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Metabolite profiling reveals the effect of drought on sorghum (*Sorghum bicolor* L. Moench) metabolism

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Abstract

Plants exposed to limited water availability respond with a series of developmental, morphological, biochemical and molecular adaptations, aiming at safeguarding basal levels of metabolic activity. Given that sorghum (*Sorghum bicolor* L. Moench) is regarded as a drought-tolerant species, it provides an ideal model to study the molecular and physiological mechanisms underlying such tolerance. Young sorghum seedlings grown under controlled conditions were subjected to drought stress, induced by polyethylene glycol (PEG) 6000 at two levels of stress (2.5% and 5% PEG), for 7 days. Non-stressed plants were also included as controls. Metabolite profiling on leaves and roots of stressed and control plants was performed by Gas-chromatography combined with Mass-spectrometry (GC-MS). For each treatment and tissue type, four biological replications were conducted. In total, the analysis yielded 143 quantifiable compounds with highly reproducible patterns. Comparative metabolite profiling of stressed *versus* control plants revealed that drought stress substantially alters the metabolite content in both leaves and roots. In leaves, most profound alterations were observed in compounds belonging to the group of sugars, including D-mannose, D-glucose, isomaltose, fructose and sucrose, but also myo-inositol and L-asparagine whereas in roots, most influencing compounds were certain sugars, such as D-glucose, fructose, sucrose and D-(+)trehalose, as well as D-mannitol. Deduced metabolomics data are discussed and suggested as functional tools towards understanding the underlying regulatory networks involved in the physiology of drought tolerance in sorghum.

Keywords: abiotic stress; drought tolerance; functional marker; GC-MS; metabolite profiling; sweet sorghum.
Abbreviations: GC_gas chromatography; MS_mass spectrometry; PCA_principal component analysis; PEG_polyethylene glycol.

Introduction

Sorghum [*Sorghum bicolor* (L.) Moench] is a C4 annual grass species of tropical origin, which is ranked the fifth most important cereal crop in the world. The crop's high water use efficiency makes it capable of successfully growing in semi-arid tropics, where other crops fail to thrive. Sorghum is characterized by its capacity to tolerate severe drought through several features including its extensive root system, its ability to reduce transpiration via leaf rolling and stomatal closure under drought, its leaf waxiness to prevent water loss and its ability to reduce metabolic processes to near dormancy under drought conditions (Martin et al., 2006; Sleper and Poelhman, 2006). Sweet sorghum shares common features with grain sorghum, yet presents a more rapid growth rate, higher biomass yields and wider adaptation. In addition, the crop's sugar-rich biomass is characterized by the availability of fermentable sugars thus, rendering it an economically advantageous feed stock over starch based crops, where the enzymatic conversion of starch to sugar is a prerequisite. Such characteristics along with the fact that sweet sorghum does not threaten food, feed and fodder value of sorghum, have rendered it one of the most attractive candidates in the search for efficient energy crops in the framework of converting biomass to energy (Rooney et al., 2007).

The determination of crops' biochemical fingerprint can greatly accelerate the identification of valuable genetic material and the understanding of plants' response to stresses and thus, may facilitate all related breeding procedures. Especially the study of complex and polygenic traits, such as drought tolerance, requires the precise determination of biochemical functions in the context of plant metabolism (Trethewey et al., 1999; Fiehn et al., 2000). To this respect, metabolite profiling offers the possibility to acquire sound knowledge on the mechanistic base of such complex traits whereas, at the same time, complements the existing functional genomics approaches by bridging the gap between gene sequences and metabolic networks (Fiehn, 2002). Gas chromatography coupled with mass spectrometry (GC-MS) is generally regarded as one of the most robust technologies applied for the purposes of profiling metabolites in plants (Fiehn et al., 2000; Roessner et al., 2001a, b, 2002; Jonsson et al., 2005; Kopka, 2006). GC-MS enables a snap-shot of plants' metabolic composition by allowing the simultaneous detection and quantification of approximately 150-200 known metabolite compounds from a single plant extract. Given the recent interest in exploiting sorghum's biomass for biofuel production and the fact that the species' drought-tolerance properties are well established, sorghum provides an ideal model to study the molecular and physiological

mechanisms underlying such tolerance. In this study, we determined through GC-MS analysis the metabolic profile of sweet sorghum plants subjected to drought stress induced by polyethylene glycol. The deduced metabolic patterns were comparatively analyzed with non-stressed sorghum plants both at the leaf and root level.

Results

Comparative leaf metabolite profiling of stressed versus control plants

Sorghum plants were subjected to drought induced by PEG, at two different levels of stress (2.5 and 5 % PEG), while non-stressed plants of the same age were included as controls. GC-MS analysis in sorghum leaf extracts yielded on average 132 quantifiable compounds that were highly reproducible among the four biological replications analyzed per category. Such compounds belong to different classes, including amino acids, organic acids, sugars, polyols, phosphates, polyamines, nitrogenous compounds, etc. Based on ANOVA, the level of 68 metabolites was significantly altered in response to stress ($P < 0.05$, Supplementary Table 1a-i). More importantly, certain compounds were detected only in leaves of stressed plants while, other compounds were exclusively found in controls. In the group of amino acids, most compounds were significantly increased in the leaves of drought-stressed plants. Substantially increased levels were found for b-alanine, aspartic acid, DL-isoleucine, glycine, L-alanine, L-asparagine, L-glutamic acid, L-homoserine, L-serine L-threonine, L-tryptophan, L-valine and tyrosine. In contrast, L-ornithine was found only in leaves of stressed plants (Supplementary Table 1a). As far as organic acids are concerned, the content of caffeic acid, citric acid, 4-hydroxycinnamic acid, DL-4-hydroxymandelic acid, D-malic acid, fumaric acid, gluconic acid, mucic acid, pipecolic acid, saccharic acid, succinic acid and trans-aconitic acid was significantly increased in stressed plants whereas, pyruvic acid decreased. Furthermore, dehydroascorbic acid and a ketoglutaric acid were only detected in leaves of control plants (Supplementary Table 1b). In addition, the level of fatty acids, including 4-guanidinobutyric acid and 2-hydroxybutyric acid, was found substantially increased in leaves of stressed plants (Supplementary Table 1c). Most sugars increased substantially under water deficit conditions. More specifically, the levels of D-glucose, D-lyxose, D-(+) trehalose, fructose, raffinose, sedoheptulose anhydride monohydrate and sucrose were found significantly higher in response to stress whereas in contrast, the level of talose significantly decreased (Supplementary Table 1d). Significantly increased concentrations of metabolites belonging to the groups of polyols (D-sorbitol, D-threitol, myo-inositol) (Supplementary Table 1e) and phosphates (phosphoric acid, o-phosphocolamine) (Supplementary Table 1f) were also found in plants subjected to stress. In the group of polyamines, the level of 1,3-diaminopropane increased whereas, the concentration of putrescine declined upon stress (Supplementary Table 1g). For N-compounds, most significantly increased levels were observed for adenine, adenosine, b-cyano-L-alanine, urethane, D-panthenol, gly-pro, 4-hydroxybenzyl cyanide, N-methylglutamic acid, 1-methyl nicotinamide, L-norleucine, N,N-dimethylglycine and pantothenic acid while, hypoxanthine was only detected in control plants (Supplementary Table 1h). Among other compounds, most profound changes were observed in concentration of galacturonic acid and isopropyl b-D-1-thiogalactopyranoside, both being significantly increased under water deficit stress. More importantly, 2-keto-L-gulonic acid was produced exclusively in response to stress while, acetol was found only in control plants (Supplementary Table 1i).

Comparative root metabolite profiling of stressed versus control plants

Sorghum root extracts yielded on average 111 quantifiable compounds which were highly reproducible in samples within each category of stressed and non-stressed plants. Although the levels of many metabolites were not significantly altered in response to stress, ANOVA detected 41 metabolites that differed significantly ($P < 0.05$, Supplementary Table 1a-i). Moreover, certain compounds were differentially produced and found either in stressed or non-stressed plants. The concentration of certain amino acids was substantially altered upon drought stress. More specifically, aspartic acid, b-alanine, glycine, L-alanine, L-glutamic acid, L-lysine, L-ornithine, L-serine and L-threonine were found significantly increased in stressed roots. To the contrary, L-methionine decreased upon stress while, L-cysteine was only detected in roots of control plants. Interestingly, certain amino acids such as b-alanine, L-alanine, L-lysine, L-ornithine, L-serine and L-threonine presented significantly higher concentration upon stress induced at 2.5 % than 5 % PEG (Supplementary Table 1a). Regarding organic acids, 4-hydroxycinnamic acid, gluconic acid, glycolic acid and succinic acid significantly increased in stressed plants whereas, D-malic acid decreased. In addition, the levels of citric acid and saccharic acid were substantially increased in roots of plants that were stressed at 2.5 % PEG, yet presented a significant decrease in plants stressed by PEG at 5 % (Supplementary Table 1b). A trend of increased levels upon drought stress was further recorded for fatty acids such as capric acid and 4-guanidinobutyric acid (Supplementary Table 1c). As far as sugars are concerned, significantly altered concentrations were observed for D-lyxose, D-(+)trehalose and sophorose, with the former two being significantly increased upon stress and the latter being exclusively found in roots of control plants (Supplementary Table 1d). Significantly increased levels were further observed for compounds belonging to the group of polyols (D-mannitol, D-sorbitol) (Supplementary Table 1e) as well as phosphates (phosphoric acid) (Supplementary Table 1f). In contrast, the concentration of polyamines, including 1,3-diaminopropane and putrescine, significantly declined upon drought stress (Supplementary Table 1g). As far as N-compounds are concerned, adenine, N-acetyl-D-glucosamine and uracil were found significantly increased in roots of stressed plants whereas, acetyl-L-serine, 5-aminovaleric acid and pantothenic acid decreased upon stress. In addition, accumulation of certain N-compounds, such as gly-pro, N-acetyl-D-mannosamine and L-mimosine increased upon low level of stress and significantly decreased upon high level of stress (Supplementary Table 1h). Further, the levels of arbutin and galacturonic acid were significantly reduced upon drought stress whereas, ribonic acid-g-lactone was found only in control plants (Supplementary Table 1i).

PCA-based clustering of metabolites and treatments

PCA analysis, which allows clustering of samples into groups, revealed a better differentiation in metabolite content of control and stressed plants in roots than in leaves. For leaves, the first PCA component accounted for 100 % of the

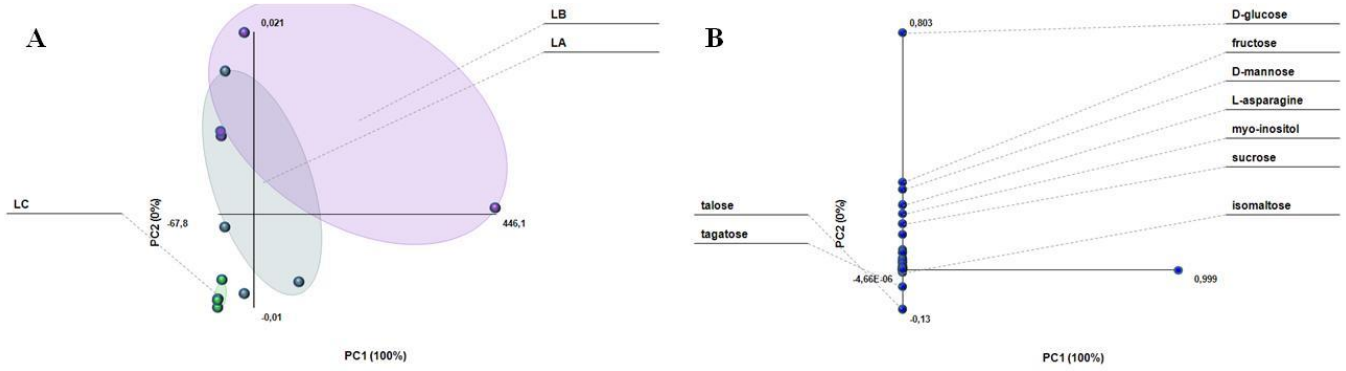

Fig 1. Principal component analysis (PCA) of GC-MS metabolite fingerprints from leaf samples harvested from *S. bicolor* control and stressed plants (*n*=4). (A) Bi-plot of principal components 1 and 2 representing 100 % of the total variance observed in the GC-MS profiles of sorghum leaves. Spots indicate individual plants of the respective treatment (LA: 2.5 % PEG, LB: 5 % PEG, LC: control). Grouping of samples within treatment categories is color-coded (a: blue, b: purple, c: green). (B) Loadings of individual identified components showing the highest variability under the studied treatments for sorghum leaves.

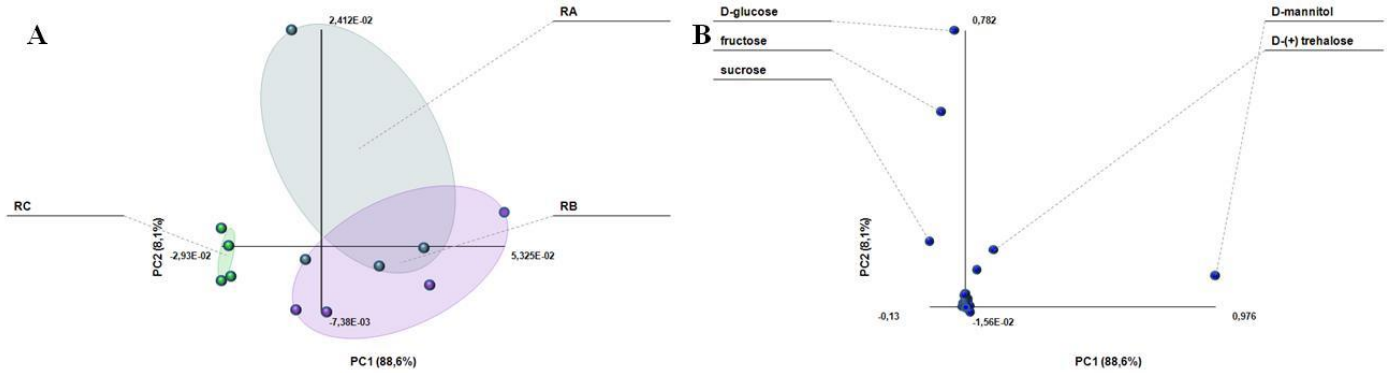

Fig 2. Principal component analysis (PCA) of GC-MS metabolite fingerprints from root samples harvested from *S. bicolor* control and stressed plants (*n*=4). (A) Bi-plot of principal components 1 and 2 representing 96.7 % of the total variance observed in the GC-MS profiles of sorghum roots. Spots indicate individual plants of the respective treatment (RA: 2.5 % PEG, RB: 5 % PEG, RC: control). Grouping of samples within treatment categories is color-coded (a: blue, b: purple, c: green). (B) Loadings of individual indentified components showing the highest variability under the studied treatments for sorghum roots.

variance in metabolite levels. In general, leaf samples originating from control plants were not separated from those originating from stressed plants that were subjected to drought induced by PEG at the level of 2.5 % or 5 %. According to the first component, samples subjected to stress induced by 2.5 % PEG exhibited similarity to control samples. In contrast, the second component allowed a more clear distinction between samples from control and 2.5 % PEG-stressed plants (Fig. 1A). PCA was further conducted to reveal the most influencing compounds among the different treatments. The first PCA component was mostly influenced by sugars, such as D-glucose, fructose, D-mannose, sucrose and isomaltose, but also myo-inositol and L-asparagine. The second component pointed as most influencing the compounds talose and tagatose (Fig. 1B). Such findings provide evidence that such sugars are differentially produced under normal and stress conditions. For roots, the first PCA component, which accounted for 88.6 % of the variance in metabolite levels, separated the root samples of control plants from those originating from stressed plants subjected to either 2.5 % or 5 % PEG. The second component however, which accounted for 8.1 % of the variance, did not allow the distinction between samples in any case (Fig. 2A). PCA analysis, performed in order to determine the most important compounds among the different treatments, pointed sugars and polyols as the compound groups that are mostly influenced by drought. According to the first component, most influencing compounds were D-mannitol and D-(+)trehalose, while the second PCA component was mostly influenced by D-glucose, fructose and sucrose (Fig. 2B).

Discussion

Understanding the complex networks underlying the crops' drought tolerance as well as all subsequent breeding efforts aiming at its improvement have been hampered by the polygenic nature of the trait and the wide environmental variation associated with it. To date, there are no or a few markers for an unambiguous screening of drought tolerance

versus susceptibility. In this context, the search for functional markers to robustly identify drought tolerant genetic material still constitutes an area necessitating further in depth research both at the basic and the applied level. These markers may be searched for in the metabolic adaptations involved under water deficit stress. Given that plants' response to abiotic stresses are dealt with a different perspective at the post-genomic era, metabolic profiling provides an amenable approach to unravel the regulatory signals of related transcriptional, translational and post-translational processes (Fiehn et al., 2000; Fiehn, 2002). In this study, we pursued the elucidation of the mechanistic base of drought tolerance through a metabolite profiling approach in sorghum, a crop species known for its ability to maintain satisfactory growth and production rates under water deficiency. To this purpose, young sorghum plants were subjected to drought stress induced by polyethylene glycol at two levels: 2.5 and 5 % PEG. Metabolite profiling was conducted through metabolite extraction performed on leaves and roots of stressed and control plants followed by GC-MS. In total, the analysis yielded 143 quantifiable compounds with highly reproducible patterns. The analysis revealed that drought stress substantially alters the metabolite content in both leaves and roots of sorghum plants. In general, metabolite changes were analogous to the level of stress induced. In several cases however, the low level of stress resulted in a more profound alteration in comparison to the high level of stress. Such pattern in response to drought stress applies mostly for metabolite content of roots and for certain amino acids, including b-alanine, L-alanine, L-lysine, L-serine and L-threonine but also for other compounds, such as citric acid and gly-pro. Substantial changes in response to stress were found in the levels of metabolites belonging to the group of sugars in both leaf and root samples. The majority of sugars significantly increased upon stress at both concentrations of PEG, with the most profound increase being found in the level of D-glucose and fructose for leaves and D-lyxose and D-(+)trehalose for roots. In particular, accumulation of D-(+)trehalose substantiates earlier findings pointing to its association with drought stress in bacteria, fungi and plants which tolerate extreme drought conditions. Such alterations are further supported by the decreased levels of D-glucose-6-phosphate which mediates trehalose synthesis (Iturriaga et al., 2000; Rontein et al., 2002). Exception to this general increasing trend of sugars was recorded for talose, whose levels were significantly reduced in leaves of stressed plants, and sophorose which was exclusively produced in the roots of control plants. Altogether, these findings are consistent with the well established role of sugars in osmotic adjustment as well as in adaptation to various abiotic stresses (Pilon-Smits et al., 1995; Holmström et al., 1996; Bohnert and Sheveleva, 1998). At the same time, sugars affect the ABA-dependent pathway, whose role is pivotal in plants' response to drought (Zhang et al., 2006), but also the synthesis of other compounds, the production of energy, the regulation of gene expression as well as the transduction of signals (Sheen et al., 1999; Smeekens, 2000; Finkelstein and Gibson, 2001; Hoekstra et al., 2001). It is worth noting however, that in contrast to other compounds, functions of essential accumulators are generally difficult to assess. In this context, sugars and amino acids, apart from their role in regulation of osmosis, also form part of basic metabolism with which their increased levels might interfere (Bohnert and Sheveleva, 1998). Sugar accumulation was further accompanied by increased concentrations in a number of organic acids such as citric acid, glycolic acid, gluconic acid and succinic acid in both leaves and roots of stressed plants. Interestingly, D-malic acid was differentially accumulated in leaves and roots of stressed plants, manifesting an increase in the former and a decrease in the latter tissue type, whereas dehydroascorbic acid was exclusively produced in leaves of control plants and was undetected in roots of all plants examined. The levels of amino acids, especially in leaves of stressed plants, follow a similar pattern of increasing accumulation, further substantiating their previously assigned role as osmoregulatory elements (Rontein et al., 2002). Changes in the content of amino acids and proteins due to water deficiency have been in the past reported in several studies. Drought stress has a profound effect on plant metabolism and, in many cases, results in reduced rate of protein synthesis. In this line, the elevated levels of most amino acids may be attributed to the reduction of protein synthesis and/or to the degradation of existing proteins. The decreased levels of L-methionine as well as the total depletion of L-cysteine in roots of stressed plants however, provides evidence that such amino acids may be assigned different roles in plant's response to drought. Although such role is not elucidated and merits further research, these findings provide evidence that these two amino acids may serve as markers for screening plants' drought tolerance. Interestingly, sorghum's response to drought was not associated with accumulation of proline. Given the fact that the latter is one of the osmolytes most frequently synthesized in plants under osmotic stress (Ashraf and Harris, 2004; Munns and Tester, 2008), its absence in sorghum exposed to drought is certainly of great interest. Accordingly, the depletion of putrescine in both leaves and roots of stressed plants is worth noting, given that polyamines are generally viewed as protective agents under various stress conditions (Chattopadhyay and Ghosh, 1998; Bouchereau et al., 1999). Furthermore, significantly increased concentrations were observed in the group of polyols. Most profound changes were found in the levels of D-sorbitol, myo-inositol and D-threitol as well as D-sorbitol and D-mannitol in leaves and roots, respectively. These findings are consistent with the increased levels of sugars since polyols may be formed under reducing conditions by their analogue sugars. Such polyol accumulating patterns in stressed plants substantiate previous findings assigning to these compounds a role in osmoregulation and maintenance of metabolic processes under stress conditions induced by water deficiency and/or other abiotic stimuli (Guo and Oosterhuis, 1995; Patonnier et al., 1999; Rizhsky et al., 2004). In this respect, inositol metabolism has been associated with osmoregulation upon salt stress in several plant species (Majee et al., 2004; Shiozaki et al., 2005) and is considered as a stress-regulated pathway that becomes the major route for carbon metabolism during the initial phase of stress adaptation period (Bohnert and Sheveleva, 1998). More importantly, the differential behavior of certain metabolic compounds points to their possible role in plants' cascade of responses under drought stress. Such differential patterns apply for 3,4-dihydroxymandelic acid, 2-hydroxybutyric acid and 2-keto-L-gulonic acid whose accumulation is exclusively induced upon stress as well as for dehydroascorbic acid, a ketoglutaric acid, acetol and hypoxanthine which are depleted in response to stress. It is therefore, tempting to hypothesize that the abovementioned compounds are of great value in the search for candidate functional markers to be employed for the purposes of screening and identifying drought tolerant genetic material. In addition, adjusting the crop's ability to accumulate such influencing compounds in response to limited water availability may lead to more drought-tolerant genotypes to meet current and future challenges.

Materials and Methods

Plant materials

Drought stress experiments were carried out using the well established sweet sorghum variety Keller, which is routinely used in the US for table syrup production. Variety Keller is a US line selection deriving from exotic material.

Drought stress treatments

In order to proceed with the drought stress assay in sorghum, a hydroponic system supplemented with aquarium pumps, so as to ensure constant aeration in roots, was established. The osmotic potential of the solution was decreased by adding polyethylene glycol (PEG) 6000. Germinated sorghum seeds were allowed to grow in plastic trays containing three different solutions: i) sterile distilled H_2O, ii) 2.5 % PEG and iii) 5 % PEG. Plants grown in distilled H_2O were included as controls. Trays were regularly monitored for the level of containing solution and, when necessary, H_2O was added in order to retain PEG concentration at constant levels. All plants were grown under identical conditions and harvested at the same time. The experimental layout was a completely random design with four replications. Each experimental plot (plastic tray) consisted of four rows and columns, of which the two middle were used to provide material for the analyses. Sampling was performed on sorghum plants subjected for 7 days at water deficit conditions. Plants were initially dried in paper towels and subsequently, leaf and root samples were separately collected and immediately frozen in liquid nitrogen and stored at –80 ^{o}C. Each plant was individually analyzed.

Metabolite extraction, derivatization and GC-MS analysis

Fresh tissue samples were ground in liquid nitrogen. Fifty mg of frozen tissue powder was extracted in 395 µl methanol and 5 µl ribitol (1 mg ml^{-1} in water), added as an internal standard. Extracts were incubated at 70 ^{o}C for 15 min with continuous shaking. Following the addition of 200 µl chloroform, samples were incubated at 37 ^{o}C for 5 min under continuous shaking. Afterwards, 400 µl of ddH_2O were added to the extracts and samples were then vortexed and centrifuged at 13,000 g for 5 min. The aqueous phase (100 µl) containing the polar metabolite fraction was transferred into new eppendorf tubes and dried using nitrogen. The dry residues were resuspended and derivatized for 90 min at 30 ^{o}C in 25 µl methoxyamine-HCl (MOX) (20 mg ml^{-1} in pyridine) with continuous gentle agitation. Afterwards, the samples were trimethylsilylated for 30 min at 37 ^{o}C by adding 75 µl of N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA). Ten µl of a retention time standard n-alkane mix were added to samples. Gas-chromatography coupled to Mass-spectrometry (GC-MS) measurement were performed in a HP6890 GC (Agilient Technologies 7890A GC System) coupled to a HP 5973 MS (Agilent Technologies 5975C Inert XL E1/C1). Four biological replications were conducted for each treatment category (control, stressed: 2.5 and 5 % PEG) and tissue type (leaves and roots). The chromatograms were automatically evaluated using the AMDIS software and metabolites were identified using the Golm metabolome database (Kopka et al., 2005, Schauer et al., 2005). The compound levels were calculated as the relative response ratio of peak areas of the target metabolite related to the peak area of the reference metabolite (ribitol, m/z 319) and normalized with respect to the sample fresh weight.

Statistical data analysis

Results are presented as average response, resulting from 4 biological replications, for all treatment categories under study: control, 2.5 % and 5 % PEG. Metabolomic data statistical analysis was performed by one-way analysis of variance (ANOVA) at a 95 % level of significance. Principal Component Analysis (PCA) was conducted using the Multibase Excel Add-in for PCA. All statistical analyses were performed using SPSS statistical software version 18 (SPSS, 2009). Multiple comparisons were performed by Tukey's test.

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