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Title: Hormonal responses to water deficit in cambial tissues of *Populus alba* L.

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Abstract. Changes of the concentration of bioactive gibberellins and abscisic acid in the cambial region of white poplar (*Populus alba* L.) were investigated in one-year-old plants, to highlight how these phytohormone signals are modulated in response to water deficit. Plants were cultivated in pots outdoor and, at the time of maximum cambial growth (T_0), irrigation was withdrawn for 8 d, inducing a mild water deficit, thus mimicking a condition that is recurrent in mediterranean climates when white poplar attains its maximum growth rate. The water deficit was suspended by resuming irrigation (T_{max}), throughout a recovery period of two weeks (T_{rec}). Cambial tissues were sampled at T_0, T_{max} and T_{rec}. Significant changes of leaf and stem relative water content, leaf water potential, stomatal conductance, transpiration, carbon assimilation, stem shrinkage and leaf number were induced by soil water shortage, which also negatively affected cambium development. Nevertheless, these responses were almost fully reversed following the resumption of irrigation. Water deficit induced the accumulation of large amounts of abscisic acid in cambial tissues, but the hormone was brought back to pre-stress levels after the recovery period. With regard to bioactive gibberellins, GA_1 was several fold more abundant than GA_4 and reached the greatest level in the plants recovering from the water status imbalance. The possible functions of gibberellins and abscisic acid in the response of cambial tissues to water deficit are discussed in view of the known physiological roles and molecular mechanisms of action of these hormonal signals.

Keywords Abscisic acid; cambium; drought; gibberellin; poplar

Introduction

Poplar (*Populus* spp.) clones and hybrids are increasingly established in fast-growing plantations worldwide to meet the expanding demand of wood from industry (Gordon 2001); moreover, they enhance soil conservation and carbon sequestration (Smith and others 2007). A survey of some of the numerous poplar hybrids obtained so far shows a marked variability in their phenology, productivity and growth rate, and a varying ability to cope with water deficit (Giovannelli and others 2007; Marron and others 2005; Monclus and others 2009; Yin and others 2004). Poplars are sensitive to drought, due

to their high transpirational demand (Marron and others 2003), therefore water availability markedly influences the natural distribution area of this genus, which thrives mostly in riparian environments (Rood and others 2003). Selection of new genotypes for fast-growing plantations must therefore take into account, besides a high productivity, the ability to withstand water deficit, because an increasing occurrence of dry periods at a global scale is expected (IPCC 2007). This requires much effort aimed at understanding the biochemical processes involved in tree secondary growth, as this is the most drought-sensitive process (Hsiao 1973). The response of radial growth to water shortage is faster and stronger than that of stem elongation (Breda and Granier 1996). The diameter increase of the trunk (hence, wood production) is due to cell divisions in a secondary meristem, i.e. the vascular cambium, and to the expansion of the newly formed cells, that differentiate into phloem (outside) and xylem (wood; inside). The cambial region includes the cambium, which is composed of meristematic cells called initials, and the phloem and xylem mother cells, both of which originate from the dividing cambial initials (Plomion and others 2001). The developmental processes of these cells may be perturbed by a water deficit, either directly or indirectly: in the former case, a loss of turgor pressure may be responsible for the decrease of cell expansion (Abe and others 2003), while in the latter it is the restraint to photosynthesis and translocation of assimilates that may lessen stem growth. The induction and the development of secondary xylem are controlled by several hormonal signals, which shape wood quality and biomass production (Aloni 2007). Cambial growth is enhanced by gibberellins (GAs), that may act synergistically with auxin. Gibberellins increase the length of xylem fibers (Eriksson and others 2000), therefore it has been suggested that their main role is to regulate early stages of xylem differentiation, including cell elongation (Israelsson and others 2005). Poplar species and hybrids may differ in their GA metabolism: the diverse activity of the early 13-hydroxylation and of the non-early 13-hydroxylation pathways determines the amount of the various GAs that are synthesized in each genotype (Pearce and others 2002). Moreover, there is evidence that this class of growth regulators may influence water use efficiency under drought (Elias and others 2012). Therefore, GAs might operate in mediating the response to water deficit, besides the primary signal abscisic acid (ABA), whose biosynthesis and accumulation has been showed increasing under abiotic stress (Zhu 2002). The response of poplar to drought has been investigated in depth in the last years.

For instance, Bogeat-Triboulot and others (2007) report that *Populus euphratica* undergoing soil water depletion shows a decline of stem diameter. However, most of the experimental work deals with the molecular mechanisms operating in leaves and roots, while the biochemical signals involved in stem growth deserve further consideration. The present work was carried out on European white poplar (*Populus alba* L.), a species widespread through the river valleys of Europe and other continents. A previous investigation performed on an analogous experimental system (Berta and others 2010) demonstrated that the expression of genes encoding a GA-regulated protein and an ABA-induced protein was altered under mild water deficit. To foster our knowledge on these biochemical signals, we analyzed changes of GAs and ABA levels in the cambial tissues induced by soil water depletion and subsequent re-hydration. We hypothesized that the concentration of the hormones in the cambial tissues might be linked to the plant water status and to the growth rate, with ABA prevailing under drought conditions and GAs upon rehydration. To our knowledge, this represents the first attempt to shed light on these relevant features, i.e. the biochemical signaling underlying the response of cambium to water deficit.

Materials and methods

Plant material

In early May 2011, 40 one-year-old *P. alba* plants, clone 'Marte', were selected for dimensional uniformity: mean height 1966 ± 294 mm, mean stem diameter 19.8 ± 3.1 mm, average leaf number per plant 51.2 ± 10. This clone was selected because its capacity to respond to water stress was yet to be ascertained (Berta and others 2010). Plants were grown in 27 l plastic pots filled with a mixture of peat-sand-perlite (50/40/10 v/v, pH 6.8), fertilized with a commercial slow release fertilizer (18:18:18, N-P-K), and irrigated every second or third day to maintain soil moisture to field capacity (soil water content = 30%). The pots were spaced 0.70 x 1.00 m. A row of one-year-old *Populus x canadensis* 'I-214' was placed around the perimeter of the experimental area. During the experiment, plants were maintained in a nursery in the open air. On July 12 (T_0, or day 0), the plants were divided into two

homogeneous groups of 20 plants each and subjected to different watering regimes for 8 d. In watered plants, soil moisture was maintained to field capacity, whereas in stressed plants, watering was suspended for 8 d (T_{max}, or day 8) and then resumed up to day 21 (T_{rec}). To avoid soil rehydration by rainfall, the pots of stressed plants were covered with water repellent plastic covers as previously reported elsewhere (Pallara and others, 2012). During the experiment the mean temperature ranged between 19.3 and 30.3 °C (meteorological data recorded at LAMMA, Laboratory of monitoring and environmental modeling for sustainable development, Florence, http://www.lamma.rete.toscana.it/eng/index.html - weather station located near the experimental site).

Soil-Plant Water Relations and Growth Measurements

Predawn and midday leaf water potential (Ψ_{pd} and Ψ_{md}, MPa) were measured each 2 d with a pressure chamber (PMS Instruments Co., Corvallis, OR, USA) on two to three fully expanded leaves per plant (leaf plastochrone index, LPI, between 5 and 7) collected from randomly selected shoots of three plants per treatment. Leaf gas exchange measurements were carried out on intact fully expanded leaves (LPI 6-7) at 13.00-14.00 h. Leaf stomatal conductance (g_s), transpiration rate (E) and net CO_2 assimilation rate (A_{max}) were measured with a portable open system (ADC-LCA3, Analytical Development, Hoddesdon, U.K.) operating at 5.7 ml s^{-1} flow rate, 33 ± 1 Pa, ambient CO_2 and a photosynthetic flux density > 1200 μmol m^{-2} s^{-1}. At the beginning of the experiment (T_0), at T_{max} and T_{rec}, four plants for each treatment were used to determine the leaf relative water content (leaf RWC) and the stem relative water content (stem RWC) following the procedure described in Berta and others (2009). Leaves were weighed immediately after collection to determine fresh weight (*Mf*), then placed in distilled water at 4 °C for 24 h before turgid weight (*Mt*) was reached. Dry weight was recorded after leaves were dried at 72 °C for 48 h. Stem pieces 50 mm long were immediately weighed within 15 min after harvest to determine the fresh mass (g). The fresh volume of stem samples (cm^3) was assessed by water displacement (Borghetti and others 1991). The dry mass (g) was measured after the samples were maintained at 72 °C for 96 h. Stem RWC was calculated following Domec and Gartner (2001):

$$\mathrm{RWC\,stem} = \frac{(Mf - Md)}{(Vf - Vs)} \times 100$$

where Mf and Md are the fresh and dry mass of the wood (g), respectively, and Vf and Vs are the volumes of fresh and solid material (cm^3), respectively. Vs was estimated by dividing Md by 1.53, assuming a density of 1.53 g cm^{-3} for dry cell wall material (Pallara and others 2012). Soil water content (SWC) was measured every day for each replicate in the watered and stressed plants by Hydro-sense probes (Campbell Scientific Inc., USA) based on time domain reflectometry (TDR). The mean SWC for each replicate was calculated from the measurements taken at two depths (150 and 300 mm). Stem radial growth was determined by point dendrometers as already reported elsewhere (Giovannelli and others 2007). Point dendrometers were placed on the stem at ¼ height from the collar. Raw data were recorded every 15 min and hourly averages were calculated. The extraction of maximum daily shrinkage (MDS) was performed by dividing the stem cycle into three distinct phases (Berta and others 2010) and identifying (1) the expansion phase, total period from the minimum to the following morning maximum; (2) the stem radius increment phase, part of the expansion phase from the time when the stem radius exceeds the morning maximum until the subsequent maximum; and (3) the contraction phase or MDS, period between morning maximum and afternoon minimum. For the determination of the leaf number per plant, positions were assigned to leaves, with the most apical leaf greater than 20 mm in length designated as Leaf 0. A consecutive number was given to each successive leaf from the apex to the base. Because the plants may shed their leaves in response to water stress, the number of leaves per plant was recorded and its changes were expressed as percentage increase with respect to the value observed prior to the onset of the experiment, viz. two weeks before T_0, on June 29.

Histological observations

Stem discs of 10–20 mm thickness were cut at 0.3 m from the collar of six plants (three irrigated and three water stressed) on January 2012, i.e. about six months after water deficit treatment. Stem discs were placed in ethanol (50 % in water) and stored at 5 °C. The discs, or their slices, were embedded in

paraffin and cross sections of 12 µm thickness were cut using a rotary microtome Leica RM 2245, dried at 50 °C for 1 h and cleaned off the residual paraffin with successive immersions in D-limonene and ethanol stained with safranin (0.04 %) and astra blue (0.15 %) with 2 % acetic acid in distilled water (Emiliani and others 2011). A Nikon Eclipse 800E light microscope was used for anatomical observations.

Cambial Region Collection

Stem discs 10-15 mm thickness and 50-100 mm length were cut at 400-500 mm from the collar (corresponding to ¼ of plant height) in early morning at T_0, T_{max} and T_{rec}. For the collection of the cambial region, the bark was immediately removed from the stem pieces with a scalpel and the cambial region was gently detached with a razor blade from the inner side of the bark and the outermost side of the sapwood. The cambial region (thin film) was weighed and immediately dipped into liquid nitrogen in order to avoid oxidation processes within the tissues.

Hormone analysis

Fresh cambial tissues (each about 0.5 g fresh weight) were homogenized with mortar and pestle on ice and extracted with 10 ml of 80 % methanol in water, supplemented with 0.6 g l^{-1} polyvinylpolypyrrolidone, 0.2 g l^{-1} butylated hydroxytoluene and 0.6 % (v/v) acetic acid. Suitable amounts (between 50 and 200 ng) of [$^2H_2$]GA_1, [$^2H_2$]GA_4 and [$^2H_6$]ABA (purity > 98 %; Olchemim, Czech Republic) were added as internal standards and the homogenates were stirred for 3 h at 4 °C. Samples were then filtered on filter paper and the extraction was repeated twice. The extracts were pooled, dried under reduced pressure at 32 °C, redissolved in 500 µl of the HPLC starting solvent and filtered with 0.45 µm GD/X syringe filters with PVDF membrane (Whatman, UK). HPLC was performed on a SpectraSystem instrument (Thermo, USA), equipped with a Hypersil ODS C18 column, 150x4.5 mm ID, 5 µm particle size, eluted as follows with solvents A (0.01 % acetic acid) and B (methanol): 0-5 min 10 % B, 5-20 min 10-30 % B, 20-21 min hold 30 % B, 21-41 min 30-100

% B, 41-51 min hold 100 % B. The detector wavelength was set at 240 nm: although GAs showed a relatively low absorbance, this wavelength allowed us to effectively dampen the background noise and to limit the absorption of light caused by methanol and acetic acid, that would have been unacceptably strong at lower wavelengths. Under these conditions, the standards of the three analytes showed the following retention times: GA_1 = 15.5 min; ABA = 22 min; GA_4 = 30.7 min. Consequently, for each sample three fractions were collected (GA_1, ABA and GA_4), that were thoroughly dried under a nitrogen stream. The fraction putatively containing ABA was methylated with ethereal diazomethane, while those of GAs were silylated with bis(trimethylsilyl)trifluoroacetamide containing 1 % trimethylchlorosilane (1 h at 70 °C). Gas chromatography-mass spectrometry (GC-MS) analysis was performed on a Saturn 2200 quadrupole ion trap mass spectrometer coupled to a CP-3800 gas chromatograph (Varian, USA). The GC column and analysis conditions were the same as in Sorce and others (2009). Full scan mass spectra were obtained in EI+ mode with an emission current of 30 mA, an axial modulation of 4 V and the electron multiplier set at -1500 V. The mass spectrometer was calibrated daily with heptacosafluorotributylamine. Data acquisition was from 150 to 300 Da (ABA) or from 250 to 600 Da (GAs) at a speed of 1.4 or 1.1 scan s^{-1}, respectively. Quantification of the derivatized analytes was based on the following ions: m\z 190 for ABA and 194 for D_6ABA; m/z 564 for GA_1 and 566 for D_2GA_1; m/z 461 for GA_4 and 463 for D_2GA_4. Data for each sample are the mean of three replicates ± SD (for each run, 1 µl of the sample was injected). Quantification was carried out by reference to a calibration plot obtained from the GC-MS analysis of a series of mixtures of each standard hormone (Olchemim, Czech Republic) with the corresponding labelled form. The results were reported as concentrations of the hormones per g of tissue fresh weight.

Statistical analyses

Differences in leaf water potential, leaf RWC, stomatal conductance, transpiration rate, CO_2 assimilation rate, stem RWC, leaf number increase, ABA and GAs concentrations per sampling date and water supply regime were compared using two-way analysis of variance (ANOVA) with a

significance cutoff of $P = 0.05$. Tukey's honestly significant difference (HSD) post hoc test was applied to detect differences among sampling dates and irrigation regimes.

Results

The experimental conditions of the present study mimicked the effects of a short period (8 d) of water depletion, a situation that is recurrent in mediterranean climates at the time when white poplar attains its maximum growth rate. The recovery from the imposed water deficit was evaluated two weeks after the resumption of irrigation and six months later we analyzed the effect of the treatment on the anatomy of stem wood. During the experiment, the SWC of the irrigated pots oscillated between 30 and 40 %, i.e. field capacity, while for the stressed plants it fell below 20 % at day 2, soon after the withdrawing of irrigation. From day 2 to T_{max} (day 8) the SWC in non irrigated plants was maintained at 12.5 % in mean (corresponding to 65 % of the soil water depletion maintained during 8 d). After resumption of the irrigation the SWC in stressed plants recovered to the values of the irrigated ones at day 21 (Online Resource 1). This treatment resulted in a mild stress, which did not cause any permanent damage to the plants and did not hamper their recovery. Indeed, the water deficit induced only a slight change of the xylem features: the xylem of the stressed plants (Fig. 1) underwent an altered pattern of development that was restricted to a thin ring made of few cell layers (nearly 4-5), while the bulk of the tissue formed during the rest of the growing season did not display any particular feature in comparison with the xylem of the irrigated plants.

Water relations, leaf gas exchange and CO_2 assimilation

Leaf Ψ_{pd} fell below -2 MPa at T_{max} in non irrigated plants (Fig. 2a), thus proving that the withdrawing of irrigation had induced an imbalance of plant water status. This time course was in agreement with that of leaf RWC, whose lowest value was attained at day 7 (Online Resource 2). Our *P. alba* clone exhibited an isohydric behavior, because the Ψ_{md} (Fig. 2b) in droughted plants was only slightly lower than in watered ones, owing to the reduction of stomatal conductance occurring during the day (Fig.

2c), as suggested by Brodribb and McAdam (2013). The Ψ_{pd} in both irrigation regimes, except for the stressed plants at T_{max}, showed similar values. At T_{rec} there were no significant differences between treatments, either for Ψ_{pd}, or for Ψ_{md}. These data suggest that the recovery of plant water balance was reached at most 15 d after the resumption of irrigation.

Water deficit strongly restricted stomatal conductance (Fig. 2c and Online Resource 3a) and leaf gas exchange varied accordingly: both transpiration and A_{max} (Online Resource 3b and 3c, respectively) reached the lowest values at T_{max}, but 2 weeks after the resumption of irrigation the treated plants had fully recovered their functionality.

Stem diameter MDS is a reliable index of stem water status: the higher its value, the greater the contraction. In the irrigated plants, MDS was almost constant (< 20 μm day^{-1}), while in the stressed plants it started to rise shortly after the withdrawing of irrigation, peaked at T_{max}, in coincidence with the lowest value of stem RWC (Online Resource 4b) and quickly recovered thereafter (Online Resource 4a).

Production of new leaves occurred throughout the experimental period in the irrigated plants, while the process slowed as a consequence of the water deficit (Online Resource 5). Indeed, in the non irrigated plants the number of leaves slightly decreased between T_{max} and T_{rec}: although such decrease was not statistically significant, it is in striking contrast with the parallel increase recorded in the irrigated ones.

Hormonal signals

The results of our analysis are expressed as concentration of each hormone per unit of fresh mass. We took into account that the fluctuations of water content (i.e. imbalance of stem water status) might affect the hormone concentration, therefore we calculated also the amount of GAs and of ABA per g of cambial tissue at full turgor (data not shown). Nevertheless, the differences were not substantial, consequently we expressed the concentration on a fresh weight basis. Owing to the limited amount of cambial tissues available, the determination of the dry weight was not possible.

In our poplar clone, we identified both GA_4 and GA_1 in the cambial tissues. Figure 3 and Online Resources 6 to 8 are examples of the results of the GC-MS analyses of selected samples, showing the chromatographic traces of the total ion current, from which the main ions of the trimethylsilylated analytes and of the deuterated internal standard were extracted. In the upper part of each figure it is reported the mass spectrum recorded at the retention time of the respective analyte, that had been previously assessed through GC-MS runs of the derivatized pure standards. The mass spectra display the fragmentation pattern of the coeluting TMS derivatives of the endogenous hormone and of the deuterated internal standard. Online Resources 6 and 7 show the analysis for the determination of GA_4 in samples from a droughted and an irrigated plant, respectively; likewise, Figure 3 and Online Resource 8 illustrate analytical runs for the determination of GA_1. There was a strong predominance of GA_1: in all samples the concentration of this molecule largely exceeded that of GA_4, which was barely detectable in most cases. Given this wide difference and considering the relatively low amounts of GA_4 that were detected, it may be hypothesized that this molecule could merely play a minor role, if any, in the response of cambial tissues to water deficit. The concentration of GA_1 rose considerably throughout the course of the experiment in both treatments (Fig. 4), probably because the trees were actively growing (plant height increased by an average of 6 % and 0.5 % in irrigated and non irrigated plants, respectively, from T_0 to T_{rec}; data not shown). Water deficit did not have a prompt effect on the hormone level, because at T_{max} the treatments did not differ significantly, but at T_{rec} the non irrigated plants showed a GA_1 concentration 36 % higher than the irrigated ones. It is worth noting that, in the non irrigated plants, the overcoming of the negative consequences of water status imbalance was accompanied by a considerable rise of GA_1 concentration, well beyond the small increase that was recorded in the irrigated ones.

Examples of GC-MS runs for the determination of ABA are shown in figure 5 and Online Resource 9: each reports the chromatographic traces of the total ion current, from which the main ions of methylated ABA and of the deuterated internal standard were extracted, as well as the mass spectra recorded at the retention time of the pure methylated standard, displaying the fragmentation pattern of the coeluting methyl derivatives of the endogenous hormone and of the labeled molecule. Figure 5 shows the analysis for the determination of ABA in samples from a droughted plant and Online

Resource 9 from an irrigated one, respectively. The concentration of ABA (Fig. 6) increased sharply from T_0 to T_{max}, attaining, in the cambial tissues of the non irrigated plants, an extremely high value (nearly 9 µg ABA per g of tissue fresh weight). Hence, water shortage elicited a strong hormonal response in the cambial region. Also in the irrigated plants the highest amount of ABA was recorded at T_{max}, although it was more than seven times lower than in the treated ones. Apparently, the irrigated plants did not undergo an appreciable imbalance of water status, as their leaf and stem water relations and leaf gas exchange seem to rule out this possibility. Nevertheless, it is tempting to speculate that the transient accumulation of ABA in the cambial tissues of the irrigated plants might be ascribed to the harsh environmental conditions. The experiment was performed in late July, when the high evaporative demand of the atmosphere could have triggered this hormonal signal in response to a mild water deficit, which could have transiently occurred during the hottest part of the days, despite irrigation effectively kept SWC close to field capacity: indeed, temperatures and VPD reached their highest values between day 0 and day 8 (Online Resource 10). In both groups, the concentration of ABA declined after T_{max}, attaining, at T_{rec}, values similar to that of T_0, again suggesting that the non irrigated plants successfully recovered from the water status imbalance.

Discussion

The response of the cambial tissues to water deficit in *P. alba* involves modulation of the concentrations of ABA and GAs. The dramatic rise of ABA in the non irrigated plants is accompanied by many physiological changes in the whole organism. Although we do not know the origin of the hormone that accumulated in the stem, i.e. in the cambial region, it is well known that soil water depletion enhances its biosynthesis and export from the roots, thus increasing the concentration of the molecule in the xylem stream and the leaves (Zhang and others 1987). The hormone is conceivably the signal for the reduction of stomatal conductance, transpiration, CO_2 assimilation and leaf formation that we observed in our poplar under reduced soil water availability. Therefore, water deficit may restrain stem growth, i.e. cambial activity, at least partly, through a chain of ABA-mediated events that repress photosynthesis (Lindoo and Noodén 1978; Nobel 2009). The response of plants to water

deficit was not sufficient to prevent dehydration: at T_{max}, leaf RWC was lower than that of the irrigated plants and Ψ_{pd} had fallen below -2 MPa, a value that may entail severe vessel embolism (Awad and others 2010). At the same time, soil water depletion caused a substantial dehydration of the stem, as demonstrated by the decline of its water content and the rise of MDS. Therefore, discontinuing irrigation upsets the balance of plant water status and this in turn will exacerbate stem growth retardation, owing to the reduction of cell expansion that follows the loss of turgor pressure, as suggested by Abe and Nakai (1999). By comparing our results with those of Berta and others (2010), it is tempting to speculate that the expression of some aquaporin genes might have been downregulated by ABA, as the hormone may function to regulate the transcription of several of these genes (Kaldenhoff and others 2008) and its concentration followed a time course which agrees with its hypothesized function.

Despite all these effects, the intensity of the water deficit was not severe enough to permanently impair the development of the non irrigated plants, as shown by our anatomical observations. Moreover, from T_{max} to T_{rec} the analyzed parameters recovered relatively fast to the values of the irrigated plants, and the concomitant decline of ABA content may have contributed to foster these changes. The ability to quickly restore carbon fixation after rewatering suggests that the photosynthetic apparatus was not damaged, which is a relevant trait of drought resistance, and that CO_2 assimilation was limited mainly by stomatal closure (Liu and Dickmann 1993), hence primarily by the dynamics of ABA. Nevertheless, the evaluation of the potential productivity of 'Marte' clone should take into account also the changes of leaf area: after T_{max} the development of new leaves slowed in the non irrigated plants and this could negatively affect their primary production. Beyond the photosynthesis- and the putatively aquaporin-mediated mechanisms of action, there are further ways through which ABA may lower stem growth. As a stress signal, it likely reduces the concentration of auxin in the stem, or interferes with auxin signal transduction (Popko and others 2010). Since auxin regulates the density and size of xylem vessels (Lovisolo and others 2002), ABA may alter growth and anatomical traits of wood through cross-talking with the above mentioned hormone. Abscisic acid may lower cell expansion, because it inhibits the expression of several genes involved in the hydrolysis of various cell wall polymers (Gimeno-Gilles and others 2009). In our

poplar clone, the water shortage led to the formation of a layer of xylem cells that were remarkably smaller than the surrounding ones. Furthemore, the time course of ABA concentration agreed with the expression pattern of some of these genes, according to the data of Berta and others (2010): at T_{max}, the maximum ABA content coincided with downregulation of a pectin-esterase, one expansin and three fasciclin-like proteins, while the decline of the hormone during the recovery period is parallel to the upregulation of the aforesaid genes, plus a further expansin, a xyloglucan-1,4-beta-D-glucanase, four arabinogalactan-proteins and three further fasciclin-like proteins. These correlations may provide a partial explanation for the molecular mechanism of ABA-mediated response of cambial tissues to water deficit.

Besides a stress signal, we had planned to study also a promoter of cambial growth, i.e. GAs. These hormones operate in synergy with auxin, stimulating division and expansion of cambial zone cells, xylem differentiation and, more specifically, xylem fiber elongation (Björklund and others 2007). Apparently, in *P. alba* this role should be assigned to GA_1, whose concentration largely exceeded that of GA_4. This is in agreement with the data of Rood and others (2000), who investigated the role of GAs in the response to water table decline in *Populus trichocarpa* by analyzing, besides the bioactive hormones, also their biosynthesis precursors and the main catabolite. Although they found that GA metabolism was altered by soil drying, there was not any significant correlation between hormone changes and root growth rate. In poplar F1 hybrids, increased GA concentration in cambial tissues has been linked to hybrid vigor for radial growth, with the fast growing clones that showed a four-fold higher concentration of bioactive GA_1 than the slow ones (Bate and others 1988; Pearce and others 2004).

In our work, the greatest increase of GA_1 concentration was recorded during the recovery (from T_{max} to T_{rec}). Given the well documented promoting effect of GA_1 on wood growth, we may infer that the hormone substantially enhanced the recovery from water stress, by helping to restore the activity and development of cambial tissues. Many genes involved in cell expansion were upregulated in *P. alba* after the resumption of irrigation (Berta and others 2010). The concomitant, strong increase of GA_1 may have been a primary signal for enhancing the expression of these growth-related genes, as observed also in other species (Voegele and others 2011). Further signal transduction pathways might

have been activated by the rise of GA_1, as suggested by the upregulation of the gene encoding the GA-related Gip1-like protein.

Drought affects the development of cambial tissues, whose response to water shortage and ability to overcome the consequences of water stress will have a critical impact on poplar growth. Therefore, the role of GA_1 and ABA in modulating the aforesaid responses will be a major determinant of wood yield and quality. This highlights the importance of a deeper knowledge of the molecular mechanisms of these hormones and of the cross-talking with other growth regulators, in cambial tissues development.

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Figure legends

Fig. 1 Cross-sectional images of white poplar annual rings collected 6 months after the experiment; well irrigated (a, 4x) and water stressed plants (b, 4x and c, 10x). Arrows into photomicrographs a and c indicate the altered structure of the xylem cells (fibers and vessels) formed under water deficit. C, cambial zone; Vs, vessel; Pr, parenchymatic ray

Fig. 2 Pre-dawn (a) and midday (b) leaf water potential (MPa) and (c) stomatal conductance measured around midday (gs; mol m^{-2} s^{-1}) of irrigated (empty bars) and non irrigated (filled bars) plants throughout the experimental period. Mean (n = 20). Bars denote SD. Means that differ at the 0.05 level are noted with different letters or with an asterisk

Fig. 3 Chromatographic traces and mass spectrum at TMS-GA_1 retention time of a cambium sample from a water stressed poplar plant at T_{max}. (a), mass spectrum; (b), reconstructed total ion current; (c), ion trace, m/z 566 (deuterated TMS-GA_1); (d), ion trace, m/z 564 (endogenous TMS-GA_1)

Fig. 4 Concentration of GA_1 (ng g^{-1} of tissue fresh weight) in cambial tissues from irrigated (empty bars) and non irrigated (filled bars) plants throughout the experimental period. Mean (n = 20). Bars denote SD. Means that differ at the 0.05 level are noted with different letters

Fig. 5 Chromatographic traces and mass spectra at Me-ABA retention time of a cambium sample from a water stressed poplar plant at T_{max}. (a), mass spectrum of deuterated Me-ABA, with traces of endogenous Me-ABA; (b), mass spectrum of endogenous Me-ABA; (c) reconstructed total ion current; (d), ion trace, m/z 194 (deuterated Me-ABA); (e), ion trace, m/z 190 (endogenous Me-ABA)

Fig. 6 Concentration of ABA (ng g^{-1} of tissue fresh weight) in cambial tissues from irrigated (empty bars) and non irrigated (filled bars) plants throughout the experimental period. Mean (n = 20). Bars denote SD. Means that differ at the 0.05 level are noted with different letters

Legends of supplementary material

ESM 1 SWC (%) for irrigated (empty symbols) and non irrigated (filled symbols) plants throughout the experimental period. Mean (n = 20). Bars denote SD

ESM 2 Leaf RWC (%) of irrigated (empty bars) and non irrigated (filled bars) plants throughout the experimental period. Mean (n = 20). Bars denote SD. Means that differ at the 0.05 level are noted with an asterisk

ESM 3 (a), stomatal conductance (gs; mol m^{-2} s^{-1}), (b) transpiration rate (E; mol m^{-2} s^{-1}) and (c) maximum photosynthetic rate (A_{max}; µmol m^{-2} s^{-1}) of leaves from irrigated (empty bars) and non irrigated (filled bars) plants throughout the experimental period. Mean (n = 20). Bars denote SD. Means that differ at the 0.05 level are noted with different letters or with an asterisk

ESM 4 (a) MDS (µm) and (b) stem RWC (%) of irrigated (empty circles or bars) and non irrigated (cross symbols or filled bars) plants throughout the experimental period. Mean (n = 20). Bars denote SD. Means that differ at the 0.05 level are noted with an asterisk in (b)

ESM 5 Changes (%) of leaf number of irrigated (empty bars) and non irrigated (filled bars) plants during the experimental period. Measurements started on June 29, i.e. 13 d before T_0. Mean (n = 20). Bars denote SD. Means that differ at the 0.05 level are noted with different letters

ESM 6 Chromatographic traces and mass spectrum at TMS-GA_4 retention time of a cambium sample from a water stressed poplar plant at T_{max}. (a), mass spectrum; (b), reconstructed total ion current; (c), ion trace, m/z 463 (deuterated TMS-GA_4); (d), ion trace, m/z 461 (endogenous TMS-GA_4)

ESM 7 Chromatographic traces and mass spectrum at TMS-GA_4 retention time of a cambium sample from an irrigated poplar plant at T_{max}. (a), mass spectrum; (b), reconstructed total ion current; (c), ion trace, m/z 463 (deuterated TMS-GA_4); (d), ion trace, m/z 461 (endogenous TMS-GA_4)

ESM 8 Chromatographic traces and mass spectrum at TMS-GA_1 retention time of a cambium sample from an irrigated poplar plant at T_{max}. (a), mass spectrum; (b), reconstructed total ion current; (c), ion trace, m/z 566 (deuterated TMS-GA_1); (d), ion trace, m/z 564 (endogenous TMS-GA_1)

ESM 9 Chromatographic traces and mass spectra at Me-ABA retention time of a cambium sample from an irrigated poplar plant at T_{max}. (a), mass spectrum of deuterated Me-ABA, with traces of endogenous Me-ABA; (b), mass spectrum of endogenous Me-ABA; (c) reconstructed total ion current; (d), ion trace, m/z 194 (deuterated Me-ABA); (e), ion trace, m/z 190 (endogenous Me-ABA)

ESM 10 (a) temperature (line) and rainfall (bars); (b) VPD. Data were measured with a frequency of 1 hour throughout the experimental period. Downward arrows indicate T_{max} (day 8) and T_{rec} (day 21)

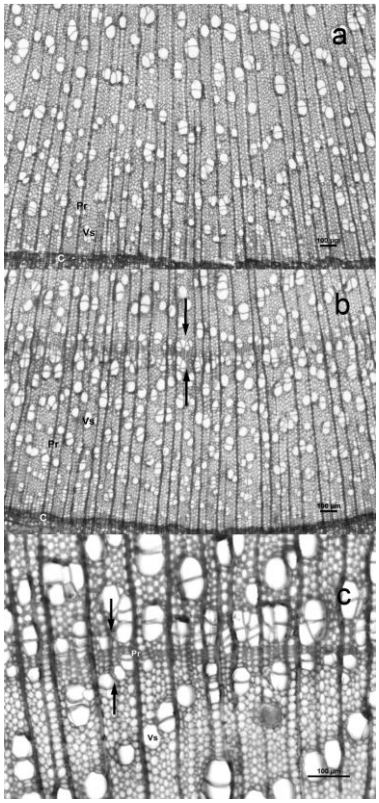

Fig. 1

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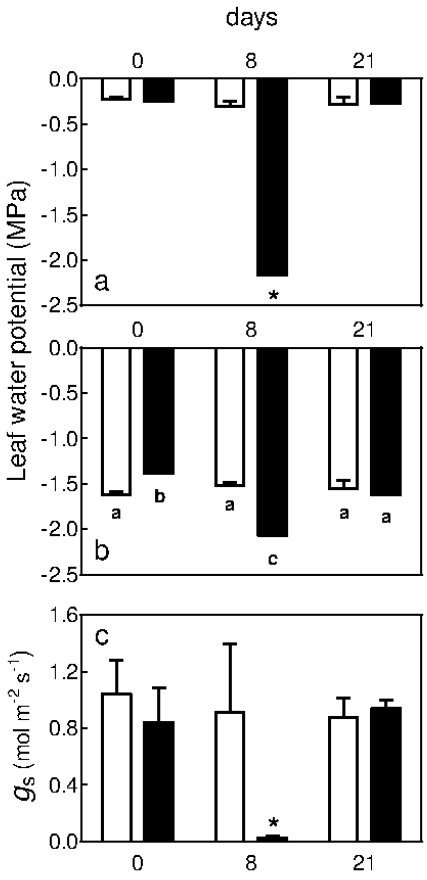
14 Fig. 2



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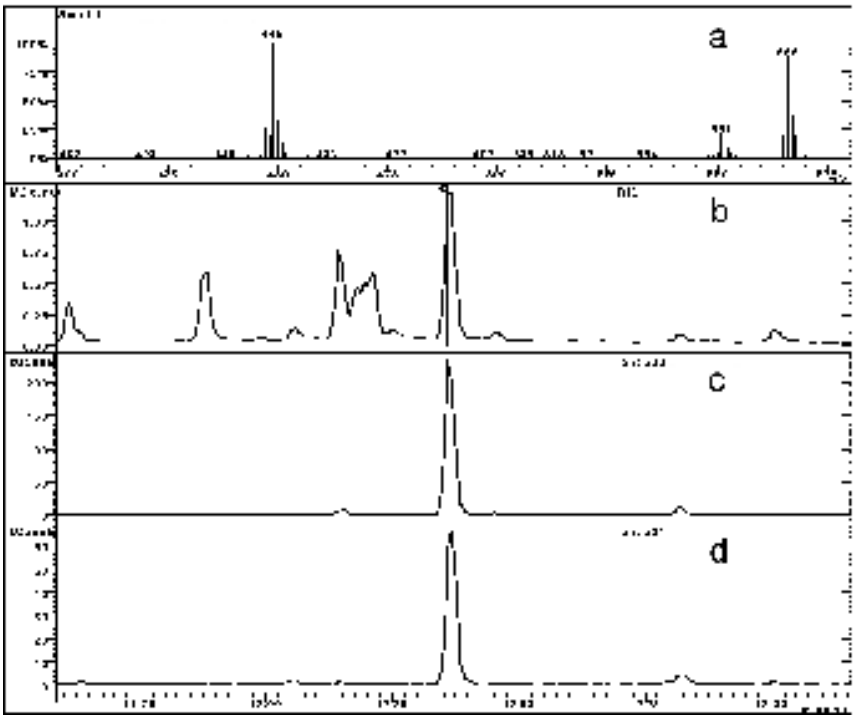

Fig. 3

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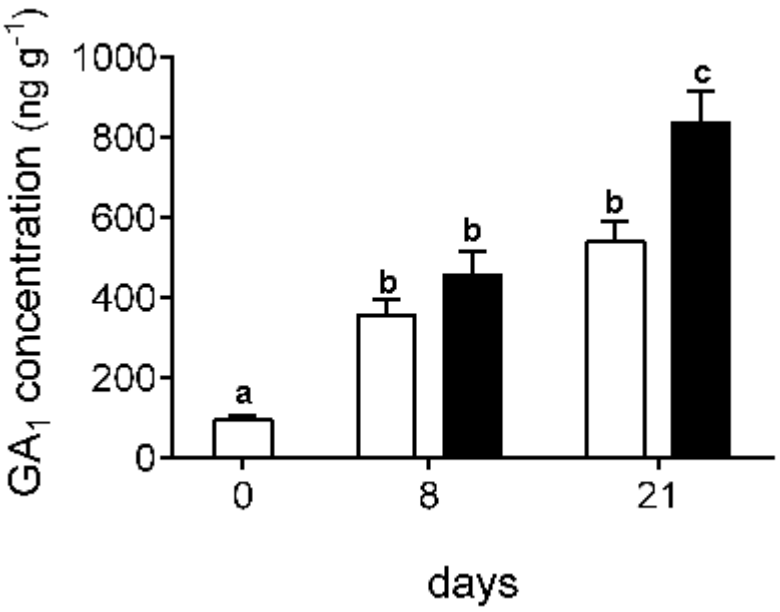

Fig. 4

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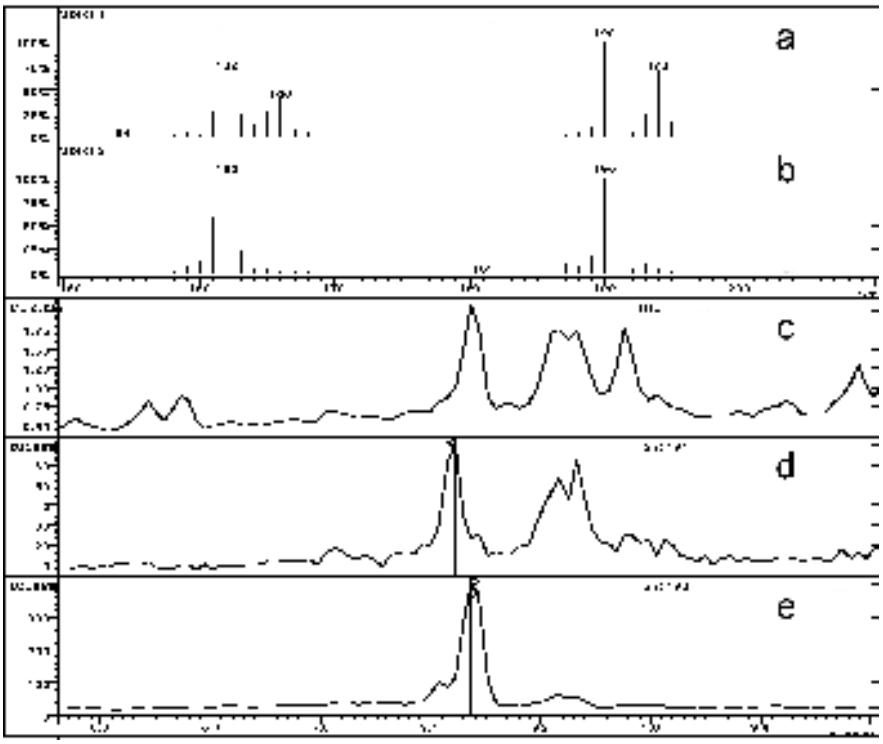

a
b
c
d
e

Fig. 5

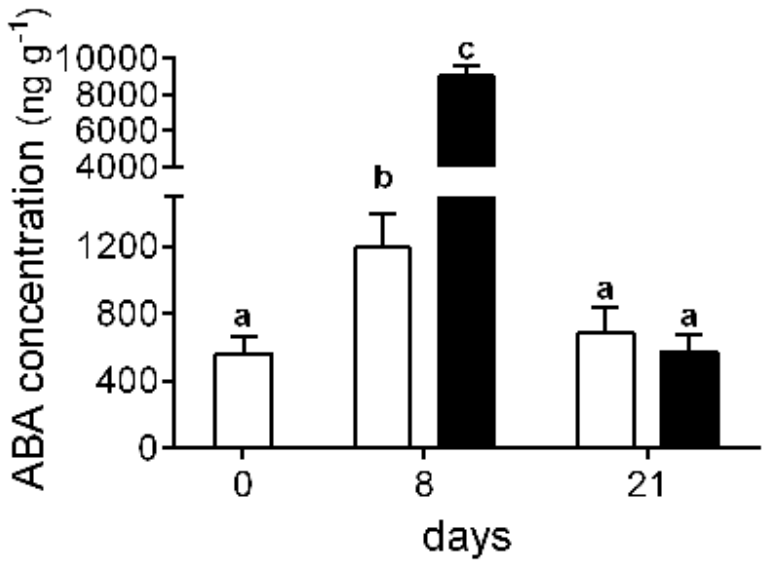
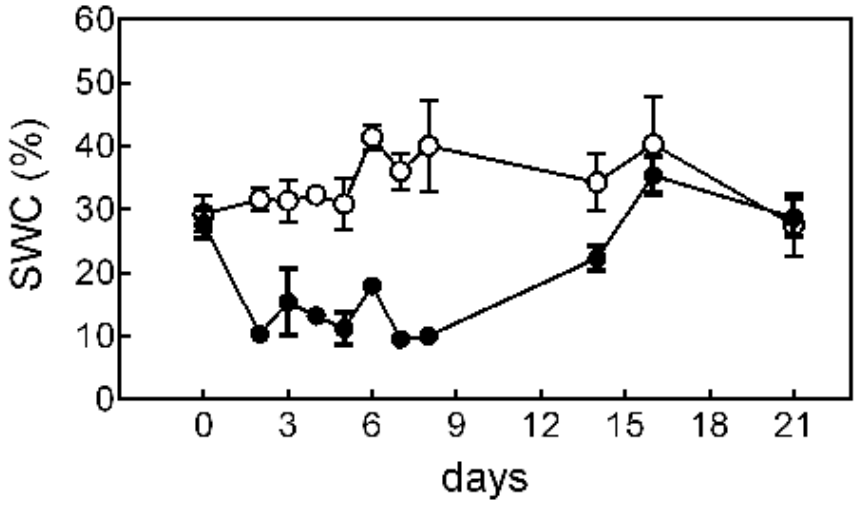
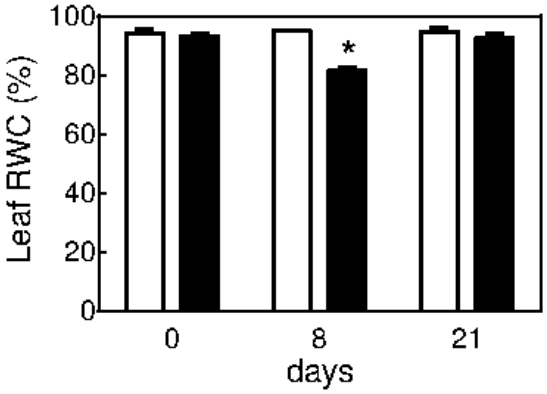

ABA concentration (ng g-1)
10000
8000
6000
4000
1200
800
400
0
a
b
c
a
a
0
8
21
days

Fig. 6

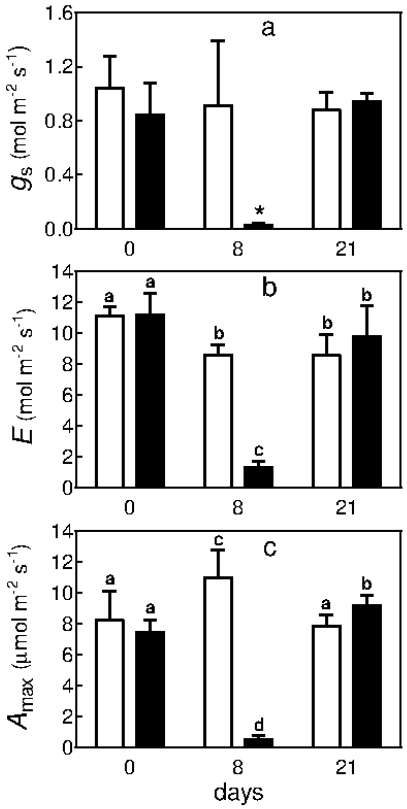
ESM 1


SWC (%)
60
50
40
30
20
10
0
0
3
6
9
12
15
18
21
days

ESM 2

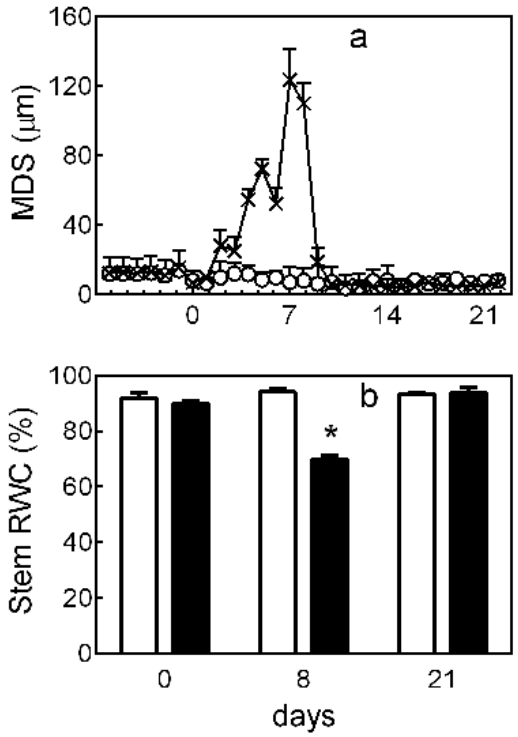

Leaf RWC (%)
100
80
60
40
20
0
*
0
8
21
days

ESM 3

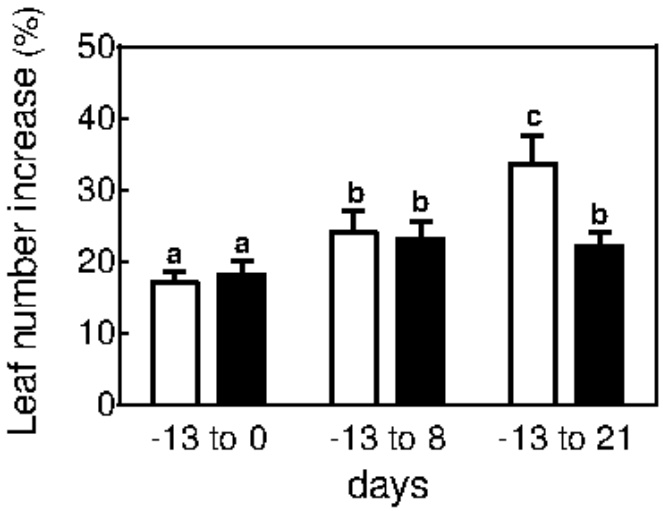

a
b
c
g_s (mol m^{-2} s^{-1})
E (mol m^{-2} s^{-1})
A_{max} (µmol m^{-2} s^{-1})
days

14

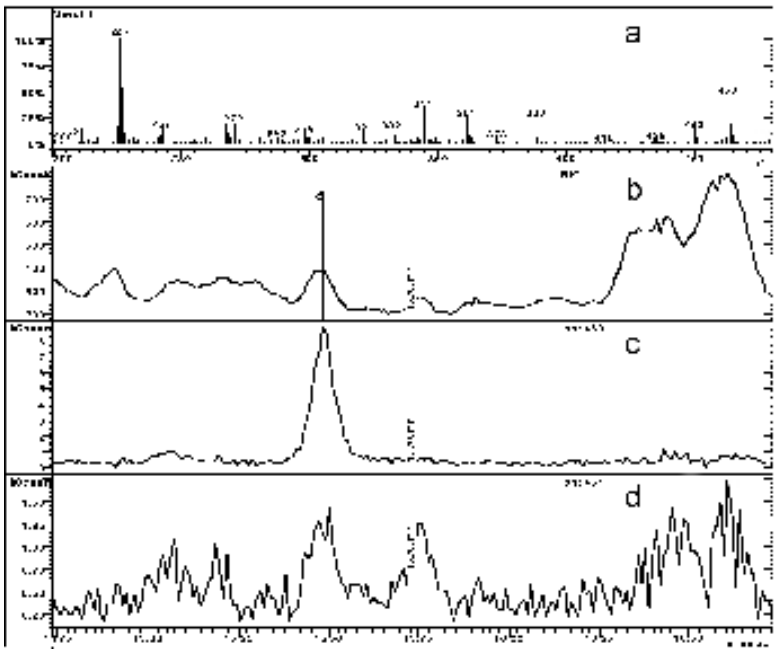
ESM 4


a
b
MDS (µm)
Stem RWC (%)
days

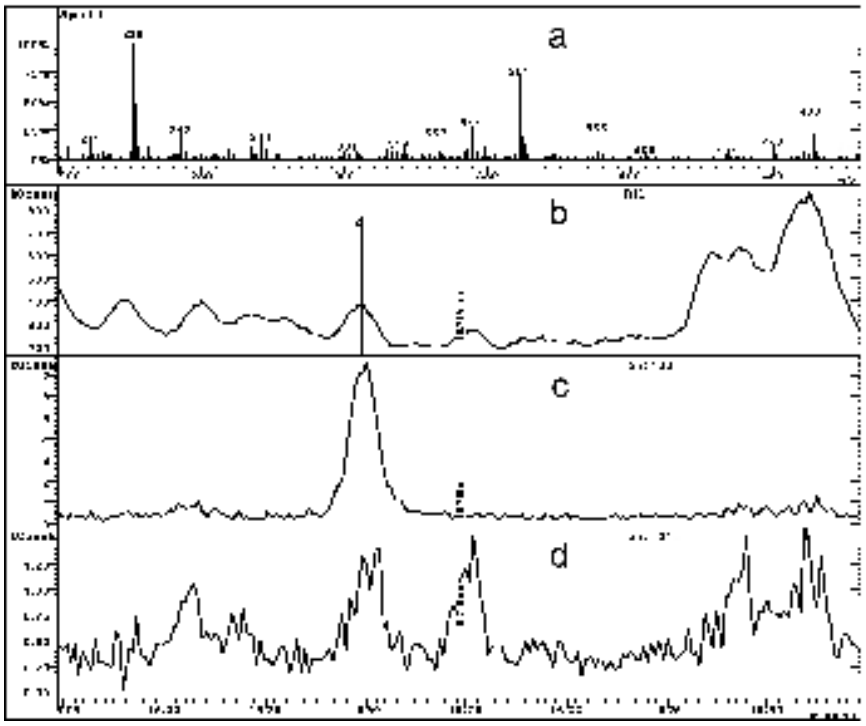
ESM 5


Leaf number increase (%)
50
40
30
20
10
0
a
a
b
b
c
b
-13 to 0
-13 to 8
-13 to 21
days

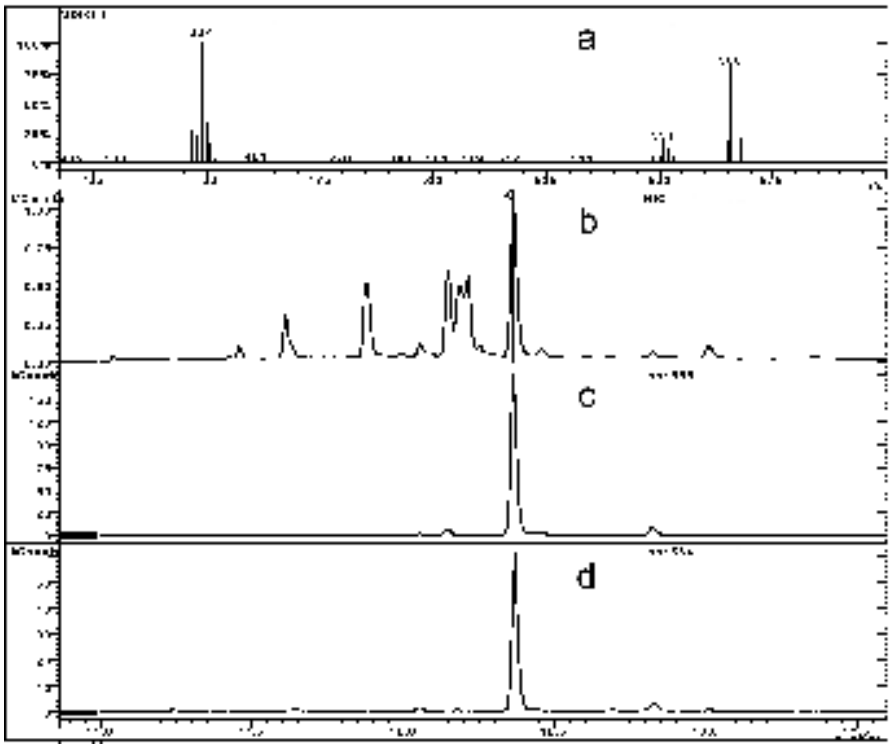
ESM 6


a
b
c
d

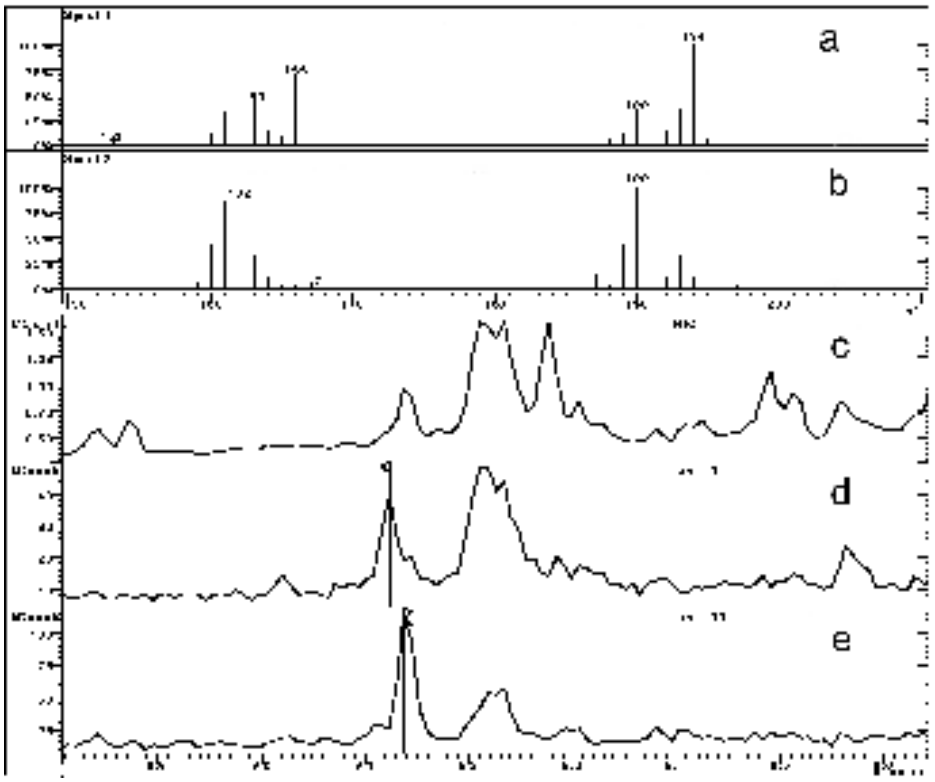
ESM 7



ESM 8



ESM 9



ESM 10