Targeting sources of drought tolerance within an *Avena* spp collection through multivariate approaches.

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Abstract

In this study we find and characterise sources of tolerance to drought amongst an oat (*Avena sativa* L.) germplasm collection of 174 landraces and cultivars. We used multivariate analysis, non-supervised Principal Component Analyses (PCA) and supervised Discriminant Functional Analyses (DFA) to suggest the key mechanism/s responsible for coping with drought stress. Following initial assessment of drought symptoms and area under the drought progress curve a subset of 14 accessions were selected for further analysis. The collection was assessed for relative water content, cell membrane stability, stomatal conductance, leaf temperature, water use efficiency, lipid peroxidation, lipoxygenase activity, chlorophyll levels and antioxidant capacity during a drought time-course experiment. Without the use of multivariate approaches it proved difficult to unequivocally link drought tolerance to specific physiological processes in the different resistant oat accessions. These approaches allowed the ranking of many supposed drought tolerance traits in order of degree of importance within this crop thereby highlighting those with a causal relationship to drought stress tolerance. Analyses of the loading vectors used to derive the PCA and DFA models indicated that two traits involved in water relations; temperature and relative water content, together with the area of drought curves were important indicators of drought tolerance. However, other parameters involved in water use such as stomatal conductance and water use efficiency were less able to discriminate between the accessions. These observations validate our approach which should be seen as representing a cost-effective initial screen which could be subsequently employed to target drought tolerance in segregating populations.

Keywords *Avena* · Drought · Multivariate approaches · Oat · Tolerance mechanisms

Abbreviations

AUCPC	Area under the conductance progress curve
AUDPC	Area under the drought progress curve
CMS	Cell membrane stability
daww	Days after withholding water
DFA	Discriminant function analysis
g_l	Stomatal conductance
IR	Infrared
LOX	Lipoxygenase activity
LP	Lipid peroxidation
OA	Osmotic adjustment
PCA	Principal components analysis

1	RWC	Relative water content
2	SCMR	SPAD chlorophyll meter reading
3	WUE	Water use efficiency
4		

Introduction

Oat (*Avena sativa* L.) ranks sixth in world cereal production statistics, following wheat, maize, rice, barley and sorghum (FAO 2011). It is widely grown in temperate areas, with an increasing interest to expand the crop to subtropical areas, Mediterranean countries (Stevens et al. 2004) and northeast China (Islam et al. 2011). This is mainly due to its good adaptation to a wide range of soil types and because on marginal soils oats can perform better than other small-grain cereals (Stevens et al. 2004). However, oats can be sensitive to hot, dry weather and hence, in most Mediterranean and dry regions drought is the main limiting factor for oat yield probably exceeding losses from all other causes (Stevens et al. 2004). Thus, new sources of oat tolerance must be exploited which may be introgressed into elite cultivars.

Plant breeding has an important role in improving germplasm to fit the agroclimatic conditions of drought-prone areas (Chaouki et al. 2004). However, cereal breeding in general, and oat breeding in particular, has been mainly based on empirical selection for yield but this is characterized by a low heritability and a high genotype x environment interaction making it a poor assessment criterion (Araus et al. 2002). As a result, modern breeding strategies attempt to include assessments of physiological, biochemical and molecular characteristics which may better reflect lineage productivity and responses to environmental stress (Araus 1996; Richards 1996; Slafer and Araus 1998). A corollary of this approach is a better understanding of drought tolerance mechanisms which in turn will further define targets in germplasm screens.

Several morphological, physiological and molecular plant responses can contribute for coping with drought stress either increasing its ability to avoid damage (avoidance mechanisms) and/or to maintain its metabolic functions under water limiting conditions (tolerance mechanisms). In this work we focussed in the tolerance mechanisms, where the physiological bases of genetic variation are far from being clear. Key features may be the capacity to maintain cell/tissue water, cell membrane stability, and to avoid oxidative damage through antioxidant machinery (Farooq et al. 2009). Thus, water related features such as relative water content (RWC), leaf water potential (LWP), stomatal conductance (g_l), transpiration rate and leaf/canopy temperature have been studied in different species under drought stress. In general water stressed plants have lower relative water content, leaf water potential and transpiration rate with a concomitant increase in leaf temperature. Furthermore, a positive correlation between grain yield and RWC has been observed in durum and bread wheat (Singh and Patel 1996; Merah 2001). Particularly in oat, decreases in g_l, in the difference between air and leaf temperatures and in RWC were associated to water deficit. However none of these indices by themselves were associated with degree of yield losses (Peltonen-Sainio and Makela 1995).

Improved tissue water status may be also achieved through osmotic adjustment (OA). This involve accumulation of specific compounds such as sugars (i.e. from the raffinose family oligosaccharides) sugar alcohols (such as mannitol), amino acids (such as proline) and amines (such as glycine, betaine and polyamines) which allows the cell to decrease osmotic potential and hence increase the gradient for water influx and turgor. Thus, OA has been related to grain yield under water deficit environments (Moinuddin et al. 2005) and considered as a selection criterion for drought tolerance in wheat (Morgan 1983).

In addition to water related features, physiological traits indicative of oxidative damage and antioxidant defence have been well documented under drought stress conditions (Farooq et al. 2009 and references therein). Altogether these changes are thought to be associated with protecting cellular functions or with maintaining the structure of cellular components (Seki et al. 2007). Particularly in oat it has been shown that as part of acclimation to drought stress, the lipid composition of root plasma membranes is selectively modified, possibly to increase their flexibility (Larsson et al. 2006). Molecular targets contributing drought tolerance, including changes in gene expression, synthesis of stress proteins and activation of molecular signalling have been only recently dissected disclosing the intricate complexity of the resistance responses to this stress (Seki et al. 2007; Kavar et al. 2008; Farooq et al. 2009).

This complexity is an important handicap for breeding. Efforts have been made to produce drought tolerant genotypes based on the knowledge of plant responses to drought and the mechanisms involved described above. However these were not always successful since although changes in water related features have been described during drought stress, not all of these features are suitable for discriminating tolerant from sensitive genotypes and not all plant species respond in a similar manner. For instance, leaf water potential was able to discriminate drought resistant and susceptible barley cultivars (Matin et al. 1989) and it has been reported to be the main trait responsible of the drought tolerance phenotype in chickpea which allowed its use as phenotypic marker in breeding programmes (Pannu et al. 1993); however, it is not a defining feature of tolerance in bread wheat (Schonfeld et al. 1988) or faba bean (Ricciardi et al. 2001). On the other hand, in bread wheat, RWC differed significantly among susceptible and resistant populations under increasing drought (Schonfeld et al. 1988). Thus, it remains uncertain in a given species which is the best feature(s) indicative of drought tolerance and/or when these should be assessed. In the present study in addition to seeking to characterise new sources of drought tolerance, we use multivariate analysis in a range of drought linked features and genotypes to reveal the key physiological mechanism/s in the oats for coping with drought stress.

Materials and methods

Plant material and treatments

For the resistance screening we used a germplasm collection of landraces consisting in 107 Spanish accessions of *A. sativa* L. and 31 of *A. byzantina* K. Koch kindly provided by the "Centro de Recursos Fitogenéticos", INIA, Madrid, Spain, and 36 commercial cultivars supplied by the Andalusian Network of Agriculture Experimentation (RAEA). For easier comparison among landraces and manuscript reading, germplasm bank codes were substituted for others codes easier to read (Sánchez-Martín et al. 2011). Oat cultivars studied were: Ac1, Acebeda, Adamo, Aintree, Alcudia, Anchuela, Araceli, Brawi, Caleche, Cannele, Chambord, Chappline, Charming, Cobeña, Condor, Cory, Edelprinz, Flega, Fringante, Fuwi, Hammel, Kankan, Kantora, Karmela, Kassandra, Kazmina, Mirabel, Mojacar, Norly, Orblanche, Pallini, Patones, Prevision, Primula, Rappidena and Saia.

As in previous cereal drought related studies, experiments were carried out at seedling stage (3 week old plants) (Xiao et al. 2007; Hao et al. 2009; Gong et al. 2010). Seedlings were grown in 0.5 L pots filled with peat:sand (3:1) in a growth chamber with 20 ºC, 65 % relative humidity and under 12 h dark/12 h light with 150 μmol m^{-2} s^{-1} photon flux density supplied by high-output white fluorescent tubes (OSRAM). During growth trays carrying the pots were freely watered with a thin layer of water of approximately 1 cm continuously present in the tray. At day 21, in those plants subjected to drought, water was withheld (Hao et al. 2009; Gong et al. 2010) for 19 days at which experiment finished. Control plants were watered as above mentioned during the whole experiment. During the drought time course the relative water content of the soil was monitored daily reaching a level of 20% by the end of the experiment as in previously reported oat work related to drought (Gong et al. 2010).

Additionally selected accessions were growth during the 2010-2011 crop season in a field plot in Salamanca, Spain (40º 55' 28.2'' N, 5º 21' 45.54''W) in water prone conditions (although not severe stress) i.e. 242.36 mm water in the growth season compared with the 600 mm by mean of north European oat growing areas. The soil of the experimental field, around 0.8 m deep, is a sandy loam or sandy-clay-loam Vertic Luvisol (FAO 2007). Plants were sown on 01/11/2010. Each accession/replication was represented by 3 rows of 1 m long, with each row consisting of 30 plants. The distance between rows was 0.5 m. Three replicates were grown in a randomized complete block design.

Visual assessment of drought symptoms

From the time at which water was withheld for drought treatment (from now on T_0) all plants were visually evaluated daily according to the following scale: 0 = vigorous plant, no leaves shows drought symptoms; 1 = one or two leaves show slight drought symptoms (less turgor) but most leaves remain erect; 2 = most leaves show slight levels of drought stress, however one or two leaves still show no drought symptoms; 3 = all leaves show drought symptoms but these are no severe; 4 = all leaves show severe drought symptoms including incipient wilting; 5 = the whole plant is wilted with all leaves starting to dry, rolled and or shrunken (Online Resource 1). Five plants per accession were assessed. Drought severity values daily assessed according to this scale were used to calculate the area under the drought progress curve (AUDPC) for each oat accession similarly to the area under the disease progress curve widely used to disease screenings (Jeger and Viljanen-Rollinson 2001) using the formula:

$$AUDPC= \sum_k^{i=1} \tfrac{1}{2} [(S_i+S_{i+1})(t_{i+1}-t_i)]$$

where S_i is the drought severity at assessment date i, t_i is the number of days after the first observation on assessment date i and k is the number of successive observations.

Relative water content

RWC was measured in ten plants per accession according to Barrs and Weatherley (1962). Measurements were carried out in the second leaves at time 0, 6, 9, 12, 15 and 18 days after withholding water (daww). Six hours after the onset of the light period, leaf blade segments were weighed (fresh weight; FW), floated on distilled water at 4 ºC overnight and weighed again (turgid weight; TW). They were then dried at 80 ºC for 48 h. After this, the dry weight (DW) was determined. RWC was then calculated as $RWC = (FW - DW) (TW - DW)^{-1} \times 100$.

Cell membrane stability

CMS was measured in ten plants per accession according to Tripathy et al. (2000). Measurements were carried out in the second leaves at 0, 6, 9, 12, 15 and 18 daww. Samples collected were washed three times in deionized water to remove electrolytes adhered on the surface. The samples were then kept in a capped vial (20 mL) containing 10 mL of deionized water and incubated in the dark for 24 h at room temperature. The conductance was measured with a conductivity meter (CMD 510, WPA, UK). After the first measurement the vials were autoclaved for 15 min to kill the leaf tissue and release the electrolytes. After cooling, the second conductivity reading was taken. These two measurements were carried out individually for all the samples from both the control and stress treatments. The control gave a measure of leakage solely due to the cutting and incubation of

leaf discs. The conductance of the stress sample was a measure of electrolyte leakage due to water stress and was assumed to be proportional to the degree of injury to the membranes. CMS was calculated as the reciprocal of cell-membrane injury after Blum and Ebercon (1981): CMS% = [(1-(T1/T2)) / (1-(C1/C2))] x 100, where T and C refer to the treated and control samples, respectively; the subscripts 1 and 2 refer to the initial and final conductance readings, respectively.

Stomatal conductance

g_l was measured in ten plants per accession with an AP4 cycling porometer (Delta-T Devices Ltd, Cambridge, UK). g_l is the sum of epidermal and stomatal conductance, but as epidermal conductance of oat is low, changes in g_l largely reflect changes in stomatal aperture. The porometer allows rapid measurement that is non-destructive and samples a relatively large area (17.5 x 2.5 mm) of leaf. It was used on the centre of the adaxial surface of leaf laminae. Measurements were carried out in the second leaves during the first 10 daww. After this period stomata were strongly closed at all hours of the day with similar readings of that at dark period. Measurements were taken three times per day, two hours after onset of the light period, at the middle of the light period and two hours before the onset of the dark period. The area of the conductance progress curve (AUCPC) of drought treated plants with respect to the control curve of non-stressed plants was calculated using the formula above mentioned for AUDPC.

Infrared temperature

Leaf temperature was estimated on the second leaves of five plants per accession, using an infrared camera (FLIR i50, FLIR Systems Inc,). The final measurement of each plant was the mean of four measurements per leaf. Measurements were taken 6 hours after the onset of the light period at 6, 12 and 18 daww in control and stressed plants.

Water use efficiency

Water use efficiency (WUE) expressed in terms of plant production per water consumed was measured gravimetrically in 5 plants of each of the 14 selected accessions according to (Xin et al. 2008). Briefly, pots were filled with the above mentioned substrate and watered until water dripped from the bottom. Three seeds were planted per pot and thinned to one plant at 7 days after emergence. The pots were then covered from both ends with 2 polythene bags that were fixed to the pot with elastic bands. A small slit was made in the top bag to allow the plant to grow through. Control pots without plants showed minimum water loss. The initial and final (after five weeks) pot weight was taken and water used was calculated by subtracting the final pot weight from the initial weight. Roots

were collected by washing the potting mix core on a wire mesh. Dry weight measurements of roots and shoots were taken after a minimum of 72 h of drying at 80ºC when the samples reached a constant weight. WUE was calculated by dividing the total dry biomass by the amount of water transpired.

Lipid peroxidation and lipoxygenase activity

Lipid peroxidation and lipoxygenase activity was measured in five plants per accession in the second leaves of control and stressed plants at time 9 and 12 daww.

Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content following the method of Rosales et al. (2006) with slight modification. For the assay of MDA, second leaves were ground with a mortar and pestle with liquid nitrogen, homogenized in 50 mmol L^{-1} potassium phosphate buffer (pH 6.0) (1:5 (p/v)) and centrifuged at 20 000 *g* for 25 min at 4ºC. For measurement of MDA content, 200 µL of 200 g L^{-1} trichloroacetic acid containing 5 g L^{-1} thiobarbituric acid was added to 50 µL aliquots of the supernatant. The mixture was heated at 95 ºC for 30 min and then quickly cooled in an ice-bath. Subsequently, the samples were centrifuged at 10000 *g* for 10 min at 4 ºC and the absorbance of the supernatant was read at 532 nm. The value for the non-specific absorption at 600 nm was subtracted from the A_{532} reading. The concentration of MDA was calculated using a calibration curve (0.1-1.2 µg μL^{-1}) of MDA.

Lipoxygenase enzyme (LOX, EC 1.13.11.12) activity was measured according to (Minguez-Mosquera et al. 1993) using 50 mmol L^{-1} potassium phosphate buffer (pH 6.0) for extraction. The reaction mixture carried out in microwells consisted of 285 µL of 50 mmol L^{-1} potassium phosphate buffer (pH 6.0), 10µL of crude extract and 5 µL of 0.5 mmol L^{-1} linoleic acid in 50 mmol L^{-1} potassium phosphate buffer (pH 6.0). The LOX activity was calculated following the increase in the extinction at 234 nm using an extinction coefficient of 25 000 L mol^{-1}. For preparation of substrate, linoleic acid (0.5 g) of higher than 99 % purity (Sigma) and 0.5 g Tween 20 were dissolved in deionized and deoxygenated H_2O. In the case of turbidity, a few drops of 2 M NaOH were added until complete transparency. The final volume of the mixture was taken to 25 mL. Aliquots of 2 mL were put into flasks which were closed under N_2.

Leaf chlorophyll content

Leaf chlorophyll was indirectly estimated on the second leaves of five plants per accession, using a SPAD-502 chlorophyll meter (Minolta Co., LTD., Japan) (Zhao et al. 2010). The final measurement of each plant was the mean of three measurements per leaf, the adaxial side of the leaves

was always placed toward the emitting window of the instrument. Measurements were taken 6 hours after the onset of the light period at 6, 9, 12, 15 and 18 daww in control and stressed plants.

Antioxidant activity

Antioxidant activity was measured in five plants per accession in the second leaves of control and stressed plants at time 9 and 12 daww. Antioxidant activity was measured in the leaves using the Ferric Reducing Ability of Plasma (FRAP) assay according to Rosales et al. (2006). The FRAP assay was performed with FRAP reagent, i.e. 1 mmol L^{-1} 2, 4, 6-tripyridyl-2-triazine (TPTZ) and 20 mmol L^{-1} ferric chloride in 0.25 mol L^{-1} sodium acetate (pH 3.6). An aliquot of 50 µL of leaf extract (10 mg per mL in methanol) was added to 1 mL of FRAP reagent and mixed thoroughly. After the mixture had been left at ambient temperature (20 ºC) for 5 min, the absorbance at 593 nm was measured. Calibration was against a calibration curve (25–1600 mol L^{-1} ferrous ion) constructed using freshly prepared ammonium ferrous sulphate.

Statistical analysis

All experiments were designed in a randomized complete block design. For ease of understanding, means of raw percentage data are presented in tables and figures. However, for statistical analysis, data recorded as percentages were transformed to arcsine square roots (transformed value = 180/π x arcsine [√(%/100)]) to normalize data and stabilize variances throughout the data range, and subjected to analysis of variance using GenStat 7th Edition, after which residual plots were inspected to confirm data conformed to normality. In addition Shapiro-Wilk test and Bartlett's test were performed to test normality and homogeneity of variances, respectively. Significance of differences between means was determined by contrast analysis (Scheffe's). In addition least significant difference (LSD) values were added to tables and figures for comparison between each two accessions. When appropriate, tukey test ($P < 0.05$) were used for multiple comparison among accessions.

For multivariate analysis the data were first analyzed using principal components analysis (PCA; Causton 1987). Briefly, this involves projecting a ('X') matrix formed from set of (N) data onto multidimensional space. Principal components (PCs) are linear combinations of original variables (known as loadings) which are used in the projection of the X matrix. Individual PCs are ranked (PC1, PC2, etc.) on the basis of the variance within the original dataset that is explained. PCA is an unsupervised method where no *a priori* knowledge of experimental structure is given. Thus, if there is clustering of either 2D or 3D projections of PCA from replicate data, this indicates that the original experimental parameters are the sources of maximal variation. PCA was followed by discriminant

function analysis (DFA) which is a supervised projection method (Manly 1994). DFA then discriminated between groups on the basis of the retained PCs and the a priori knowledge of which values were replicates (either biological or machine). DFA was programmed to maximize the Fisher ratio (i.e. the within-class to between-class variance) and the similarity between different classes reflects the optimal number of PCs that are fed into the DFA algorithm. All calculations were performed in Pychem 2.0 (Jarvis et al. 2006).

PC-DFA models were created to identify the variables or parameters associated with the differences between the susceptible and resistant accessions. The approach was based on deriving robust models where differences between classes were separated along a particular PC-DF axis. This allowed plotting the contributions of individual variables measured to the model ("loading vectors"). Those parameters that appeared > ± 1 standard deviation (STD) from the mean value loading were recorded as 'discriminatory' variables associated with the differences.

Due to the complex experimental structure and the need to derive PC-DFA models where classes were separated along particular PC-DF axes, differing approaches were followed, each generating a list of key parameters. Firstly PC-DFA plots were constructed for each individual time point (6, 9, 12, 15 and 18 daww). Since none of the models discriminate well between accessions, PC-DFA plots were constructed grouping different time points. In addition models were derived by grouping the accessions (i.e. most resistant vs. most susceptible or moderately resistant vs. themselves). This identified the key time points and parameters to discriminate susceptible and resistant accessions.

Results

Screening for drought resistance sources

Following daily visual assessment of drought symptoms, AUDPC values were calculated for the 174 *Avena* accessions which could be grouped into 7 classes overall conforming to a normal distribution (Fig. 1a). Accessions from the most frequent class, with AUDPC values of between 31 and 35, were considered neither resistant nor susceptible to drought stress. Accessions with values lower than 20 were considered to be highly resistant to drought, with others with scores between 21 and 31 considered to be resistant or moderately resistant. Accessions with AUDPC values higher than 46 were considered to be highly susceptible but those between 35 - 45 to exhibit moderate susceptibility. Thus, 31.6% of the accessions were regarded as moderately resistant, and 0.5% as highly resistant (Fig. 1a) with these classes including 44 *A. sativa* and 4 *A. byzantina* landraces and 8 commercial cultivars. Fig. 1b represents the drought progress curves along the drought time course of

Patones and Flega, the most resistant and susceptible accessions, respectively. For all accessions the drought progress curve was a sigmoidal which in susceptible accessions scores rapidly (by ~1-2 days) started to increase and reached 5 by day sixteen. In contrast, the most resistant accessions maintained a score of 0 for at least 2-3 days and did not achieve a score of 5 score until at least 18 days after the imposition of drought stress. For further analysis of drought resistance components 14 accessions were selected according to their AUDPC values, including 1 highly resistant (Patones), 1 resistant (Gen16), 9 moderately resistant (Mirabel, Anchuela, Gen17, Gen76, Gen100, Gen122, Gen124, Gen125 and Gen 135), 1 moderately susceptible (Rapidena) and 2 highly susceptible (Flega and Alcudia) accessions. Statistical comparison of the specific AUDPC values of these accessions showed that the highly susceptible accessions significantly differed from the moderately resistant, resistant and highly resistant ones (Tukey $P < 0.05$: Table 1).

Field assessment of yield of selected accessions grew in water prone conditions showed a high correlation with the water stress tolerance showed under the assayed controlled conditions (Online Resource 2).

Characterisation of resistance mechanisms underlying responses to drought

Assessment of RWC (Fig. 2a) showed values of approximately 90% in all accessions before withholding water with no significant differences amongst accessions. Following the imposition of drought conditions there was a decline in RWC in all accessions although those accessions classified as highly susceptible (i.e. Alcudia and Flega) exhibited a more rapid decline compared to the most resistant ones (i.e. Patones and Gen124). Overall statistical analysis showed significant differences among accessions ($P < 0.001$), time points after withholding water ($P < 0.001$) and interaction between these factors ($P < 0.001$). Interestingly, accessions such as Gen125, Anchuela and Gen17 considered moderately resistant rapidly reduced their RWC and at day 15 and 18 exhibited no significant difference to Alcudia and Flega (Fig. 2a).

A similar pattern was observed following assessment of CMS (Fig. 2b). Thus, overall analysis showed significant differences among accessions ($P < 0.001$), time points ($P < 0.001$) and interaction between these factors ($P < 0.001$). CMS values ranged from 100 in non-stressed plants to 20% in the most susceptible accessions after 18 days of drought treatment. This reduction in CMS with time was observed in all accessions. However, we did not find a significant linear correlation between RWC and CMS values. This suggested that not all accessions maintained membrane integrity under drought stress in the same way. To further investigate this behaviour we derived model curves based on observed values. RWC and CMS values fitted with negative gompertz curves (non-symmetrical) in all accessions, except Gen100 and Gen125 for RWC and Patones for CMS that fitted with negative

logistic type curves ($P < 0.001$). Fig. 2c, d shows the observed points and derived curves for the most susceptible, Flega, and most resistant accession, Patones. Table 2 shows the point of inflexion, and the lower and upper asymptotes (floor and ceiling) for all accessions for RWC and CMS. Regarding to RWC, susceptible accessions such as Flega showed the earlier inflexion point and also the lowest floor together with Alcudia. By contrary Patones and also Gen135 showed inflexion points at longer times and high floor values. All accessions shower similar ceiling values of approximately 85% of RWC (Table 2). Regarding CMS, Gen 122, Gen125 and Gen135 showed the earliest inflexion points but not the lowest floor values that were shown by Flega, Alcudia, Gen 100 and Gen 17. Interestingly Patones showed a later inflexion point together with a high floor value (Table 2). CMS values at a limit RWC of 45% calculated from curves showed that Patones had the higher CMS. However other accessions, such as Gen16 and Gen124, considered moderately resistant exhibited high membrane damage at 45% RWC (Table2).

WUE in terms of dry biomass per litre of water consumed ranged between 1.55 (Gen 16) and 2.63 (Mirabel), respectively (Table 3). Data showed differences among accessions with Mirabel and Gen 17 showing significant higher values compared to the other accessions.

Stomatal conductance

In most accessions measurements taken two hours after the onset of the light period and the in the middle of the light period tended to be higher than those taken two hours before the end of the light period (Fig. 3a). Water-stressed plants showed a rapid reduction in g_l with respect to the controls, especially in susceptible accessions such as Flega and Alcudia (at ~ 134 h after withholding water). Interestingly, other accessions considered moderately susceptible and resistant such as Rapidena and Anchuela, respectively also showed this trend (between 110-158 h after withholding water) (Fig. 3a). In order to compare the patterns of g_l between accessions we calculated the difference between the area under the conductance progress curve (AUCPC) of control and stressed plants. The values depicted in Table 3 shows that Flega, Alcudia (both classed as susceptible), Rapidena (moderately susceptible) and Anchuela (moderately resistant) exhibited a reduction in AUCPC values which were significantly greater than that observed in the most resistant accessions such as Patones ($P < 0.001$). However, other accessions such as Gen76 and Gen100 considered moderately resistant also showed significant differences with Patones ($P < 0.001$) (Fig. 3b). Such observations indicated mechanisms of drought resistance amongst the germplasm collection that were not only associated with stomatal regulation.

Assessing infrared temperature

Analysis of infrared (IR) temperatures of control plants indicated significant differences between accessions ($P < 0.001$), sampling times ($P < 0.001$) and interaction between these factors ($P < 0.001$). This indicated that the accessions were at very different physiological statuses even at optimal environmental conditions. For example, accessions such as Flega, Gen76 and Anchuela had the lowest temperature values in most of the sampling times assessed whereas Gen124 had the highest values (data not shown). Interestingly, water stressed plants exhibited a dramatic increase in leaf temperature with time after withholding water when compared to controls ($P < 0.001$) with highly significant differences between accessions ($P < 0.001$; Fig. 4a). The overall mean increase in temperature in water stressed plants was 1.73 ºC but was more dramatic at later time points. Thus, whilst between T_6 and T_{12} the mean increase in temperature of water stressed plants respect to the controls was by 1.27 ºC between T_{12} and T_{18} this was 3.45 ºC. However not all accessions responded in the same manner to the drought stress during the time course as indicated the interaction between these two factors ($P < 0.001$). At T_6 Gen122, Gen135 and Rapidena showed the lowest differences with respect to their controls whilst Gen17 showed the highest increase ($P < 0.001$). At T_{12}, Patones and Rapidena showed the lowest temperature increase but Anchuela, Gen 122, Mirabel, Alcudia and Flega had the highest increases ($P < 0.001$) compared to controls. At T_{18} Patones showed the lowest temperature increase of any of the accessions (Fig. 4a). Fig. 4b shows the infrared pictures of controls and drought stressed Flega and Patones plants at T_{15} and indicates the higher increase in temperature with respect to the control observed in the susceptible Flega compared to that in Patones.

Lipid peroxidation and lipoxygenase activity

All accessions exhibited significantly greater MDA content at both, 9 and 12 daww ($P < 0.001$) compared with their controls. However, differences amongst accessions were highly significant ($P < 0.001$) as were those between time points ($P = 0.022$) with lower relative MDA content 9 than 12 daww (Fig. 6a). We also observed significant accession x time points ($P = 0.004$) and accession x treatment ($P = 0.005$) interactions. Thus, at 9 daww highly resistant accessions such as Patones, Mirabel, and Gen122 had the lower MDA content whereas the most susceptible or moderately susceptible accessions such as Flega and Rapidena had the highest levels (Fig. 5a). Nevertheless, other accessions such as Alcudia which were considered highly susceptible did not significant differ in MDA content when compared with resistant Patones or Mirabel. In the same way resistant accessions such as Gen16 and Gen17 had similar concentrations to that found in the susceptible Flega.

Analysis of lipoxygenase (LOX) activity in non-stressed plants showed no significant differences among accessions. However, we observed a significant increase in LOX activity in water stressed plants respect to their controls, which differed amongst accessions ($P < 0.001$) and time points ($P < 0.001$) which at 9 daww was lower than that at 12 daww (Fig. 5b). The lack of interaction

accession x time point indicated that all accessions increase LOX activity with time after withholding water in a similar manner. We observed very high levels of LOX activity in the highly susceptible and moderately susceptible accessions Flega, Alcudia and Rapidena, but also in Anchuela considered moderately resistant (Fig. 5b). The LOX activity of the other accessions did not significantly differed to that from Patones except Gen125 at 12 daww. There was an overall correlation between LOX activity and MDA content (r= 0.856; $P < 0.001$) but not at the level of all individual genotypes. Thus, we found a significant correlation between LOX and lipid peroxidation in Alcudia (r=0.874; $P = 0.018$), Anchuela (r=0.847; $P < 0.001$), Flega (r=0.904; $P = 0.021$), Gen122 (r=0.850; $P = 0.002$), Gen125 (r=0.864; $P = 0.03$) and Gen17 (r=0.834; $P < 0.001$) but not in the rest of accessions.

Leaf chlorophyll content

Analysis of SPAD chlorophyll meter reading (SCMR) showed significant differences between accessions ($P < 0.001$), treatment ($P < 0.001$) and time points ($P < 0.001$) and interaction between all factors ($P < 0.001$). This indicated that accessions respond very differently to water stress along the course of the drought experiment. Thus, Patones, Mirabel, Gen135 and Gen122 had a very low decrease in chlorophyll following the imposition of water stress compared to their controls, whilst accessions such as Flega, Rapidena, Alcudia, Gen17 and Gen124, showed a rapid decrease ($P < 0.001$) (Fig. 6). The highest overall differences between accessions respect to SCMR were observed from 15 days after withholding water. However, significant genotypic differences were observed at each time point after withholding water (P between 0.01 and 0.001). Interestingly when we assessed the SCMR in controls plants we also observed differences between accessions ($P < 0.001$), time points ($P < 0.001$) and interaction between these factors. Differences among control plants were observed at all timepoints, with Mirabel and Patones showing the highest SCMR in 6, 9, 12 and 15 days and Flega, Gen122 and Gen125 showing the lowest values in most time points assessed.

Antioxidant activity

Antioxidant activity significantly increased in leaves of water stressed plants compared to their controls ($P < 0.001$) with overall means of 49.7 and 75.5 μmol g^{-1} fresh weight for control and drought treated plants, respectively. However, overall there were no significant differences amongst accessions, time points or interactions between these factors. Only when analyzed separately, were significant increases detected in Gen124 at 12 daww ($P = 0.04$) with respect the other accessions except Gen 16, Gen122 and Gen125 (Fig. 7).

Selecting key physiological traits linked to drought resistance using multivariate statistical approaches.

Following the assessment of the above mentioned physiological traits during drought stress, a multivariate analysis was performed in order to determine those traits that better discriminate between susceptible and resistant accessions. In total 28 variables were studied derived from the data already presented. Unsupervised Principal Component Analysis (PCA) the whole dataset including all variables could not separate between accessions; hence, supervised PC-Discriminant Function Analysis (PC-DFA) was performed on 19 PCs (explaining 99% of the total variation) but this still failed to discriminate between accessions. To simplify the analyses, those variables from most extreme sampling times were removed; i.e. 6 daww (since at this time water stress was very low and most accessions had not registered any physiological change) and 18 daww (where most accessions exhibited the most extreme damage). PC-DFA plots (Fig. 8a) showed that it was now possible to distinguish between the highly resistant, the resistant and the susceptible accessions. PC-DFA indicated that leaf temperature at 12 and 15 daww, (based on PC loading vectors) following by AUDPC, and RWC at day 15 were the individual parameters that contributed most to the derived projections (Online Resource 3A). In addition, hierarchical cluster analysis (HCA) based on the PCs separated the most susceptible accessions, Flega and Alcudia and also the most resistant accession; Patones, from other resistant and moderately resistant accessions (Fig. 8b). The model did not discriminate between the considered moderately susceptible accession, Rapidena, and the moderately resistant accessions (Fig. 8a, b).

PCAs and DFAs projections of the trait data showed a very different trend depending on the sampling time assessed (Fig. 9). When analyzed those parameters recorded at 6 and 9 daww none of the PC-DF axes explained differences among any of the accessions (Fig. 9). By 12 daww it was possible to discriminate between the most susceptible accessions, Flega and Alcudia, from other accessions with examination of the PC loading vectors indicating that infrared temperature was the parameter that most explained this variation following by AUCPC and LOX (Online Resource3B). Analysis of parameters recorded at 15 daww allowed the discrimination between the most resistant, Patones, and the remaining accessions with again infrared temperature and also AUCPC as the main loading vectors explaining this difference (Fig. 9), (Online Resource 3c). A similar trend could be observed when traits taken at 18 daww were analyzed, with infrared temperature and SCMR measurements as main parameters explaining the model (Online Resource 3d).

Since the parameters that most contributed to the differences among accessions in the general model (Fig. 8a), were leaf temperature at 12 and 15 daww, AUDPC and RWC at T_{15} these were also analysed together and separately from the others variables (Fig. 10). In these analyses PCA results differed greatly from the general model (Fig. 8) and showed good discrimination among accessions. In addition, when focussed only on these parameters, DFA analysis could also discriminate between the highly resistant, the moderately resistant and the highly susceptible accessions (Fig. 10).

Since from the breeding point of view it would be highly desirable selection of resistant accessions in the absence of the stress, those parameters from which genotypic differences were found among control plants were also analysed. However, in this analysis, neither PCA nor DFA allowed discrimination among accessions (Fig. 11). We also compared several of the accessions in order to determine other key traits discriminating between them (Online Resource 4, 5). PCA and DFA models clearly discriminated between the most susceptible, Flega, and most resistant, Patones, accession when analyzed separately from the rest (Online Resource 4). SCMR9, AUCPC, LOX12, RWC12, IR12, IR15, SCMR15 and LP12 were the parameters that most contributed to the discrimination between these two accessions. When comparing only the moderately or resistant accessions three different clusters were observed, the first one including Gen135, Gen122, Gen124, Gen125, Gen100 and Gen17, the second one with Gen16, Gen 76 and Rapidena, and the third one with Mirabel, and Anchuela (Online Resource 4). The significant parameters explaining this grouping were leaf temperature at 12, 15 and 18 daww, SCMR at 18 daww and LOX at 9 daww.

Discussion

Drought is currently one of the main constrains preventing crops plants from expressing their full genetic potential. The identification of sources of drought tolerance in germplasm which is sexually compatible with elite crops is crucial to secure productivity. Thus, we sought to find novel sources of tolerance to drought in an oat germplasm collection consisting of 174 accessions. Following a visual assessment of the oat collection during a time course of withholding water, 11 accessions of *A. sativa* and *A. byzantina* including landraces and commercial cultivars were identified as being either highly resistant, resistant or moderately resistant to drought. *Avena sativa* and *A. byzantina,* sometimes known, respectively as the white and red oats, are the main cultivated oats. They are self-pollinating hexaploids and sexually compatible with hybridizing techniques (Stevens et al. 2004) so that our identified germplasm could be readily introduced into breeding programmes. Crucially, field assessment of yield under water prone conditions correlated with the drought tolerance observed under controlled conditions thereby indicating the usefulness of the assay in young plants. Small differences between field and controlled conditions might be due to other drought resistance mechanisms expressed in field and/or older plants such as specific avoidance mechanisms (root architecture, date of flowering etc.) which were not assessed in this work. In addition experimental factors such as pot size for controlled experiments and/or row spacing for field experiments could influence the observed differences.

Further, we characterize the selected accessions assessing several physiological drought tolerance mechanisms and used a multivariate approach in order to determine the key features/responses explaining the drought tolerance and susceptibility in oat. This is of high practical importance for breeding since the lack of effective selection criteria is considered to be a major impediment to breeding for drought-prone environments (Araus et al. 2002; Ouk et al. 2006; Venuprasad et al. 2007). Many drought associated markers have been extensively studied by many authors. However, many are based solely on correlation – i.e. they simply occur at the same time as drought stress. However, the causal relationship to drought stress tolerance is often not fully assessed. In line with this, we observed that several of the traits usually associated with drought resistance were present in many, but not in all, of the resistant accessions. Indeed, even susceptible accessions possessed some features associated with drought resistance. Such observations, ably demonstrated how difficult it has been to best discriminate drought tolerance within a group of oat accessions.

Among previously reported drought-linked physiological features, we assessed several associated to water relations such as RWC, g_l, leaf temperature and WUE. RWC is an appropriate measure of plant water status in terms of the physiological consequence of cellular water deficit. Other parameters such as water potential is useful in dealing with water transport in the soil-plant-atmosphere continuum, but does not account for OA which is a powerful mechanism of conserving cellular hydration (Islam et al. 2011). In contrast, RWC takes into account the possible effect of both leaf water potential and OA (Blum 1999; Islam et al. 2011). Predictably, RWC declined in all accessions after water was withheld but the rates of RWC reduction differed among accessions. Susceptible accessions rapidly reduced their RWC but some of the moderately resistant accessions were not very different in their RWC loss rates. In these latter accessions specific mechanisms such as the preservation of cell membrane integrity might allow the maintenance of metabolic activity even at low RWC. Indeed, we observed that most of these latter accessions had high values of CMS at low (45%) RWC. Cell membrane is one of the main cellular targets to different stresses (Levitt 1980) and maintenance of membrane stability during drought is important for normal physiological metabolism to continue under low water potential (Tripathy et al. 2000). However, phenotype selection only for CMS may not always be accurate for breeding purposes because of its complex nature and its strong interaction with the environment (Tripathy et al. 2000). Ideally, it is important to evaluate this trait under controlled environment and equal RWC for accurate comparisons as demonstrated in this study and these are not readily transferred to a field situation.

In addition to RWC, g_l is an important component contributing water relations during drought stress. Stomata close progressively as drought progresses, followed by parallel decreases of net photosynthesis. Indeed, stomatal closure has been suggested as the main determinant for decreased photosynthesis under mild to moderate drought (Cornic and Massacci 1996; Medrano et al. 2002),

although some authors disagree (Tezara et al. 1999). We observed a reduction in g_s in all assessed accessions during the water stress experiment although differences in stomatal conductance of stressed and controls plants differed greatly among accessions. As previously reported (Medrano et al. 2002), stomata of most accessions closed in response to drought before any change in leaf water content was detectable. This is attributed to the abscisic acid (ABA) root-to-leaf signalling promoted as the soil dries. Thus, g_s is responsive to several external (soil water availability, vapour pressure deficit) and internal (ABA, leaf water status) factors related to drought and can be considered as an integrative parameter reflecting photosynthetic response during water stress (Medrano et al. 2002). Accordingly, accessions such as Alcudia, Flega, Anchuela and Rapidena with the highest decreases in stomatal conductance compared to their controls during the water stress (AUCPC values) would be photosynthetically more affected than Patones, Gen16, Gen17 Gen122, Gen124 and Gen135. Stomatal movements are very dynamic due to complex regulation stated above. For this reason three measurements during the light time course were performed. This was preferred to solely midday g_s readings because, as drought becomes progressively intense, the daily peak conductance is displaced (Flexas et al. 2000) and only one measurement might not display accurate g_s curves.

When water evaporates from the surface of the leaf, it becomes cooler due to stomatal conductance (beside vapour pressure deficit). Thus, leaf temperature and temperature depression compared to ambient air temperature is a good indicator of a genotypes' physiological fitness (Araus et al. 2002). Since measurements performed in this study were achieved under controlled environmental conditions, differences in leaf temperature among accessions could be easily compared in stressed and non-stressed plants. Most moderately resistant accessions had very small increases in leaf temperature 12 daww suggesting good physiological homeostasis since cooling improves the photosynthetic activity and prevents premature senescence. Clearly, such homeostatic mechanisms are compromised in most accessions by 18 daww.

Regarding WUE, although it is one of the most studied parameter related to drought resistance, there is a constant debate of 'putative' drought resistance mechanisms, 'water-use efficiency', and their interrelationship and associations with yield potential. WUE for yield is often equated in a simplistic manner with drought resistance. However, several authors (i.e Condon et al. 2002; Blum 2005) suggested that selection for higher WUE assuming that it equated with improved drought resistance or improved yield under stress may in fact lead to the selection of negatively acting factors. Thus, genotypic variations in WUE are mainly driven by variations in water use rather than by variations in plant production or assimilation per given amount of water use. In line with the suggestions of such as Blum (2005) our data did not show a clear correlation of WUE with drought tolerance suggesting that WUE did not highly contribute to the observed genotypic variation to drought.

The above reported mechanisms directed to cell/tissue water conservation are tightly linked to physiological traits associated with oxidative damage and/or activation of antioxidant mechanisms. Under drought, stomata close and this limits CO_2 fixation in the chloroplast so that electron flow in the light reactions exceeds that required for CO_2 assimilation. This leads to the over-reduction of photosynthetic components and the resulting production of reactive oxygen species (ROS), such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), or the hydroxyl radical (OH^-) (Cruz et al. 2004). These ROS, if not quenched by antioxidant machinery, can seriously disrupt metabolism through membrane lipid peroxidation, chlorophyll loss and protein carbonylation (Basu et al. 2010). During the lipid peroxidation, protons are abstracted from for example, phospholipids to initiate a lipid radical (L^-)–lipid hydroperoxide (LOOH) chain reaction ($LH + {}^{\cdot}OH \rightarrow L^{\cdot} + H_2O$; $L^{\cdot} + O_2 \rightarrow LOO^{\cdot}$; $LOO^{\cdot} + LH. \rightarrow LOOH + L^{\cdot}$ etc. in a propagative cycle). The peroxidation of non-saturated groups within acyl chains [also known as polyunsaturated fatty acids (PUFA)] in a membrane would severely disrupt its integrity. Our results shows an increase in the PUFA breakdown product MDA content, in all accessions with time after withholding water with higher increases in susceptible accessions. However, several of the moderately resistant accessions had similar MDA content to the susceptible ones. Further, in several of the accessions we found a correlation between the MDA content and the lipoxygenase activity. This could indicate that in these accessions most of the peroxidation of the membrane polyunsaturated fatty acids was mediated by this enzymatic reaction rather than free radical chemistry. Furthermore, in several of the accessions with high MDA content we also observed considerable chlorophyll-loss in stressed compared to control plants as indicated by SCMR values (Yadava 1986) which can also suggests transpiration efficiency (Nageswara-Rao et al. 2001). Our results showed that several of the resistant accessions maintained chlorophyll levels upon drought stress indicating the maintenance of photosynthetic related activities. Interestingly, we also observed significant differences among controls in all time points. Control plants of accessions showing low SCMR in most time points assessed might indicate an early senescence of the leaves, than in the case of i.e. Flega would add to the chlorophyll loss due to drought stress. Oxidative damage in the plant tissue above reported may be alleviated by a concerted action of both enzymatic and non-enzymatic antioxidant mechanisms. These mechanisms include scavenge of radicals by β-carotenoids, α-tocopherol, ascorbate, glutathione, anthocyanins, flavonoids, carotenoids (Mittler and Blumwald 2010), and enzymes including superoxide dismutase, catalase, glutathione reductase, and ascorbate peroxidase (Halliwell 1987; Asada 1992). The total non-enzymatic antioxidant activity estimated by the FRAP assay revealed no differences amongst accessions in the present study either at 9 or at 12 daww. This does not rule out the possibility of significant differences between accessions in specific non-enzymatic antioxidants or antioxidant enzymes which should be studied in detail. However, antioxidant activity was not further assessed in the present study since it is more difficult to test in a breeding population in a realistic manner.

Considered individually, it is difficult to determine which of the above stated drought linked physiological features is the crucial one/s responsible for the tolerance phenotype in oat. However, by screening large germplasm pools and following PC-DFA models based on all these variables assessed together, we identified specific water use related features such as leaf temperature and RWC as the main traits indicative of drought tolerance in oat. Other physiological processes involved in cell/tissue water maintenance including g_l and those reflecting oxidative damage and antioxidant defence albeit linked with the resistance responses might be considered as weakly correlated events not suitable for discriminating among oat accessions.

Interestingly not all measurement of leaf temperature and RWC discriminated among accessions but only the intermediates taken at time 12 and 15 daww together with the AUDPC assessed during the whole time course of water stress. With selected variables, both the unsupervised PCA and the DFA analyses were useful in discriminating between the most susceptible, the moderate and most resistant accessions indicating its appropriateness for breeding selection. Furthermore, the very different nature of the plant material assessed (landraces, and commercial cultivars from *A. sativa* and *A. byzantina* species) point to the strength of this approach as it was used in populations with very different genetic backgrounds. The study also show that the physiological status of the control plants is not enough to discriminate among accessions and parameters for selection need to be also measured under the drought stress in order to perform an accurate selection.

Overall, this work allowed for the first time the ranking of many supposed drought resistance traits in order of degree of importance within oat, highlighting those with a causal relationship to drought stress tolerance and not only correlated with it in determinate accessions. Our approach was to encompass as much oat biodiversity as possible but screened under controlled conditions. Screening under natural stress conditions is difficult because of the irregular and erratic drought response (Venuprasad et al. 2007) whereas controlled conditions allowed the inexpensive and robust screening of large populations with optimized protocols for selection of plants carrying specific physiological mechanisms that can be coupled later with yield assessments in the field for selected accessions. As such, this initial stage can be readily adopted by crop breeders. By combining information on the basis of yield limitation under contrasting environments with the new physiological/ biochemical /molecular selection tools, the probability of accelerating the rate of genetic progress through plant breeding will be significantly increased (Araus et al. 2002). This study shows the potential of multivariate analysis as robust approach to target key mechanisms responsible for drought tolerance in oat. In addition multivariate analysis can help breeders by speeding genotype selection from large breeding populations otherwise difficult and expensive to test.

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Supplementary material

Additional Supplementary material may be found in the online version of this article:

Online Resource 1 Visual assessment of drought symptoms during a 19 days time course of drought.
Online Resource 2.Yield of selected accessions in prone water conditions.
Online Resource 3 PC-DFA loading vectors contributing to the derived projections that discriminated between accessions
Online Resource 4 Scatterplot of Principal Component Analysis (PCA) and Discriminant Function Analysis (PCA) scores of components 1 and 2 of the most susceptible (Flega) and Most resistant (Patones) accessions based on all variables assessed
Online Resource 5 Scatterplot of Principal Component Analysis (PCA) and Discriminant Function Analysis (PCA) scores of components 1 and 2 of the moderately resistant accessions based on all variables assessed.

Figure Legends

Fig.1 Visual assessment of drought symptoms during a 19 days time course of drought. **a** Classification of *A. sativa* (■) and *A. byzantina* (▣) wild accessions and in commercial oat cultivars (□) plants according to the Area Under the Drought Progression Curve (AUDPC) based on a 5-0 visual scale of drought damages where 0= completely healthy plant and 5= completely wilted plant. According to this, accessions were classified as highly susceptible, moderate susceptible, moderate resistant, resistant and highly resistant. **b** AUDPC curves of the most susceptible, Flega (solid circles), and most resistant, Patones (open circles), accession. Data are based on five plants per accession distributed in randomized blocks + standard error

Fig. 2 Relative Water Content and Cell Membrane Stability assessment. **a,b** RWC and CMS of the selected oat accessions along a time course of drought. Data are mean of ten replicates per accession and treatment. L.S.D bar ($p<0.05$) is represented for accession comparison in any of the times points. Alcudia (◇); Anchuela (■); Flega (●); Mirabel (▲); Patones (○); Rapidena (△); Gen16 (▢); Gen17 (▲); Gen76 (◆); Gen100 (◍); Gen122 (◇); Gen124 (□); Gen125 (△); Gen135 (●). **b,c** Original (solid line) and model derived (dotted line) of RWC and CMS curves of the most susceptible, Flega, and most resistant, Patones, accessions. Equation of the fitted curve and the correlation coefficient are also depicted in the figure

Fig. 3 Stomatal conductance (mmol m^{-2} s^{-1}) of the selected oat accessions along a time course of drought. Three measurement were taken during 10 days: two hours after beginning of light period, in the middle, or two hours before the end of light period. Data are based on ten replicates per accession and treatment. Control are represented by open circles and drought treatment by solid triangles

Fig. 4 Infrared Temperature assessment. **a** Increase in Infrared Temperature (IRT) in ºC, in the selected oat accessions at day 6 (white bars) and day 12 (grey bars) and day 18 (black bars) after withholding water. Data are based on five plants per accession and treatment and four IR readings, each in one different leaf, per plant. **b** Infrared temperature images of the most susceptible, Flega, and most resistant, Patones, accession, 12 days after withholding water

Fig. 5 Lipid peroxidation of the selected oat accessions at day 9 (open bars) and day 12 (solid bars) after withholding water. **a** Relative content of malondialdehyde in second leaves respect to its corresponding controls. Data are based on five replicates per accession and treatment $\pm$ standard error. **b** Relative lipoxygenase activity in the second leaf respect to its corresponding controls. Data are based on five replicates per accession and treatment $\pm$ standard error.

Fig. 6 Decrease of the SPAD Chlorophyll Meter Readings (SCMR) respect to the control in the selected oat accessions. SCMR were assessed in the second leaf along a time course of drought. Data are based on five replicates per accession and treatment and three SCMR per leaf. L.S.D bar ($p<0.05$) is represented for accession comparison in any of the times points. Alcudia (◇); Anchuela (■); Flega (●); Mirabel (▲); Patones (○); Rapidena (△); Gen16 (□); Gen17 (◭); Gen76 (◆); Gen100 (○); Gen122 (◇); Gen124 (□); Gen125 (△); Gen135 (●).

Fig. 7 Test FRAP of antioxidant activity in the second leaf of the selected oat accessions at day 9 (open bars) and day 12 (solid bars) after withholding water. Activity is expressed in percent respect to its corresponding controls. Data are based on five replicates per accession and treatment ± standard error

Fig. 8 Multivariate analysis of selected oat accessions according to the different parameters assessed. **a** Scatterplot of Discriminant Function Analysis scores of components 1 and 2 based on the different parameters assessed (AUDPC, RWC, CMS, Stomatal Conductance, MDA, LOX, Antioxidants, SCMR, and IRT) at different time points after withholding water. Alcudia (▽); Anchuela (■); Flega (●); Mirabel (▲); Patones (○); Rapidena (△); Gen16 (□); Gen17 (◭); Gen76 (▼); Gen100 (○); Gen122 (▽); Gen124 (□); Gen125 (◭); Gen135 (●). **b** Hierarchical Cluster Analysis of the selected oat accessions according to the model represented in a

Fig. 9 Multivariate analysis of selected oat accessions according to the different parameters assessed at different sampling times. Alcudia (▽); Anchuela (■); Flega (●); Mirabel (▲); Patones (○); Rapidena (△) ; Gen16 (□); Gen17 (◭); Gen76 (▼); Gen100 (○); Gen122 (▽); Gen124 (□); Gen125 (△); Gen135 (●)

Fig. 10 Scatterplot of Principal Component Analysis (PCA) and Discriminant Function Analysis (PCA) scores of components 1 and 2 according to significant parameters obtained from the general model (AUDPC, RWC15, IRT12 and IRT15). Alcudia (▽); Anchuela (■); Flega (●); Mirabel (▲); Patones (○); Rapidena (△) ; Gen16 (□); Gen17 (▲); Gen76 (▼); Gen100 (○); Gen122 (▽); Gen124 (□); Gen125 (△); Gen135 (●)

Fig. 11 Scatterplot of Principal Component Analysis (PCA) and Discriminant Function Analysis (PCA) scores of components 1 and 2 according to parameters obtained from control plants. Alcudia (▽); Anchuela (■); Flega (●); Mirabel (▲); Patones (○); Rapidena (△) ; Gen16 (□); Gen17 (▲); Gen76 (▼); Gen100 (○); Gen122 (▽); Gen124 (□); Gen125 (△); Gen135 (●)

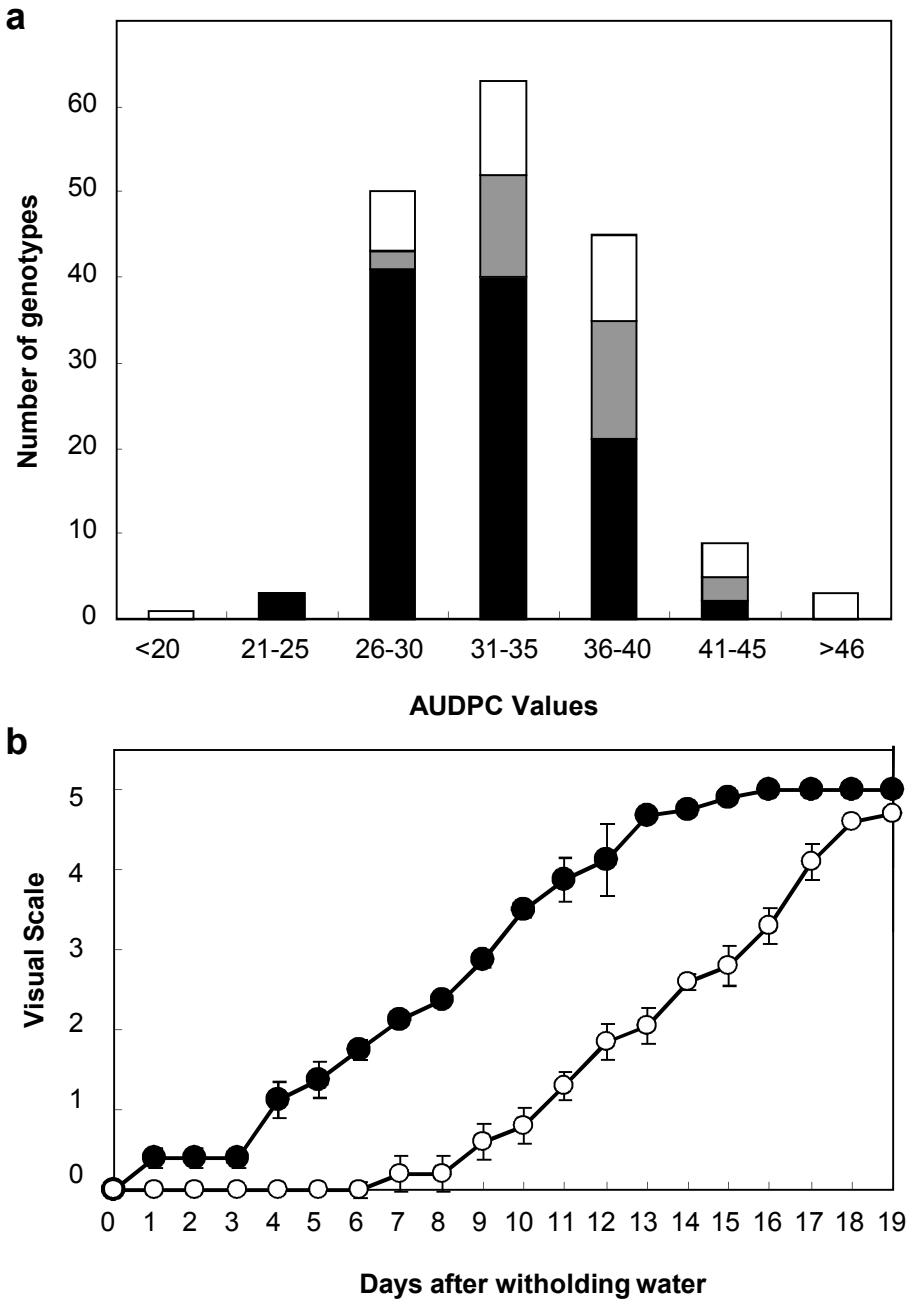


Figure 1. Visual assessment of drought symptoms during a 19 days time course of drought **A.** Classification of *A. sativa* (■) and *A. byzantina* (□) wild accessions and in commercial oat varieties (□) plants according to the Area Under the Drought Progression Curve (AUDPC) based on a 5-0 visual scale of drought damages where 0= completely healthy plant and 5= completely wilted plant. According to this, genotypes were classified as highly susceptible, moderate susceptible, moderate resistant, resistant and highly resistant. **B**. AUDPC curves of the most susceptible, Flega (solid circles), and most resistant, Patones (open circles), genotype. Data are based on five plants per genotype distributed in randomized blocks + standard error.

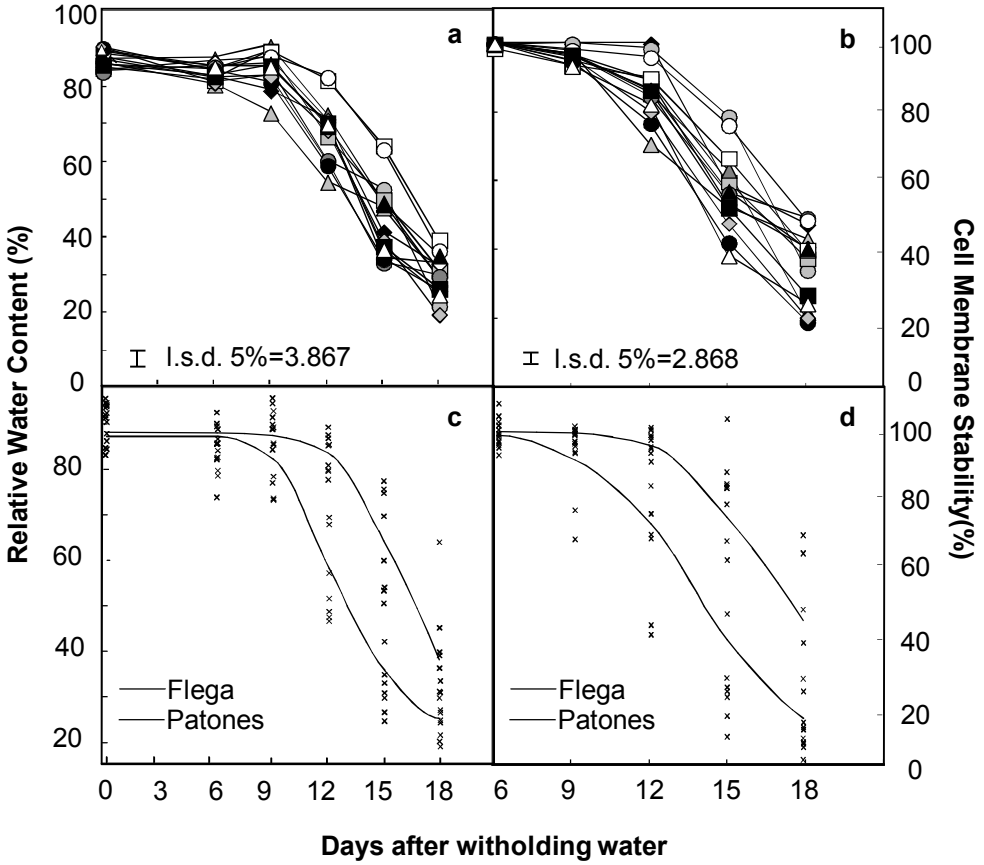


Figure 2. Relative Water Content and Cell Membrane Stability assessment. **A.** RWC and CMS of the selected oat genotypes along a time course of drought. Data are mean of ten replicates per genotype and treatment. L.S.D bar ($p<0.05$) is represented for genotype comparison in any of the times points. Alcudia (◇); Anchuela (■); Flega (●); Mirabel (▲); Patones (○); Rapidena (△); Gen16 (□); Gen17 (▲); Gen76 (◆); Gen100 (○); Gen122 (◇); Gen124 (□); Gen125 (△); Gen135 (●). **B**. Original (solid line) and model derived (dotted line) of RWC and CMS curves of the most susceptible, Flega, and most resistant, Patones, genotypes. Ecuation of the fitted curve and the correlation coeficient are also depicted in the figure.

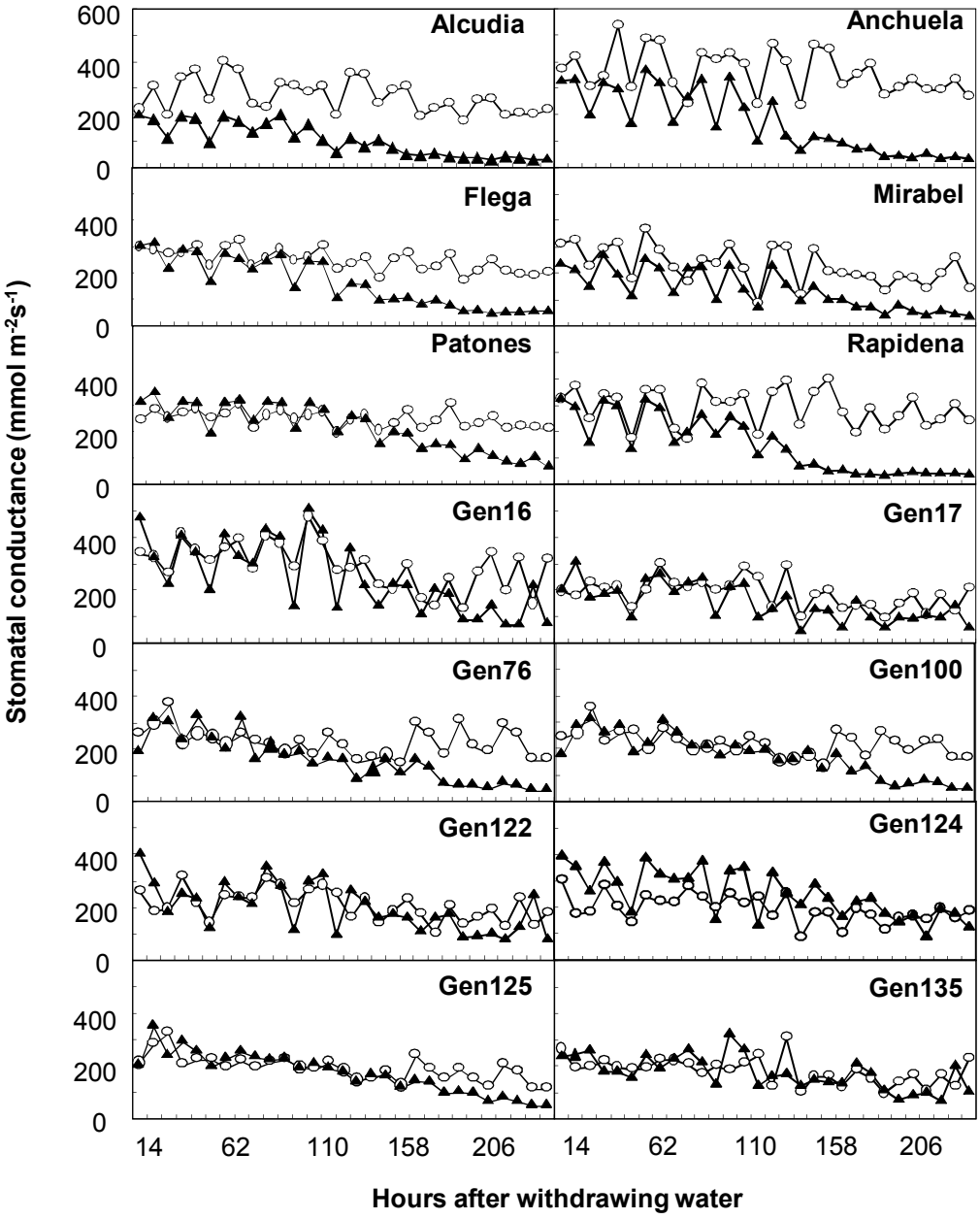


Figure 3. Stomatal conductance (mmol $m^{-2}s^{-1}$) of the selected oat genotypes along a time course of drought. Three measurement were taken during 10 days: two hours after beginning of light period, in the middle, or two hours before the end of light period. Data are based on ten replicates per genotype and treatment. Control are represented by open circles and drought treatment by solid triangles.

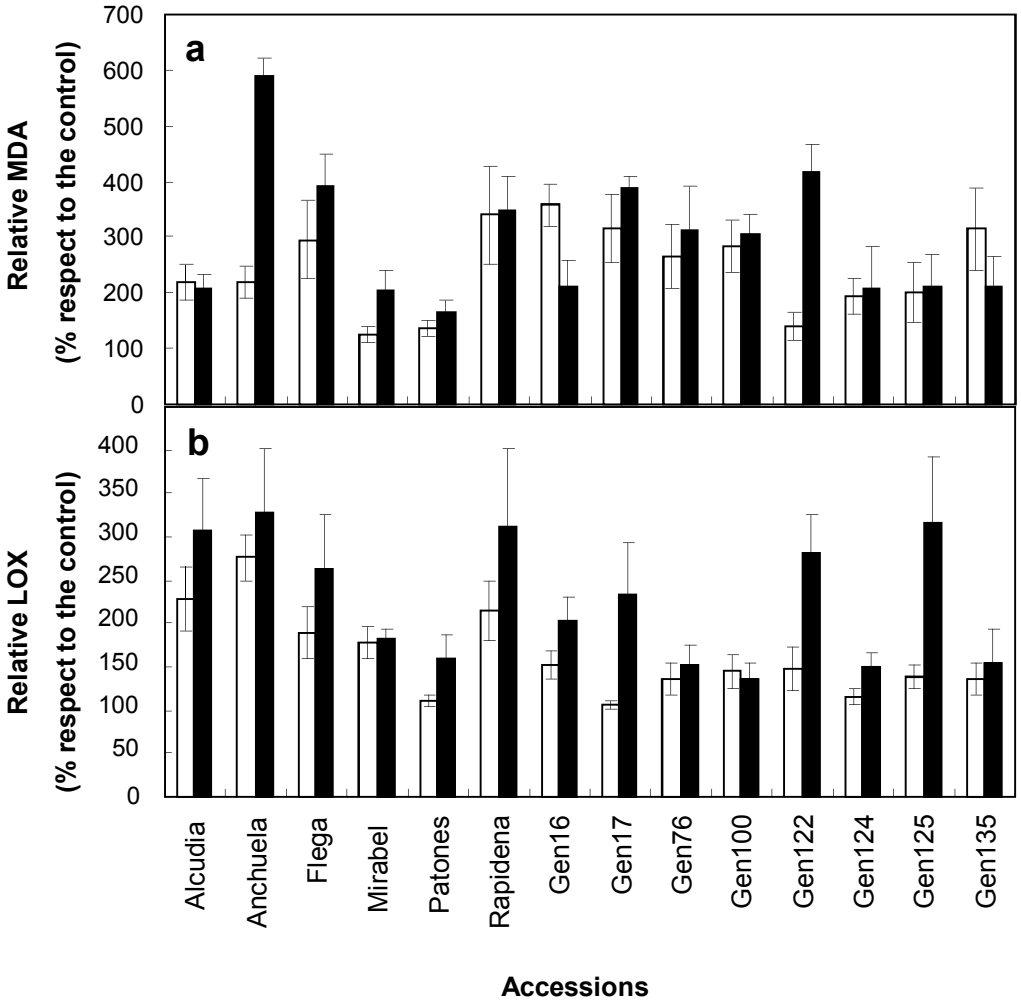


Figure 4. Lipid peroxidation of the selected oat genotypes at day 9 (open bars) and day 12 (solid bars) after withholding water. **A.** Relative content of malondialdehyde in second leaves respect to its corresponding controls. Data are based on five replicates per genotype and treatment ± standard error. **B**. Relative lipoxigenase activity in the second leaf respect to its corresponding controls. Data are based on five relicates per genotype and treatment ± standard error.

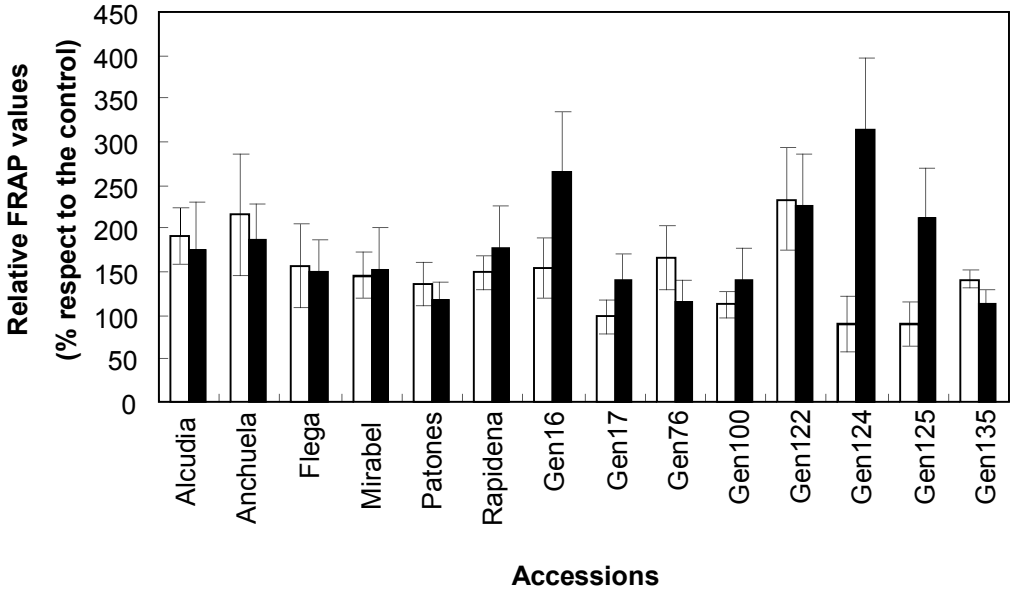


Figure 5. Test FRAP of antioxidant activity in the second leaf of the selected oat genotypes at day 9 (open bars) and day 12 (solid bars) after withholding water. Activity is expressed in percent respect to its corresponding controls. Data are based on five replicates per genotype and treatment $\pm$ standard error.

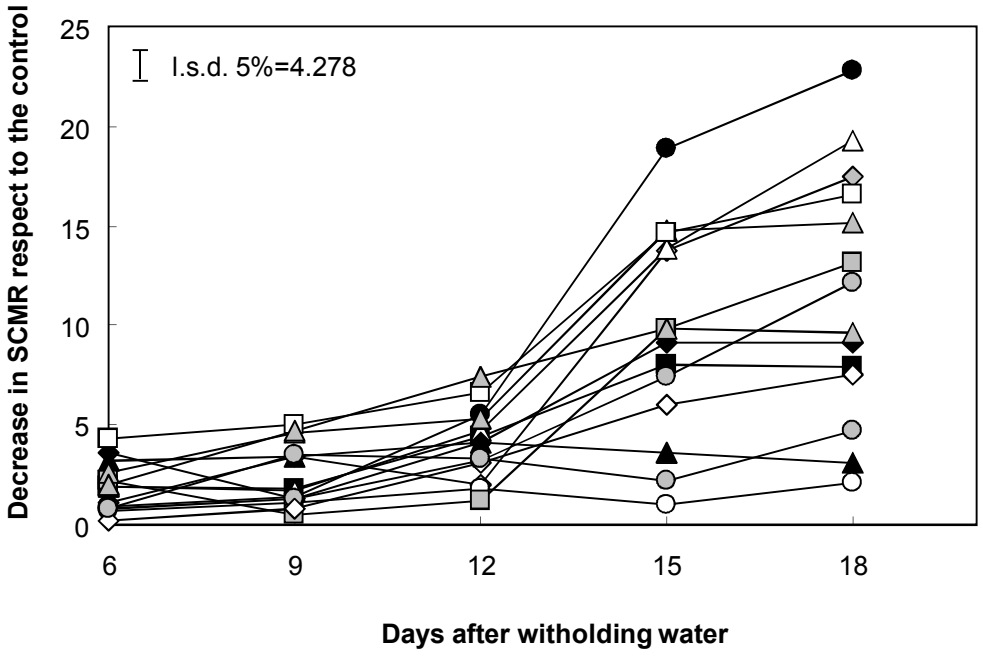
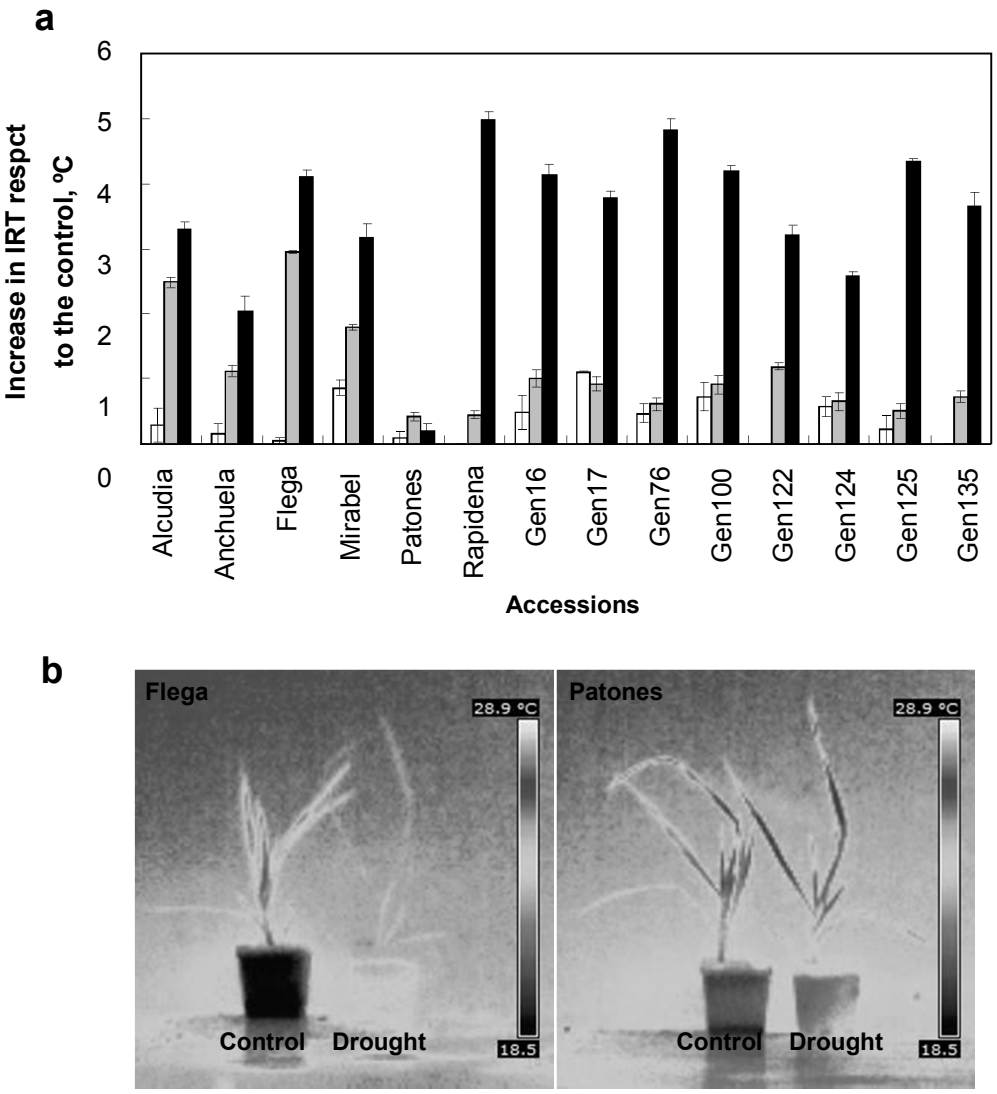


Figure 6. Decrease of the Spad Chlorophyll Meter Readings (SCMR) respect to the control in the selected oat genotypes. SCMR were assessed in the second leaf along a time course of drought. Data are based on four replicates per genotype and treatment and three SCMR per leaf. L.S.D bar ($p<0.05$) is represented for genotype comparison in any of the times points. Alcudia (◇); Anchuela (■); Flega (●); Mirabel (▲); Patones (○); Rapidena (△); Gen16 (□); Gen17 (▲); Gen76 (◆); Gen100 (○); Gen122 (◇); Gen124 (□); Gen125 (△); Gen135 (●).



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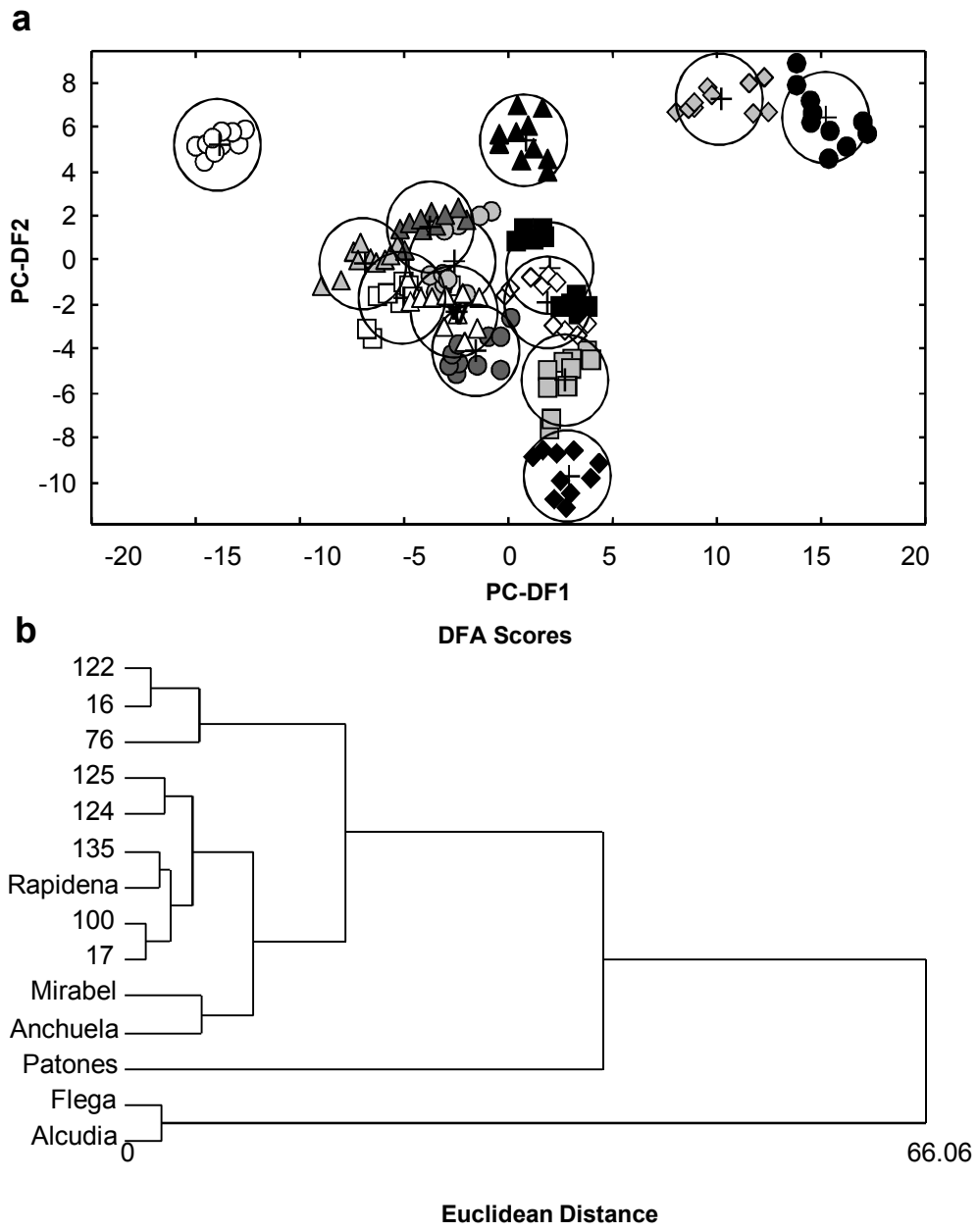


Figure 8. Multivariate analysis of selected oat genotypes according to the different parameters assessed **A.** Scatterplot of Discriminant Function Analysis scores of components 1 and 2 based on the different parameters assessed (AUDPC, RWC, CMS, Stomatal Conductance, MDA, LOX, Antioxidants, SCMR, and IRT) at different time points after witholding water. Alcudia (▽); Anchuela (■); Flega (●); Mirabel (▲); Patones (○); Rapidena (△) ; Gen16 (□); Gen17 (▲); Gen76 (▼); Gen100 (●); Gen122 (▽); Gen124 (□); Gen125 (△); Gen135 (●). **B**. Hierarchical Cluster Analysis of the selected oat genotypes according to the model represented in A.

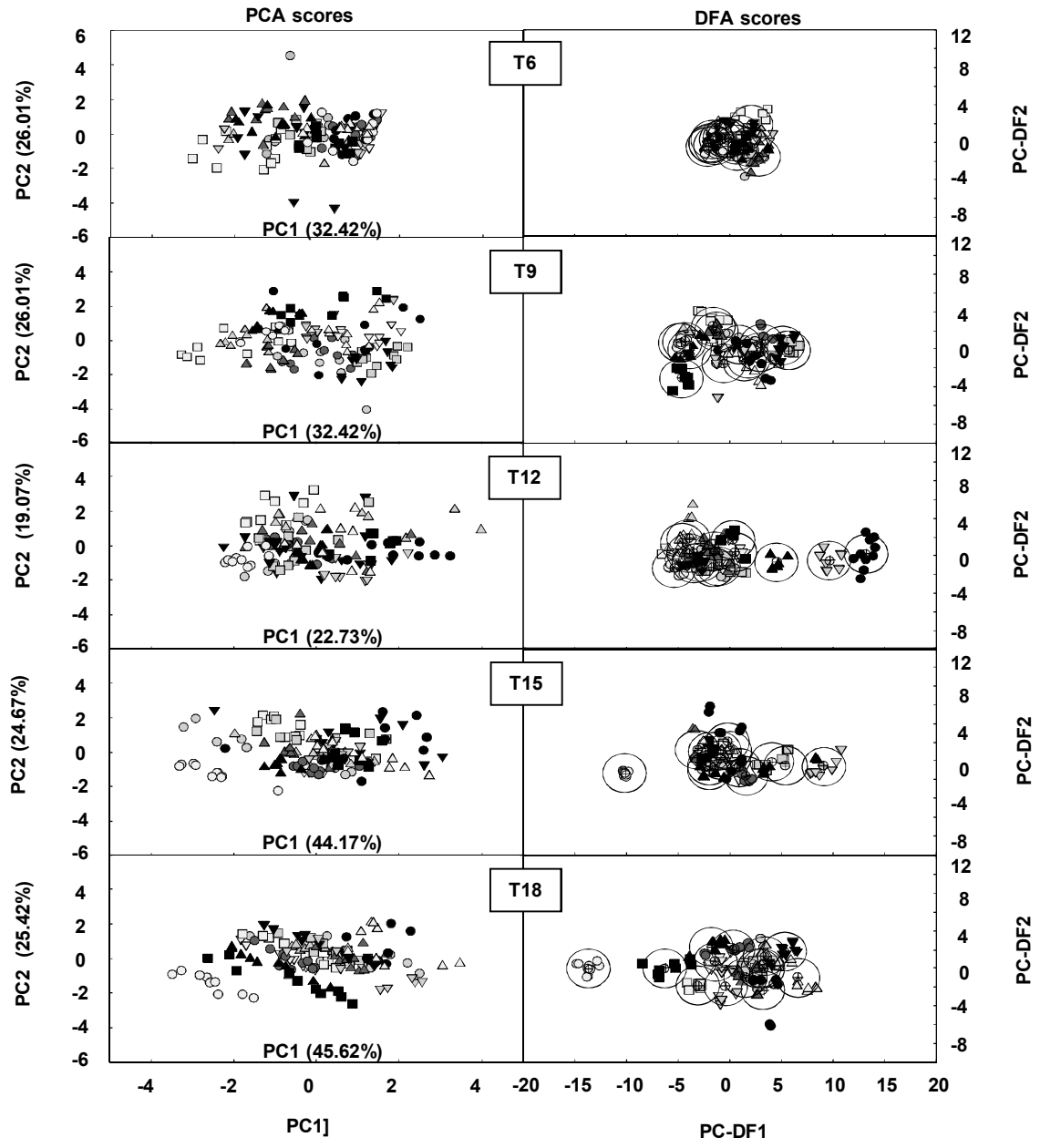
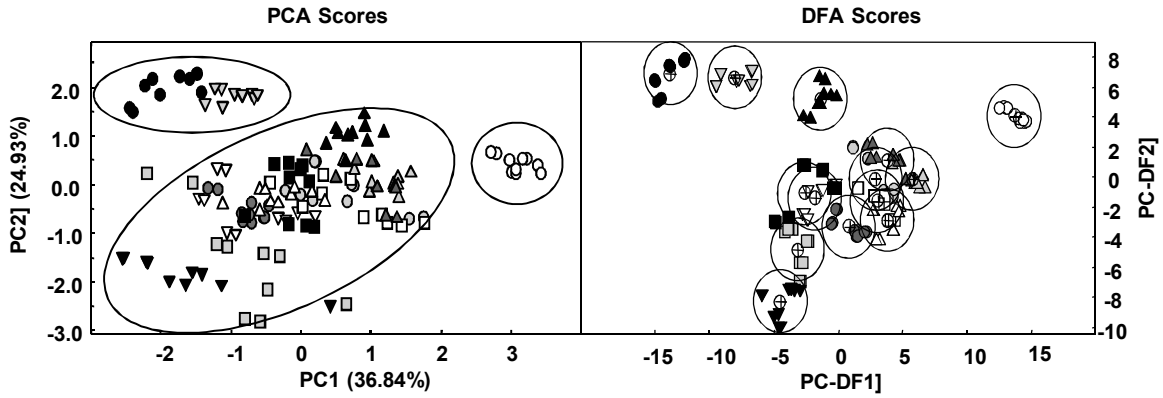


Fig. 9 Multivariate analysis of selected oat accessions according to the different parameters assessed at different sampling times. Alcudia (▽); Anchuela (■ Flega (●; Mirabel (▲); Patones (○); Rapidena (△) ; Gen16 (□ Gen17 (▲ Gen76 (▼); Gen100 (◯; Gen122 (▽); Gen124 (□); Gen125 (△); Gen135 (●)



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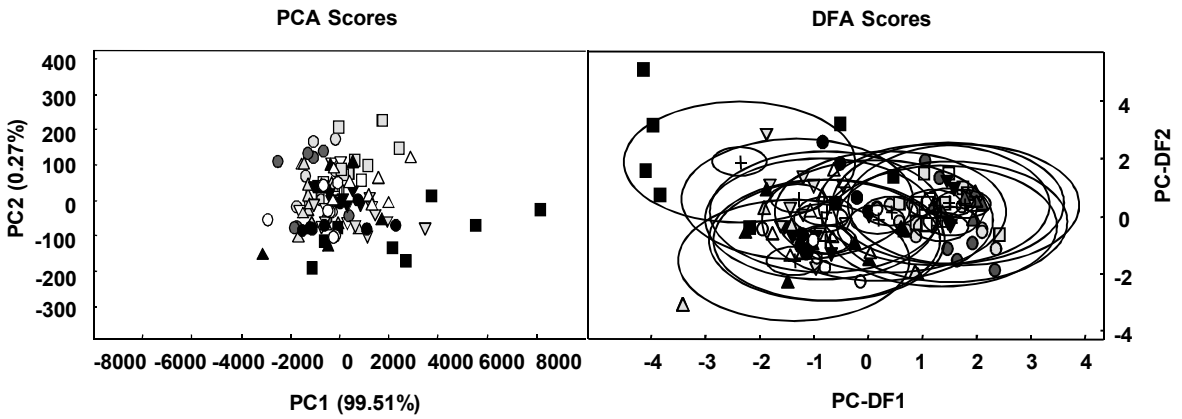


Fig. 11 Scatterplot of Principal Component Analysis (PCA) and Discriminant Function Analysis (PCA) scores of components 1 and 2 according to parameters obtained from control plants. Alcudia ( ); Anchuela ( ); Flega ( ); Mirabel ( ); Patones ( ); Rapidena ( ) ; Gen16 ( ); Gen17 ( ); Gen76 ( ); Gen100 ( ); Gen122 ( ); Gen124 ( ); Gen125 ( ); Gen135 (

Planta

Targeting sources of drought tolerance within an *Avena* spp collection through multivariate approaches.

Javier Sánchez-Martín, Luis AJ Mur, Diego Rubiales and Elena Prats.

Supplementary material

Supplementary Online Resource Legends

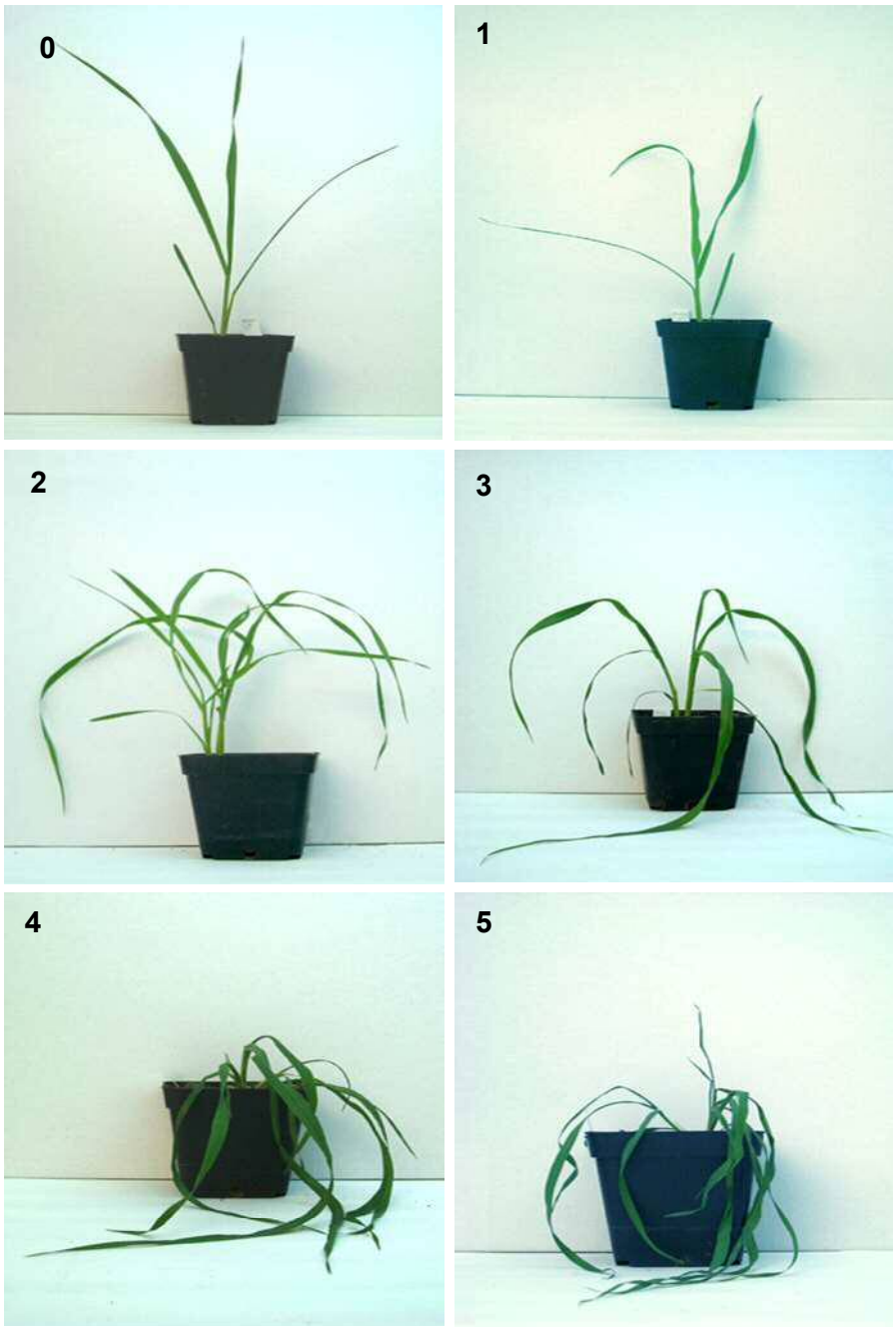
Online Resource 1 Visual assessment of drought symptoms during a 19 days time course of drought.

Online Resource 2 Yield (Kg per Ha) of selected genotypes in prone water conditions (242.36mm in the growing season.

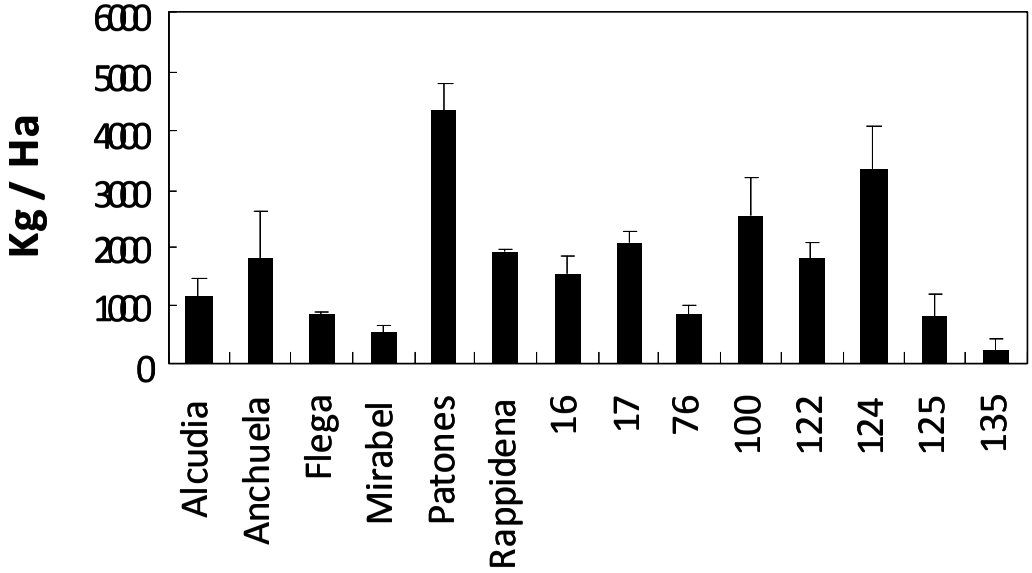
Online Resource 3 PC-DFA loading vectors contributing to the derived projections that discriminated between genotypes when assessing **a** all variables; **b,c** and **d**. variables taken at 12, 15 and 18 d. a. w. w respectively together with AUDPC and AUCPC taken during the whole time course. The inner and outer circles represent respectively one and two standard deviations from the mean (shown by a cross). Hence, the variables shown represent major sources of variation in the datasets.

Online Resource 4 Scatterplot of Principal Component Analysis (PCA) and Discriminant Function Analysis (PCA) scores of components 1 and 2 of the most susceptible (Flega) and most resistant (Patones) genotypes based on all variables assessed. Alcudia (▽); Anchuela (■); Flega (●); Mirabel (▲); Patones (○); Rapidena (△); Gen16 (□); Gen17 (△); Gen76 (▼); Gen100 (○); Gen122 (▽); Gen124 (□); Gen125 (△); Gen135 (⬭).

Online Resource 5. Scatterplot of Principal Component Analysis (PCA) and Discriminant Function Analysis (PCA) scores of components 1 and 2 of the moderately resistant genotypes based on all variables assessed. Alcudia (▽); Anchuela (■); Flega (●); Mirabel (▲); Patones (○); Rapidena (△); Gen16 (□ Gen17 (△ Gen76 (▼); Gen100 (○); Gen122 (▽); Gen124 (□); Gen125 (△); Gen135 (●).



Online Resource 1.



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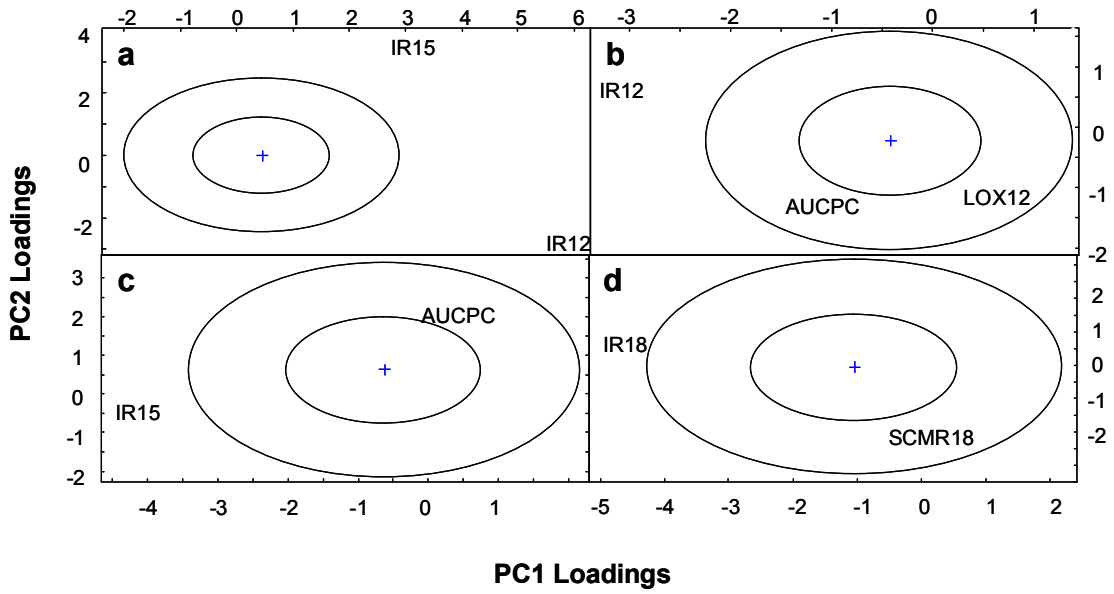

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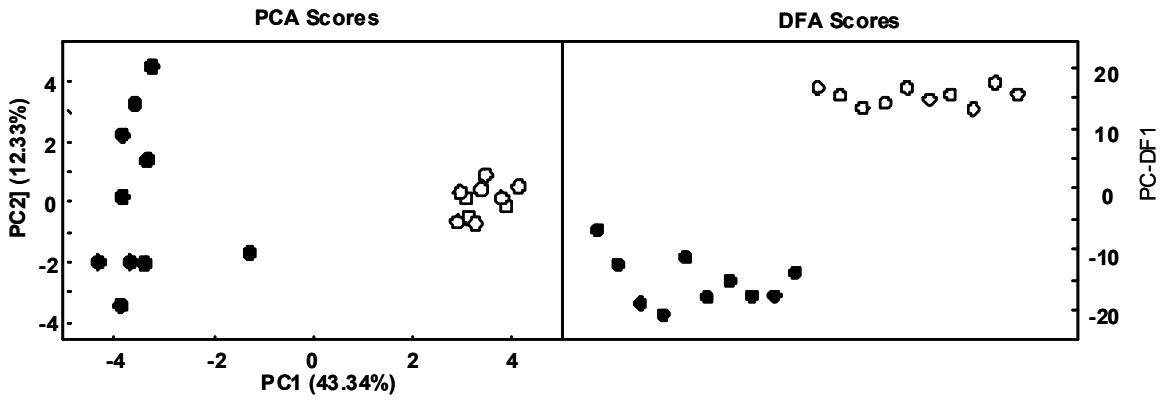

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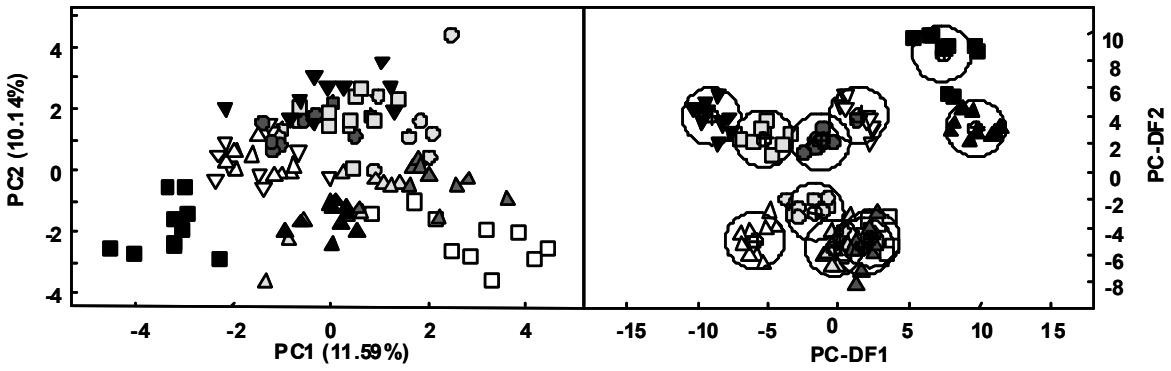

Online Resource 5.