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A simple, fast and accurate screening method to estimate maize (*Zea mays* L) tolerance to drought at early stages

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Abstract

There is a great need for the selection of plants with higher drought tolerance, so that fast and effective techniques to identify variations in drought tolerance are mandatory for screening large numbers of genotypes. This work presents a protocol for easy and reliable assessment of responses of maize genotypes to water stress conditions imposed during early stages of development. Three experiments using 11 commercial maize hybrids under four levels of water stress were carried out: i) germination, ii) seedling growth, and iii) early growth bioassays. Constant and uniform water stress was imposed using solutions of polyethylene glycol 6000 (PEG 6000). Plant material was evaluated for several morphological, physiological and biochemical traits and monitored for photosynthetic efficiency. Principal component analysis (PCA) of these joint experiments revealed that germination percentage, early root development and stomatal conductance were the most useful traits for discriminating maize hybrids according to their tolerance to water stress. A subsequent greenhouse assay performed with two hybrids with contrasting responses under soil drying conditions validated the previous results. According to our results, the key of drought tolerance was a rapid response of stomatal conductance, which allowed a longer survival to stress even under severe desiccation. This work provides the researcher with a simple and reliable screening method that could be implemented as a decision support tool in the selection of the most suitable genotypes for cultivation in areas where water availability is a problem, as well as for the selection of tolerant genotypes to early drought in breeding programs.

Keywords: *Zea mays, germination, seedling growth, early growth*

Introduction

Abiotic stresses, especially drought, are one of the main challenges of agriculture at global scale as they strongly affect the potential productivity of crops, being responsible for large yield losses worldwide (Boyer et al, 2013). Drought is an endemic problem in large agricultural areas. Furthermore, inter-annual variations in water availability can compromise crop yields even in humid areas, such as the Atlantic coast of Spain, where maize is grown under rain-fed conditions. According to current models, drought is expected to worsen with ongoing climate changes, becoming a great challenge for future maize production (Olesen et al, 2011; Supit et al, 2012; Pachauri and Meyer, 2014).

Large screenings and identification of crop genotypes with high drought tolerance are one of the main pillars of studies on drought tolerance. In the search for an appropriate screening protocol, several strategies have been proposed. Direct measurement of drought tolerance traits under realistic conditions, by means of soil drying through natural drought or exclusion of throughfall or irrigation, appears to be the most unbiased approach. But field environmental conditions are unpredictable and heterogeneous, and thus these techniques introduce remarkable errors and difficulties for interpreting and comparing results. Moreover, replicated field trials performed on a large enough scale and during long enough periods would be in most cases prohibitive in terms of cost and space requirement. Results from greenhouse pot experiments can be easier to interpret but, still, the temporal and spatial variability of soil water loss results in unpredictable and heterogeneous conditions surrounding the root system (Passioura, 2006; Verslues et al, 2006; Whitmore and Whalley, 2009; Munns et al, 2010). More recently, high-throughput phenotyping techniques have revolutionized comprehensive studies on plant performance and responses to stresses, but their expansion is being hampered by their high cost and technical complexity so they are still far from being easily accessible to growers and breeders worldwide. Thus, none of these approaches

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are feasible at the large scale that routine evaluations or breeding programs require. There is still a need for simple, reliable and affordable laboratory screening tests that allow predicting drought tolerance for large genotype collections, as well as comparing and integrating results among institutions. Ideally this protocol should serve to agronomists, plant physiologists and breeders, by being «suitable for genetic studies and rapid screening while still being relevant to stress conditions in the real world», as said in Verslues et al (2006).

When implementing an appropriate screening protocol, very controlled conditions are needed in order to ensure the maximum reliability and repeatability (Vanhove et al, 2012). Hydroponic solutions in combination with ionic or non-ionic osmotica have been frequently used to simulate water stress effects in plants. Hydroponics allows the maintenance of uniform conditions, and osmotica can lower the water potential of the solution in a controlled and precise manner. Polyethylene glycol (PEG), a neutral, non-ionic and non-toxic polymer with high water solubility, is the most widely used osmoticum to mimic decreases in soil water potential. High molecular weight PEG (6000 or above) cannot penetrate the cell wall pores, resulting in conditions closely matching the effect of reduced matric potentials and thus causing a loss of water from both the protoplast and the cell wall and the collapse of the entire cell, including the wall (cytorrhysis). As plants subjected to long-term soil water deficits also experiment cytorrhysis, the use of PEG solutions avoid metabolic interferences associated to the use of ionic or low molecular weight osmotica that penetrate into the cells causing plasmolysis (Lawlor, 1970; Oertli, 1985; Verslues et al, 2006). However, the use of PEG is a controversial issue. The main problem is that PEG solutions are highly viscous, limiting O_2 diffusion to the roots (Munns et al, 2010), but this issue can be overcome by replacing solutions frequently. The use of PEG solutions is the most feasible option for simulating drought conditions in short-term experiments, which would be useful for evaluating the suitability of new sources of germplasm, and also for screening of large populations from the early generations in breeding programs. In a more practical sense, these experiments could be easily implemented by scientific advisory institutions for the routine-based screening of commercial hybrids for drought tolerance, and for the creation of recommended lists that would help growers in the selection of the most suitable varieties for cultivation.

The first objective of this work was to establish a practical and reliable experimental method able to group maize genotypes in relation to their tolerance to drought at early stages. For this objective, we used maize commercial hybrids and solutions of PEG 6000 to simulate different drought levels. Our second objective was to verify the reliability of this method by evaluating, in a soil drying experiment under greenhouse conditions, two hybrids with contrasting tolerance to drought according to the previous method. The proposed protocol would allow the screening of large numbers of maize genotypes, leading to more reproducible results and enabling unification of criteria used for classification of maize germplasm according to its drought tolerance at early stages.

Materials and Methods

Screening of drought tolerance in maize at germination, seedling growth and early growth

Eleven commercial single cross hybrids were evaluated for their tolerance to early growth. These hybrids were chosen among a set of maize hybrids that were widely grown in Northwestern Spain, and evaluated by SERIDA (Spain) for and their good performance under temperate climate (with typically mild-humid summers), rainfed conditions. Hybrids belong to different developers and FAO cycles. Commercial names of the chosen maize hybrids have been omitted, and each hybrid was assigned a number randomly between 1 and 11 (Supplementary Table 1).

Drought tolerance was evaluated at three stages: seed germination, seedling growth (pre-germinated seeds) and early growth (young plants at V3 stage). Drought stress was imposed using aqueous solutions of PEG 6000 at concentrations simulating slight, moderate and severe stress conditions (Table 1). Concentrations were chosen according to our experience in previous assays, and considering that the germination process is more sensitive to drought, i.e., imbibition in solutions with reduced water potential delays the seed osmotic water uptake, thus lowering seed water content below a «critical» (minimum) value required for radicle emergence and growth (Bradford, 1995). All solutions were adjusted at pH 6.0. Osmotic potentials were calculated following the equations of Michel and Kaufmann (1973), and verified using a cryoscopic osmometer (Gonotec OSMOMAT 030).

Germination assays

Screening for seed germination under water stress conditions was conducted on 14 cm diameter Petri dishes by placing 30 seeds of each maize hybrid on a Whatman No. 2 filter paper layer moistened with 10 ml of the corresponding solution. Petri dishes were sealed with Parafilm and incubated in the dark at 27ºC in a growth chamber. The number of germinated seeds was counted every 12 h until no new germination events were observed. A seed was considered to be germinated when the seed coat was ruptured and the root emerged $\geq$ 1 mm. Total germination index (Gt) was calculated from the cumulative germination data as described in Chiapusio et al (1997). Additionally, other germination indices were derived from primary germination data to obtain information about the effects on the ontogeny of germination: speed of germination (S), speed of accumulated

Table 1 - PEG concentrations, and their corresponding osmotic potentials (Ψo), used to evaluate responses to induced drought stress for maize commercial hybrids.

Stress level	PEG 6000 (g l^{-1})	Ψo (MPa)†
Germination		
Control	0	0
Slight	100	- 0.15
Moderate	150	- 0.30
Severe	200	- 0.49
Seedling establishment / Early growth		
Control	0	0
Slight	150	- 0.30
Moderate	200	- 0.49
Severe	300	- 1.03

†Osmotic potentials of PEG 6000 solutions at 25 ºC calculated according to Michel and Kauffman (1973).

germination (AS), coefficient of rate of germination (CRG) and mean germination time (MGT), following Chiapusio et al (1997) and de Bertoldi et al (2009). For each hybrid and treatment, five replicates, randomly distributed in the growth chamber, were used.

Seedling growth assays

For the evaluation of seedling growth, seeds of each hybrid were pre-germinated on containers with moistened filter paper at 27ºC in the dark. Then 20 pre-germinated seeds of each hybrid (radicle length 1-3 mm and no coleoptiles emerged) were placed on 14 cm diameter Petri dishes with the corresponding solution (Table 1) and incubated in a growth chamber as described above. After 72 h, primary root and coleoptile lengths, as well as the number of secondary roots, were recorded for all seedlings on each Petri dish. Then the primary roots, coleoptiles and secondary roots harvested from each Petri dish were collected and, for each of these three samples, the fresh weight (FW) and dry weight (DW) after drying 72 hours at 60ºC were obtained. The dry weight/fresh weight (DW/FW) ratios for each sample, total root weight and shoot/root ratio were also calculated. Five replicates per hybrid and treatment were used.

Early growth assays

Early growth assays were performed in a greenhouse located at the SERIDA experimental station 'La Mata' in Grado, Asturias (43°32'N;7°00'W, 65 masl) under natural light conditions (14 h of natural light and 10 h without light) and controlled temperatures (10 - 35ºC) and relative humidity (80 ± 10%). Maize seeds were sown in 1 liter pots containing a mixture of perlite and vermiculite (2:1 v:v), and pots were placed in large plastic trays for optimization of irrigation. Pots were irrigated with tap water until coleoptile emergence, and thereafter with half-strength Hoagland nutrient solution. All pots were irrigated daily by adding nutrient solution to each tray, and replacing the solution every 2 days. When plants reached the V3 stage (three collared leaves), drought stress treatments were imposed by adding the corresponding concentration of PEG 6000 to the Hoagland solution (Table 1). Control plants were maintained in the Hoagland solution. Treatments were maintained for 72 h, and solutions were replaced daily to maintain constant concentrations.

From the beginning of treatments and every 24 h, net photosynthetic rate (measured as CO_2 assimilation, µmol CO_2 m^{-2} s^{-1}), stomatal conductance to water vapor (mol H_2O m^{-2} s^{-1}) and transpiration rate (mol H_2O m^{-2} s^{-1}) were recorded in the third leaf of five leaves per hybrid and treatment by using a LI-6400XT portable photosynthesis system (Li-Cor Inc, Lincoln, NE, USA). Water use efficiency (WUE) was calculated as the ratio CO_2 assimilation/stomatal conductance (Xu and Hsiao, 2004). Measurements were performed under constant light conditions (photon flux 1,000 µmol m^{-2} s^{-1}) and CO_2 concentrations (400 µmol CO_2 m^{-2} s^{-1}) to minimize effects of environmental variations. Irrigation and daily measurements were made at the same hour to minimize interactions with circadian cycles and environmental conditions (Berger et al, 2010).

At the end of the assay (after 72 h under stress conditions), FW of roots and aerial parts were immediately recorded, as well as the DW after drying each part during 72 h at 60°C. The third leaf of each plant was detached and weighed separately, and for each of them the leaf area (LA) was recorded with a Leaf Area Meter CI-202 (CID Bio-Science Inc, Camas, WA, USA), and the specific leaf area (SLA) was calculated as the ratio LA/DW of the leaf (Garnier et al, 2001). Leaf relative water content (RWC) was determined in one leaf per plant following the equation [(fresh weight – dry weight)/(turgid weight – dry weight)] × 100 (Turner, 1981). The remaining upper leaves were used to calculate free proline content using a modified Bates method (Ramos and Pedrol, 2001) and total protein concentration according to a modified Bradford method (Pedrol and Ramos, 2001). For each maize hybrid and treatment, five replicate pots with one plant per pot were evaluated.

Soil drying experiment

Based on the previous results, we identified two maize hybrids with contrasting responses to PEG treatment: the tolerant hybrid named 5 and the susceptible hybrid named 6. These two hybrids were evaluated in a greenhouse under natural light conditions (14 h natural light/10 h dark) and controlled temperatures (10 - 35ºC) and relative humidity (80 ± 10%). Each seed was sown in a 1 liter pot filled with commercial substrate (Gramoflor, GmbH & Co, Vechta, Germany) at 100% water availability (WA), previously calculated by the gravimetric method. Pots were maintained under these conditions by daily top irrigation until target weight until plants reached V3 stage. At this moment, two treatments were established for each hybrid: half of the pots were maintained at 100% WA (control) throughout the experiment, and the other half was subjected to drought stress by withholding irrigation. All pots were weighed every day to monitor WA; in control pots,

water lost was replaced by watering every two days until target weight. From here on, measurements of gas exchange and chlorophyll a fluorescence were carried out every 24 h to monitor the photosynthetic performance of plants. Gas exchange-related parameters were measured with a LI-6400XT portable photosynthesis system, as described before. Parameters related to chlorophyll a fluorescence (efficiency of the photosynthetic system PSII [Y(II)], dissipated energy as heat [Y(NPQ)], dissipated energy as fluorescence [Y(NO)], non-photochemistry quenching [qN], photochemistry quenching [qL], and electron transport rate [ETR]) were measured using a modulated pulse fluorimeter Maxi Imaging PAM (Walz, Effeltrich, Germany). A detailed review of these parameters and their biological significance can be found in Maxwell and Johnson (2000) and Baker (2008). All these measurements were made in the third leaf of the same plants, in at least three plants per hybrid and treatment, and at the same hour.

When the drought stressed pots reached 50% WA three plants per hybrid, along with three control pots, were harvested. Subsequent harvests were made when the non-irrigated plants reached 35%, 25%, and finally < 5% WA, which was considered the point of maximum stress. Immediately after each harvest, FW of aerial parts was recorded, as well as the DW after drying at 60°C 72 h. LA, SLA, and RWC were determined on the third leaf of each harvested plant as described before. Material from the remaining upper leaves of at least four plants per hybrid and treatment were used to determine free proline and total protein contents as described before. Using the same material, cellular osmolarity measurements were performed with a Gonotec OSMOMAT 030 cryoscopic osmometer (Gonotec, Berlin, Germany).

Statistical analyses

The statistical package IBM SPSS Statistics v.22 (IBM Corp, NY, USA) was used for all statistical analyses. For each hybrid, all data was expressed in percentage with respect to the corresponding control in order to allow a standardized comparison among hybrids, beyond differences in plant growth and productivity. Two-way analyses of variance were made with stress and hybrids as main sources and variation and the corresponding interactions. Repetitions and their interactions were considered random effects. Data were tested for normality by Kolmogorov-

Table 2 - Summary statistics with minimum (Min), maximum (Max), mean (Mean) and standard deviation (SD) values of parameters measured on 11 maize commercial hybrids after germination, seedling growth and early growth bioassays.

	Min	Max	Mean	SD
Germination				
Percentage of germinated seeds (Gt)	10.00	100.00	76.99	23.44
Speed of germination (S)	0.60	15.00	6.18	3.09
Speed of accumulated germination (AS)	0.94	47.79	17.75	10.70
Coefficient of rate of germination (CRG)	0.83	1.56	1.19	0.15
Mean germination time (MGT)	24.00	128.57	64.15	22.28
Seedling establishment				
Root length (mm)	8.14	144.78	53.31	31.70
Root dry weight (mg)	1.52	14.95	5.99	3.05
Root dry weight/fresh weight	0.08	0.27	0.16	0.05
Shoot length (mm)	0.00	78.83	25.63	21.48
Shoot dry weight (mg)	0.00	26.64	8.50	7.07
Shoot dry weight/fresh weight	0.07	0.30	0.15	0.05
Secondary roots (number)	0.00	6.36	2.88	1.45
Secondary roots dry weight (mg)	0.00	18.10	5.42	3.62
Secondary roots dry weight/fresh weight	0.08	0.36	0.17	0.06
Total root dry weight (mg)	1.52	30.66	11.40	5.94
Shoot/root ratio	0.00	3.02	0.72	0.57
Early growth				
Total root dry weight (mg)	0.55	2.69	1.48	0.45
Total root dry weight/fresh weight	0.07	0.17	0.11	0.50
Shoot dry weight (mg)	0.43	2.69	1.46	0.03
Shoot dry weight/fresh weight	0.07	0.14	0.09	0.01
Shoot/root ratio	0.36	1.859	1.01	0.30
Relative water content (RWC) (%)	66.73	95.48	86.73	7.08
Leaf area (LA) (cm^2)	28.95	180.49	90.56	28.70
Specific leaf area (SLA) (cm^2 g dw^{-1})	463.99	988.83	627.27	91.60
Protein content (mg g dw^{-1})	10.24	90.29	43.00	17.08
Proline content (μmol g dw^{-1})	0.36	8.81	2.17	1.20
Photosynthetic rate (72h) (μmol CO_2 m^{-2} s^{-1})	-0.31	9.89	3.56	2.04
Stomatal conductance (72h) (mol H_2O m^{-2} s^{-1})	-0.03	0.39	0.07	0.08
Transpiration rate (72h) (mmol H_2O m^{-2} s^{-1})	0.10	1.86	0.65	0.35
Water use efficiency (WUE) (72h)	-1.34	14.25	5.90	2.76

Smirnov test and homogeneity of variances by Levene's test. When variances were homogeneous, the effects of treatments on each parameter were determined with one-way analyses of variance, and least significant differences (LSD) test was used for post hoc mean comparisons. In the case of heteroscedasticity, variance was analyzed by Kruskall-Wallis H test and Tamhane's T2 for *post hoc* multiple comparisons. Gas exchange variables were subjected to covariance analyses (ANCOVA) with environmental parameters previous to their analysis. A principal component analysis (PCA) was performed with data from the PEG 6000 screening assays, in order to synthesize all the measured variables into a limited number of principal components (PCs). Percentage data were first standardized. PCs with eigenvalues above 1 were selected and used for a hierarchical cluster analysis (CA), allowing hybrids to be organized into distinct groups with similar responses to early drought. Data from soil drying experiments were analyzed by independent samples t-test, comparing each drying treatment with the 100% WA control for each hybrid.

Results

Screening of drought tolerance at germination, seedling growth and early growth

The commercial maize hybrids used in this study showed large variability for their responses to drought stress, particularly for S, AS, root length, shoot length, DW of secondary roots, total root DW, photosynthetic rate, and stomatal conductance. Conversely, variability was limited for RWC, CRG, specific leaf area, and the ratios root DW/FW and shoot DW/FW (Table 2). Traits with wide or narrow variability were distributed among the three stages of development evaluated.

Differences among hybrids were highly significant for all traits (data not shown) and differences among stress levels were as well highly significant except for shoot DW. Analyses of variance for the parameters measured along time (photosynthetic rate, stomatal conductance, transpiration rate, and water use efficiency) showed significant differences among hybrids only for water use efficiency, while differences among stress levels or among times were always highly significant (data not shown). Genotype × stress level interactions were highly significant for all traits except MGT; interactions were generally of rank rather than of magnitude; therefore, results and discussion will focus on genotype × stress level when pertinent.

Total germination at slight water deficit was not significantly reduced except for the hybrid 10 (Supplementary Table 2). Significant reductions of germination began at moderate stress level, and reached 50% at the higher stress level, except for hybrids 2, 4 and 5, which were able to maintain germination levels around 100% throughout the stress conditions. Kinetics of germination, measured by the indices S, AS, CRG and MGT was also different among hybrids, being affected by all stress levels for all genotypes except for hybrid 5.

Seedling growth of all hybrids was significantly affected at all stress levels, particularly for coleoptile-related traits as coleoptile growth was strongly affected by all stress treatments, being completely inhibited at 300 g l^{-1} (Supplementary Table 2). Slight stress conditions strongly affected the seedling development, with reductions in growth and weight of primary roots of around 50-65%. Exceptions were hybrids 2, which was less affected, and 3, in which the coleoptile growth was stimulated. At severe stress conditions, all hybrids were negatively affected. Conversely, secondary root development was stimulated for all hybrids at slight stress conditions, not significantly affected at moderate stress and strongly inhibited under severe stress conditions. Roots were strongly reduced in hybrids 6 and 10 at the slight stress level. Total root biomass was generally balanced between the reduction of main root growth and the stimulation of secondary roots development due to water stress. Hybrids 1, 2, 5, and 8 had outstanding root development while hybrid 6 showed the worst response. On the other hand, root biomass was stimulated in hybrid 3 at slight and moderate stress levels, whereas hybrid 5 had higher secondary root biomass at all stress levels when compared to control.

In the early growth assay, morphological measurements revealed that stress conditions stimulated root growth for all hybrids (Supplementary Table 2), showing increases in root biomass (with the exception of hybrids 7, 10, and 11) and decreases in shoot/root ratios (excepting hybrids 5, in which this ratio remained constant, and 6, in which aerial growth prevailed). No clear trends were found for aerial biomass. The DW/FW ratios for roots and aerial parts also increased with stress intensity, again with the exceptions of hybrids 5 (for which shoot DW/FW remained constant), 10 and 11. Variations in leaf area did not show specific trends, although in most cases reductions were found at the higher stress levels. Punctual exceptions were found for hybrids 2, 3, and 6, while hybrid 4 showed consistent increases at all stress levels. A general reduction in the SLA was also observed at all stress levels compared to the control. The leaf RWC decreased with PEG 6000 treatments in a dose-dependent manner in all cases, but the magnitude of these reductions differed among genotypes. Hybrids 1, 4, and 7 showed reductions around 25% at the highest PEG concentration, whereas these reductions were lower (around 10%) for hybrids 5 and 6.

The concentrations of stress-related metabolites (proline and soluble proteins) had a negative relationship with stress level for most hybrids, particularly for moderate and severe stress levels. Proline increased clearly with stress intensity in hybrid 2, whereas hybrid 5 had low and stable proline levels. The total concentration of soluble proteins generally decreased as stress intensity increased, although at slight and

Table 3 - Principal component analysis of germination. seedling establishment and early growth parameters measured on 11 maize commercial hybrids. Eigenvalues and variance of the first 6 principal components (PC) are given.

		PC 1	PC 2	PC 3	PC 4	PC 5	PC 6
Eigenvalue		12.74	3.23	2.33	1.88	1.49	1.21
Explained variance (%)		45.49	11.53	8.33	6.70	5.32	4.32
Accumulated explained variance (%)		45.49	57.02	65.35	72.05	77.37	81.69
Germination							
	Gt	0.71	0.31	0.06	0.09	0.44	-0.15
	S	0.94	-0.05	0.06	0.04	0.10	-0.10
	AS	0.93	-0.09	0.07	0.07	0.08	-0.09
	CRG	0.92	-0.12	0.11	0.03	-0.06	-0.05
	MGT	-0.81	0.21	-0.19	0.11	0.17	-0.06
Seedling establishment							
	Root length	0.84	-0.06	-0.18	-0.01	0.00	0.16
	Root DW	0.70	0.27	-0.34	0.29	0.00	0.22
	Root DW/FW	-0.87	0.22	-0.11	0.15	-0.04	-0.18
	Coleoptile length	0.93	-0.19	-0.07	-0.12	-0.07	-0.01
	Coleoptile DW	0.91	-0.06	-0.13	-0.06	-0.08	0.04
	Secondary roots	0.81	0.23	0.08	-0.17	0.06	-0.06
	Secondary roots DW	0.63	0.38	-0.28	0.25	0.15	0.25
	Total root DW	0.71	0.36	-0.33	0.29	0.11	0.26
	Shoot/Root ratio	0.87	-0.13	0.08	-0.23	-0.09	-0.14
Early growth							
	Root DW	-0.32	0.43	-0.05	0.73	0.15	-0.13
	Root DW/FW	-0.81	0.26	-0.38	-0.07	0.12	-0.02
	Shoot DW	0.08	-0.06	0.40	0.84	-0.28	-0.04
	Shoot DW/FW	-0.71	-0.24	0.06	-0.05	0.08	0.26
	Shoot/Root ratio	0.39	-0.47	0.49	0.35	-0.38	0.06
	RWC	0.88	0.16	0.01	-0.01	-0.15	-0.08
	LA	0.06	-0.19	0.58	0.09	0.58	-0.05
	SLA	0.08	-0.31	0.38	0.01	0.71	0.19
	Protein	0.61	0.12	0.06	-0.10	0.06	-0.49
	Proline	0.32	0.51	-0.23	-0.02	0.06	-0.46
	Photosynthetic rate (T3)	0.14	0.73	0.37	-0.18	-0.07	0.34
	Conductance (T3)	-0.07	0.68	0.58	-0.22	-0.20	0.08
	Transpiration (T3)	-0.18	0.77	0.53	-0.16	-0.08	0.05
	WUE (T3)	0.55	-0.01	-0.22	-0.08	0.00	0.43

The most important parameters contributing to each PC (|correlation| ≥ 0.4) are remarked.

moderate stress conditions protein concentration increased in all hybrids excepting 1, 7, and 9. Under severe stress conditions, the protein concentration decreased for all hybrids. Strong decreases with respect to their controls were found for hybrids 6 and 7.

Differences among hybrids for gas exchange measurements increased with the time of exposure to PEG, and data obtained 72 h after the beginning of PEG treatments (T3) were clearly discriminant. All hybrids showed the highest values of CO_2 assimilation and stomatal conductance at moderate stress levels, whereas WUE decreased as stress intensity increased. The magnitude of these responses showed a great variability among hybrids.

Principal components and cluster analyses were carried out in order to synthesize all the measured variables. The first six principal components (PCs) had eigenvalues above 1 and explained 81.7% of the variability. PC1 explained 45.5% and joined most of the parameters related with germination, seedling growth and plant water status (DW/FW, RWC, and WUE); therefore, we can consider PC1 as a Growth component (Table 3). PC2 explained 11.5% of the variability, and had positive loadings for gas exchange parameters as well as root development in young plants. Thus, PC2 can be considered a Photosynthesis component. PC3 to PC6 explained low percentages of variability (less than 10% each), and their significance was less clear. PC3 was associated to aerial growth, leaf conductance and transpiration; PC4 to early growth parameters; PC5 to leaf growth, and finally PC6 had positive contributions from WUE and negative from proline and protein contents.

To clarify the comparison between hybrids, PC scores were used in a hierarchical cluster analysis, allowing the identification of homogeneous groups of population samples (Figure 1). Responses of hybrids were grouped into five clusters, corresponding to four functional units. The first cluster comprised the largest number of cases including all controls, but it was not particularly similar to any functional profile. Clusters 2 and 5 distinguished those individuals tending to maintain high productivities and high conductance and transpiration rates even at high levels of stress. The third cluster grouped the lowest number of cases, maintaining a good photosynthetic performance

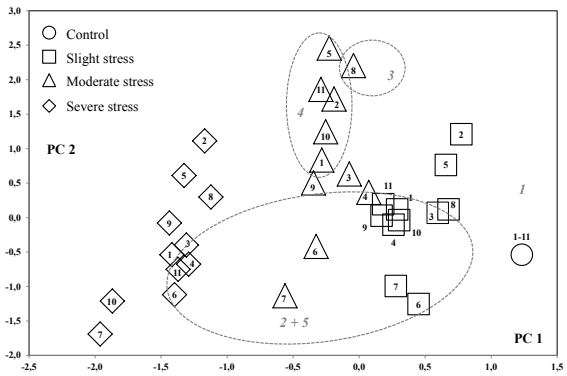

Figure 1 - Principal component analysis of germination, seedling establishment and early growth parameters measured on 11 maize commercial hybrids. Hybrids are labelled with numbers between 1 and 11. Symbols are means of each experimental replicate. Samples were distributed according to the scores of the principal components 1 (PC 1) and 2 (PC 2). Dashed lines combine homogeneous groups of samples according to hierarchical cluster analysis.

under stress with a concomitant decrease in early growth. Finally, cluster 4 included cases with high photosynthetic performance and high productivities under stress conditions. PC1 and PC2 were used for graphical representation of hybrids and clusters (Figure 1). Hybrids with high scores in PC1 had a good growth response, and those with high scores in PC2 maintained high photosynthetic activities at the corresponding stress level. Therefore, hybrids with high scores in PC1 and PC2 (2, 3, 5, and 8) are considered tolerant to early drought while those with low scores (hybrids 6, 7 and 10) are considered sensitive.

Soil drying experiment

Hybrids 5 and 6 were considered as representatives of tolerant and susceptible hybrids based on their responses to increasing concentrations of PEG 6000, and both had shown high yields in previous field evaluations by SERIDA in northwestern Spain. Young plants showed visible drought symptoms when water availability (WA) dropped below 50%. The experiment continued until WA fell below 5% and plant damage was irreversible (i.e. CO_2 uptake below 2.0 µmol CO_2 m^{-2} s^{-1}). This critical point was reached by hybrid 5 in 15 days and by hybrid 6 in 11 days. Both hybrids also differed for the time required to reach 50%, 35% and 25% WA, which was shorter for hybrid 6 in all cases.

The higher rates of CO2 assimilation and stomatal conductance were found during the first days of drought stress, when an increase of photosynthetic-related parameters was observed for both hybrids (Figure 2). Hybrid 5 showed a faster and more efficient response to drought stress, decreasing its stomatal conductance and consequently reducing CO2 assimilation, but increasing its WUE and thus the survival period. Conversely, a delayed reduction in stomatal conductance was observed in hybrid 6, leading to a faster desiccation and a reduced number of days at which plants reached the irreversible point when compared to hybrid 5. As expected, in both cases the decrease in stomatal opening is coupled with a progressive and sharp decrease in CO2 assimilation rates. Transpiration rates also followed the same trend than stomatal conductance for both hybrids (data not shown). On the other hand, fluorescence-related parameters did not change significantly throughout the experiment except for slight decreases of ETR at the first days of water withholding, more evident for hybrid 6. Hybrid 6 also showed an occasional reduction of Y(II), coupled with a significant increase of Y(NPQ) and qN (Table 4).

For the early growth- and water status-related parameters, no clear effects were shown on RWC at 50% or 35% WA (data not shown), and deviations with respect to each control were not very high at 25% WA. Effects were stronger at higher stress levels, with RWC reductions of 10% and 40% with respect to the control for hybrids 5 and 6, respectively. Both hybrids showed reductions in leaf growth, greater in the case of hybrid 6. Significant reductions in root and aerial DW were also found in hybrid 6 at the higher stress level.

Increases in cellular osmolarity were observed at 25% and <5% WA for both hybrids, being these increases more pronounced in hybrid 5 (data not shown). Proline contents were analyzed in order to detect its possible role as an active osmolyte in cells.

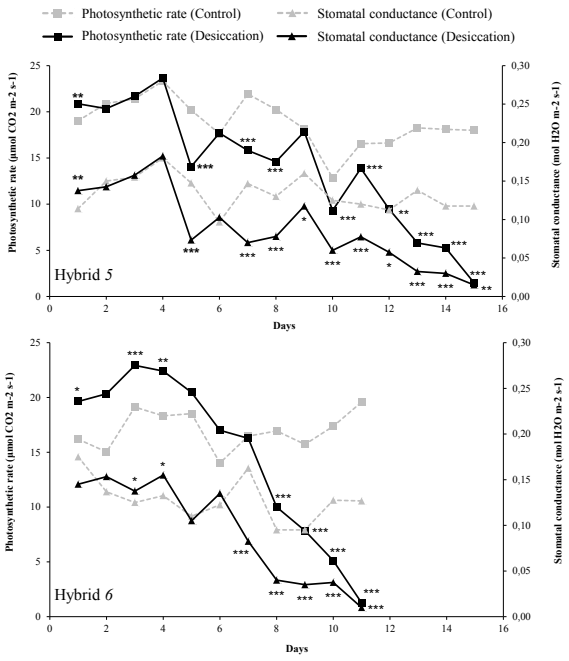

Figure 2 - Evolution of photosynthetic rate (μmol CO_2 m^{-2} s^{-1}) and stomatal conductance (mol H_2O m^{-2} s^{-1}), measured on control plants (100% water availability) and plants exposed to progressive desiccation in two maize commercial hybrids with previous contrasted sensitivity to early drought. For each parameter and time, asterisks denote significant differences between control and desiccation treatments: * - P ≤ 0.05; ** - P ≤ 0.01; *** - P ≤ 0.001; t-test.

Table 4 - Evolution of parameters related to photosynthetic performance of on plants of maize commercial hybrids 5 and 6 exposed to progressive desiccation, based on imaging measurements of chlorophyll a fluorescence.

Day	Y(II)	Y(NPQ)	Y(NO)	qN	qL	ETR
			Hybrid 5			
10						-
			Hybrid 6			
1						--
3						--
6	-					--
7	-	++		++	---	

For each parameter and time, signs denote significant reductions (-) or stimulations (+) with respect to their respective controls: one sign - $P < 0.05$; two signs - $P < 0.01$; three signs - $P < 0.001$; t-test.

Its increase was clearly correlated with the stress severity in hybrid 6, with a sharp increase at the end of desiccation. But in hybrid 5, proline levels remained lower than control values throughout the desiccation period, being significantly higher than control only at 35% WA. These values are not consistent with the increase in cellular osmolarity at the higher stress levels. Levels of soluble proteins followed a common pattern with proline in hybrid 5, showing a significant increase at 35% WA. In the case of hybrid 6, protein concentrations remain lower than in control from 35% WA on.

Discussion

Identification of genotypes with high drought tolerance is one of the main pillars for the selection of the most adequate genotype, or breeding of new ones. Therefore, our main objective was to find a simple and reliable method that allows large scale evaluations under controlled conditions.

Drought can impact plants at every developmental stage and at multiple levels, and in consequence plants have evolved complex strategies involving a large number of responses at morphological, physiological and biochemical levels of organization (Blum, 1996; Tardieu et al, 2011). In the present study, the parameters selected to explore the responses of maize hybrids to drought demonstrated to have good discriminating ability. Differences in stress response indicate that there are differences in the ability of maize hybrids for their ability to detect the stress, to respond and to tolerate the stress up to different stress levels (Chaves et al, 2009). However, the abrupt appearance of the stress and the short period under stress that characterizes this type of screenings affects the ability of the plant to respond to stress, forcing the sudden triggering of its response and allowing the discrimination of those cultivars with more efficient capacity to cope with drought stress. In the seedling growth evaluations, differences in growth patterns are due to the diverse ability for facing stress. Conversely, morphological differences found in the early growth assay might be affected by the time of stress imposition, as the influence of previous growth under non-stressed conditions is underestimated.

Right from the earliest crop stages, drought causes a great decline in germination rates and increased seedling mortality (Anjum et al, 2011). In our study, slight stress caused a delay in germination, while moderate and severe levels also decreased the percentage of germination. Total germination index (GT) provides a good overall assessment of the effects of water stress on germination, but should not be used as the only indicator. Indices S, AS, CRG, and MGT revealed differences in the dynamics and progress of the germination process between hybrids in cases where GT, that considers only the final time, did not show any effect. Hence, combination of GT with other indices (S, AS, CRG, and MGT) allow an efficient comparison of the effects of drought stress on maize hybrids (Chiapusio et al, 1997).

A direct consequence of drought is cellular dehydration that leads to a reduced cell expansion. Due to this early seedling growth, in which expansive growth processes play a key role, is largely affected (Sharp et al, 1988). This growth reduction was smaller for hybrids 2, 3, and 5 (subsequently classified as tolerant). Mild or moderate stress conditions typically reduce shoot growth in maize seedlings. In contrast, roots are less sensitive than shoots to growth inhibition at low water potentials, and thus root elongation and dry weight accumulation is less affected than for shoots (Westgate and Boyer, 1985). As a consequence, the shoot/root ratios decrease. This balance between root and shoot growth has a genetic regulation but also significant environmental effects (Ruta et al, 2010). Under optimal conditions coleoptile growth is considered a desirable trait as it is associated with a further higher yield (Bruce et al, 2002; Rebetzke et al, 2006). But under drought stress conditions, the maintenance of growth of the main root is considered to be an adaptive mechanism for optimization of soil water uptake (Sharp and Davies, 1989), enabling roots to penetrate deeper in the soil and increasing the possibilities of finding water sources. However, the initial growth of secondary roots is also necessary as they increase the surface area for water uptake and could guarantee the subsequent water supply to the main root before the water deficit reach severe values. Thus, a good development of the root system can be critical for seedling establishment, and increases the possibilities of survival under severe drought conditions. According to this, hybrids classified as sensitive had a scarce root growth and higher shoot/root ratios than the tolerant ones at all stress levels. Under severe stress conditions, although there is a strong overall reduction of seedling growth, the maintenance of root growth is still observed, at least for the primary root. Many studies of stress response focus on the aerial parts of the plant given the difficulties in accessing the root system and in establishing precise and uniform stress conditions in the environment surrounding the roots. However, in vitro evalu-

ations of the first stages of development have been proposed as a convenient and reliable approach (Ruta et al, 2010).

In young plants, changes in biomass partitioning were also expected to occur in order to optimize plant water uptake vs. water loss. The reduction of vegetative growth (plant height and leaf area) and biomass are well-known effects of drought stress (Blum, 1996). Reduction in leaf growth is a direct consequence of drought stress, but it can be also considered an adaptive response to avoid water loss by evapotranspiration. On the contrary, root growth may be favored. Thus, under drought conditions and as occurred in seedling growth, decreased shoot to root ratios can be expected (Shao et al, 2008). For those hybrids in which the strongest reductions in seedling root growth were observed (6, 7, and 10), young plants maintained high shoot/root ratios under stress in greenhouse assays. Those sensitive hybrids also suffered the strongest reductions in germination rates under drought stress conditions. This relation between morphological traits and drought stress tolerance from the early stages of development were reported previously in maize (Bruce et al, 2002; Ruta et al, 2010) but also in other species (Grzesiak et al, 1997; Lopes and Reynolds, 2011).

For other morphological traits such as aerial biomass or LA, changes as a response to drought stress are well known (Van Volkenburgh and Boyer, 1985; Edmeades et al, 1999). But in this experiment these parameters did not show significant differences among hybrids and stress levels, although in most cases reductions in leaf area were found at the higher stress levels. Despite its suitability for evaluations under strictly controlled conditions, water stress imposed by PEG is notably different from naturally occurring drought stress. Water stress imposed by PEG is interpreted by the plant as an instant stimulus rather than a natural long-term stimulus, allowing the observation of the early responses of the plant (Granda et al, 2011). But longer periods of stress conditions would be needed for these morphological responses to become obvious. On the other hand, physiological parameters with a «water status» component (SLA and RWC) showed clear differences between treatments with an overall reduction as stress level increased, although RWC was able to discriminate hybrid tolerance only under severe stress, as in Sucre and Suárez (2011). For these parameters, and as water loss caused by PEG treatment occur soon after inducing the stress, significant differences appear after a short period under stress.

Drought also induces a metabolic re-programming that results in changes in the whole transcriptome and metabolome (Niinemets, 2016) and, consequently, in an increased protein expression and accumulation that can be quantified as an estimation of the magnitude of the response to drought. At a biochemical level, osmotic adjustment mediated by the accumulation of certain metabolites is considered one of the most conspicuous responses to drought (Muscolo et al, 2015). This osmotic adjustment helps plants to maintain an adequate leaf turgor, and is mediated by organic solutes such as proline, glycine betaine or soluble carbohydrates that can also contribute to other functions (Farooq et al, 2009). Although some of the most sensitive and tolerant hybrids showed a consistent response for these parameters, changes in leaf proline and protein concentrations did not show very clear trends among all hybrids and stress levels, similarly to findings of Chimenti et al (2006) in young and flowering maize populations.

Another well-known effect of drought is stomatal closure, which limits intercellular CO_2 availability, reducing photosynthetic carbon fixation. Stomatal closure represents one of the earliest responses to drought, protecting the plant from transpirative water loss and increasing its water use efficiency. On the contrary, the primary photochemical events of PSII are considered to be very resilient to drought, so it is widely accepted that under moderate water deficits photosynthetic capacity is maintained. Under severe drought conditions, non-stomatal constraints to photosynthesis appear as a consequence of the prolonged decrease in CO_2 availability, which causes impairment between light energy captured and conversion. Biochemical limitations, consisting on the down-regulation of enzymes of the photosynthetic metabolism, appear as a consequence of the metabolic impairment caused by the lower intercellular CO_2, and result in a reduced but still reversible photosynthetic capacity. Moreover, photochemical limitations to photosynthesis appear when there is an excess of energy that cannot be used for CO_2 fixation and that needs to be dissipated. If dissipation mechanisms fail, an irreversible damage to photosystems can occur (Flexas and Medrano, 2002; Flexas et al, 2004; Chaves et al, 2009). In our first experiment, consistent and significant results were obtained for measurements made 48 and 72 h after the onset of stress treatments. In general terms, hybrids able to reduce stomatal conductance can, subsequently, reduce transpiration values and limit water loss. Values for net photosynthetic rate follow the opposite trend, as stomatal closure implies limited CO_2 availability. In the case of hybrid 10 stomatal opening did not involve higher CO_2 availability, perhaps due to a damage of the photosynthetic machinery.

Drought tolerance is a complex trait. In consequence, a large number of morphological, physiological and agronomical traits can be used to assess responses to drought (Farooq et al, 2009). For dealing with these complex data matrices, multivariate analyses are the best approach. Multivariate analysis methods such as PCA and CA are powerful tools for the joint analysis of large sets of variables. PCA is mainly used as a tool in exploratory data analysis and for making predictive models, as this method allows

transforming a number of possible correlated variables into a limited number of uncorrelated variables or principal components (PCs). In our work PCA provided a global perspective of hybrids response, and allowed us to identify those traits with the highest discriminating ability for drought tolerance. CA was also a valuable method to classify maize genotypes into groups that share similar responses and levels of stress tolerance.

In the experiments included in our protocol, photosynthesis-related traits had the highest selective value, suggesting promising opportunities for selection at this level. It would be worthwhile investigating the mechanisms underlying the response of maize to drought at the photosynthetic level with a more appropriate experimental design simulating real drought conditions.

In the soil drying experiment, the study of the response of photosynthesis-related traits was completed with the study of the response of chlorophyll fluorescence parameters, as chlorophyll fluorescence analysis is considered a reference method for studying stress response (Baker, 2008). Leaves capture part of the light for photosynthesis, part of the light is dissipated as heat and part returns as fluorescence; thus, the increase of one of these fractions is associated with the reduction of the others (Maxwell and Johnson, 2000). The efficiency of the photosynthetic system PSII [Y(II)], the photochemistry quenching (qL) and the electron transport rate (ETR) inform of the photosynthetic activity that can be affected by CO_2 availability. But these parameters showed a null predictive value of early drought conditions and a poor discriminating ability, if compared to gas exchange-related measurements. Our results are in agreement with those reviewed in Baker and Rosenqvist (2004) and Berger et al (2010), who pointed out that relevant changes in fluorescence-related parameters are often achieved only under mild or severe drought conditions, appearing as a consequence of the decrease of intracellular CO_2 concentration derived from stomatal closure. These stomatal limitations are responsible for the early decline in photosynthetic rate and stomatal conductance of hybrid 6, which can be related to the punctual decreases detected in ETR (Flexas et al, 2002), whereas subsequent decreases in these parameters did not result in significant decreases in ETR. No consistent differences were found for other fluorescence parameters related to possible damages in the photosynthetic machinery. Thus, in this experiment fluorescence-related parameters were not good indicators of drought stress response. On the contrary, the best indication of drought stress response was obtained by monitoring gas exchange.

The final increase in cell osmolarity found in both hybrids can be partially related to the decrease in RWC due to desiccation and, in the case of hybrid 6, proline synthesis. But the magnitude of this increase in hybrid 5, which is not associated with an increase in proline levels, suggest that other metabolites different from proline may be contributing to this higher osmolarity (Ashraf and Foolad, 2007).

Separately, PEG-based screening and soil drying experiments gave the same results as they both considered hybrids 5 and 6 as tolerant and sensitive to drought, respectively. But when comparing parameters measured in screening and soil drying experiments, consistent relationships can be found for several parameters, whereas trends are less obvious in other cases. For both hybrids, the same trends were observed for LA, SLA, and RWC in both assays but with different magnitudes. Same trends but different magnitudes were also observed in the case of photosynthetic-related parameters (photosynthetic rate and stomatal conductance). On the other hand, different trends were found in the responses of root DW, aerial DW and, consequently, shoot/root ratio. Whereas at the end of the soil drying experiments reductions in both root and aerial biomasses can be found as a consequence of drought, these reductions are not observed in the PEG-based screening experiment. In the case of proline and soluble protein contents, no clear relations were found when comparing the results of each hybrid between both assays. As mentioned before, the different timing and intensity of stress imposition in these two experiments can explain the differences when no clear trends are found.

As proline and protein contents had the lowest discriminant ability in PEG-based experiments, the utility of these parameters seem to be limited for screening purposes. But when performing soil drying experiments for a better understanding of drought responses, their utility have been demonstrated (Hare and Cress, 1998). In the case of morphological parameters (root and aerial biomasses), although the results may not be coincident in both experiments, they have demonstrated to have modest discriminant ability and, therefore, their measurement is useful for screening purposes.

In this work, we describe a method based on the use of PEG 6000 solutions to characterize a set of maize hybrids according to their tolerance to drought at early stages of development. Our results highlighted different responses of morphological, physiological and biochemical characters that, when considered together, allowed an efficient discrimination of maize genotypes. The subsequent assay performed under soil drying conditions validated our previous results, but highlighted similarities and differences in the response of the parameters evaluated. We can conclude that this method is useful for screening maize genotypes for drought tolerance. This method also enables the rapid assessment and comparison of the responses of morphological and physiological traits potentially involved in drought stress tolerance of germplasm, complementing more detailed physiological and agronomic studies.

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