynthetic activity and as a consequence an increase in excess energy, which leads to the formation of ROS (Chaves et al. 2009). Additionally, the increased carotenoid content observed both during mild and severe drought in $CaCl_2$-treated plants may serve as a line of defense against oxidative damage caused by ROS.

Drought stress significantly reduces photosynthetic rate (Chaves et al. 2009), whereas $CaCl_2$ pretreatment ameliorates this decrease (Iqbal and Ashraf 2007a). Here, we observed genotype-dependent changes in $CaCl_2$-treated plants as P_N increased in Cam/B1/C1 and Sebastian during mild drought and decreased in severe drought. In Georgie P_N decreased at mild drought and stayed at almost the same level in severe drought. The decrease of P_N in mild drought conditions was reported before in barley (de Mezer et al. 2014). The basis of the reduction in net carbon (P_N) efficiency during increasing water deficit has not been fully explained yet. As reported by Chaves et al. (2009), when water availability is low, diffusion of CO_2 through the stomata is reduced, and it apparently reduces mesophyll conductance to CO_2. Our data suggest that the phenomena can be affected by $CaCl_2$-treatment. Gas exchange parameters relating to stomatal factors were all negatively affected by $CaCl_2$ treatment at the initial stages of drought, and as drought progressed only Cam/B1/C1 gained from osmopriming. WUE_{inst} and WUE_{intr} values were highest for H_2O-treated Georgie during mild drought and Sebastian during severe drought. Cam/B1/C1 WUE_{intr} values were positively affected by osmopriming under severe drought conditions; the substantial increase recorded was due to a decrease in G_S values compared to the H_2O-treated control.

The physiological mechanisms through which plants respond to salinity and drought are very similar, suggesting that both stresses must be perceived by the plant cell as deprivation of water (Munns 2002). Both drought and salinity disturb the mineral-nutrient relations in plants through their effects on nutrient availability, transport, and partitioning. However, high salinity in comparison to drought deprivation includes both an ionic (chemical) and an osmotic (physical) component (Huang et al. 2012). Additionally, salinity stress also induces ion deficiency or imbalance due to the competition of nutrients such as K^+, Ca^{2+}, and NO_3^- with the toxic ions Na^+ and Cl^- (Hu and Schmidhalter 2005). Although Ca^{2+} is a key signal messenger for regulating plant resistance to both drought and salinity, the interaction between Ca^{2+} and each stress has been studied more intensively for salinity than for drought stress (Hu and Schmidhalter 2005).

Conclusion

This report details an experiment conducted on three barley varieties, in which differential drought responses were tested previously, and seeks to provide clarification on whether seed pretreatment with $CaCl_2$ results in better tolerance to drought stress. $CaCl_2$ pretreatment beneficial effect on drought stress adaptation in barley is manifested by: elevation of some divalent cations (Mg^{2+}, Zn^{2+}) which play a major role in calcium-related adaptation to drought, higher seedling vigor and maintenance of high leaf water potential which correlate with a better drought recovery and thus a better performance of plants at early season drought, higher transpiration rate without negative impact on turgor as revealed by water content in leaves, higher stomatal aperture, and accelerated intracellular CO_2 content resulting in increased carboxylation in tolerant genotype opposite to susceptible one. We conclude that $CaCl_2$ priming improves drought tolerance in several barley cultivars, including stress-tolerant and drought-susceptible varieties. Furthermore, our study shows that the beneficial effects of calcium preconditioning interact significantly with genetically determined drought tolerance.

Author contribution statement MK—planning, conducting experiments, preparing manuscript; KG—measurement of gas exchange parameters, participation in preparing manuscript; AW—measurement of ion content; OF-S—conducting experiments; JS—discussion of results and participation in preparing manuscript.

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