INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
1560–8530/2007/09–2–226–230
http://www.fspublishers.org

Apoplastic Protein Pattern, Hydrolases and Peroxidase Activity of *Vicia faba* Cultivars as Influenced by Drought

M.A. EL-TAYEB[1] AND M.K. AHMED
Botany Department, Faculty of Science, South Valley University, Qena 83523, Egypt
[1]Corresponding author's email: mohattia1210@yahoo.com

ABSTRACT

Effect of drought on apoplastic protein content, protein pattern, enzymic activiteis and minieral concentration of *Vicia faba* cv Giza 40 (relatively drought-tolerant) and *Vicia faba* cv Giza 667 (relatively drought-sensitive) was studied. While drought decreased Na^+, K^+, Ca^{2+} concentration in leaf apoplast of Giza 40, it increased Na^+ and Ca^{2+} concentraion in case of Giza 667. Giza 40 has K^+:Na^+ ratio higher than that of cv Giza 667 under drought condition. The protein content of intercellular wasing fluid (IWF) increased in cv Giza 40 and decreased in cv Giza 667 when subjected to drought. The elevated protein content in cv Giza 40 was the result of *de novo* synthesis of 13 specific polypeptides with apparent molecular mass range of 0.54 - 84.61 kDa. The declined protein content was associated with 10 newly synthesied polypeptides with molecular mass of 0.54 - 169.87 kDa. The changes in protein content were accompanied by a specific alterations in the activities of apoplastic enzymes in the cultivars. In general, the activities of apoplastic peroxidase and hydrolases were higher in Giza 40 than that of Giza 667 under either conditions. The results indicated that the changes in leaf apoplast in response to drought are cultivar specific.

Key Words: Apoplast; Drought; Hydrolases; Peroxidase; *Vicia faba*

INTRODUCTION

Drought is an important limiting factor for growth and crop production in arid and semiarid zones of the world. Some plants have a set of physiological adaptaions that allow them to tolerate water stress conditions. The degree of adaptations to a decrease of water potential caused by drought may vary considerably among species (Savé *et al*., 1995) and also within a species (Parker & Pallardy, 1985). Most physiological researches have mainly focused on three topics related to drought, namely water relations, photosynthesis and the accumulation of specific metabolites such as the compatible solutes glycinebetaine and proline. These processes have been invistigated in detail in respect to their contribution to the described limitations of growth and to tolerance under drought conditions (Chaves *et al*., 2003).

Physiological phenomena in the apoplast are important for growth and defence. The contents of the xylem stream first spread over the leaf apoplast in most species before the leaf cells specifically import mutrient elements from the apoplast (Dietz, 1996). The physio-chemical properties of the cell wall and the biochemical activities in the apoplast determine the capacity and the rate of cell expansion (Grignon & Sentenac, 1991). The apoplast is a major site, where plants express proteins invoved in the defence against pathogens (Jung *et al*., 1993). In addition to these functions some studies have also established the role of the apoplast in adaptation to environmental stress (Dietz, 1996; Chikov *et al*., 2001; Sattelmacher, 2001). Those roles include detoxification of SO_2 (Pfanz *et al*., 1990) and ozone (Luwe & Heber *et al*., 1995) and aluminium (Horst, 1995). The apoplast is an important site for cold acclimation (Marentes *et al*., 1993).

The apoplastic polypeptides in barley exhibit qualitatively similar but quantitatively distinct response towards various heavy metals (Blinda *et al*., 1997). Specific enzymes such as extracellular peroxidases and hydrolases have been investigated in barley under various stress regimes (Ramanjulu *et al*., 1999; Dietz *et al*., 2000). However, an analysis comparing the apoplastic responses in cultivars with different tolerance to drought is not available. Therefore, this work was conducted to study how drought affects mineral and protein content, qualitative and quntitative polypeptide composition and enzymes activities in leaf apoplast of two *Vicia faba* cultivars differing in drought tolerance.

MATERIALS AND METHODS

Two *Vicia faba* cultivars (Giza 40 & Giza 667) were grown in plastic pots containing 2 kg air-dried soil (sand:clay 1:1 v/v) per pot. The seeds were sown (10 per pot) in the pots and watered with tap water in order to adjust soil water content to 90% of the maximum field capacity. The seedlings were grown for two weeks under normal conditions. Pots from each cultivar were divided randomly into two groups (4 pots each). One group was continuously watered to 90% field capacity and served as control. Drought stress in the second group was developed by withholding water until the soil water content became 40% of the maximum field capacity. The plants were irrigated two times weekly with ½ strength Hoagland solution to

desired soil moisture. After two weeks the fully expanded leaves were eexcised. Intercellular washing fluid (IWF) was extracted after infiltration of the excised leaves with a solution containing 100 mm sorbitol and Triton X-100 (0.1% v/v) by centrifugation of the leaves at 1000 xg for 5 min. Apoplastic protein content was determined by a modified Lowry procedure as described by Brune *et al*. (1994).

Analysis of ion content. IWF was extracted in HNO_3 (10% v/v). The extracts were analyzed for Na^+, K^+ and Ca^{2+} with a flamephotometer (Cornning 410). Each measurement was performed in triplicate. The element contents were quantified by comparison with standards solutions at appropriate dilutions: ion concentration was calculated as mg mL^{-1} IWF.

Analysis of polypeptide composition. For the analysis by one-dimensional SDS-PAGE, intercellular washing fluid was diluted with identical volumes of a two–fold concentrated loading buffer to final concentrations of 2.5% SDS, 125 mm Tris-HCl pH 6.8, 10 mm dithiothreitol (DTT) and 10% glycerol. After at heating at 95°C for 5 min, polypeptides were concentrated in a 6% stacking gel and separated in 12% resolving polyacrylamide gel (Dietz & Bogorad, 1987). Polypeptides were visualized by staining with silver nitrate. Data were ananlysed and identified by gel documentaion system; molecular protein markers, percentages of band intensity, molecular weight of each polypeptide in relation to standard markers using gel analyser (Phoretix 1D V 2).

Determination of enzyme activities. Activities of acid hydrolases were determined by liberation of p-nitrophenol from synthetic substrates as described by Boller and Kende (1979). The incubation assay contained 100 mm citric acid-KOH, pH 4.6, 2 mg p-nitrophenol substrates and extracts (5 - 50 μL) in a final volume 400 μL. After incubation at 37°C for 60 min, the reaction was terminated by alkalinization with 1 mL of 100 mm Na_2CO_3. The amount of liberated p-nitrophenol was quantified spectrophotometrically at 405 nm using the molar extinction coefficient of ε =18.400 m cm^{-1}. Apoplastic peroxidase activity was measured using the tetramerization reaction of guaiacol. The spectrophotometric assay contained 100 mm phosphate buffer, pH 6.5, 2 mm guaiacol, 1 mm H_2O_2 and sample. Changes in absorption were monitored at 436 nm. Tetramerization rate was calculated using the molar extinction. (ε = 2550 m cm^{-1}).

RESULTS AND DISCUSSION

The effects of both biotic and a biotic stress on apoplastic space have been studied by some authors. The reports suggest that apoplastic compartment could be important in the plant cells response to stresses (Vanacker *et al*., 1998, 99; Ranieri *et al*., 1996; 2000; Minibaeva *et al*., 2001). Therefore, in the present study, the effect of drought on apoplastic minerals, protein pattern and activities of apoplastic peroxidase (POX) and hydrolases were investigated in the leaves of two *Vicia faba* cultivars differing in drought tolerance. In previous study, among five *Vicia faba* cultivars Giza 40 showed the highest and Giza 667 the lowest drought tolerance (El-Tayeb, 2006). Therefore, cv Giza 40 and cv Giza 667 were used to test the effect of drought (40% field capacity) on the leaf apoplast.

Effect of drought on minerals and protein contents in the IWF. Plants of the dought tolerant cultivar (*V. faba* Giza 40) grown under drought showed lower Na^+, K^+ and Ca^{2+} concentration in the apoplastic space of leaves than those of control plants by about 25, 21.9 and 42.2%, respectively (Table I). In cv. Giza 667, drought decreased the apoplastic K^+ concentration by about 16.5% and increased Na^+ and Ca^{2+} concentration by 128.6% and 58%, respectively. The drought-tolerant cv Giza 40 exhibited low salt concentration in apoplast when compared with those of cv. Giza 667 under drought stress. In agreement with this Munns (1993) related apoplastic salt accumulation with damage and low apoplastic salt accumulation with tolerance. It is worthy to notice that K^+:Na^+ ratio in leaf apoplast slightly affected in cv Giza 40 by drought, while a significant decrase in case of cv Giza 667 was observed. It appears that drought tolerance in cv Giza 40 was associated by higher K^+:Na^+ although it had lower concentration of these ions. Joshi *et al.* (1979) suggested that stress tolerance of plants could be characterised by higher K^+:Na^+ ratio, which may be used to predict drought tolerance in wheat varieties.

The apoplastic protein content exhibited oppisite response to drought stress in the two cultivars (Table I). While the protein content of the IWF strongly increased (86.8%) in the leaves of cv Giaz 40 (relatively drought-tolerant), it sharply decreased (52.3%) in case of cv Giza 667 (relatively drought-sensitive) in response to drought. Ramanjulu *et al.* (1999) found that drought induced a significant decrease in the apoplastic protein content in the leaves of moderatly salt-tolerant barley. The accumulation of proteins in leaf apoplast has been reported in stress tolerant species including winter rye (Yu & Griffith, 2001) and *Deschampsia antarctica* (Pravo & Griffith, 2005). The data indicate that, accumulation of proteins in the apoplast could be used to predict drought tolerance of *Vicia faba* cultivars.

Effect of drought on proteins composition of IWF. In order to look for polypeptides responsive to drought, apopalstic fluids were isolated from leaves and analysed for polypeptide composition by SDS PAGE (Fig. 1). Known amounts of IWF were loaded on each lane. In control plants, analysis of leaf apoplatic proteins revealed the presence of 16 protein bands in the molecular weight range of 179.81 to 0.82 kDa and 11 bands with molecular weight range of 209.3 to 1.078 kDa in realtively drought-sensitive cv Giza 667 and drought-tolerant cv Giza 40, respectively (Table II). Drought diminished the protein bands in the apoplast of cv Giza 667 leaves from 16 to 12, while these bands increased from 11 to 13 in case of drought tolerant cv Giza 40. In addition, in case of former cv. 14 apoplastic proteins

Table I. Protein, Na^+, K^+, K^+/Na^+ and Ca^{2+} contents of intercellular washing fluid (IWF) isolated from leaves of two *Vicia faba* cultivars subjected drought (40% Field Capacity). The data are means of 4 independent replicates ± SD, respectively

Cultivar	Field Capacity (F.C%)	Protein content (µg/µl-IWF^{-1})	Na^+ (mg ml-IWF^{-1})	K^+ (mg ml-IWF^{-1})	Ca^{2+} (mg ml-IWF^{-1})	K^+/Na^+
cv Giza 40	90% (Control)	13.22 ± 0.69	0.024 ± 0.007	0.234 ± 0.041	0.161 ± 0.016	9.844 ± 1.397
	40%	24.69 ± 2.05	0.018 ± 0.006	0.183 ± 0.012	0.093 ± 0.026	8.991 ± 0.857
		(86.8)	(-25.0)	(-21.9)	(-42.2)	(-8.7)
cv Giza 667	90% (Control)	16.30 ± 2.78	0.017 ± 0.003	0.220 ± 0.002	0.088 ± 0.019	13.177 ± 2.483
	40%	7.77 ± 2.43	0.039 ± 0.008	0.184 ±0.010	0.139 ±0.022	4.628 ±0.952
		(-52.3)	(128.6)	(-16.5)	(58.0)	(-64.9)

disappeared, 10 polypeptides were *de novo* syntheized and two polypeptides at 80.36 and 12.87 kDa increased in band intensity in response to drought stress. With regarde to cv Giza 40, all apoplastic proteins 11 disappeared and 13 proteins were newely sybthesized under drought conditions. A detailed analysis comparing the response of apoplastic proteins in different cultivars of a species differeing in drought tolerance is not available. However, Robinson *et al.* (1990) suggested that the disappearance of polypeptides during stress were compensated by the increased synthesis of others. Moreover, under salt stress, despite the reduction in protein levels (Singla & Grover, 1994), the cells preferentially synthesised a few specific proteins that are termed stress proteins (Pureek *et al*., 1995). It appears that one of the most important mechanisms involved in the cell protection against stress is the induction of *de novo* synthesis of a set of proteins (Kermode, 1997). In the present study, drought in general, induced *de novo* synthesis of 13 polypeptides (Mwt: 84.61, 26.22, 15.54, 15.05, 13.31, 9.86, 7.56, 5.78, 3.25, 2.78, 2.56, 1.71 & 0.54 kDa) and 10 new polypeptides (Mwt: 169.87, 14.91, 8.60, 7.00, 5.37, 3.09, 2.66, 2.18, 1.61 & 0.54 kDa) in the leaf apoplast of drought-tolerant Giza 40 and drought-sensitive cv Giza 667, respectively (Table II). In consistent with this, Kuznetsov and Shevyakova (1997) obseved that the salt tolerant cells could be related with selective phosphorylation of several poplypeptides The data reveal that cv Giza 40 had higher number of drought responsive proteins than cv Giza 667. Also the polypeptides of leaf apoplast from drought treated cv Giza 40 (Drought-tolerant) was characterized by the apperance of *de novo* synthesis proteins of molecular masses: 15.05, 15.54 and 26.22 kDa (Table II). Yu and Griffith (2001) reported that, two thaumatin-like proteins (16 & 26 kDa) are drought-induced apoplastic proteins in winter rye leaves. They concluded that cold acclimation and drought induced antifreeze activity in winter rye plants. Hwang *et al*. (2004) found that thaumatin-like proteins of 16, 26 kDa were increased in their amounts in apoplast as cold treatment being longer. They also observed a correlation among the amounts of those proteins accumulated in apoplast and freezing tolerance in five cultivars of barley. These proteins may play a role in osmorgulation in leaf apoplast of cv Giza 40. In consistent with this, Bressan *et al.* (1987) showed the induction of 26 kDa polypeptide, which was increased with the addaption of cultured tobacco cell to salt stress.

The above findings suggest that drought may activate different regulatory genes in various genotypes of a species. The data also indicated a direct or indirect role for some the drought-induced proteins in cellular adaptiaton to stress. Detailed characterization of these proteins in the two contrasting broad bean genotypes would aid in further understanding of the molecular regulation of drought tolerance and sensitivity in plant cultivars.

Effect of drought on apoplastic peroxidase and hydrolases. The changes in the apoplastic protein content brought by drought stress were further followed with regard to specific enzyme activity known to be expressed in the apoplast. In case of drought sensitive cv Giza 667, drought increased the activities of peroxidase by up to 118.5%, acid phosphatase by 92.7% and β-glucosidase by 17.4%, whereas decreased the activities of α-mannosidase by 54.5%, phosphodiestrease by 48.3% and β-galactosidase by 38.5%. Drought stimulated the activities of acid phosphotase by up to 247.6%, peroxidase to 174.2%, β-glucosidase by 85.4%, α-mannosidase by 70.3%, phosphodiestrase by 34.9% and β-galactosidase by 21.8% (Table III). The results revealed that, cv Giza 40 has apoplastic enzymes with higher activity than those in cv Giza 667 under both control and drought conditions. The increase of peroxidase activity was higher in cv Giza 40 than in Giza 667. It has been reported that apoplastic peroxidase activity increased under various stress conditions (Cipollini, 1998; Ramanjulu *et al*., 1999; Ranieri *et al*., 2000; Taşgın *et al*., 2006). Gay and Trezum (1995) found that, resistant varity of cabbage has more peroxdase isozymes in hydathode fluid under biotic stress than susceptible one. This may indicate that, apoplastic peroxidase playes a role in drought tolerance, which is more pronounced in case of drought-tolerant cv Giza 40. In agreement with this, Minibaeva and Gordon (2003) reported that some soluble peroxidase forms are easily secreted into the apoplast and supposed to induce the plant defense response to reactive oxygen species.

Hydrolytic enzymes are typical constituents of extraplasmic compartments of plant cells (Holden & Rohringer, 1985). Under drought, the stimulation of apoplastic β-galactosidase was higher in Giza 40 than in Giza 667. β-galactosidase is involved in breakdown of pectic polymers of galactose during cell growth (Konno *et*

Table II. Effect of drought (40% FC) on molecular weight and relative amount of the different types of protein bands of the leaf apoplast of two *Vicia faba* cultivars

Band No	MW (kDa)	cv Giza 40 Control	0% FC	MW (kDa)	cv Giza 667 Control	40% FC
1	209.30	0.12	-	179.81	0.48	-
2	84.61	-	2.52*	169.87	-	0.48*
3	80.36	2.58	-	80.37	2.64	2.94
4	26.22	-	0.48*	34.09	0.48	-
5	15.54	-	1.2*	23.79	0.48	-
6	15.20	2.1	-	14.91	-	1.62*
7	15.05	-	0.6*	14.78	1.62	-
8	13.31	-	1.14*	12.87	1.08	1.44
9	13.16	1.44	-	10.83	0.48	-
10	9.86	-	1.08*	9.07	0.78	-
11	8.60	1.2	-	8.60	-	0.9*
12	7.56	-	0.48*	7.00	-	0.36*
13	7.42	0.48	-	5.57	1.74	-
14	5.78	-	0.48*	5.37	-	1.38*
15	5.57	0.84	-	3.64	0.36	-
16	3.79	0.54	-	3.21	0.6	-
17	3.37	1.08		3.09	-	1.2*
18	3.25	-	1.62*	2.99	0.54	-
19	2.78	-	0.42*	2.66	-	0.66*
20	2.56	-	1.26*	2.63	0.48	-
21	2.53	1.56	-	2.43	1.26	-
22	1.71	-	1.02*	2.18	-	0.78*
23	1.68	0.78	-	1.61	-	0.96*
24	0.54	-	1.8*	1.54	0.96	-
				0.82	1.08	-
				0.54	-	1.32*
Total number		11	13		16	12
No of responsive proteins			13			10

al., 1986) and in breakdown the bonds between cell wall polysaccharides during cell wall loosening (Murry & Bandurski, 1975). The activity of β-glucosidase stimulated by drought stress in the leaf apoplast of both cultivars. However, this stemulation was higher in cv Giza 40 than in case of Giza 667 (Table III). In consistence with this, β-glucosidase activity is expressed in barley leaves, which hydrolyses ABA glucopyranoside in the apoplast thereby releasing the physiologically active free ABA (Dietz *et al*., 2000). The liberated ABA may be taken up into the cells and trigger responses involved in the adaptation of plant to the prevailing stress conditions, in addition to free ABA. In the present study, the activity of α-mannosidase increased in cv Giza 40, while it was declined in case of Giza 667 in response to drought α-mannosidase is involved in glycosylation and deglycosylation of proteins and thereby in protein routing and protection against hydrolytic degaradation (Faye *et al*., 1988). It is suggested that the increased α-mannosidase activity in drought-tolerant cv Giza 40 could be due to the stimulated vesicular transport from cytoplam to the apoplast by exocytosis. Water stress is known to activate extracellular phosphatases (Stephan *et al*., 1994; Shih & Kao, 1998). Phospatases are involved in the acquisition and recycling of Pi. A drought-induced stimulation of acid phosphatse activity was obseved in this study, which was more pronounced in case of drought-tolerant cv Giza 40.

In conclusion, the results clearly demostrate the different responsiveness of leaf apoplast in two *Vicia faba* cultivars differing in tolerance to drought. Drought tolerance in cv Giza 40 was associated with higher K^+:Na^+ ratio, protein content and the activity of peroxidase and hydrolases in leaf apoplast. Further work on these lines is necessary to test is the amount of the protien induced by drought stress and the activity of peroxidase and hydrolases could be used as an indicator for drought tolerance in genotypes of *Vicia faba*.

Fig. 1. SDS PAGE of apoplastic proteins of two *Vicia faba* cultivars (Giza 40 & Giza 667) grown under control or subjected to drought (40% Field capacity). Identical amounts of IWF were loaded in each lane of 12.5% PAGE and stained with silver nitrate

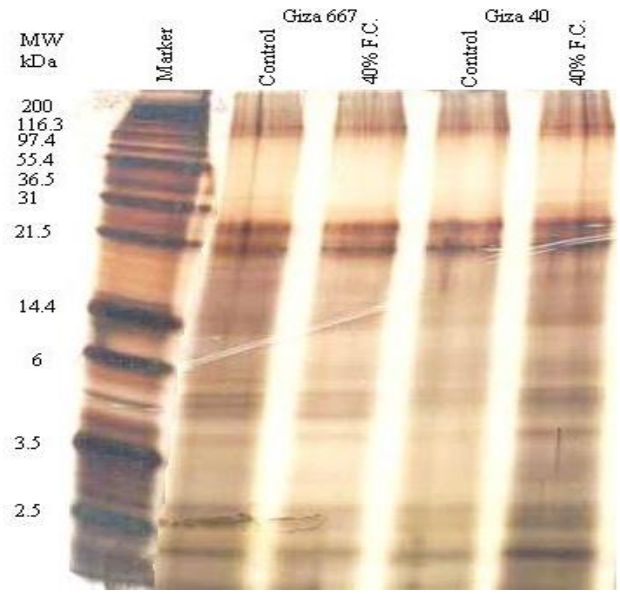

Table III. Activity of acidic hydrolases and peroxidases in the IWF in the leaves of two *Vicia faba* cultivars subjected to drought (40% F.C.). The data are means of 8 (Peroxidase) and 4 (Hydrolases) determinations, replicates ± SD, respectively. The number in bracets give the percent increase as compared to the control

	Giza 40		Giza 667	
	Control	40% F.C.	Control	40% F.C.
Enzyme	Enzyme activity [nmol-ml IWF^{-1} min^{-1}]			
Peroxidase	786.0 ± 75.1 (0)	2155.5 ± 62.0 (174.2)	513.3 ± 70.2 (0)	1121.5 ± 94.0 (118.5)
Phosphatase	333.2 ± 24.8 (0)	1158.7 ± 116.8 (247.6)	310.8 ± 2.6 (0)	598.8 ± 53.8 (92.7)
β-Glucosidase	82.13 ± 5.96 (0)	152.24 ± 26.67 (85.4)	81.20 ± 8.74 (0)	95.36 ± 7.43 (17.4)
β-Galactosidase	135.1 ± 5.3 (0)	164.6 ± 11.7 (21.8)	125.6 ± 6.8 (0)	77.2 ± 10.1 (-38.5)
α-Mannosidase	28.0 ± 1.9 (0)	47.7 ± 5.3 (70.3)	21.9 ± 1.1 (0)	10.0 ± 1.3 (-54.5)
Phosphodiestrase	9.71 ± 0.44 (0)	13.11 ± 0.47 (34.9)	4.93 ± 0.93 (0)	2.55 ± 0.44 (-48.3)

Acknowledgment. Special thanks to Prof. Dr. Karl-Josef Dietz, Department of Plant Physiology and Biochemistry, Bielefeld University, Germany for providing lab facilities.

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(Received 22 June 2006; Accepted 20 September 2006)