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Identification of Discriminant Factors after Exposure of Maize and Common Bean Plantlets to Abiotic Stresses

Lázaro HERNÁNDEZ[1], Octavio LOYOLA-GONZÁLEZ[1], Bárbara VALLE[1], Julia MARTÍNEZ[1], Leyanes DÍAZ-LÓPEZ[1], Carlos ARAGÓN[1], Oscar VICENTE[2], Jutta PAPENBROCK[3], Richard TRETHOWAN[4], Lourdes YABOR[1], José Carlos LORENZO[1*]

[1]*Universidad de Ciego de Ávila, Centro de Bioplantas, Ciego de Ávila 69450, Cuba; lazaro@bioplantas.cu; octavioloyola@bioplantas.cu; bvalle@bioplantas.cu; jmartinez@bioplantas.cu; leyanes@bioplantas.cu; cearagon79@gmail.com; lyabor@bioplantas.cu; jclorenzo@bioplantas.cu (*corresponding author)*
[2]*Universitat Politècnica de València, Institute of Plant Molecular and Cellular Biology (IBMCP, UPV-CSIC), Camino de Vera s/n, 46022 Valencia, Spain; ovicente@ibmcp.upv.es*
[3]*Leibniz University Hannover, Institute of Botany, Herrenhaeuser Str. 2, D-30419 Hannover, Germany; Jutta.Papenbrock@botanik.uni-hannover.de*
[4]*The University of Sydney, Plant Breeding Institute, Faculty of Agriculture and Environment, Australia; richard.trethowan@sydney.edu.au*

Abstract

Adverse environmental conditions limit crop yield and better understanding of plant response to stress will assist the development of more tolerant cultivars. Maize and common bean plantlets were evaluated under salinity, high temperature, drought and waterlogged conditions to identify biochemical markers which could be useful for rapid identification of putative stress tolerant plants. The levels of phenolics (free, cell wall-linked, total), aldehydes including malondialdehyde and chlorophylls (*a*, *b*, total) were measured on stressed plantlets. Only two indicators were statistically non-significant: chlorophyll *b* in maize plantlets stressed with sodium chloride and malondialdehyde content in drought stressed maize. The most remarkable effects of abiotic stresses can be summarized as follows: (i) salinity increased levels of free phenolics in maize plantlets and chlorophylls (*a*, *b*, total) in common bean; (ii) high temperature (40 °C) elevated levels of chlorophylls (*a*, *b*, total) in maize but decreased chlorophylls (*a*, *b*, total) and free phenolics in common bean; (iii) drought increased phenolics and decreased chlorophylls (*a*, *b*, total) in maize and increased chlorophyll pigments (*a*, *b*, total) in common bean; (iv) waterlogging increased free phenolics and decreased chlorophylls (*a*, *b*, total) in maize and increased chlorophyll (*a*, total) in common bean. Free phenolics and chlorophylls, especially *a*, were the most responsive indicators to stress and can, therefore, be considered putative biochemical markers for abiotic stress tolerance in maize and common bean. The use of Fisher's linear discriminant analysis to differentiate non-stressed and stressed plants in breeding programs is also a novel aspect of this report. Fisher's linear discriminant functions classified correctly 100% of non-stressed or stressed originally grouped plants.

Keywords: abiotic stress, biochemical markers, genetic improvement, *Phaseolus vulgaris*, *Zea mays*

Introduction

Adverse environmental conditions, such as salinity, high temperature, drought and waterlogging, limit the geographical distribution of plant species and crop yield (Osmond *et al.*, 1987). Predicted climatic change, population growth and the importance of sustainable food production makes the development of stress tolerant crop cultivars a high-priority globally (Zhu, 2001). Maize is the second most important agricultural crop globally. It is a human and livestock food and also used in the processing of industrial goods (Qing *et al.*, 2009). Global maize production in 2011 exceeded 700 million tons (FAOSTAT, 2013). However, legumes also play a critical role in human and animal diets and contribute to sustainability by maintaining soil fertility (Tilman *et al.*, 2002). The protein content of grain legumes can be three times that of cereal grains, thus a significant proportion of human protein and nutritional requirements can be supplied by legumes (Gepts *et al.*, 2005). Common bean (*Phaseolus vulgaris* L.), one of the world's most important grain legumes, is consumed as a dietary staple worldwide, particularly in Latin America and Africa (FAOSTAT, 2013).

Efficient and effective genetic improvement of stress tolerance of crops such as maize and common bean requires easy to measure markers that have a higher heritability than

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the targeted abiotic stress trait (William *et al.*, 2007). A number of biochemical markers have been reported for abiotic stresses. For example, salinity is associated with increases in abscisic acid (Shafi *et al.*, 2011), proline (Benhassaini *et al.*, 2012), glycine-betaine (Quan *et al.*, 2004), polylols, sugar alcohols and soluble sugar concentrations (Gurmani *et al.*, 2007). Salinity stress also decreases plant growth (Munns, 2005), nutrient uptake (Abdelgadir *et al.*, 2005), K^+: Na^+ ratio (Díaz-López *et al.*, 2012a), stomatal aperture and density (Huang *et al.*, 2009), hexoses, sucrose and starch (Arbona *et al.*, 2005) and chlorophyll contents (Rivelli *et al.*, 2012).

Moreover, high temperature stress is associated with increased lipid peroxidation (Silva *et al.*, 2010b) and decreased photosynthesis (Ribeiro *et al.*, 2009), CO_2:O_2 ratio in chloroplasts (Foyer and Noctor, 2000) and stomatal aperture (Ribeiro *et al.*, 2004). Whereas drought stress is linked with increased abscisic acid (Gurmani *et al.*, 2007), myo-inositol (Díaz-López *et al.*, 2012b) and glycine-betaine levels (Quan *et al.*, 2004); and decreased CO_2 assimilation (Gindaba *et al.*, 2004), relative water content (Galle *et al.*, 2007), leaf turgor pressure (Schachtman and Goodger, 2008), osmotic potential (Silva *et al.*, 2010a), starch content (Chao *et al.*, 2006) and sugars and oligosaccharides (Anderson and Kohorn, 2001). Likewise, waterlogging is associated with increased free amino acids (Medina *et al.*, 2009), abscisic acid (Xu *et al.*, 2007), and Na^+ and Cl^- concentrations (Wetson and Flowers, 2010), and decreased total biomass (Colmer and Voesenek, 2009), relative growth rate (Mielke *et al.*, 2003), stomatal conductance and photosynthesis (Lopez and Kursar, 2003), CO_2 assimilation (Gimeno *et al.*, 2012), soluble sugars and starch concentration (Gimeno *et al.*, 2012).

In a study of maize under salinity stress, Omoto *et al.* (2012) found that the activities of pyruvate orthophosphate dikinase, phosphoenolpyruvate carboxylase, NADP-dependent malate dehydrogenase and NAD-dependent malate dehydrogenase, which are derived mainly from mesophyll cells, increased, whereas those of NADP-malic enzyme and ribulose-1,5-bisphosphate carboxylase / oxygenase, which are derived mainly from bundle sheath cells, decreased. In salt-treated plants, the photosynthetic metabolites malate, pyruvate and starch decreased by 40, 89 and 81%, respectively. Gas-exchange analysis revealed that the net photosynthetic rate, the transpiration rate, stomatal conductance and the intercellular CO_2 concentration decreased strongly in salt-treated plants. Moreover, maize net photosynthesis was inhibited at leaf temperatures above 38ºC, transpiration rate increased progressively while nonphotochemical fluorescence quenching increased (Crafts-Brandner and Salvucci, 2002). However, under drought stress a substantial decrease in gas exchange attributes (net photosynthetic rate, transpiration rate, stomatal conductance, water use efficiency, instantaneous water use efficiency and intercellular CO_2) was observed in maize (Anjum *et al.*, 2011).

Anaerobic treatment dramatically altered the patterns of gene expression in maize seedlings (Subbaiah and Sachs, 2002). During anaerobiosis pre - existing protein synthesis is immediately repressed, with the concurrent initiation of selective synthesis of approximately 20 proteins. Among these anaerobic proteins were enzymes involved in glycolysis and related processes. However, inducible genes that have different functions were also found; these may function in other, perhaps more long - term, processes of adaptations to flooding, such as aerenchyma formation and root - tip death (Subbaiah and Sachs, 2002).

In common bean, salinity had adverse effects not only on biomass yield and relative growth rate, but also on other morphological parameters such as plant height, number of leaves, root length and shoot/root weight ratio. Photosynthesis, transpiration rate and stomatal conductance were also adversely affected (Gama *et al.*, 2007). In contrast, high temperature that exceeds optimal growth conditions tends to decrease both NO_3^- uptake and N_2 fixation (Hungria and Kaschuk, 2014). Drought stress reduces leaf water potential and gas-exchange characteristics (CO_2 assimilation, stomatal conductance) (Fenta *et al.*, 2014). It has been suggested that nodule characteristics and symbiotic nitrogen fixation ability should be included with above- and below-ground traits as phenotypic markers in germplasm evaluation and breeding programs aimed at improving drought tolerance in common bean (Fenta *et al.*, 2014). Flooding tends to reduce root dry weight, leaf area and total chlorophyll content in common bean (Celik and Turhan, 2011).

This work focuses on two of the most important grain crops in Cuba and many other countries: maize and common bean. Our aim was to identify previously unreported biochemical markers for tolerance to salinity, high temperature, drought and waterlogging, which could be used for the rapid identification of putative stress tolerant maize and common bean plants in crop breeding programs. Stress screening was conducted on young plantlets; a method that allows large numbers of plants to be inexpensively screened. These protocols will therefore be attractive to crop breeding programs. We measured phenolics, aldehydes and chlorophylls to examine their expression in maize and common bean under abiotic stress. Data collected were used to generate Fisher's linear discriminant functions and differentiate non-stressed and stressed plants.

Materials and Methods

Survival response to different levels of stress

After harvesting in Ciego de Avila, Cuba (2012), maize (cv. 'Tuzón') and common bean (cv. 'Milagro Villaclareño') seeds were stored at 4 °C in the dark in hermetically closed containers. Seeds at 12% moisture content based on fresh weight (ISTA, 2005) were stored. Seeds of maize and common bean were sown in plant containers (200 cm^3 of ferralitic red soil collected in Ciego de Avila, Cuba, pH 6.8, conductivity: 0.88 S cm^{-1}, 3 seeds per container) and allowed to germinate and grow in a growth chamber at 28 °C before the imposition of stress treatments. The photosynthetic photon flux density was 800 µmol m^{-2} s^{-1}. Chemical fertilizers were not used and each plant container was irrigated with 25 ml water daily for 10 d. After 10 d the plantlets were subjected to different stress treatments using five containers per treatment.

Salt stress was imposed by irrigating each pot daily with 25 ml of NaCl solution at increasing concentrations (200, 400, 600 and 800 mM) – or with water in the non-stressed controls – and

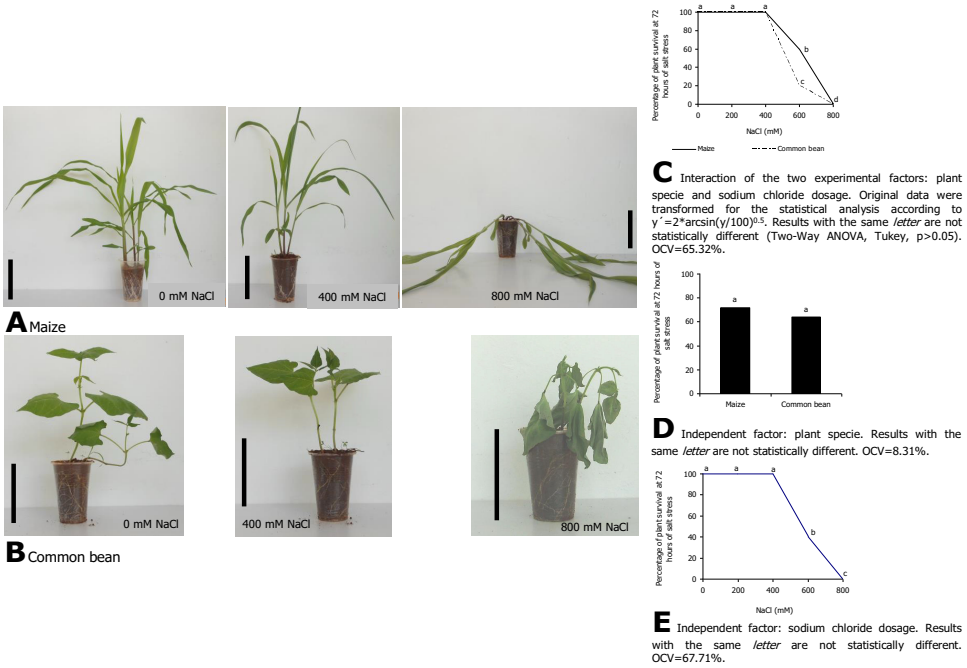

Fig. 1. Effect of sodium chloride on maize and common bean plantlets. Seeds were allowed to germinate and grow without salt stress during 10 days, then plantlets were stressed during 72 hours. Each plant container was irrigated every day with 25 ml water (without or with NaCl). In each photograph, black vertical bars represent 10 cm. Pot volume = 200 cm^3. Substrate: Ferralitic red soil. In C, D and E, OCV means Overall Coefficient of Variation = (Standard deviation/Average)* 100. To calculate this coefficient, average values of each treatment were considered. The higher difference between the treatments compared, the higher the OCV

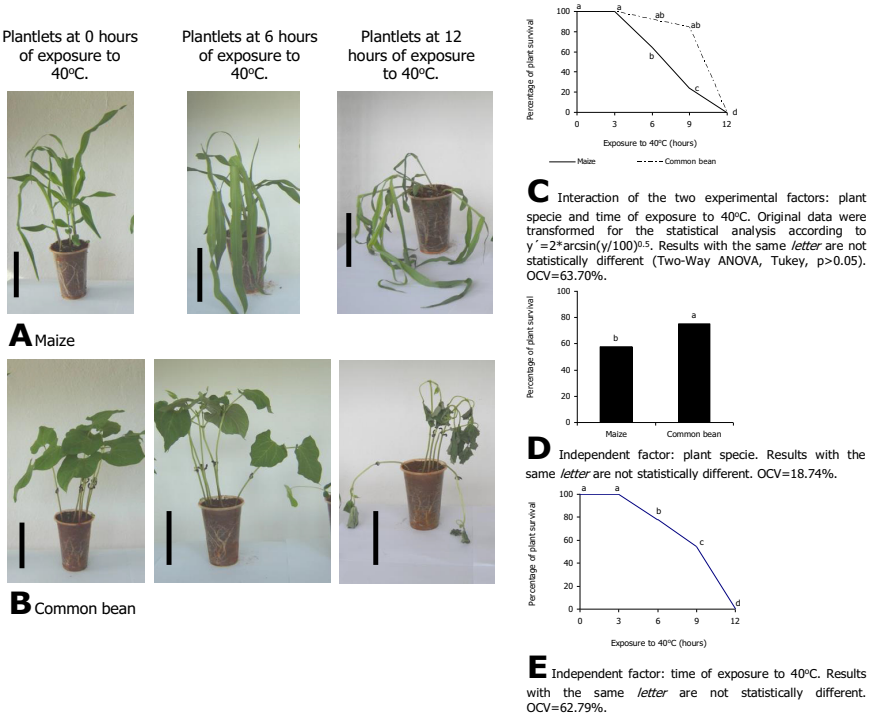

Fig. 2. Effect of exposure to high temperature (40 °C) on maize and common bean plantlets. Seeds were allowed to germinate and grow without high temperature stress (28 °C) during 10 days, then plantlets were exposed to 40 °C during 12 hours. In each photograph, black vertical bars represent 10 cm. Pot volume = 200 cm^3. Substrate: Ferralitic red soil. In C, D and E, OCV means Overall Coefficient of Variation = (Standard deviation/Average)* 100. To calculate this coefficient, average values of each treatment were considered. The higher difference between the treatments compared, the higher the OCV

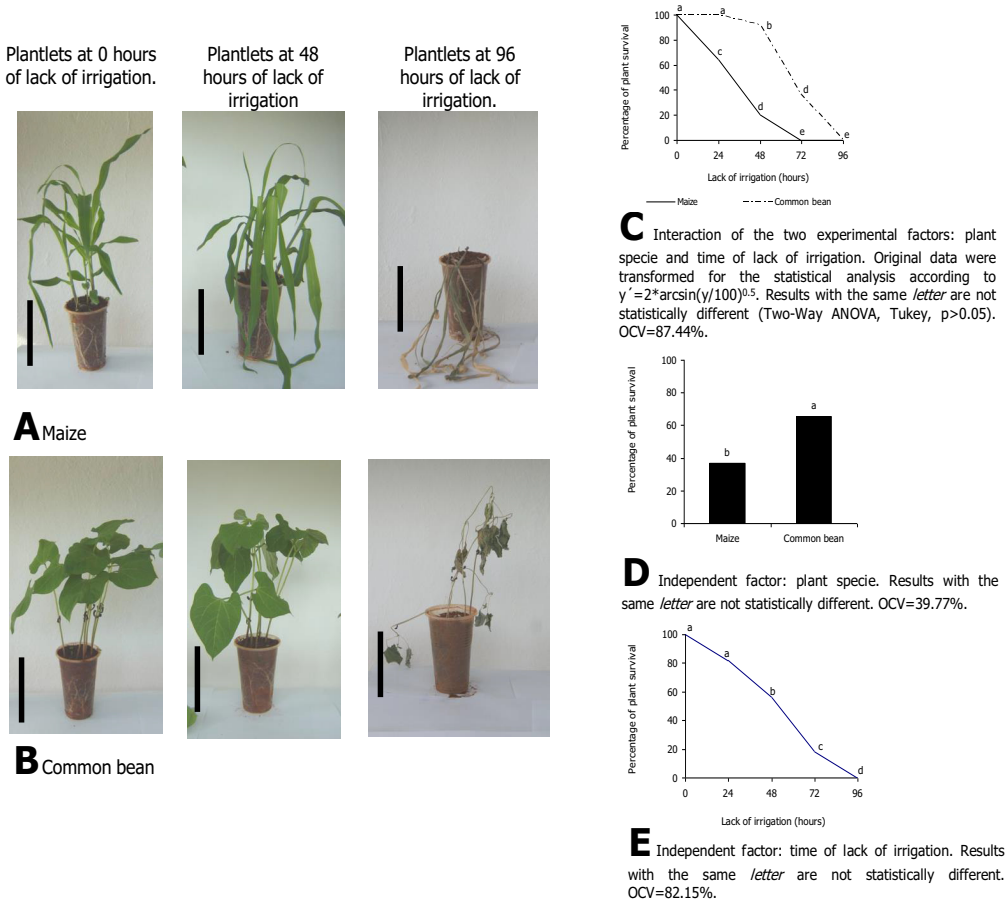

Fig. 3. Effect of lack of irrigation on maize and common bean plantlets. Pots were irrigated every day with 25 ml water during 10 days, then watering was suspended. In each photograph, black vertical bars represent 10 cm. Pot volume = 200 cm^3. Substrate: Ferralitic red soil. In C, D and E, OCV means Overall Coefficient of Variation = (Standard deviation/Average)* 100. To calculate this coefficient, average values of each treatment were considered. The higher difference between the treatments compared, the higher the OCV

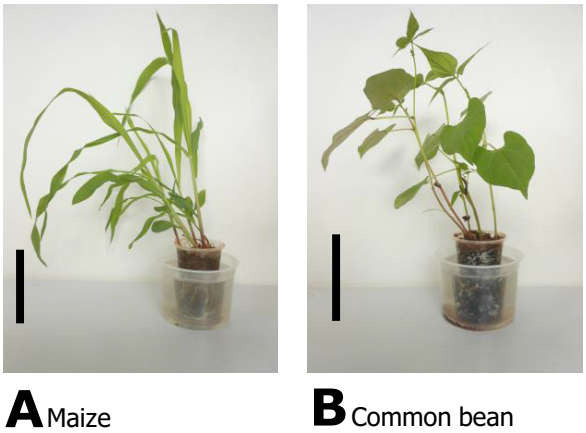

Fig. 4. Effect of flooding on maize and common bean plantlets. Pots were irrigated every day with 25 ml water during 10 days, then pots were immersed into 350 ml water during 10 days but plant survival was not affected as shown in these photographs. In each photograph, black vertical bars represent 10 cm. Plant pot volume = 200 cm^3. Substrate: Ferralitic red soil

percentage plantlet survival was recorded after 72 h. Heat stress was generated by exposing plantlets to 40 ºC for 12 h and survival was assessed every 3 h of treatment. Drought stress was imposed by suspending watering for 96 h; during this period, plantlet survival was registered every 24 h. Finally, to assess the effect of waterlogging, pots were immersed in 350 ml water for an additional 10 d and survival rates were determined every 24 h during this period.

Biochemical changes induced by stresses

Phenolics, aldehydes and chlorophylls were assessed in stress-treated maize and common bean plantlets ten days after sowing. Plantlets otherwise maintained under the conditions described above, were either treated with 567 mM NaCl for 72 h (salt stress), exposed to 40 ºC for 9.3 h (heat stress), kept without irrigation for 51.8 h (water stress), or immersed in water (350 ml per pot) for 51.8 h. After treatment, middle-aged leaves were collected from the three plantlets of each container, pooled and ground in liquid nitrogen to a fine powder. Leaf material was similarly collected from the corresponding non-stressed controls. Three independent samples (1 g powder each) per treatment were used for all biochemical assays.

Chlorophylls (*a*, *b*, total) were quantified following Porra (2002), phenolics (free, cell wall-linked, total) by the method of Gurr *et al.* (1992), and malondialdehyde and other aldehydes as described

Table 1. Effect of sodium chloride on maize and common bean plantlets at 72 h of salt stress (567 mM NaCl)

Indicators evaluated in middle-aged leaves*	Maize		Common bean	
	0 mM NaCl	567 mM NaCl	0 mM NaCl	567 mM NaCl
Free phenolics (mg gallic acid equivalents/g fresh mass)	1.80b	14.94a	9.69b	10.14a
Cell wall-linked phenolics (mg gallic acid equivalents/g fresh mass)	86.03a	45.07b	43.82b	52.70a
Total content of phenolics (mg gallic acid equivalents/g fresh mass)	87.82a	60.01b	53.52b	62.84a
Malondialdehyde (µM/g fresh mass)	22.72b	34.66a	55.30a	43.65b
Other aldehydes (µM/g fresh mass)	81.14b	192.97a	129.54b	157.94a
Chlorophyll *a* (mg/g fresh mass)	2.96a	2.60b	0.23b	0.78a
Chlorophyll *b* (mg/g fresh mass)	1.78a	1.82a	0.53b	2.08a
Total content of chlorophyll (mg/g fresh mass)	4.67a	4.21b	0.79b	2.80a

*In each crop, results with the same *letter* are not statistically different (t-test, p>0.05).

Table 2. Effect of exposure to high temperature (40 °C) on maize and common bean plantlets at 9.3 h of stress

Indicators evaluated in middle-aged leaves*	Maize		Common bean	
	28 °C	40 °C	28 °C	40 °C
Free phenolics (mg gallic acid equivalents/g fresh mass)	14.40a	9.04b	6.54a	1.12b
Cell wall-linked phenolics (mg gallic acid equivalents/g fresh mass)	82.39a	47.96b	37.82a	19.10b
Total content of phenolics (mg gallic acid equivalents/g fresh mass)	96.79a	57.00b	44.37a	20.22b
Malondialdehyde (µM/g fresh mass)	27.58a	23.33b	80.37b	84.82a
Other aldehydes (µM/g fresh mass)	164.62a	88.63b	154.95b	365.73a
Chlorophyll *a* (mg/g fresh mass)	0.33b	3.05a	4.18a	0.25b
Chlorophyll *b* (mg/g fresh mass)	0.38b	1.85a	3.08a	0.13b
Total content of chlorophyll (mg/g fresh mass)	0.77b	4.81a	7.27a	0.35b

*In each crop, results with the same *letter* are not statistically different (t-test, p>0.05).

Table 3. Effect of drought on maize and common bean plantlets at 51.8 h of stress

Indicators evaluated in middle-aged leaves*	Maize		Common bean	
	25 ml water/day	Lack of irrigation	25 ml water/day	Lack of irrigation
Free phenolics (mg gallic acid equivalents/g fresh mass)	1.80b	11.96a	9.69b	12.15a
Cell wall-linked phenolics (mg gallic acid equivalents/g fresh mass)	86.03a	56.02b	43.82b	46.21a
Total content of phenolics (mg gallic acid equivalents/g fresh mass)	87.82a	67.97b	53.52b	58.36a
Malondialdehyde (µM/g fresh mass)	22.72a	23.67a	55.30b	95.98a
Other aldehydes (µM/g fresh mass)	81.14b	148.36a	129.54b	278.79a
Chlorophyll *a* (mg/g fresh mass)	2.96a	0.55b	0.23b	3.83a
Chlorophyll *b* (mg/g fresh mass)	1.78a	0.33b	0.53b	4.32a
Total content of chlorophyll (mg/g fresh mass)	4.67a	0.85b	0.79b	8.17a

*In each crop, results with the same *letter* are not statistically different (t-test, p>0.05).

in Heath and Packer (1968). To determine the levels of chlorophyll pigments, extraction was carried out with 5.0 ml acetone (80%, v:v). The samples were centrifuged (12,000 rpm, 4 °C, 15 min) and supernatants collected and absorbances at 647 and 664 nm recorded.

Phenolic compounds were extracted and quantified using a spectrophotometer by a colorimetric method based on reaction with Folin Ciocalteu reagent (mg gallic acid equivalents per g fresh mass). Malondialdehyde and other aldehydes were quantified by a colorimetric method based on reaction with thiobarbituric acid.

Statistical analyses

SPSS (Version 17.0 for Windows) was used to perform t-, ANOVA and Tukey test ($p \leq 0.05$). For the statistical analysis only, percentages of plant survival were transformed according to $y' = 2*\arcsin(y/100)^{05}$ to reach normality (Kolmogorov-Smirnov) and variance homogeneity (Levene).

Fisher's linear discriminant functions were generated from the data matrix recorded in this research (48 cases = 4 types of stress (salinity, high temperature, drought and waterlogging) x 2 plants (maize and common bean) x 2 experimental conditions (control and stressing treatment) x 3 replications). Eight variables were considered: levels of phenolics (free, cell-wall linked and total), malondialdehyde, other aldehydes and chlorophylls (*a*, *b* and total). SPSS was also used to obtain the Fisher's linear discriminant functions.

Results

Survival response to different levels of stress

The effect of salt stress on maize and common bean is shown in Fig. 1. Both crop species are susceptible to dosages higher than 400 mM NaCl (Fig. 1C, E). Although plant survival at 600 mM NaCl was lower in common bean than in maize this was not significant (Fig. 1D). In contrast, common bean plantlets showed higher tolerance to heat stress than maize (Fig. 2). In maize, plant survival decreased significantly with exposure to 40 °C for more than 3 h; whereas this reduction was very slight in common bean plantlets up to 9 h of treatment (Fig. 2C). When the time of exposure to heat stress is considered an independent factor; that is plantlets of both species are pooled, 50% survival was observed after 9.3 h of treatment (Fig. 2E). Additionally, common bean plantlets tolerated longer periods without irrigation than maize (Fig. 3C). A significant reduction in plant survival was observed after 24 h in maize and 48 h in common bean (Fig. 3E). Finally, plantlet survival was not affected up to 10 d waterlogging conditions in either species (Fig. 4).

Biochemical changes produced by stresses

Phenolic compounds, aldehydes and chlorophylls were assessed in middle-aged leaves from plants surviving 72 hours of treatment with 567 mM NaCl conditions which caused 50%

Table 4. Effect of waterlogging on maize and common bean plantlets at 51.8 h of stress

Indicators evaluated in middle-aged leaves*	Maize		Common bean	
	25 ml water/day	Flooded	25 ml water/day	Flooded
Free phenolics (mg gallic acid equivalents/g fresh mass)	1.80b	12.14a	9.69b	10.33a
Cell wall-linked phenolics (mg gallic acid equivalents/g fresh mass)	86.03a	43.54b	43.82b	57.47a
Total content of phenolics (mg gallic acid equivalents/g fresh mass)	87.82a	55.68b	53.52b	67.80a
Malondialdehyde (μM/g fresh mass)	22.72a	20.67b	55.30a	51.61b
Other aldehydes (μM/g fresh mass)	81.14b	83.52a	129.54b	272.39a
Chlorophyll *a* (mg/g fresh mass)	2.96a	0.34b	0.23b	1.84a
Chlorophyll *b* (mg/g fresh mass)	1.78a	0.22b	0.53b	1.14a
Total content of chlorophyll (mg/g fresh mass)	4.67a	0.52b	0.79b	2.95a

* In each crop, results with the same *letter* are not statistically different (t-test, p>0.05).

Table 5. Classification as non-stressed or stressed made by Fisher's discriminant functions

Type of stress evaluated	Plant		Results of discriminant functions				
			Function for non-stressed		Function for stressed	Classification according to discriminant functions	
Salinity	Maize	0 mM NaCl	89.35	>	74.70	Non-stressed	Correct
			97.74	>	80.02	Non-stressed	Correct
			100.32	>	81.59	Non-stressed	Correct
		567 mM NaCl	5.71	<	34.58	Stressed	Correct
			3.44	<	33.39	Stressed	Correct
			9.04	<	36.93	Stressed	Correct
	Common bean	0 mM NaCl	96.55	>	79.93	Non-stressed	Correct
			92.18	>	77.22	Non-stressed	Correct
			96.26	>	79.50	Non-stressed	Correct
		567 mM NaCl	12.91	<	32.76	Stressed	Correct
			9.91	<	30.82	Stressed	Correct
			6.45	<	28.77	Stressed	Correct
High temperature	Maize	28 °C	98.78	>	88.65	Non-stressed	Correct
			101.28	>	90.14	Non-stressed	Correct
			101.31	>	90.22	Non-stressed	Correct
		40 °C	40.23	<	48.48	Stressed	Correct
			39.85	<	48.40	Stressed	Correct
			-8.25	<	18.61	Stressed	Correct
	Common bean	28 °C	98.33	>	82.27	Non-stressed	Correct
			96.65	>	81.33	Non-stressed	Correct
			93.10	>	79.22	Non-stressed	Correct
		40 °C	51.01	<	62.14	Stressed	Correct
			49.55	<	61.20	Stressed	Correct
			51.42	<	62.22	Stressed	Correct
Drought	Maize	25 ml water/day	89.35	>	74.70	Non-stressed	Correct
			97.74	>	80.02	Non-stressed	Correct
			100.32	>	81.59	Non-stressed	Correct
		Lack of irrigation	45.85	<	54.35	Stressed	Correct
			36.74	<	48.78	Stressed	Correct
			41.29	<	51.41	Stressed	Correct
	Common bean	25 ml water/day	96.55	>	79.93	Non-stressed	Correct
			92.18	>	77.22	Non-stressed	Correct
			96.26	>	79.50	Non-stressed	Correct
		Lack of irrigation	51.01	<	62.14	Stressed	Correct
			49.55	<	61.20	Stressed	Correct
			51.42	<	62.22	Stressed	Correct
Waterlogging	Maize	25 ml water/day	89.35	>	74.70	Non-stressed	Correct
			97.74	>	80.02	Non-stressed	Correct
			100.32	>	81.59	Non-stressed	Correct
		Flooded	40.09	<	47.17	Stressed	Correct
			38.04	<	46.03	Stressed	Correct
			32.66	<	42.45	Stressed	Correct
	Common bean	25 ml water/day	96.55	>	79.93	Non-stressed	Correct
			92.18	>	77.22	Non-stressed	Correct
			96.26	>	79.50	Non-stressed	Correct
		Flooded	58.89	<	67.09	Stressed	Correct
			56.57	<	65.83	Stressed	Correct
			40.44	<	55.68	Stressed	Correct

Function for non-stressed = 1,879*A + 2,167*B + 2,861*C - 0,461*D + 1,869*E - 4,453*F - 97,446

Function for stressed = 1,661*A + 1,338*B + 1,657*C - 0,226*D + 1,22*E - 2,685*F - 46,834

Legend:
A: Free phenolics (mg gallic acid equivalents/g fresh mass)
B: Cell wall-linked phenolics (mg gallic acid equivalents/g fresh mass)
C: Malondialdehyde (µM/g fresh mass)
D: Other aldehydes (µM/g fresh mass)
E: Chlorophyll a (mg/g fresh mass)
F: Chlorophyll b (mg/g fresh mass)

Fig. 5. Fisher's linear discriminant functions to differentiate non-stressed and stressed materials

plantlet death as calculated from Fig. 1E and compared to levels in the corresponding non-stressed controls. In general, salt stress induced statistically significant changes in the levels of all indicators with the exception of chlorophyll *b* contents in maize, although in most cases these changes were relatively small. The most remarkable effects of abiotic stresses can be summarized as follows. The salinity stress treatment produced an 8-fold increase in free phenolics in maize and between 3 and 4-fold increases in chlorophyll content (*a*, *b*, and total) in common bean (Table 1). On the other hand, maize and common bean responded differently to heat stress in the levels of biochemical markers (Table 2). In common bean, when biochemical marker expression was assessed after 40ºC treatment for 9.3 h a 6-fold decrease in free phenolics and a 20-fold reduction of chlorophylls (*a*, *b*, and total) compared to the control was observed. In contrast, chlorophyll levels increased in maize between 5 and 10-fold under the same conditions. Moreover, fifty percent of all plantlets died 51.8 h after irrigation was suspended (Fig. 3E). Under these conditions, the most relevant observed changes in biochemical marker levels were a 7-fold increase in free phenolic compounds and a 5-fold reduction in chlorophyll contents (*a*, *b*, and total) in maize, and an increased in chlorophyll levels in common bean (Table 3). In the waterlogging stress experiment, biochemical evaluations were made 51.8 h post water immersion; a time that coincided with 50% plant death under drought. Similar to drought stress, waterlogging induced an increase in free phenolics and a decrease in chlorophyll levels in maize, while chlorophylls increased in common bean (Table 4).

In general, free phenolics tended to increase in maize under stress (salinity, drought and waterlogging) whereas chlorophylls decreased, particularly under drought and waterlogging. In contrast, chlorophyll levels in common bean increased under stress (salinity, drought and waterlogging). However, heat stress elicited a different response in both maize and common bean. Under high temperature, chlorophyll increased in maize while free phenolics decreased in common bean.

The statistical package-generated discriminant functions are shown in Fig. 5. Total contents of phenolics and chlorophylls were disregarded by SPSS. Requirements of this kind of analysis were met. Groups of the dependent variable were mutually excluded: plants were submitted or not to stress. Therefore, the dependent variable was not metrical but categorical. Independent variables (biochemical compound levels) were all metrical. The number of cases (48) was higher than twice the number of variables (8). Equality of covariance matrixes was reached (Box test, $p>0.05$). Results from both functions were compared and they classified correctly 100% of non-stressed or stressed originally grouped plants (Table 5).

Discussion

One of the first and more general responses of plants to abiotic stress is the inhibition of growth, since plants redirect all their resources (energy and metabolic precursors) to defense reactions against stress (Baker, 1993; Grattan and Grieve, 1999; Ullrich, 2002; Xiong and Zhu, 2002). This phenomenon has been reported under salinity stress (Munns, 2005), waterlogging (Lopez and Kursar, 2003; Mielke *et al.*, 2003), drought (Chao *et al.*, 2006) and high temperature (Ribeiro *et al.*, 2004). Furthermore, biochemical and physiological responses to the above stresses have been observed.

In our experiments, free phenolics and chlorophylls, especially *a*, were the most affected indicators and therefore can be regarded as potential abiotic stress biochemical markers. Leaf chlorophyll content was affected by salinity in tetraploid wheat (Munns and James, 2003), rice (Sultana *et al.*, 1999), *Brassica oleracea* (Bhattacharya *et al.*, 2004), *Brassica juncea* (Qasim, 1998) and *Brassica napus* (Pak *et al.*, 2009). Salinity can affect chlorophyll content through inhibition of chlorophyll synthesis or an acceleration of its degradation (Zhao *et al.*, 2007). Thioyapong *et al.* (2004) found that the chlorophyll losses due to salinity stress is consistent with possible differences in reactive oxygen species (ROS) production among the genotypes and suggested that in salt sensitive genotypes, ROS scavenging systems were unable to detoxify ROS generated. Our results do not support these findings as common bean chlorophyll levels increased under salinity (Table 1).

According to Baker (1993), changes in the photochemical efficiency of plants under drought may be assessed by the analysis of chlorophyll *a* fluorescence efficiency associated with photosystem II. Under stress, a decrease in the ratio of variable fluorescence / maximum fluorescence has been attributed to the inactivity of the photosystem II reaction centers due to the degradation of the D1 and D2 proteins responsible for the transfer of water electrons to chlorophyll *a* associated with the photosystem II reaction center (Hao *et al.*, 1999; Lazár, 1999). Chlorophyll content could therefore, be correlated to chlorophyll fluorescence thus indicating its suitability as a future biochemical marker. Abiotic stresses decrease photosynthesis, mainly by limiting CO_2 entrance to leaves through stomatal closure. Moreover, membrane systems containing chlorophylls are destabilized affecting the luminous phase thus leading to increased synthesis of chlorophylls that are unable to fix more CO_2 (Hörtensteiner, 2006; Hörtensteiner and Kräutler, 2011).

A consequence of the abiotic stress-induced limitation of photosynthesis is the exposure of plants to excess energy, which, if not safely dissipated, may be harmful to photosystem II because of over reduction of the reaction centers (Demmig-Adams and Adams, 1992) and increased production of ROS in the chloroplasts (Smirnoff, 1993). On the other hand excess energy could be used to synthesize secondary metabolites as suggested by Selmar and Kleinwächter (2013).

Phenolic compounds and flavonoids are among the most influential and widely distributed secondary products in the plant kingdom (Ali and Abbas, 2003). Many play important physiological and ecological roles and are involved in resistance to different types of stress (Ayaz *et al.*, 2000). These metabolites have several defense functions and, therefore, their biosynthesis in plants is generally induced in response to biotic and abiotic stimuli such as UV-B radiation, drought, chilling, ozone, heavy metals, and attacks by pathogens, wounding, or nutrient deficiency (Bettaieb *et al.*, 2011; Dixon and Paiva, 1995; Grace, 2005)

Our results indicated that free phenolics and chlorophylls, especially *a*, were the most responsive indicators. The differences recorded between maize and common bean is to some extent a

function of their differences in photosynthetic efficiency, however there may also be a genotype within species effect. We have hypothesized that, in stressed plants, levels of free phenolics and chlorophylls first increase and subsequently decrease. However, such changes take place in different time frames depending on the plant species and genotype. In the experiments shown here, moderate and severe stress conditions were applied that did not necessarily represent any specific natural environment, but were used for selection purposes only. In follow-up experiments, mild to moderate stress conditions may enable plant metabolism to respond properly to the respective stress conditions. Further studies are in progress to determine the practical use of free phenolics, chlorophylls and other biochemical markers for stress tolerance in breeding programs.

The Fisher's linear discriminant functions shown in this paper (Fig. 5) are important tools for those breeding programs focused on the production of abiotic stress-tolerant plants. Maize and common bean seeds of new genotypes can be grown for 10 days and then stressed or not as described here. Levels of phenolics (free and cell-wall linked), malondialdehyde, other aldehydes and chlorophylls (*a*, *b*) are determined. Such new data are evaluated in both discriminant functions. If the resulting value of the stressed discriminant function is similar to that of the non-stressed discriminant function, the new genotype can be regarded as putatively tolerant, as it shows similar physiology under either non-stressing or stressing conditions. Although the new genotype tolerance still requires additional confirmation under a field environment, the results described here allow some research cost reductions because there is no inclusion of a large number of susceptible cultivars in expensive field trials. At present, this research group is carrying out further experiments to know if these discriminant functions can be used in other plant species.

Discriminant analysis is useful for situations where the building of a predictive model of group membership based on observed characteristics of each case is desirable. The procedure generates discriminant functions based on linear combinations of the predictor variables, which provide the best discrimination between groups. The functions are generated from a sample of cases for which group membership is known. The functions can then be applied to new cases with measurements for the predictor variables but unknown group membership (Bantte and Prasanna, 2003; Cardi, 1998; Daoyu and Lawes, 2000; Figlivolo *et al.*, 2001; Somersalo, 1998; Teshome *et al.*, 1997). The use of this kind of analysis for differentiation of non-stressed or stressed plants is a novel aspect of this report that can be applied for early selection of plant tolerance to abiotic factors.

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