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Research article

Change in desiccation tolerance of maize embryos during development and germination at different water potential PEG-6000 in relation to oxidative process

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ABSTRACT

Desiccation tolerance is one of the most important traits determining seed survival during storage and under stress conditions. However, the mechanism of seed desiccation tolerance is still unclear in detail. In the present study, we used a combined model system, desiccation-tolerant and -sensitive maize embryos with identical genetic background, to investigate the changes in desiccation tolerance, malonyldialdehyde (MDA) level, hydrogen peroxide (H_2O_2) content and antioxidant enzyme activity during seed development and germination in 0, −0.6 and −1.2 MPa polyethylene glycol (PEG)-6000 solutions. Our results indicated that maize embryos gradually acquired and lost desiccation tolerance during development and germination, respectively. The acquirement and loss of desiccation tolerance of embryos during development and germination were related to the ability of antioxidant enzymes including superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11), catalase (CAT, EC 1.11.1.6), glutathione reductase (GR, EC 1.6.4.2) and dehydroascorbate reductase (DHAR, EC 1.8.5.1) to scavenge reactive oxygen species (ROS) and to control MDA content. Compared with treatment in water, PEG-6000 treatment could markedly delay the loss of desiccation tolerance of germinating embryos by delaying water uptake and time course of germination, increasing GR activity and decreasing MDA content. Our data showed the combination of antioxidant enzyme activity and MDA content is a good parameter for assessing the desiccation tolerance of maize embryos. In addition, H_2O_2 accumulated in mature embryos and PEG-treated embryos after drying, which was at least partially related to a longer embryo/seedling length in rehydration and the physiological mechanisms of priming.

1. Introduction

Desiccation tolerance is one of the most important traits determining seed survival during storage and under stress conditions. Orthodox seeds acquire gradually desiccation tolerance during development. After undergoing a maturation drying phase, the seeds pass into a metabolically inactive or quiescent state and could be stored for an extended period of time. Once these seeds germinate, desiccation tolerance is rapidly lost after only a few hours of germination [1]. During desiccation sensitive phase, re-drying may seriously impair subsequent germination and seedling establishment [2]. Conversely, recalcitrant seeds originated from tropical and subtropical plant species are characterized by the absence of maturation drying, and most of them have a high water content and active metabolism when they are shed from the mother plant. They are sensitive to drying and low temperatures, and quickly lose viability during storage [1,3]. Seeds of many important economical plants are recalcitrant, including many important tropical plantation crop species such as rubber and cocoa, tropical fruit crops such as mango, lychee and longan, and tropical timber species which belong to the families Dipterocarpaceae and Araucariaceae, etc. It is now difficult to find a suitable strategy to preserve these species' seeds. Thus, it is essential to understand the mechanisms of seed desiccation tolerance in more detail.

Abbreviations: APX, ascorbate peroxidase; AsA, ascorbic acid; CAT, catalase; DAP, days after pollination; DHAR, dehydroascorbate reductase; DW, dry weight; EDTA, ethylenediamine tetraacetic acid; EFGS embryo, embryo excised from germinating seeds; g g^{-1}, g H_2O g^{-1} DW; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; MGT, mean germination time; MDA, malonyldialdehyde; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NBT, nitroblue tetrazolium; O_2^-, superoxide radical; PEG, polyethylene glycol; PVPP, polyvinylpolypyrrolidone; ROS, reactive oxygen species; SOD, superoxide dismutase.

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Oxidative processes and free radicals are induced by a wide range of stresses, including dehydration [4]. Reactive oxygen species (ROS) cause lipid peroxidation, protein oxidation and DNA damage, all of which contribute to cell death [5,6]. It has been proposed that desiccation tolerance is associated with a capacity to effectively scavenge ROS because it involves increased antioxidant enzyme activities [7,8], such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) and dehydroascorbate reductase (DHAR). Antioxidant defense systems accumulate in orthodox seed when desiccation tolerance is acquired, and degrade when desiccation tolerance is lost [9,10]. The relationship between changes in ROS content and the acquisition and loss of seed desiccation tolerance has been reported in maize [6], silver maple [11], *Trichilia dregeana* [12], and wheat [13]. However, these studies were only involved in individual development or germination process, or in diverse species with different genetic background such as recalcitrant or orthodox seeds, which make understanding the mechanism of desiccation tolerance difficult.

The operation and controlling of hydration conditions might be an effective pathway to decrease the rate of desiccation tolerance loss of seeds during germination. Hydration under controlled conditions, achieved by incubation in solution containing an osmotic agent, causes the transient activation of antioxidant response and DNA repair systems that preserve genome integrity and seed quality. Primed seeds are subsequently re-dried for storage, distribution, and planting. This technique, known as osmopriming, is routinely used by seed companies and germplasm banks to improve seed vigor [14]. Polyethylene glycol (PEG) molecule with a $M_r \geq 6000$ (PEG-6000) is inert, non-ionic, and virtually impermeable chains that have frequently been used to induce water stress and maintain uniform water potential throughout the experimental period [15]. PEG-6000 is small enough to influence the osmotic potential, but large enough not penetrate membranes and stays in apoplast fluid [15]. Chen et al. [16] reported that PEG osmopriming enhanced desiccation tolerance of spinach seeds.

Germinating orthodox seed is sometime studied instead of recalcitrant seed because both are desiccation sensitive and have a high degree of subcellular development and metabolic activity [17]. In the present study, we used a combined model system, desiccation-tolerant and -sensitive maize embryos with identical genetic background, to investigate the changes in desiccation tolerance, malonyldialdehyde (MDA) level, hydrogen peroxide (H_2O_2) content and antioxidant enzyme activity during seed development and germination in PEG solutions with different water potentials. Our results indicated that the combination of antioxidant enzyme activity and MDA content might be a good parameter for assessing seed desiccation tolerance under drying stress. PEG treatment can delay the loss of desiccation tolerance of germinating maize embryos by delaying water uptake and time course of germination.

2. Results

2.1. Acquisition of desiccation tolerance during development

During seed development, the water content of embryos decreased from 3.28 g H_2O g^{-1} DW (g g^{-1}) at 24 days after pollination (DAP) to 1.32 g g^{-1} at 52 DAP; and dry weight of embryos gradually increased, for example, the mean dry weight was 2.0 mg $embryo^{-1}$ at 28 DAP and 15.8 mg $embryo^{-1}$ at 44 DAP (Fig. 1a). The maize embryos began to acquire desiccation tolerance (% survival) during 32–36 DAP (Fig. 1b), and their survival increased from zero at 28 DAP to 100% at 52 DAP. We have observed that embryo/seedling length produced by dried embryos significantly increased with increasing development periods of time. For example, seedling length produced by dried embryos at 44 and 52 DAP was 17.8 and 57.1 mm $embryo^{-1}$, respectively (Fig. 1b).

2.2. MDA and H_2O_2 content during development

Malonyldialdehyde is a product of lipid peroxidation, which has considerable potential to damage membranes and may be a principal cause of deterioration in orthodox seeds [5]. To test the relationship between lipid peroxidation and drying injury, we comparatively assayed the changes in MDA contents of fresh and dried maize embryos at different developmental stage. MDA content of fresh embryos gradually decreased from 24 (0.32 μmol g^{-1} DW) to 40 DAP (0.08 μmol g^{-1} DW), and then clearly increased from 40 to 52 DAP (0.24 μmol g^{-1} DW) (Fig. 2a). Compared with fresh embryos at different developmental stage, the MDA content of dried embryos decreased with seed development, and was lower than that of fresh ones at 24, 28, 48 and 52 DAP, respectively (Fig. 2a).

H_2O_2 is a ROS, their contents of fresh embryos markedly decreased with seed development (Fig. 2b). Drying decreased H_2O_2 content of embryos at 24 DAP and increased after 36 DAP as compared with fresh embryos at different developmental stage. We have noted that H_2O_2 contents of dried embryos have a little changes from 24 (1.06 μmol g^{-1} DW) to 52 (0.96 μmol g^{-1} DW) DAP (Fig. 2b).

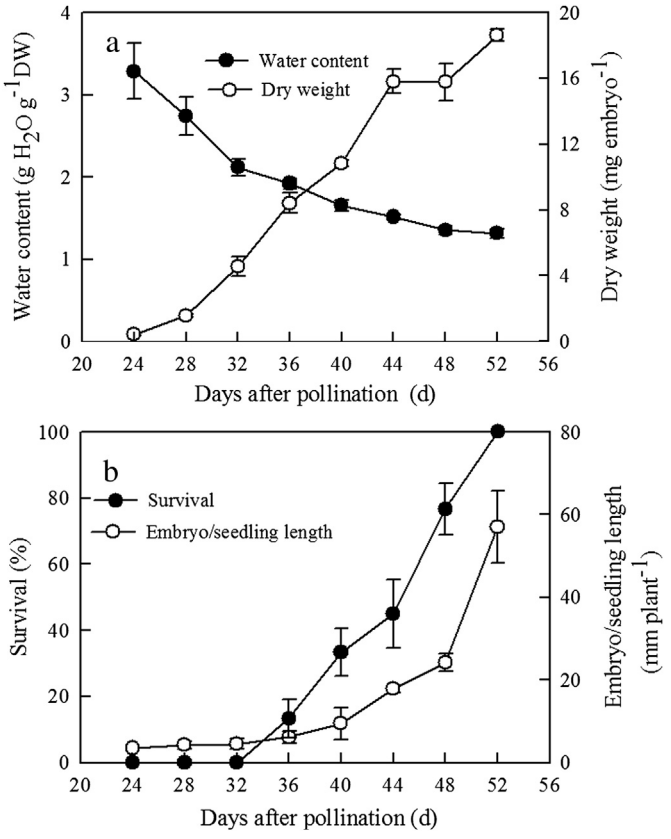

Fig. 1. Changes in water content and dry weight of embryos (a), and in survival and length of embryo/seedling of dried embryos (b) during maize seed development. a, After maize seeds at different days after pollination (DAP) were collected, and the embryos were immediately excised, and the water content and dry weight of fresh embryos were measured. b, The excised embryos at different DAP were dried to a water content of 0.07 ± 0.01 g g^{-1}, and then the survival of dried embryos and length of embryo/seedling produced by dried embryos were assayed. All values are means ± SD of three replicates of 20 embryos each.

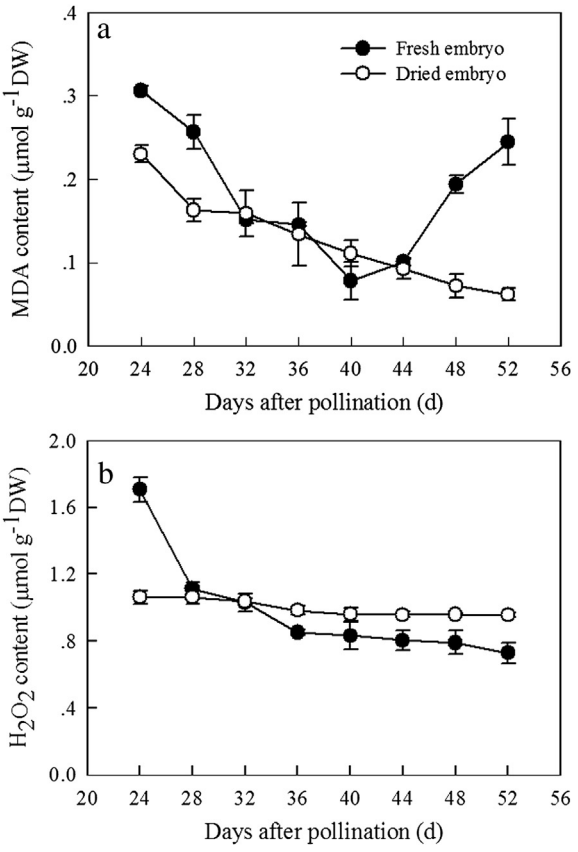

Fig. 2. Changes in MDA (a) and H_2O_2 (b) contents of fresh and dried embryos during maize seed development. The excised embryos at different time after pollination were dried to a water content of 0.07 ± 0.01 g g^{-1}, and the MDA and H_2O_2 contents of fresh and dried embryos were determined. All values are means ± SD of three replicates of 20 embryos each.

2.3. Antioxidant enzyme activity during development

SOD activity of fresh embryos increased from 7.6 units mg^{-1} protein at 24 DAP to 9.1 units mg^{-1} protein at 52 DAP (Fig. 3a). APX activity markedly decreased to 68.4% of its initial value at 52 DAP (Fig. 3b). CAT activity decreased during 24–36 DAP and then increased after 36 DAP (Fig. 3c). GR activity slightly decreased during development (Fig. 3d), and DHAR activity increased before 36 DAP and then decreased to 58% of its original value at 52 DAP (Fig. 3e).

Compared with fresh embryos at different developmental stage, SOD, CAT, GR and DHAR activities were enhanced by drying, but APX activity was not (Fig. 3). Furthermore, SOD, APX and DHAR activities of dried embryos decreased from 24 to 52 DAP, while CAT and GR activities increased (Fig. 3).

2.4. Loss of desiccation tolerance during germination

To investigate the effect of different water potential PEGs on the water uptake and germination of maize seeds, the dry seeds were germinated in 0 (water), −0.6 and −1.2 MPa PEG for different periods of time, respectively. The results showed that the water uptake rate of seeds was much faster in 0 MPa PEG than in −0.6 and −1.2 MPa PEG (Fig. 4a). For example, water contents of seeds germinated in 0, −0.6 and −1.2 MPa PEG for 72 h were 4.01, 1.24 and 0.82 g g^{-1}, respectively. And water content of seeds germinated in −0.6 and −1.2 MPa PEG for 216 h were still lower than that in 0 MPa PEG for 72 h (2.24 and 1.24 versus 4.01 g g^{-1}, respectively) (Fig. 4a). Moreover, PEG at −0.6 and −1.2 MPa obviously delayed germination of maize seeds. For example, germination of seeds in 0, −0.6 and −1.2 MPa PEG were 94.8, 64.5 and 2.0%, respectively, at 72 h of germination. And the germination of seeds in −0.6 and −1.2 MPa PEG at 216 h of germination were also lower than that in 0 MPa PEG at 72 h (82.9 and 51.7 versus 94.8%, respectively) (Fig. 4b). The mean germination time (MGT) of seeds was 35.8, 77.6 and 131.3 h in 0, −0.6 and −1.2 MPa PEG, respectively (Fig. 4b).

To test effect of re-drying on survival of embryo excised from germinating seeds (EFGS embryo), the dry maize seeds were germinated in 0, −0.6 and −1.2 MPa PEG for different periods of time, respectively, and then the embryos were excised and were re-dried to a water content of 0.07 ± 0.01 g g^{-1} and were re-germinated. It has been observed that the survival of re-dried embryos was dependent on PEG water potentials in which maize seeds were germinated and germination time (Fig. 5a). When seeds were germinated in 0 MPa PEG, the survival of re-dried embryos increased from 0 to 24 h of germination, and then decreased with increasing germination time. The optimal germination time in which desiccation tolerance of maize embryos was not lost by subsequent re-drying was about 24 h in 0 MPa PEG. When germinated in −0.6 and −1.2 MPa PEG, the survival of re-dried embryos markedly increased, especially in longer than 36-h germination as compared with germination in 0 MPa PEG. The promoting effect in −1.2 MPa PEG was larger than that in −0.6 MPa PEG when germination time was ≥56 h. For example, when germinated for 72 h, the survival of re-dried embryos was zero in 0 MPa PEG, while that was larger than 80% in −0.6 and −1.2 MPa PEG; and for 216 h, that was zero in −0.6 MPa PEG and was 2.5% in −1.2 MPa PEG (Fig. 5a). For desiccation tolerance of re-dried embryos, the optimal germination time was 24 h in −0.6 MPa PEG and was 24–72 h in −1.2 MPa PEG. We have also observed that except for 4 h germination, the changes in shoot length produced by re-dried embryos were in accordance with those in embryo survival, but changes in radicle length have a little difference. The radicle length produced by re-dried embryos gradually decreased with increasing germination time in 0 MPa PEG. The desiccation tolerance of shoots was larger than that of radicles (Figs. 5b and c).

2.5. MDA and H_2O_2 contents during germination

The MDA content of EFGS embryos and re-dried embryos increased with increasing germination time in 0, −0.6 and −1.2 MPa PEG solutions. For example, the MDA content of EFGS embryos increased by 228, 81 and 36% at 72 h of germination in 0, −0.6, and −1.2 MPa PEG, respectively, and re-drying slightly decreased MDA content. PEG at −0.6 and −1.2 MPa obviously decreased the MDA contents of EFGS embryos and re-dried embryos as compared with 0 MPa PEG (Figs. 6a-1, a-2 and a-3).

H_2O_2 content of EFGS embryos and re-dried embryos notably increased during germination in 0, −0.6 and −1.2 MPa PEG, especially in the early phase of germination. PEG at −0.6 and −1.2 MPa decreased H_2O_2 content of EFGS embryos and increased H_2O_2 content of re-dried embryos. For example, at 72 h of germination, H_2O_2 levels were 1.4 and 1.2 μmol g^{-1} DW in re-dried embryos treated with −0.6 and −1.2 MPa PEG, which were 1.8 and 1.6 times of re-dried embryos in 0 MPa PEG, respectively (Figs. 6b-1, b-2 and b-3).

2.6. Antioxidant enzyme activity during germination

To assay the effect of different water potential PEGs on antioxidant enzyme activity, we monitored the changes in SOD, APX, CAT, GR and DHAR activities of EFGS embryos and re-dried embryos during germination of maize seeds in 0, −0.6 and −1.2 MPa PEG

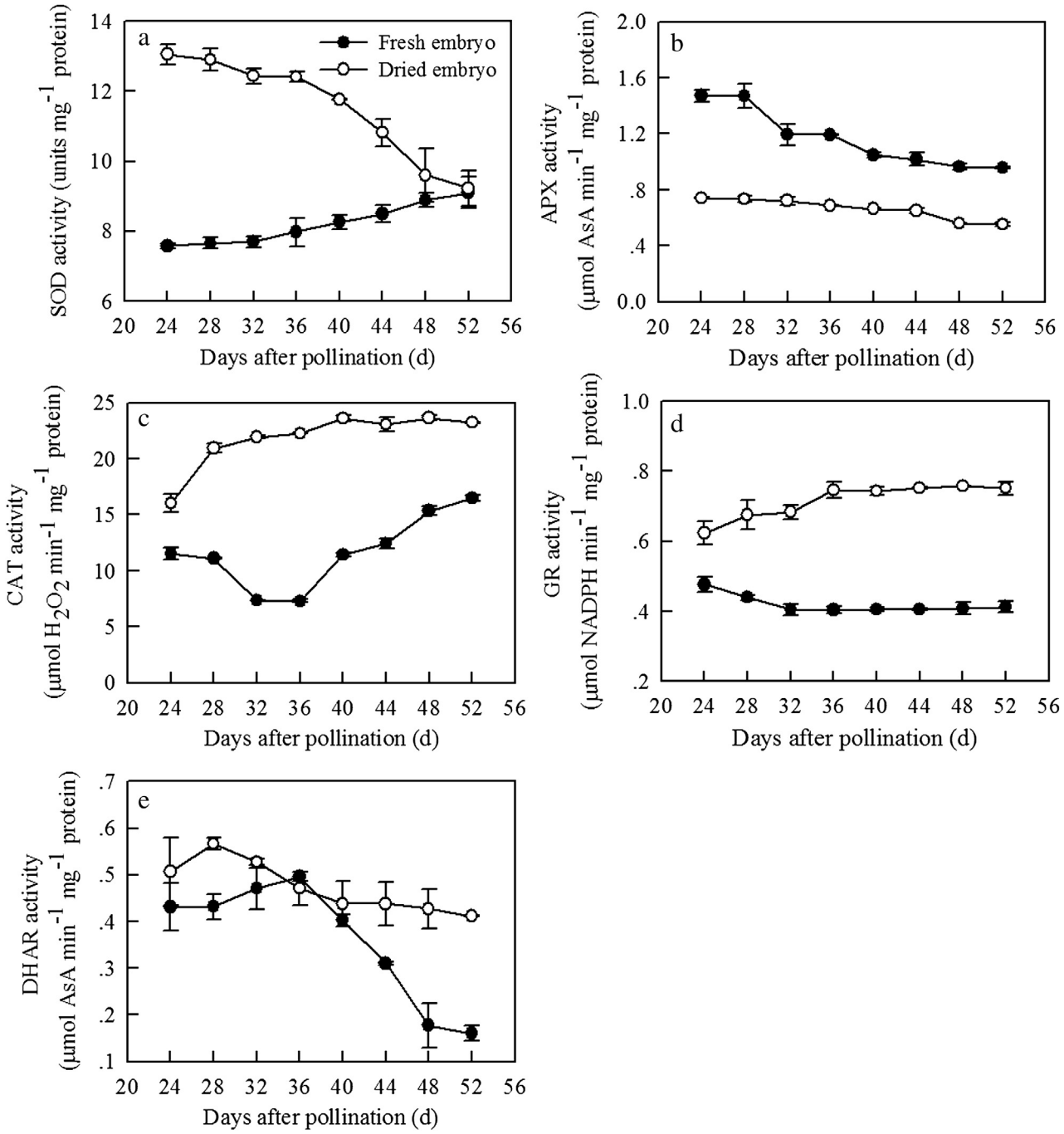

Fig. 3. Changes in activities of SOD (a), APX (b), CAT (c), GR (d) and DHAR (e) of fresh and dried embryos during maize seed development. The excised embryos at different time after pollination were dried to a water content of 0.07 ± 0.01 g g^{-1}, and these enzyme activities of fresh and dried embryos were determined. All values are means ± SD of three replicates of 40–50 embryos each.

(Fig. 7). The results showed that SOD, APX and CAT activities of EFGS embryos and re-dried embryos in 0, −0.6 and −1.2 MPa PEG, GR and DHAR activities of EFGS embryos and re-dried embryos in 0 MPa PEG and GR activities in −0.6 MPa PEG clearly increased with germination. And GR activities in −1.2 MPa PEG and DHAR activities in −0.6 and −1.2 MPa PEG decreased during early phase of germination and then increased (Fig. 7). We have also observed that these five enzyme activities in −0.6 and −1.2 MPa PEG were lower than those in 0 MPa PEG in the same germination time (Fig. 7).

As compared with EFGS embryos, re-drying decreased the activities of SOD, APX and CAT in 0, −0.6 and −1.2 MPa PEG and of GR in 0 MPa PEG. Interestingly, the activities of GR in −0.6 and −1.2 MPa PEG and of DHAR in 0, −0.6 and −1.2 MPa PEG were increased by re-drying (Fig. 7).

3. Discussion

3.1. Acquisition of desiccation tolerance of embryos during development in relation to changes in MDA and H_2O_2 content and antioxidant enzyme activity

Desiccation tolerance of maize embryos was assayed by assessing their capability to germinate and produce seedlings after drying. As in other species such as bean [10], soybean [18] and

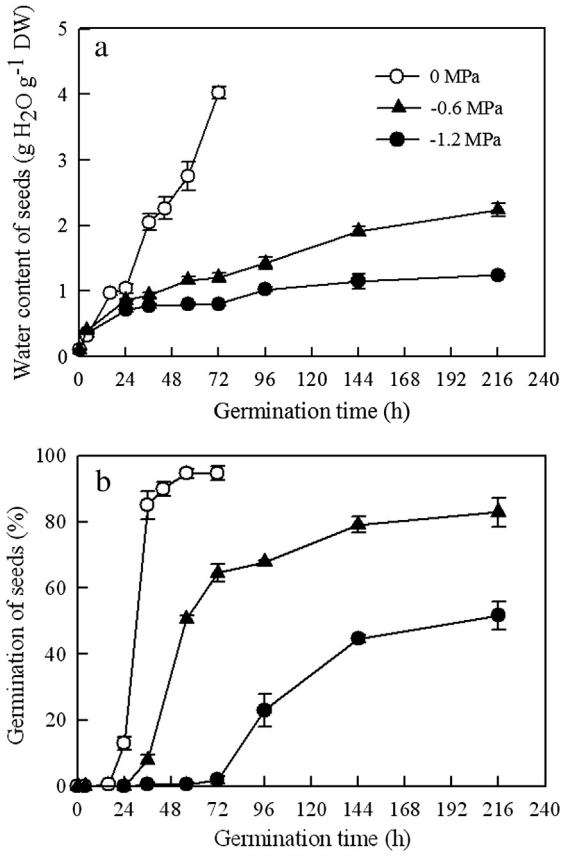

Fig. 4. Changes in water content (a) and germination percentage (b) of maize seeds during germination in different water potential PEG-6000 solutions. Seeds were germinated in 0, −0.6 and −1.2 MPa PEG-6000 solutions for indicated periods of time, and then water content and germination percentage of seeds were measured. All values are means ± SD of three replicates of 50 seeds each.

lupine [19], maize embryos started to acquire desiccation tolerance at 32–36 DAP, and completely acquired desiccation tolerance at 52 DAP (Fig. 1).

Dehydration usually induces oxidative processes and subsequent free radical production [4]. In non-photosynthetic organs like seeds, ROS are mainly generated through mitochondrial activity and electron leakage [20]. It has been suggested that ROS production increased during the desiccation-intolerant stage of seed development and germination in which water content and metabolic activity of seeds were relative higher and dehydration could disrupt cellular membranes [21], thereby increasing electron leakage. Leprince et al. [22] proposed that ROS production decreased when respiration is reduced, as is the case during maturation drying of orthodox seeds. Therefore, shutting down of respiration could decrease drying injury [6,7,17]. Vertucci and Farrant [1] reported that mitochondrial respiration decrease when water content is lower than 0.25 g g^{-1} and increase with increasing water content above 0.25 g g^{-1}.

MDA is formed by the reaction of ROS with lipid molecules of tissues, as an indicator of lipid peroxidation [23]. MDA is also toxic for biomacromolecule, and is usually used as a parameter of cell damage [5,12]. MDA content of fresh embryos at 24 to 40 DAP and of dried embryos at 24 to 52 DAP decreased (Fig. 2a). These results were consistent with decrease in water content of fresh and dried embryos during development (Fig. 1a). It is noted that the decrease in water content of embryos is the result of dry matter accumulation, and has not a direct relationship with the decrease of MDA. Wu et al. [24] reported that respiratory rate of mitochondria of maize embryos decreased with seed development. Such decrease in MDA contents (Fig. 2a) might be a result of respiratory rate decrease, which might be also associated with metabolic shutdown. In addition, we have observed that respiration rate of embryo decreased with seed development (data not shown). However, to our knowledge, we cannot explain the reason why MDA content increased from 40 to 52 DAP, which remains to further study. Walters et al. [25] suggested that at intermediate water content, uncontrolled oxidative reactions that are dependent upon metabolism can occur, leading to desiccation damage. In the present study, drying of maize embryos was rapid by placing them over activated silica gel, these embryos spent insufficient time at intermediate water content for damage consequent upon the deleterious aqueous-based reactions to accumulate [8,18]. Therefore, we have observed that MDA contents of dried embryos were lower than those of fresh ones at 24, 28, 48 and 52 DAP, respectively (Fig. 2a). Moreover, drying could change cell permeability, thereby some MDAs were leaked out from the embryos because of volatility of MDA [8]. H_2O_2 content of fresh embryos markedly decreased from 24 to 52 DAP (Fig. 2b), which might be also related with decrease in respiration caused by metabolic shutdown. Rolletschek et al. [26] suggested that most parts of maize kernels are subjected to low oxygen during development. The low oxygen condition strongly inhibits mitochondrial respiration because NADH oxidation along the electron transport chain is blocked when the final acceptor, oxygen, is missing. Although MDA and H_2O_2 contents on a dry weight basis were widely accepted [27,28], the expression may be a misleading, because of the changes in dry weight of seeds with development. The same can be attributed to seed germination, when dry weight decreases due to storage substances mobilization [29].

Antioxidant enzymes are an important pathway of ROS detoxification. Activities of SOD and CAT increased in fresh maize embryos with development (Fig. 3), showing that acquirement of desiccation tolerance was related with the increase of SOD and CAT activity. Compared with fresh embryos at different developmental stage, drying increased activities of SOD, CAT, GR and DHAR. Furthermore, the activities of CAT and GR increased with development age (Fig. 3). Although activities of SOD and DHAR of dried embryos decreased from 24 to 52 DAP, their activities were still higher than those of fresh embryos (Fig. 3). These results indicated that increase in CAT and GR activities might be one of reasons for increasing desiccation tolerance, and were in agreement with the finding of Bailly et al. [10] who found that desiccation-tolerant dried seeds have high CAT and GR activities.

3.2. Loss of desiccation tolerance of embryos during germination in relation to changes in MDA and H_2O_2 content and antioxidant enzyme activity

Mature dry seeds contain mitochondria with poorly differentiated inner membranes and functional enzymes necessary for respiration such as those of the Krebs cycle, and terminal oxidases. An early event during phase I imbibition is the resumption of energy metabolism [30]. With increasing water uptake and germination percentage of mature dry seeds (Fig. 4), MDA and H_2O_2 contents (Figs. 6a-1 and b-1) and SOD, APX, CAT, GR and DHAR activities (Figs. 7a-1, b-1, c-1, d-1 and e-1) of germinating maize embryos rapidly increased, indicating that germinating maize seeds have high metabolic activity.

Desiccation tolerance of embryos was usually expressed in survival percentage and/or seedling growth from embryos, which were excised from germinating seeds, re-dried, and then allowed to germinate again. The survival and shoot length (besides 4 h-germination) of re-dried embryos increased from 0 to 24 h of germination, and decreased after 36 h of germination (Figs. 5a and c). We inferred that before and a few hours after radicle protrusion

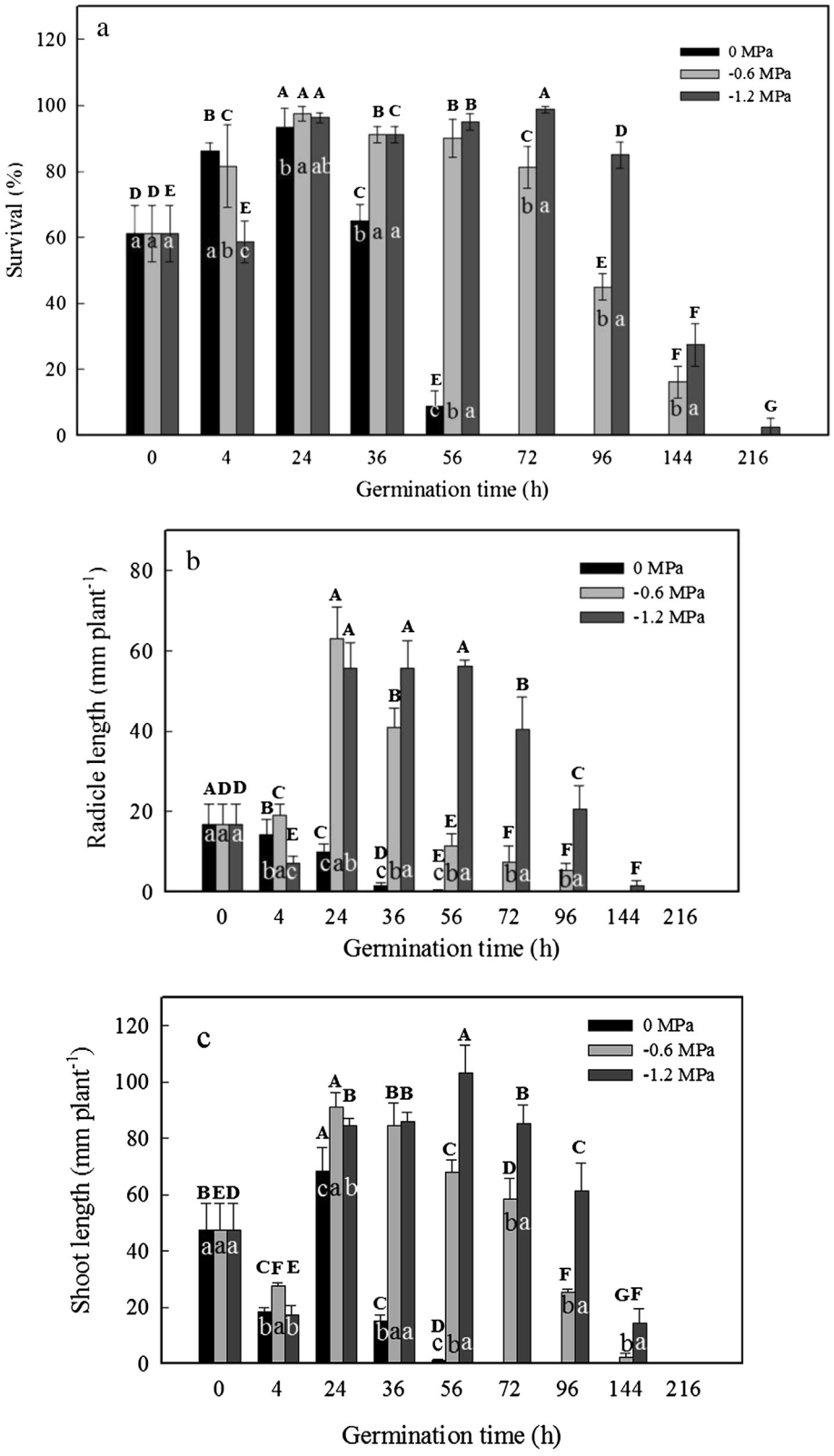

Fig. 5. The response of embryos excised from germinating maize seeds (EFGS embryos) incubated in different water potential PEG-6000 solutions to re-drying. After seeds were germinated in 0, −0.6 and −1.2 MPa PEG-6000 solutions, respectively, for indicated periods of time, the embryos were excised and re-dried to a water content of 0.07 ± 0.01 g g^{-1}, and then survival of re-dried embryos (a) and radicle (b) and shoot (c) length produced by re-dried embryos were determined at 5th day of germination of embryos. All values are means ± SD of three replicates of 20 embryos each. Bars with different uppercase letters are significantly different within re-dried embryos germinated at same water potential PEG and different germination time, and those with different lowercase letters are significantly different within re-dried embryos germinated at same germination time and different water potential PEGs (Student's *t*-test, $P < 0.05$).

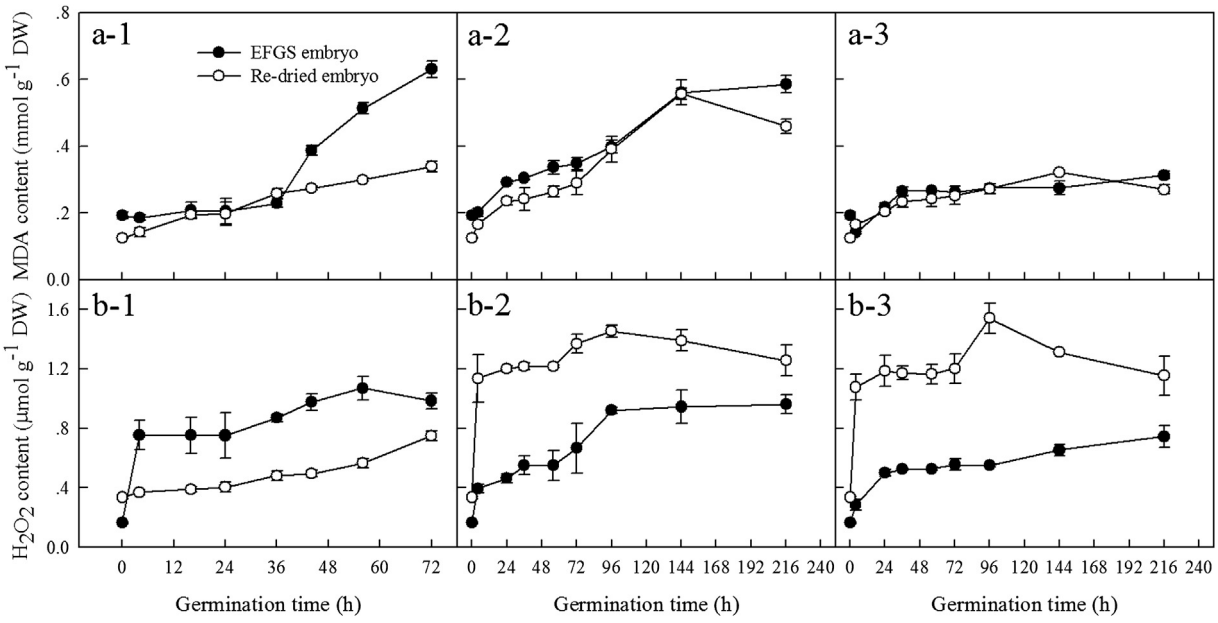

Fig. 6. Effect of PEG-6000 treatment on MDA (a-1, 2, 3) and H_2O_2 (b-1, 2, 3) contents of embryos excised from germinating seeds (EFGS embryos) and re-dried embryos. After maize seeds were germinated in 0, −0.6 and −1.2 MPa PEG-6000 solutions, respectively, for indicated periods of time, the embryos were excised and re-dried to a water content of 0.07 ± 0.01 g g^{-1}. And then MDA and H_2O_2 contents of EFGS embryos and re-dried embryos were measured. 1, 2 and 3 indicate that maize seeds were germinated in 0, −0.6 and −1.2 MPa PEG-6000 solutions, respectively. All values are means ± SD of three replicates of 20 embryos each.

(0–24 h of germination), the increasing antioxidant enzymes (Fig. 7) could effectively scavenge ROS (Fig. 6a-1) and made those embryos desiccation-tolerant (Fig. 5). After 36 h of germination, the loss of desiccation tolerance might be related to an obvious decrease of SOD and CAT activity and a notable increase of MDA content (Figs. 6a-1 and 7a-1 and c-1). Sun [31] reported that the desiccation tolerance loss of *Vigna radiata* seeds was correlated with the resumption of active growth of embryonic axes and that was rapidly increased with an increasing germination time. In addition, we have observed that desiccation tolerance of maize embryos during germination was gradually lost, which is in agreement with the hypothesis that desiccation tolerance is a quantitative feature [1] rather than an all-or-nothing event [32].

3.3. Effect of PEG treatment on desiccation tolerance, MDA and H_2O_2 content and antioxidant enzyme activity of germinating embryos

The physiological and biochemical mechanisms of priming have been exploited to accelerate synchronized seed germination, promote vigorous seedling establishment, and stimulate vegetative growth [14]. Compared with maize seeds germinated in 0 MPa PEG, water uptake and germination of seeds in −0.6 and −1.2 MPa PEG were clearly delayed (Figs. 4a and b), which delay the loss of desiccation tolerance of maize embryos by preventing radicle protrusion, as indicated by survival and radicle and shoot length (Fig. 5). These results suggested that increase in desiccation tolerance of maize embryos might be owing to a decreasing metabolic activity by PEG treatment.

Compared with treatment in water, PEG at −0.6 and −1.2 MPa decreased the MDA content in EFGS embryos and re-dried embryos and H_2O_2 content in EFGS embryos (Fig. 6). This might be due to the reduced metabolism activity of maize embryos germinated in −0.6 and −1.2 MPa PEG. Interestingly, re-drying decreased H_2O_2 content of maize embryos germinated in 0 MPa PEG, and increased H_2O_2 content in −0.6 and −1.2 MPa PEG (Figs. 6b-1, b-2 and b-3). Although the cause of these results is unclear, they at least were partially related to a longer embryo/seedling length in re-germination and the physiological mechanisms of priming. The faster seedling growth of primed seeds related to increased rate of seed germination after priming. Many studies indicated that H_2O_2 can lead to cell-wall modification required for radicle elongation and promotes seed germination [33,34].

The activities of SOD, APX, CAT, GR and DHAR of EFGS embryos were decreased by −0.6 and −1.2 MPa PEG as compared with germination in 0 MPa PEG (Fig. 7), showing that PEG treatments decrease metabolic activity of germinating maize embryos. A clear increasing GR activity by −1.2 MPa PEG (Fig. 7d-3) may be related with increase in desiccation tolerance of maize embryos. It has been reported that there is a close relationship between GR activity and drought tolerance [35], and GR promotes glutathione transferase accumulation which contributes to tolerance against osmotic stress in wheat [36]. The reduced metabolism activity and decreased ROS content make the increasing antioxidant enzymes effectively scavenge ROS, which led to the higher survival of PEG-treated embryos than that of water-treated embryos. However, prolonged exposure to PEG-6000 would increased subsequent drying injury (Fig. 5), which would irreversibly affect seed viability and lead to unpredictable desiccation sensitivity due to an increasing oxidative DNA damage [37].

In conclusion, drying stress leads to ROS production and lipid peroxidation. Low levels of ROS induce antioxidant enzyme expression as a stress signal, which can function cooperatively to control ROS overproduction and avoid oxidative damage. However, once ROS is massively produced (e.g., when radicle protrusion), antioxidant enzyme activity cannot effectively scavenge ROS, which disrupts metabolic balance and leads to membrane lipid degradation and cell death. Membrane damage is the main reason for the loss of the germination capacity during desiccation [11]. Desiccation-tolerant seeds, such as those seeds at late development stage and treated by PEG, have low metabolic activity including low ROS content and antioxidant enzyme activity. This might imply an important survival strategy that may preserve soluble carbohydrates, which helps seeds to avoid carbohydrate starvation and energy-consumption [38]. Drying increase the SOD, CAT, GR and DHAR activities and decrease MDA content of desiccation-tolerant embryos, and decrease the SOD and CAT activities of desiccation-

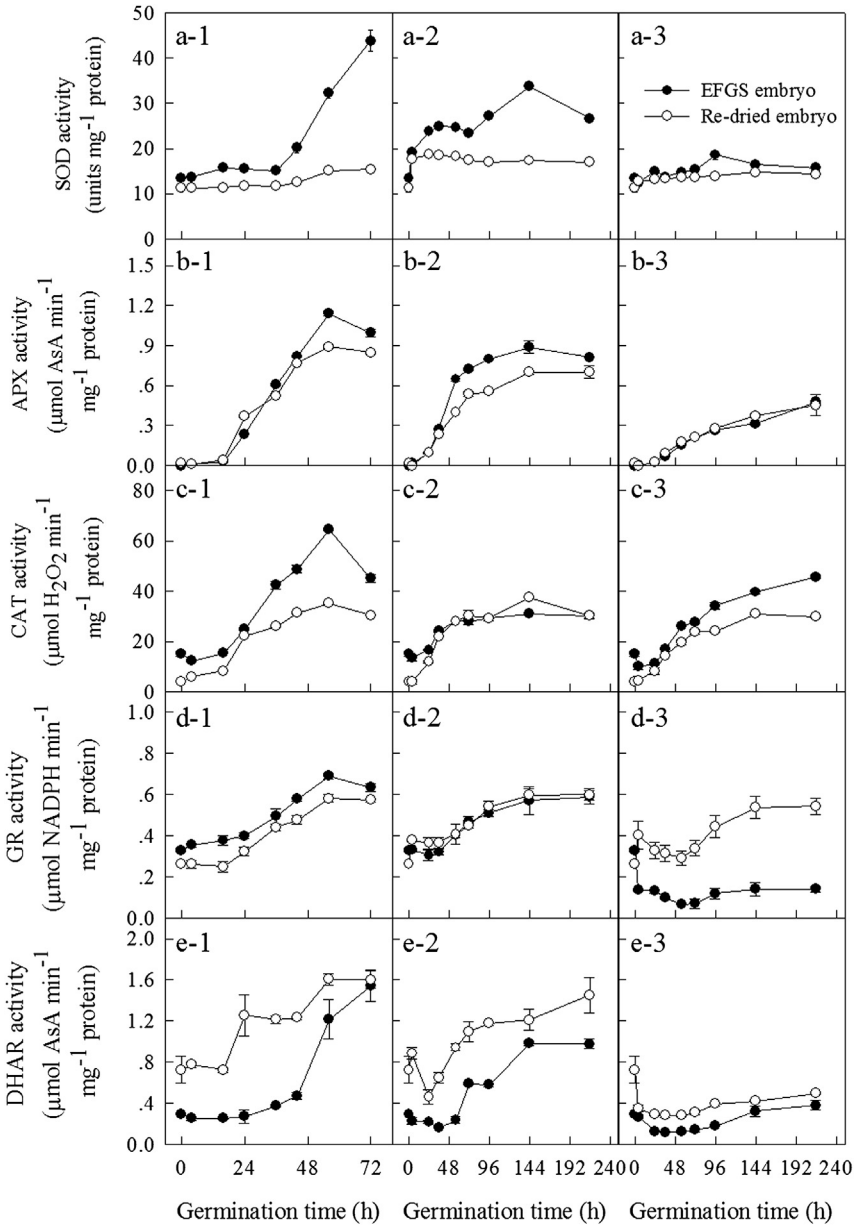

Fig. 7. Effect of PEG-6000 treatment on SOD (a), APX (b), CAT (c), GR (d) and DHAR (e) activities of embryos excised from germinating seeds (EFGS embryos) and re-dried embryos. After maize seeds were germinated in 0, −0.6 and −1.2 MPa PEG-6000 solutions, respectively, for indicated periods of time, the embryos were excised and re-dried to a water content of 0.07 ± 0.01 g g^{-1}. And then these enzyme activities of EFGS embryos and re-dried embryos were assayed. 1, 2 and 3 indicate that maize seeds were germinated in 0, −0.6 and −1.2 MPa PEG-6000 solutions, respectively. All values are means ± SD of three replicates of 40–50 embryos each.

intolerant (geminated in water) embryos. The combination of antioxidant enzyme activity and MDA content is a good parameter for assessing the desiccation tolerance of maize embryos. PEG treatment can clearly delay the loss of desiccation tolerance of germinated maize embryos by delaying water uptake and time course of germination, increasing GR activity and decreasing MDA content. It is noted that PEG treatment can also induce the expression of late embryogenesis abundant genes and proteins, thereby increase the desiccation tolerance of germinating seeds [39].

4. Materials and methods

4.1. Plant material

Maize (*Zea mays* L. 'Nongda 108') seeds were purchased from Beijing Baofeng Seed Co., Ltd (Beijing, China). To analyze acquisition of desiccation tolerance during development of maize embryos, maize seeds were manually collected at different DAP from plants growing in Xishuangbanna Tropical Botanical Garden in July 2007. After excision from seeds, some of fresh embryos were immediately frozen in liquid nitrogen for enzyme analysis, the others were dried to a water content of (0.07 ± 0.01) g g^{-1} and then were either frozen rapidly in liquid nitrogen for enzyme analysis or germinated for measuring survival (Fig. 8).

For analysis of change in embryo desiccation tolerance during germination of maize seeds, mature dry seeds were surface sterilized in 0.1% (w/v) $HgCl_2$ for 3 min, and then rinsed three times in sterilized water. These seeds were germinated in 0 (water), −0.6 and −1.2 MPa PEG, respectively, at 25 °C and in the dark. After germinated for different periods of time, the embryos were excised from the seeds. Some of fresh embryos were frozen rapidly in liquid nitrogen for enzyme analysis, the others were re-dried to a water

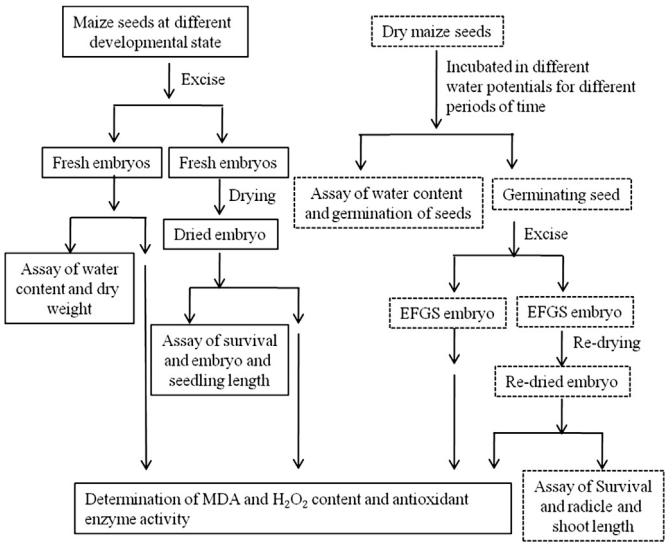

Fig. 8. Schematic drawing of analysis for desiccation tolerance of maize embryos during development and germination at different water potential PEG-6000 in relation to oxidative process. EFGS embryo, embryo excised from germinating seeds.

content of (0.07 ± 0.01) g g^{-1} and then were either frozen rapidly in liquid nitrogen for enzyme analysis or re-germinated for measuring survival and radicle and shoot length (Fig. 8).

The fresh and dried embryos during development, and EFGS embryos and re-dried embryos during germination were used as assay for MDA and H_2O_2 contents and enzyme activity (Fig. 8).

4.2. Drying or re-drying of embryos and determination of water content

Drying or re-drying of embryos was rapidly achieved by placing them on the filter paper that was then placed over activated silica gel for different periods of time within closed desiccators at 25 °C.

Water content of embryos was determined gravimetrically at 80 °C for 48 h, and is expressed on a dry mass basis (g H_2O (g DW)$^{-1}$, g g^{-1}).

4.3. Assessment of seed germination, and determination of embryo survival and shoot and radicle length

For assessment of seed germination, batches of twenty maize seeds were germinated on moist filter paper in closed Petri dishes at 25 °C in the dark for 5 days. A seed was considered to have germinated when the emerging radicle elongated to 2 mm. Mean germination time (MGT) was calculated according to the equation of Ellis and Roberts [40]:

$$\mathrm{MGT} = \sum Dn \Big/ \sum n$$

Where n is the number of seeds completing germination on day D and D is the number of days counted from the beginning of germination.

For determination of embryo survival, batches of twenty fresh and/or (re-) dried embryos were germinated on moist filter paper in closed Petri dishes at 25 °C in the dark for 5 days. The embryos showing measurable increase in length and volume and appearing light green were counted as surviving, those showing no increase in length and volume and appearing dark brown were counted as dead. The length of radicle and shoot was determined at 5 days after germination.

4.4. Determination of MDA and H_2O_2 contents

The MDA content of embryos was determined as described by Huang et al. [8], and is expressed in μmol g^{-1} DW.

The H_2O_2 content of embryos was assayed based on the method of Brennan and Frenkel [41], but modified as follows: twenty embryos were homogenized in 5 mL of cold acetone (−20 °C). A titanium reagent (20% titanic tetrachloride in concentrated HCl, v/v), NH_4OH and H_2SO_4 were added to the homogenate. After centrifugation at 15,000 g at 4 °C for 15 min, the absorbance of the supernatant was determined immediately at 415 nm. The H_2O_2 content is expressed in μmol g^{-1} DW.

4.5. Determination of antioxidant enzyme activity

Assay of SOD activity. Forty embryos were homogenized to a fine powder with a mortar and pestle under liquid nitrogen. Subsequently soluble proteins were extracted by grinding the powder in an extraction mixture composed of 50 mM Tris–HCl (pH 7.8), 1.0 mM ethylenediamine tetraacetic acid (EDTA), 0.05% (v/v) Triton X-100, 2% (w/v) polyvinylpolypyrrolidone (PVPP) and 1 mM ascorbic acid (ASA). The homogenate was centrifuged at 16,000 g at 4 °C for 15 min and the supernatant was transferred to new tube for enzyme assay.

SOD (EC 1.15.1.1) assay was based on the method of Huang et al. [8], who measured inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm, but which was modified as follows. The reaction mixture contained 3 mL of 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 75 μM NBT, 16.7 μM riboflavin and enzyme extract. SOD activity is presented as absorbance of sample divided by absorbance of blank, giving the percentage of inhibition. Riboflavin was added at last, and the reaction was initiated by placing the tubes under two 15-W fluorescent lamps and terminated after 15 min by removal from the light source (50 μmol m^{-2} s^{-1}). An illuminated blank without protein gave the maximum reduction of NBT, therefore, the maximum absorbance at 560 nm. In this assay, 1 unit of SOD is defined as the amount required inhibiting the photoreduction of NBT by 50%. The specific activity of SOD was expressed in unit of SOD mg^{-1} protein.

Assay of APX, CAT, GR and DHAR activity. A fine powder of fifty embryos homogenized under liquid nitrogen was extracted by grinding in 5 mL of 50 mM Tris–HCl (pH 7.0), containing 20% (v/v) glycerol, 1 mM AsA, 1 mM dithiothreitol, 1 mM EDTA, 1 mM reduced glutathione (GSH), 5 mM $MgCl_2$ and 1% (w/v) PVPP. After two centrifugation steps at 4 °C (10 min at 12,000 g and 15 min at 25,000 g, respectively), the supernatant was collected for determination of APX, CAT, GR and DHAR activities.

APX (EC 1.11.1.11) was assayed as the decrease in absorbance at 290 nm due to AsA oxidation, according to the method of Huang et al. [8]. The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 1 mM AsA and the enzyme extract in a final volume of 3 mL at 25 °C.

CAT (EC 1.11.1.6) activity was determined by directly measuring the decomposition of H_2O_2 at 240 nm, as described by Huang et al. [8]. The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 10 mM H_2O_2 and the enzyme extract in a final volume of 3 mL at 25 °C.

GR (EC 1.6.4.2) was determined as the decrease in absorbance at 340 nm due to the oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH), according to Song et al. [12]. The reaction mixture contained 50 mM Tris–HCl buffer (pH 7.5), 5 mM $MgCl_2$, 0.5 mM oxidized glutathione, 0.2 mM NADPH and enzyme extract in a final volume of 0.2 mL at 25 °C.

DHAR (EC 1.8.5.1) was assayed directly by following the formation of AsA at 265 nm, according to Song et al. [12]. The reaction

mixture contained 50 mM phosphate buffer (pH 7.0), 0.5 mM dehydroascorbic acid, 2.5 mM GSH and enzyme extract in a final volume of 0.2 mL at 25 °C.

The protein content of crude enzyme extract was measured according to Huang et al. [8], using bovine serum albumin as standard.

4.6. Statistical analysis

Data are presented as means ± standard deviation of three replicates. The statistical differences of survival of re-dried embryos and length of radicle and shoot produced by re-dried embryos were tested using an analysis of variance (ANOVA) using the statistical package software JMP and means were compared by the Student's *t*-test. Figures show significant differences using letter designations, where A/a is the highest mean, B/b indicates the next highest mean, etc. Data points with different letters show significant differences ($P < 0.05$).

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