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Alleviation of negative effects of water stress in two contrasting wheat genotypes by calcium and abscisic acid

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Abstract

The individual and interactive role of calcium and abscisic acid (ABA) in amelioration of water stress simulated by polyethylene glycol (PEG) 6000 was investigated in two contrasting wheat genotypes. PEG solution (osmotic potential -1.5 MPa) was applied to 10-d-old seedlings growing under controlled conditions and changes in photosynthetic rate, activities of ribulose-1,5-bisphosphate carboxylase and phosphoenolpyruvate carboxylase, water potential and stomatal conductance were observed in the presence of 0.1 mM ABA, 5 mM calcium chloride, 1 mM verapamil (Ca^{2+} channel blocker), and 1 mM fluridone (inhibitor of ABA biosynthesis). ABA and calcium chloride ameliorated the effects of water stress and the combination of the two was more effective. The two genotypes varied for their sensitivity to ABA and Ca^{2+} under stress. As was evident from application of their inhibitors, ABA caused more alleviation in C 306 (drought tolerant) while HD 2380 (drought susceptible) was more sensitive to Ca^{2+}.

Additional key words: fluridone, net photosynthetic rate, phosphoenolpyruvate carboxylase, ribulose-1,5-bisphosphate carboxylase, stomatal conductance, *Triticum aestivum*, verapamil.

Introduction

Plants acclimate in response to prevailing conditions by making non-heritable alterations at various levels of their organization. Water stress is frequently experienced in field grown plants and the responses to water stress have been characterized at physiological, molecular and genetic level (Bray 1997). Abscisic acid (ABA) and calcium figure as key participants in stress related mechanisms (Chandler and Robertson 1994, Sanders *et al.* 1999) and both are reported to accumulate under various kinds of stresses (Cowan *et al.* 1997). The interaction between the two has a pivotal role in mediation of stress induced stimulus-response coupling mechanisms (Cousson and Vavasseur 1998). Increase in ABA due to stress has been reported to elevate intracellular calcium, which has been suggested to act as a secondary messenger and transducer of stress signal (Leung and Giraudat 1998). The participation of ABA and calcium in regulation of aquaporins has recently been indicated in xylem parenchyma cells (Netting 2000). Exogenous application of ABA and calcium induces stress tolerance (Singer *et al.* 1996, Ingram and Bartels 1996, Jiang and Huang 2001). The mechanism of action of calcium and ABA involve protein kinases in signal cascade (Leung and Giraudat 1998) and their functioning during stress requires to be explored. The contrasting wheat genotypes evoke different mechanisms of tolerance in response to water stress (Sairam *et al.* 1998).

Keeping in view the involvement of ABA and calcium in stress responses, an experiment was designed to investigate their role in mediation of carboxylation during water stress using two differentially sensitive wheat genotypes.

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Abbreviations: ABA - abscisic acid; FL - fluridone; PEG - polyethylene glycol; PEPC - phosphoenolpyruvate carboxylase; RUBPC - ribulose-1,5-bisphosphate carboxylase; VP - verapamil.
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Materials and methods

Two wheat (*Triticum aestivum* L.) genotypes, C 306 (drought tolerant) and HD 2380 (drought susceptible) were used for investigation. Seeds of each genotype were surface sterilized with 0.1 % mercuric chloride for 2 min and thoroughly washed with sterilized distilled water. The seeds were germinated in autoclaved 10 cm glass Petri dishes lined with two layers of germination paper moistened with 3.5 cm^3 of sterilized distilled water at alternating temperature of 23/20 °C in dark for 72 h. Thereafter light/dark cycle of 16/8h was maintained in growth chamber (*SEW*, New Delhi, India) having irradiance of 400 μmol m^{-2} s^{-1} near the surface of plants. After the formation of first leaf, water stress was imposed using polyethylene glycol-6000 solution of osmotic potential -1.5 MPa. In a preliminary experiment involving stress levels, -1.5 MPa was opted as it caused 50 % inhibition of seedling growth without any visible damage to the seedlings. The medium was also supplemented with calcium chloride (5 mM) or ABA (0.1 mM), either individually or in combination. Simultaneously, a calcium channel blocker, verapamil (VP, 1 mM) and ABA biosynthesis inhibitor, fluridone (FL, 1 mM) were also used separately or in combination. The concentrations selected for the present study were standardized by having a range of these and evaluated using membrane stability as indicator of stress injury to leaf tissues of seedlings growing under PEG-simulated water stress. The concentrations of ABA and $CaCl_2$ which minimized the membrane injury, measured as electrical conductivity and the concentrations of fluridone and verapamil which caused maximum membrane leakage under stress were chosen for the present study. Observations were taken on 2nd, 4th and 6th day. Net photosynthetic rate (P_N) and stomatal conductance (g_s) were measured in fully expanded leaves using infrared gas analyser (*CID*, Washington, USA). Measurements were performed at 10:00 using an external light source to provide uniform irradiance of 1 200 μmol m^{-2} s^{-1} at ambient temperature of 26 ± 2 °C and CO_2 concentration of 350 ± 10 μmol mol^{-1}. Water potential of leaves was measured in pressure chamber apparatus (*PMS*, Corvallis, USA). The pressure was increased at the rate of 0.1 MPa s^{-1}.

Ribulose 1,5 bisphosphate carboxylase (RUBPC) was assayed as per Lilley and Walker (1974). The assay mixture contained 50 mM HEPES buffer (pH 7.8), 10 mM KCl, 1 mM EDTA, 15 mM $MgCl_2$, 5 mM ATP, 5 mM dithiothreitol, 0.2 mM NADH, 70 mM $NaHCO_3$, 5 mM phosphocreatine, 2 units each of glucose-6-phosphate dehydrogenase, ribulose-5-isomerase, phosphocreatine kinase, 3 PGA kinase and 0.2 cm^3 of enzyme extract. Reaction was started by adding ribulose-5-phosphate and absorbance was read at 340 nm in a *Shimadzu* (Kyoto, Japan) UV-visible spectrophotometer. Phosphoenolpyruvate carboxylase (PEPC) was assayed according to Meyer *et al.* (1988). The sample was homogenized in 10 volumes of extraction medium containing 50 mg polyvinylpyrrolidone in 50 mM glycylglycine buffer, pH 7.4, with 10 mM of 2-mercapto-ethanol. Samples were centrifuged at 65 000 *g* at 4 °C for 40 min. The samples were assayed in a mixture including 50 mM ACES, pH 7.0, 5 mM free Mg^{2+}, 5 mM $NaHCO_3$, 160 μM NADH, 12 international units (IU) of malate dehydrogenase and 5.5 IU of lactate dehydrogenase. Enzyme activity was initiated after addition of 1 mM of phosphoenolpyruvate and calculated from oxidation of NADH as decrease in absorbance at 340 nm.

All the observations were replicated thrice and analysed statistically for standard error and analysis of variance using *Microstat* software.

Results

On 2nd day of PEG-induced stress, a reduction in P_N, RUBPC and PEPC activities, water potential (ψ_w) and stomatal conductance (g_s) was observed in both the genotypes from their pre-stress levels (Tables 1 - 5). C 306 had higher P_N, RUBPC and ψ_w than HD 2380, which showed higher PEPC and g_s. In the presence of ABA, $CaCl_2$, VP and FL, both the genotypes showed decline in these parameters. On 4th day, the control plants recorded decrease in these parameters that continued on 6th day too. ABA, $CaCl_2$ and their combination caused marked increase in P_N, RUBPC, and PEPC while ψ_w slightly decreased on 4th day and increased subsequently on 6th day. The g_s increased slightly on 4th day in treatments with ABA and $CaCl_2$ while a marked increase was noticed in treatment with their combination. On 6th day, P_N, enzyme activities, ψ_w, and g_s were higher in treatments with ABA and $CaCl_2$ as compared to control. The differences between their individual and combined application were significant on 6th day for P_N and PEPC. From the perusal of data, it appeared that the two genotypes reacted differently to the presence of ABA or calcium that was also evident from use of their inhibitors. On 6th day, the water stress amelioration was noticed to be more with ABA in C 306 while calcium was more effective in HD 2380 for all the parameters. It was further observed that HD 2380 showed higher reduction in the presence of Ca^{2+} inhibitor (VP) while C 306 was affected more with ABA inhibitor (FL). The combined application of VP and FL caused significant decrease in all the parameters as compared to ABA and $CaCl_2$.

Table 1. Photosynthetic rate [μmol (CO_2) m^{-2} s^{-1}] in tolerant (C 306) and susceptible (HD 2380) genotypes of wheat during PEG-6000 simulated water stress (-1.5 MPa) in the presence of ABA and $CaCl_2$. Fluridone and verapamil were used as inhibitors of ABA and calcium, respectively (means ± SE, $n = 3$). Pre-stress P_N: C 306 - 14.8; HD 2380 - 12.3.

Treatments	2nd day		4th day		6th day	
	C 306	HD 2380	C 306	HD 2380	C 306	HD 2380
Control	8.6 ± 0.12	6.4 ± 0.09	6.4 ± 0.13	5.2 ± 0.11	5.4 ± 0.11	3.2 ± 0.11
ABA (0.1 mM)	6.6 ± 0.09	5.8 ± 0.11	9.1 ± 0.10	7.2 ± 0.14	11.1 ± 0.13	8.4 ± 0.14
$CaCl_2$ (1 mM)	6.1 ± 0.08	4.1 ± 0.14	8.2 ± 0.12	8.5 ± 0.16	9.4 ± 0.11	9.2 ± 0.14
$CaCl_2$+ABA	5.2 ± 0.11	3.6 ± 0.11	8.9 ± 0.12	8.8 ± 0.12	13.8 ± 0.12	10.4 ± 0.16
ABA + verapamil (1 mM)	4.4 ± 0.14	2.7 ± 0.11	6.7 ± 0.13	3.5 ± 0.14	5.8 ± 0.10	3.2 ± 0.14
$CaCl_2$ + fluridone (1 mM)	3.8 ± 0.11	2.1 ± 0.13	4.2 ± 0.10	4.6 ± 0.14	4.6 ± 0.12	5.8 ± 0.11
Fluridone + verapamil	2.8 ± 0.13	1.4 ± 0.14	3.0 ±0.12	2.1 ± 0.12	4.1 ± 0.12	2.0 ± 0.13
$CD_{0.05}$ genotype	0.6		0.4		0.6	
$CD_{0.05}$ treatments	0.8		0.9		0.8	

Table 2. RUBPC activity [mol kg^{-1}(Chl) s^{-1}] in tolerant (C 306) and susceptible (HD 2380) genotypes of wheat during PEG-6000 simulated water stress in the presence of ABA, $CaCl_2$, fluridone and verapamil (means ± SE, $n = 3$). Pre-stress activity: C 306 - 7.1; HD 2380 - 6.5.

Treatments	2nd day		4th day		6th day	
	C 306	HD 2380	C 306	HD 2380	C 306	HD 2380
Control	5.2 ± 0.11	4.1 ± 0.12	4.3 ± 0.11	3.2 ± 0.11	2.8 ± 0.11	1.4 ± 0.11
ABA (0.1 mM)	3.5 ± 0.10	2.8 ± 0.11	4.1 ± 0.14	3.4 ± 0.12	5.8 ± 0.14	4.2 ± 0.10
$CaCl_2$ (1 mM)	3.1 ± 0.12	2.0 ± 0.12	3.8 ± 0.12	4.8 ± 0.11	6.2 ± 0.12	5.6 ± 0.14
$CaCl_2$+ABA	3.4 ± 0.14	2.6 ± 0.10	4.5 ± 0.14	5.1 ± 0.14	6.5 ± 0.12	5.8 ± 0.11
ABA + verapamil (1 mM)	2.6 ± 0.14	1.4 ± 0.12	3.1 ± 0.10	1.8 ± 0.12	2.5 ± 0.14	1.4 ± 0.12
$CaCl_2$ + fluridone (1 mM)	2.1 ± 0.12	1.9 ± 0.11	2.2 ± 0.12	2.8 ± 0.11	2.0 ± 0.12	2.1 ± 0.16
Fluridone + verapamil	1.3 ± 0.13	0.8 ± 0.12	1.6 ± 0.14	1.2 ± 0.14	1.1 ± 0.11	0.9 ± 0.12
$CD_{0.05}$ genotype	0.5		0.4		0.6	
$CD_{0.05}$ treatments	0.7		0.9		0.7	

Table 3. PEPC activity [mol kg^{-1}(Chl) s^{-1}] in tolerant (C 306) and susceptible (HD2380) genotypes of wheat during PEG-6000 simulated water stress in the presence of ABA, $CaCl_2$, fluridone and verapamil (means ± SE, $n = 3$). Pre-stress activity: C306 - 9.1; HD 2380 - 11.2.

Treatments	2nd day		4th day		6th day	
	C 306	HD 2380	C 306	HD 2380	C 306	HD 2380
Control	6.6 ± 0.13	7.2 ± 0.11	4.6 ± 0.11	5.2 ± 0.12	3.1 ± 0.14	4.2 ± 0.11
ABA (0.1 mM)	5.1 ± 0.14	5.8 ± 0.14	6.2 ± 0.13	6.8 ± 0.12	7.1 ± 0.11	7.9 ± 0.12
$CaCl_2$ (1 mM)	4.5 ± 0.11	6.5 ± 0.12	5.9 ± 0.10	7.1 ± 0.10	6.5 ± 0.12	9.4 ± 0.14
$CaCl_2$+ABA	6.2 ± 0.12	7.1 ± 0.11	7.1 ± 0.11	7.8 ± 0.12	8.2 ± 0.14	8.9 ± 0.11
ABA + verapamil (1 mM)	3.4 ± 0.11	2.6 ± 0.10	5.2 ± 0.12	3.1 ± 0.14	5.4 ± 0.11	3.4 ± 0.12
$CaCl_2$ + fluridone (1 mM)	2.9 ± 0.14	1.9 ± 0.12	4.1 ± 0.14	4.2 ± 0.12	4.4 ± 0.10	5.6 ± 0.14
Fluridone + verapamil	1.2 ± 0.12	0.9 ± 0.14	1.8 ± 0.11	1.5 ± 0.11	1.4 ± 0.12	1.1 ± 0.12
$CD_{0.05}$ genotype						
$CD_{0.05}$ treatments						

Table 4. Water potential [-MPa] in tolerant (C 306) and susceptible (HD2380) genotypes of wheat during PEG-6000 simulated water stress in the presence of ABA, $CaCl_2$, fluridone and verapamil (means ± SE, $n = 3$). Pre-stress level: C 306 - 0.5; HD 2380 - 0.6.

Treatments	2nd day		4th day		6th day	
	C 306	HD 2380	C 306	HD 2380	C 306	HD 2380
Control	0.5 ± 0.02	0.7 ± 0.02	0.7 ± 0.02	0.8 ± 0.02	0.9 ± 0.02	1.5 ± 0.02
ABA (0.1 mM)	0.6 ± 0.03	0.8 ± 0.03	0.6 ± 0.03	0.7 ± 0.02	0.6 ± 0.02	0.6 ± 0.03
$CaCl_2$ (1 mM)	0.5 ± 0.02	0.8 ± 0.02	0.6 ± 0.02	0.7 ± 0.03	0.6 ± 0.03	0.4 ± 0.02
$CaCl_2$+ABA	0.5 ± 0.02	0.7 ± 0.03	0.6 ± 0.02	0.7 ± 0.02	0.5 ± 0.03	0.4 ± 0.03
ABA + verapamil (1 mM)	0.6 ± 0.03	0.8 ± 0.04	0.8 ± 0.04	0.9 ± 0.03	0.5 ± 0.02	1.0 ± 0.03
$CaCl_2$ + fluridone (1 mM)	0.6 ± 0.05	0.8 ± 0.02	0.7 ± 0.04	0.9 ± 0.04	1.0 ± 0.03	0.9 ± 0.02
Fluridone + verapamil	0.6 ± 0.02	0.8 ± 0.03	0.9 ± 0.03	1.0 ± 0.02	1.0 ± 0.02	1.2 ± 0.02
$CD_{0.05}$ genotype	0.023		0.041		0.045	
$CD_{0.05}$ treatments	0.048		0.046		0.058	

Table 5. Stomatal conductance [mmol m^{-2} s^{-1}] in tolerant (C 306) and susceptible (HD 2380) genotypes of wheat during PEG-6000 simulated water stress in the presence of ABA, $CaCl_2$, fluridone and verapamil (means ± SE, $n = 3$). Pre-stress level: C 306 - 512; HD 2380 - 560.

Treatments	2nd day		4th day		6th day	
	C 306	HD 2380	C 306	HD 2380	C 306	HD 2380
Control	420 ± 10.2	504 ± 9.6	124 ± 7.4	168 ± 8.2	104 ± 6.5	132 ± 8.2
ABA (0.1 mM)	140 ± 8.5	184 ± 10.1	164 ± 8.6	196 ± 7.4	200 ± 8.9	232 ± 7.6
$CaCl_2$ (1 mM)	204 ± 9.2	180 ± 7.5	228 ± 7.2	204 ± 6.8	220 ± 7.6	192 ± 7.3
$CaCl_2$+ABA	124 ± 9.1	156 ± 8.1	248 ± 9.2	288 ± 7.6	260 ± 9.1	300 ± 6.8
ABA + verapamil (1 mM)	276 ± 10.5	364 ± 9.5	268 ± 7.3	312 ± 6.9	212 ± 8.5	312 ± 7.1
$CaCl_2$ + fluridone (1 mM)	348 ± 8.3	232 ± 7.1	380 ± 8.2	224 ± 6.8	380 ± 8.2	272 ± 9.2
Fluridone + verapamil	156 ± 7.6	124 ± 6.5	152 ± 6.2	136 ± 7.6	128 ± 6.5	116 ± 4.8
$CD_{0.05}$ genotype	11.5		11.1		9.4	
$CD_{0.05}$ treatments	12.8		8.8		12.4	

Discussion

Water stress caused immediate reduction in P_N, RUBPC and PEPC activities, and g_s though water potential showed a slow decline in both the genotypes. The photosynthetic gas exchange and water use efficiency have been reported to be reduced during water stress in wheat (Shangguan *et al.* 2000). The initial decrease in P_N and activity of carboxylases on 2nd day seem to be associated with stomatal limitation as indicated by reduction in g_s rather than reduced availability of water as ψ_w did not show significant change. Hafid *et al.* (1997) also attributed decrease in P_N in wheat soon after drought stress was imposed to reduced stomatal conductance. Although stomatal closure seems to be the main cause of photosynthesis decrease (Kaiser 1987), a reduction in utilization of assimilates due to stress may also cause feedback inhibition of carboxylation (Stitt 1991). In potato plants exposed to PEG-induced water deficit stress, Buiss *et al.* (1998) noticed that while water use efficiency remain unchanged, the reduction in carboxylation efficiency and the P_N was observed to be non-stomatally limited. Our observations on immediate reduction in g_s and slow decline of water potential under stress correspond with those of Socias *et al.* (1997) in *Trifolium subterraneum* L., where a 50 % reduction in stomatal conductance was observed under stress before any substantial change in water potential was detected.

The tolerant genotype, C306 showed higher P_N, RUBPC activity, and ψ_w than the susceptible genotype HD2380, which had higher PEPC activity and g_s than C 306 under stress. PEPC activity was noticed to be higher than RUBPC activity under water stress in both the genotypes and it increased in the presence of ABA and calcium. PEPC was recorded to increase in response to osmotic stress (Tang *et al.* 1997) and with exogenous application of ABA and calcium ionophores (Taybi and Cushman 1999). High PEPC activity under stress may

contribute to generation of malate, an osmoregulant and calcium has been reported to increase malate content under osmotic stress (Ortiz *et al.* 1994). The higher ψ_w in HD 2380 with calcium application as observed on 6th day of stress may be ascribed to increased malate generation through elevated content of PEPC.

The effect of water stress in both the genotypes was ameliorated in the presence of exogenous ABA and calcium, though the response was not immediate. The initial decline in P_N and RUBPC activity as observed on 2nd day in presence of ABA and calcium is probably due to their high internal contents and redistribution which has been reported to occur quickly under water stress (Daeter and Hartung 1995, Sorooshzadeh *et al.* 1999). However, as the stress progressed the treated plants showed improvement. Stress tolerance in plants has been reported to be induced by application of ABA or calcium due to their effect on pressure potential (Singer *et al.* 1996, Netting 2000), membrane integrity (Abdel-Basset 1998), stomatal regulation (Cousson and Vavasseur 1998), oxidative metabolism (Jiang and Huang 2001), and up- or down-regulation of gene(s) encoding specific proteins (Swami and Smith 1999, Taybi and Cushman 1999). Soybean seedlings treated with calcium and subjected to water stress showed a higher P_N than non-sprayed ones (GenPing *et al.* 1995). Our results pointed towards the interdependence of ABA and Ca^{2+} during stress, which was apparent from inhibitor studies. Blocking the response to one affected the sensitivity of the plants to the presence of other. Suppression of both ABA and Ca^{2+} using their inhibitors in the growth medium was observed to be extremely inhibitory. The interactive functioning of the two has been reported and calcium has been indicated as signal transducer in ABA mediated stress response (Frandsen *et al.* 1996, Leung and Giraudat 1998). In sunflower, Quintero *et al.* (1999) observed increase in hydraulic conductivity and exudation rate in roots treated with ABA and calcium.

Presence of calcium and ABA alleviated the stress injury but the response of two genotypes varied as was apparent from use of inhibitors. While C 306 was found to be more sensitive to ABA, HD 2380 appeared to show more dependence on calcium for responses to water stress. The differential sensitivity of contrasting genotypes to water stress may exist at endogenous regulation of ABA and calcium content and redistribution. An insight into their internal status may lead to identification of some finer controls of stress sensitivity. The maize genotypes varying in drought susceptibility were observed to differ with respect to ABA transport mechanisms (Jovanović *et al.* 2000).

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