



GERMINATION, α -, β -AMYLASE AND TOTAL DEHYDROGENASE ACTIVITIES OF *AMARANTHUS CAUDATUS* SEEDS UNDER WATER STRESS IN THE PRESENCE OF ETHEPHON OR GIBBERELLIN A₃

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Amaranthus caudatus L. seed germination was studied under different levels of water deficit induced by PEG 6000 in laboratory conditions. PEG at osmotic potentials -0.2 to -0.3 MPa at 24°C in darkness delayed germination and reduced final germination percentage. PEG solutions at osmotic potential lower than -0.3 MPa almost totally blocked seed germination. Ethephon was much more effective than GA₃ in reversing PEG-caused inhibition of *A. caudatus* seed germination. PEG decreased α -amylase activity after 14 h incubation. It decreased β -amylase activity after 14 and 20 h, and caused an increase in total dehydrogenase activity only after 20 h of incubation. Unlike GA₃, ethephon increased α -amylase activity in seeds after 12 and 14 h of incubation under water deficit. After 20 h of incubation there was no difference in α -amylase activity in any of the treatments. Neither ethephon nor GA₃ affected the activity of β -amylase and dehydrogenase.

Key words: *Amaranthus caudatus* seeds, α -, β -amylase, dehydrogenase, ethylene, gibberellin A₃, polyethylene glycol.

INTRODUCTION

Plants are exposed to many types of environmental stress. Water deficit is one of the most serious problems for germination, a crucial phase of plant life (Gill et al., 2003). The sequence of events leading to the emergence of the radicle through the seed coat is governed by water uptake from the external medium (Kaur et al., 1998). Water availability plays a significant role in enzymatic reactions, in solubilization and transportation of metabolites, and as a reagent in the hydrolytic breakdown of proteins, lipids and carbohydrates in the storage tissues of germinating seeds (Bewley and Black, 1994). The activity of some enzymes such as α -amylase in *Cicer arietinum* cotyledons (Gupta et al., 1993; Kaur et al., 1998; 2000) or α - and β -amylase in *Medicago sativa* germinating seeds (Zeid and Shedeed, 2006) is reduced by water stress. On the other hand, α -amylase activity in *Hordeum vulgare* seedlings (Jacobsen et al., 1986), β -amylase activity in *Cucumis sativus* cotyledons (Todaka et al., 2000), cytosolic glyceraldehyde-3-phosphate dehydrogenase activity in *Cratero-*

stigma plantagineum plants (Velasco et al., 1994) and protease activity in *Oryza sativa* seedlings (Pandey et al., 2004) may be increased by stress. Water deficit may affect seed germination by delaying its beginning or decreasing the final germination percentage (Hardegree and Ermmerich, 1990). In germination studies, polyethylene glycol (PEG 6000) as osmotic agent has often been used to simulate water deficit. It has been effective in research work because it does not penetrate the cells, is not degraded, and does not cause toxicity due to its high molecular weight (Hasegawa et al., 1984). In earlier work, PEG-induced water stress inhibited *Amaranthus caudatus* seed germination, an effect overcome by ethephon and a precursor of ethylene biosynthesis, 1-aminocyclopropane-1-carboxylic acid (ACC) (Kępczyński, 1986b). Exogenous ethylene was also effective in reversing PEG-caused inhibition of germination in *Amaranthus retroflexus* seeds (Schonbeck and Egley, 1980). Gibberellic acid (GA₃) has been reported to increase the germination percentage in *Cicer arietinum* in solution of PEG (Kaur et al., 1998, 2000).

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This study compares the effect of ethephon and gibberellin A₃ on germination *Amaranthus caudatus* seeds and on α - or β -amylase activity during incubation under PEG-induced water stress. Since seed imbibition and germination are correlated with an increase of respiration, the activity of total dehydrogenase (respiratory enzymes) was also determined.

MATERIALS AND METHODS

PLANT MATERIAL

The experiments used *Amaranthus caudatus* cv. atropurpureus seeds harvested in 2003 and stored dry at -20°C. These seeds were obtained from the W. Legutko Ornamental and Vegetable Seed Station (Kobylin, Poland).

GERMINATION TESTS

Seeds were incubated in 5 cm Petri dishes on one layer of filter paper moistened with 1.5 ml distilled water or the same volume of water solutions of polyethylene glycol (PEG) 6000 with osmotic potentials from -0.2 to -0.45 MPa at -0.05 MPa intervals. Osmotic potential was determined according to Michel and Kaufmann (1973). The effect of ethephon (liberating ethylene) or GA₃ at 10⁻⁵-3×10⁻⁴ M on seed germination was studied in treatments with PEG at osmotic potentials of -0.25, -0.3 or -0.35 MPa in five replicates of 50 seeds. The seeds were incubated in darkness at 24°C. All assays were performed under green safelight (0.5 $\mu\text{M m}^{-2} \text{s}^{-1}$). A seed was regarded as germinated when its radicle was ~1 mm in length.

ENZYME EXTRACTION AND ASSAY

For determination of enzyme activity, three replicates of 100 whole seeds (~0.1 g) were incubated in distilled water with PEG at -0.3 MPa and -0.3 MPa with 10⁻⁴ M ethephon or 10⁻⁴ M GA₃ for 12, 14 and 20 h. Fresh weight was determined before enzyme extraction.

α -amylase activity was measured according to Black et al. (1996). Seeds were homogenized in an Eppendorf tube with 4 ml ice-cold 20 mM TRIS-maleate buffer (pH 6.2) containing 1 mM CaCl₂. The homogenate was centrifuged at 12,000 g for 5 min. The clear supernatant was used for assaying α -amylase activity. 1.2 ml buffer with 1.2 ml enzyme extract was incubated for 2 min at 37°C. To 2.4 ml diluted enzyme extract, 0.6 ml suspension (25 mg ml⁻¹) of Phadebas blue starch (Pharmacia, Uppsala, Sweden) was added, vortexed and incubated with shaking for 30 min at 37°C. Adding 0.6 ml 0.5 M

NaOH stopped the reaction. The reaction mixture was centrifuged at 8000 g for 5 min and the absorbance of the supernatant was measured at 620 nm. A calibration curve using barley malt α -amylase was prepared. Enzyme activity was expressed in units g⁻¹ FW; one unit is equivalent to the amount of enzyme liberating 1 mg maltose from starch at 37°C and pH 6.2.

β -amylase was measured by the method of Bernfeld (1955). Seeds were homogenized with 4 ml ice-cold 16 mM sodium acetate buffer, pH 4.8. The homogenate was centrifuged at 12,000 g for 15 min, and the supernatant was used for determining β -amylase activity. To 0.5 ml 1% potato starch in 16 mM sodium acetate buffer equilibrated at 37°C for 2 min, 0.5 ml enzyme extract was added, vortexed and incubated with shaking for 5 min at 37°C. To the reaction mixture, 0.5 ml 3,5-dinitrosalicylic acid (DNSA) reagent was added and then boiled for 5 min. Absorbance at 540 nm was read after adding 4.5 ml distilled water. DNSA reagent consisted of 1% 3,5-dinitrosalicylic acid, 0.4 M NaOH and 1 M potassium sodium tartrate. A standard curve using maltose solution was prepared. β -amylase activity was expressed in units g⁻¹ FW; one unit is defined as the amount of enzyme liberating 1 mg maltose from starch in 5 min at 37°C and pH 4.8.

Total dehydrogenase activity was determined by homogenizing seeds with 2 ml ice-cold 0.1 M sodium phosphate buffer, pH 7.2, containing 1.5 % (w/v) 2,3,5-triphenyltetrazolium chloride. Samples were incubated at 25°C for 24 h. Then the samples were centrifuged for 6 min at 12,000 g and the pellet was extracted with 7 ml acetone. The absorbance of the supernatant was measured at 510 nm. A calibration curve using 1,3,5-triphenylformazan was prepared. Enzyme activity was expressed in mg g⁻¹ FW formazan liberated from 2,3,5-triphenyltetrazolium chloride in the determined conditions.

DATA ANALYSIS

Germination data are expressed as means (\pm SD) of five replicates; enzyme activity data are expressed as means (\pm SD) of three replicates. Statistical analyses were done with Statistica for Windows 7.1 (StatSoft Inc., Tulsa, U.S.A.). Two-way ANOVA was used to determine the effect of each PEG treatment at each incubation time. Three-way ANOVA was used to compare the effects of different concentrations of plant growth regulators (PGRs), PEG treatments and incubation time. Differences between means were considered to be significant at $P \leq 0.05$ by Duncan's multiple range test. Prior to analyses the data were tested for normality of distribution with the Shapiro-Wilk test. Germination data were arcsine-transformed to ensure homogeneity of variance. Enzyme activity data were log(x)-transformed.

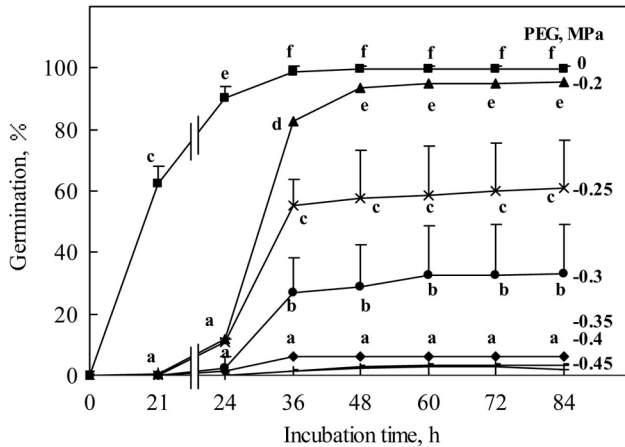


Fig. 1. Effect of PEG on *Amaranthus caudatus* seed germination at different incubation times at 25°C in the dark. Two-way ANOVA with Duncan's test on arcsine-transformed data was used to determine significant differences. Points with different letters differ significantly at $P \leq 0.05$.

RESULTS

SEED GERMINATION

Amaranthus caudatus L. seeds germinated almost completely after 24 h incubation in distilled water (Fig. 1). PEG solutions at osmotic potential -0.2 to -0.45 MPa significantly inhibited germination, manifested in delayed initiation of germination and/or reduction of the final germination percentage (Fig. 1). PEG at -0.25 and -0.3 MPa inhibited germination completely or almost completely after 24 h; after 84 h, reduction of germination by ~40% (-0.25 MPa) and 67% (-0.3 MPa) was noted. In PEG solutions with osmotic potentials lower than -0.3 MPa, seed germination did not exceed 6% during the whole incubation period. Nearly all seeds incubated in water in the presence of ethephon or GA₃ germinated after 24 h (Figs. 2, 3). Ethephon was highly effective in reducing the inhibitory effect of water stress (-0.25, -0.3 and -0.35 MPa) on seed germination after 24 h (Fig. 2a). As many as 90% of the seeds could germinate at osmotic potential -0.25 MPa due to the application of 10⁻⁵ M ethephon, the lowest concentration used. At its highest concentration, 80-90% of the seeds germinated at -0.3 and -0.35 MPa. After 48 h, ethephon at all applied concentrations additionally increased seed germination in the presence of PEG (Fig. 2b). GA₃ was not so effective as ethephon. Application of GA₃ simultaneously with PEG at -0.25 MPa increased the germination percentage after 24 h incubation up to only ~40% (Fig. 3a). Unlike ethephon, this hor-

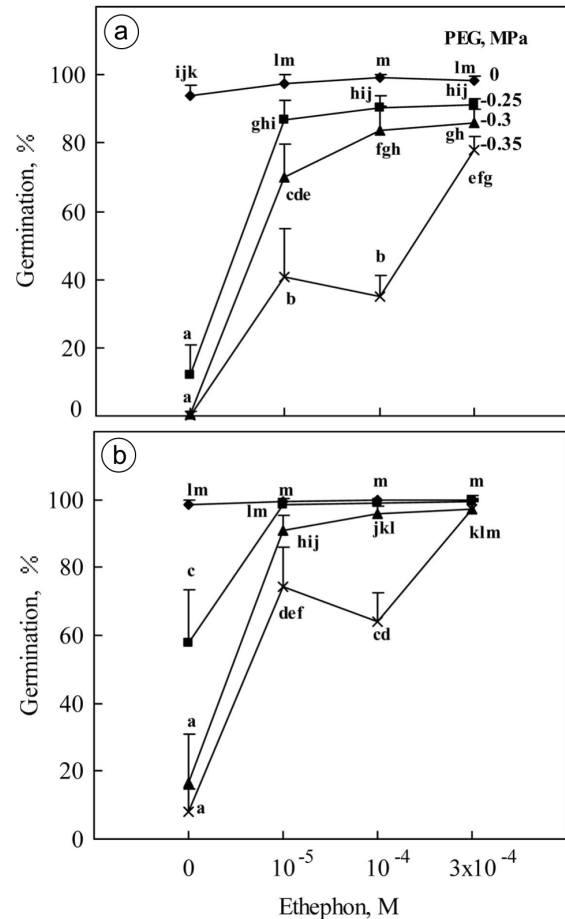


Fig. 2. Effect of ethephon on *Amaranthus caudatus* seed germination in the presence of PEG after 24 h (a) or 48 h (b) incubation at 25°C in the dark. Three-way ANOVA with Duncan's test on arcsine-transformed data was used to determine significant differences. Points with different letters differ significantly at $P \leq 0.05$.

mone did not affect germination in the presence of PEG at -0.3 and -0.35 MPa. After 48 h, GA₃ significantly alleviated PEG-induced inhibition at all osmotic potentials, with the most effective concentration at 10⁻⁴ M (Fig. 3b). At this concentration of gibberellin about 90% (-0.25 MPa) and 70% (-0.3 MPa) of the seeds were able to germinate.

ENZYME ACTIVITY

PEG at -0.3 MPa had no significant effect on α -amylase activity after 12 h and 20 h (Fig. 4). After 14 h, however, PEG significantly decreased enzyme activity versus the control. Combined application of ethephon and PEG showed significantly higher α -amylase activity versus the controls and PEG alone after 12 and 14 h incubation. α -amylase activ-

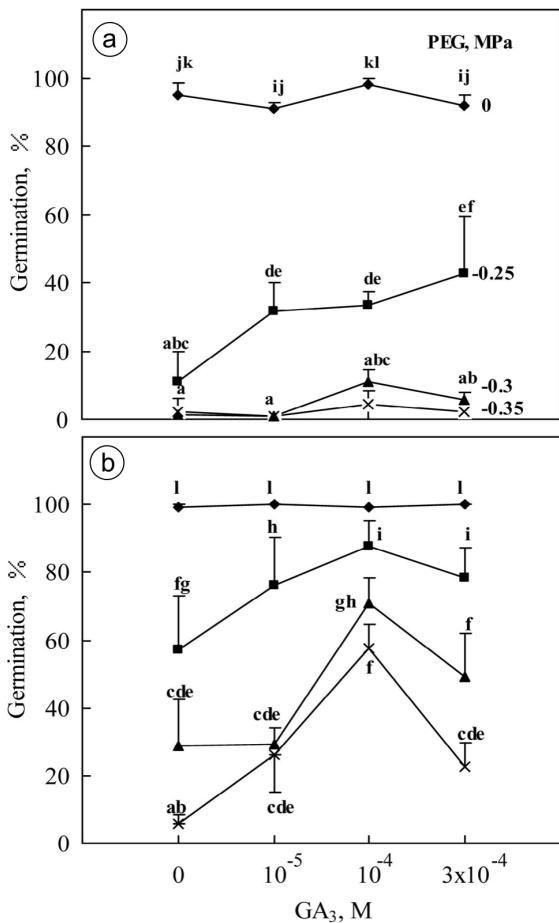


Fig. 3. Effect of GA_3 on *Amaranthus caudatus* seed germination in the presence of PEG after 24 (a) or 48 h (b) incubation at 25°C in the dark. Three-way ANOVA with Duncan's test on arcsine-transformed data was used to determine significant differences. Points with different letters differ significantly at $P \leq 0.05$.

ity after 20 h was similar when seeds were incubated in PEG or PEG with ethephon (Fig. 4) despite the difference in seed germination under those two conditions. GA_3 did not affect α -amylase activity during the incubation period. Its activity in the presence of GA_3 under water stress was low at 12 and 14 h; it increased after 20 h to reach levels similar to that in seeds incubated in water or PEG. PEG significantly decreased β -amylase activity at 14 and 20 h but not at 12 h. Ethephon and GA_3 applied simultaneously with PEG did not affect β -amylase activity during the incubation period. PEG did not affect dehydrogenase activity after 12 and 14 h, and significantly increased dehydrogenase activity versus the control after 20 h. Neither ethephon nor GA_3 affected dehydrogenase activity during the incubation period.

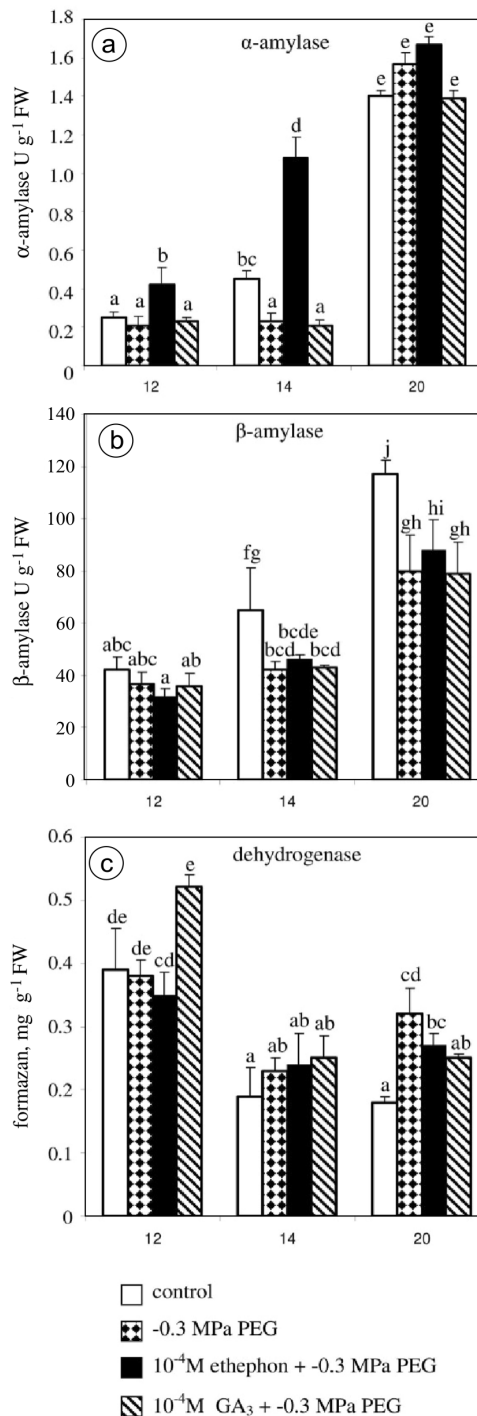


Fig. 4. Effect of PEG (-0.3 MPa) in the presence of ethephon at 10^{-4} M or GA_3 at 10^{-4} M on α -amylase (a), β -amylase (b) or total dehydrogenase (c) activity in *Amaranthus caudatus* seeds after different incubation times at 25°C in the dark. Three-way ANOVA with Duncan's test on $\log(x)$ -transformed data was used to determine significant differences. Numbers above columns indicate seed germination (data not transformed). Columns with different letters differ significantly at $P \leq 0.05$.

DISCUSSION

In our work, polyethylene glycol 6000 inhibited *Amaranthus caudatus* seed germination, in accord with earlier results from experiments with this species (Kępczyński, 1986b). Poor seed germination under PEG-induced water deficit has also been observed in *Bowdichia virgilioides* (Silva et al., 2001), *Cicer arietinum* (Gupta et al., 1993; Kaur et al., 1998), and *Medicago sativa* (Zeid and Shedeed, 2006). As in previous experiments with *A. caudatus* seeds (Kępczyński, 1986b), the inhibitory effect of PEG was strongly reversed by ethephon. Ethylene and its biosynthesis precursor ACC can also successfully counteract osmotic inhibition of *A. caudatus* seed germination (Kępczyński and Karssen, 1985). In many species, inhibition of seed germination due not only to various stress conditions but also to dormancy can be partially or completely reversed by ethylene or ethephon (Kępczyński and Kępczyńska, 1997). In earlier work, PEG-caused water stress inhibited ethylene biosynthesis, and reversal of PEG-inhibited *A. caudatus* seed germination was associated with increased ethylene production (Kępczyński and Karssen, 1985). Since the inhibitory effect of PEG was also antagonized by GA₃, it is possible that water deficit also affects gibberellin synthesis in *A. caudatus* seeds. The involvement of endogenous gibberellins in *A. caudatus* seed germination was shown in experiments using an inhibitor of gibberellin biosynthesis (Kępczyński, 1986a). In halophyte species, saline stress inhibited seed germination and reduced gibberellin production (Boucaud and Ungar, 1976). As in other work on inhibition of seed germination (by NaCl) (Białecka and Kępczyński, 2009), in the present study ethephon was more effective than GA₃ in reversing inhibition (by PEG) of *A. caudatus* seed germination. Likewise, ethephon was more efficient than GA₃ in nullifying the negative effect of the gibberellin biosynthesis inhibitors tetcyclacis (Kępczyński, 1986a) and jasmonates on seed germination of this species (Kępczyński and Białecka, 1994). The ethylene-dependent action of gibberellin in *A. caudatus* seed germination has been shown (Kępczyński, 1986a). Inhibition of α -amylase activity after 14 h incubation at -0.3 MPa PEG may suggest that *Amaranthus caudatus* seed germination requires the activity of this enzyme. At 20 h, α -amylase activity increased to levels similar to that in control seeds, showing that the increased activity of this enzyme did not result in promotion of germination. However, since seeds could germinate partially after prolonged incubation at -0.3 MPa, high activity of this enzyme may be required for germination under water stress. On the other hand, it has been argued that inhibition of α -amylase synthesis is not a mechanism by which drought prevents the germination of

Agropyron desertorum seeds (Wilson, 1971). The increase of α -amylase activity might be related to adaptive strategy to water deficit. The stimulatory effect of ethephon on α -amylase activity at 12 and 14 h may suggest that prolonged increased activity of this enzyme during early imbibition is required for solutes to accumulate, raise the negative osmotic potential, and thereby cause germination under water deficit. According to this view, GA₃, which also reversed inhibition of seed germination but with a delay versus ethylene, might be associated with increased α -amylase activity later than after 20 h incubation, or that gibberellins act in a different way than ethylene does. Our results also indicate that PEG reduced α -amylase activity in *A. caudatus* seeds, and neither ethylene nor GA₃ counteracted water-deficit-caused inhibition of germination through regulation of the activity of this enzyme. Increased total dehydrogenase activity in the PEG treatments after 20 h incubation suggests that water deficit stimulated respiratory enzyme activity in *A. caudatus* seeds. In *Craterostigma plantagineum* plants, Velasco et al. (1994) showed that cytosolic glyceraldehyde-3-phosphate dehydrogenase was induced by water stress, and suggested that changes in the glycolysis rate are important in coping with that stress. In *Triticum aestivum* seeds germinating under salinity, an increase of the respiration rate was suggested as an adaptive strategy for stress (Kasai et al., 1998). Neither ethephon nor gibberellin controlled total dehydrogenase activity in *A. caudatus* seeds under water stress.

To summarize, ethylene liberated from ethephon reversed the effect of PEG-induced water stress on *Amaranthus caudatus* seed germination much more effectively than GA₃ did. Incubation of seeds under water stress affected α -, β -amylase and total dehydrogenase activities. Reversal of the inhibitory effect of water stress by ethylene and gibberellin A₃ was not related to control of β -amylase or total dehydrogenase activity. One of the actions of exogenous ethylene in reversing the effects of water stress on seed germination might be regulation of α -amylase activity.

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