

# HYDRAULIC AND CHEMICAL MECHANISMS IN THE RESPONSE OF *Pinus pinaster* A i t. TO CONDITIONS OF WATER STRESS

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## Abstract

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The increase of temperatures at a global scale constitutes a threaten, among the others, for forest ecosystems, and imposes a deeper comprehension of plant behaviour towards drought stress, in order to better plan a sustainable management of water resources.

Young plants of *Pinus pinaster* A i t. were exposed to prolonged drought stress, during which their xylem ABA concentration, leaf water potential and stomatal conductance were measured at regular intervals on the top branches; the same measurements were taken on a sample of control, non stressed plants. At the same time, plant transpiration rate was measured in response to different ABA concentrations and light regimes. Plant responses to variations of environmental parameters were measured, too: leaf T° vs. atmospheric T°; “E” vs. VPD; “A” vs. (CO<sub>2</sub>).

The data suggest that the plant final response to drought stress, *i.e.* stomatal closure, is mediated by a combination of hydraulic and hormonal regulation; the mechanical one is triggered on the short period and is more quickly reversible, while the chemical one acts on the medium-long term, when the water stress tends to chronicize.

*Key words:* VPD, ABA, water potential, transpiration rate

*Abbreviations:* VPD – vapour pressure deficit; E – transpiration rate; A – photosynthetic activity; ABA – abscisic acid;  $\psi_w$  – leaf water potential

## Introduction

The Global Change represents a problem on a world scale, threatening the environmental resources and the economy of all countries of the world (Moffat, 1992; Reilly et al., 2007). The greenhouse effect, due to greenhouse gases emission, pollution of the oceans and a general

change in the direction of use of agricultural and urban soils, has led during the last 100 years to a general reduction of precipitations and to an average increase of the temperatures of 0.2–0.6 °C, and some projections prefigure a further increase of up to 2 °C for the end of the century (NRC, 2002).

The main effect of these changes on forest ecosystems is a higher incidence and severity of drought stress (Aber et al., 2001); it is therefore necessary to lead studies focused on the realization of a sustainable management of water resources in such environments, for example by proposing and improving experimental models for the behaviour of species that undergo water stress.

According to recent studies (e.g. Davies, Zhang, 1991; Thompson et al., 2007) abscisic acid (ABA) has a fundamental role in the regulation of gaseous exchanges, particularly in relation to the water status of the plant (Tardieu et al., 1993). Several theories, not reciprocally excluding, have been formulated to explain the stomatal response to variations of the water status of the plant, such as a direct hydraulic response to a variation of the water potential (Comstock, Mencuccini, 1998); closing of stomata following a gradient of the water potential deriving from peristomatal transpiration (Dewar, 1995); a combined response to variations of xylem ABA concentration and VPD, through stomatal and peristomatal transpiration (Jarvis, Davies, 1997); pH-dependent stomatal response to the ABA produced and sent by the roots (“anion trapping” of ABA to the mesophyll and epidermis in acid conditions) (Taiz, Zeiger, 2010; Sauter et al., 2001).

Some doubts persist about the reciprocal roles of the hormonal and the hydraulic signal in mediating the stomatal response of the plant to water stress conditions: aim of this work was to investigate the relationships between xylem ABA concentrations, water potential and stomatal conductance in pine plants undergoing water stress, in order to provide experimental models of their behaviour and better comprehend the mechanisms involved in plant response to drought conditions.

## Material and methods

### *Plant material*

Plant of *Pinus pinaster* Ait. aged 3 years, height  $80 \pm 5$  cm, diameter  $20 \pm 2$  mm, provided by the Forester Office of Garfagnana (Lucca, Italy) were used for all the experiments. The plants were transplanted into pots of polyethylene filled with silty clay soil (original composition was 10% sand, 45% silt and 45% clay) mixed with sand (soil: sand = 3:1 v/v), diameter of 16 cm, and placed into an open PVC greenhouse. The conditions were: temperature ranging between 25–31 °C at daytime, 16–20 °C at nighttime; relative humidity between 40–60% at daytime, 20–35% at nighttime; PAR (measured with a Quantum Sensor - Quantometer Radiation Sensor, Li-Cor) ranging from 500  $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  on days with heavy cloud cover to 1000  $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  on clear days. Microirrigation was provided by an automatic system, with a drip for each pot; the timer was set on 10 minutes of irrigation per day starting at h 7:00. These conditions were applied for all the following trials, except where differently specified.

### *Preliminary trial*

A preliminary trial was performed to point out the optimal duration of the water stress in order to show significant effects on the concentration of xylem ABA. Four plants were exposed to water stress and four acted as a control;

each treatment was replicated three times. Xylem sap was collected from the top branch leaves of the plants at days 0, 7, 15 and 21 from the beginning of the trial, and concentration of ABA was measured. The needles were chosen randomly among the basal, intermediate and apical ones along the branch, in order to better represent its state; the length of the selected needles was always between 10 and 15 cm. The results pointed out 15 days to be a sufficient time to induce significant increase in ABA concentration (data not shown), in accordance with the literature (Jackson et al. 1995; Netting 2000), so that 15 days was chosen as the duration of the water stress trial.

### *Response of the plant to the variation of environmental parameters*

Prior to the water stress experiment, the response of the plants to variations of environmental parameters were measured with CIRAS® (PP Systems). The instrument is a double channel infrared gas analyzer allowing measurements of CO<sub>2</sub> and H<sub>2</sub>O concentrations in up to two gas streams; the method of analysis is non-dispersive infrared, configured as an absolute absorptiometer with microprocessor control of linearization (PPSystems, 2009).

Foliar temperature vs atmospheric temperature, transpiration rate (E) vs vapour pressure deficit (VPD) and photosynthetic activity (A) vs CO<sub>2</sub> concentration were measured.

### *Transpiration rate (E)*

The levels of transpiration were measured on branches excised from the plants, in relation to variations of light level and of exogenous ABA concentration (synthetic ABA, Sigma, Poole, U.K.), by potometer. The examined branches were taken randomly from the top, second and third level, and special care was used to choose only the well developed ones, not covered by the others. The cut branch was trimmed, in immersion in distilled water, with a GEM® blade to remove resinous residues and to avoid possible embolus in the xylem water flux (i.e. the sap flow), and then inserted into a rubber collector connected with a graduated tube filled with the nutritive solution. The shifting of the meniscus, proportional to the transpiration volume in the unit of time, was measured at regular intervals. All the system was water tight to prevent water leakage. The nutritive solution used contained Ca(NO<sub>3</sub>)<sub>2</sub> 0.4 mM and KH<sub>2</sub>PO<sub>4</sub> 0.2 mM (Tardieu et al., 1993); the environmental conditions were: T = 32 °C; R.H. = 26%. In all the experiments the measurements of transpiration started after a period of 30 min of acclimation of the plants to the environmental conditions.

The transpiration rate against light intensity was monitored on four groups of three branches each, exposed to an irradiation of 1100 µmol m<sup>-2</sup> s<sup>-1</sup> during 45 min., followed by 45 min. at 10 µmol m<sup>-2</sup> s<sup>-1</sup>; measurements of the meniscus were performed every 5 min.

The effect of exogenous ABA concentration on transpiration was studied through a static and dynamic approach.

For the static experiment the transpiration on branches exposed to ABA concentrations of 0, 0.005, 0.01 and 0.02 mM was monitored every 5 min. during one hour and a half. A group of four branches, replicated three times, was used for each concentration.

In the dynamic experiment excised branches (four groups of three branches each) were exposed to a concentration of exogenous ABA of 0 mM during the first 45 min., then 0.2 mM during 75 min. and again 0 mM during 60 min. The dynamic variation of ABA concentration was allowed by a system of taps connecting the graduated tubes where the branches were set with two tubes in parallel, one with the 0 mM solution and the other with the 0.2 mM one, designed in such a way to prevent water leakage and formation of air bubbles. Transpiration rate (E) was monitored every 5 min. during the three hours of the experiment.

### *Water stress experiment*

For this experiment 15 plants were exposed to water stress by stopping the irrigation, and 15 acted as a control continuing with the same irrigation regime; both the treatments were replicated three times. Leaf water potential, xylem ABA concentration and stomatal conductance were measured on the top branch leaves at days 0, 3, 6, 9, 12

and 15 (coinciding with the end of the trial) of treatment; water percentage in the soil was measured at the same days, according to the formula:

$$\% \text{H}_2\text{O} = [(\text{FW}-\text{DW}) * 100] * \text{DW}^{-1}$$

in which %  $\text{H}_2\text{O}$  = soil water percentage in weight ; **FW** = soil fresh weight ; **DW** = soil dry weight after one week at 70 °C.

Leaf water potential was measured with a pressure chamber (Sholander et al., 1965); stomatal conductance was measured with IRGA method (CIRAS\*, PP Systems).

Xylem ABA concentration was also measured, on days 0 and 15, in leaves from the second and third branches, in order to point out a possible vertical gradient. All the measurements were performed at 8:00 a.m., after covering of the plants with black plastic sheets the evening before to simulate the predawn. Leaf water potential was also measured, on days 0 and 15, at 10:00 a.m. and 01:00 p.m., to investigate the hydraulic behaviour of stressed and non stressed plants.

### *Extraction of xylem sap and ABA measurement*

The selected leaf-buds were cut and the cortex removed along about 4 cm from the extremity of the cut, then they were placed in the pressure chamber to extract the xylem sap. The sap was collected in 1.5 ml eppendorf vials and stocked at -80 °C until the analysis.

The analysis of the ABA concentration was performed by immunoassay method (Vernieri et al., 1990) after proper dilution in distilled water.

### *Statistical analysis*

Data were subject to statistical analysis by using the SAS statistical software program (SAS INSTITUTE, 1990). Non linear regression between E and VPD was performed; t-test was performed between E in conditions of light and darkness; analysis of variance was performed to point out significant differences among treatments in E vs. (ABA); (ABA),  $\psi_{\text{tot}}$  and stomatal conductance vs. days of trial; (ABA) vs. n° of branch;  $\psi_{\text{tot}}$  vs. hour, and significance of differences was then determined with Tukey HSD test.

## **Results**

### *Plant response to variations of environmental parameters*

The plant responses to variations of temperature showed a quite punctual correspondence between the leaf and the surrounding environment (Fig. 1a): foliar temperature was following the variations of the atmospheric one, always remaining about 0.8 °C higher.

The transpiration level (E) responded almost immediately to variations of vapour pressure deficit (VPD) (Fig. 1b): higher levels of VPD were associated with higher transpiration rates. Dropping VPD to a level of 1 KPa resulted in almost complete closure of the stomata and consequent blocking of the transpiration; values of the omega factor (Jarvis, 1985) ranged between a maximum of 0.215 and a minimum of 0.105.

Photosynthetic assimilation (A) varied proportionally to  $\text{CO}_2$  concentration (Fig. 1c); the increase of A was almost instantaneously following the increase of ( $\text{CO}_2$ ). The compensation point was identified at 80 ppm  $\text{CO}_2$  concentration, at which the photosynthetic

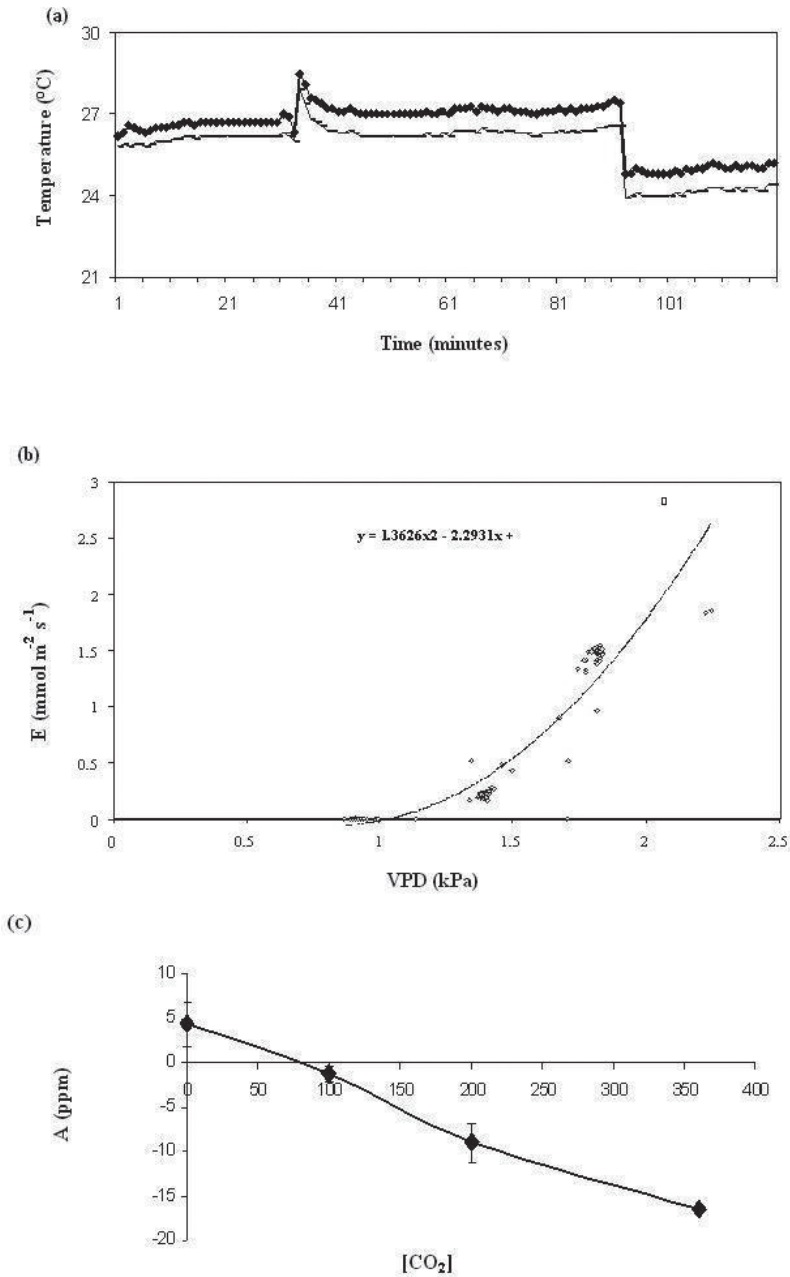


Fig. 1. (a): Trends of the temperature measured in the atmosphere (lower one) and on the leaves (upper one); (b): Transpiration ( $E$ ) as a function of Vapour Pressure Deficit (VPD); (c): Photosynthetic Assimilation ( $A$ ), expressed as - ppm of  $\text{CO}_2$ , as a function of  $[\text{CO}_2]$ .

rate equals the respiratory rate; for lower CO<sub>2</sub> concentrations an active respiratory CO<sub>2</sub> efflux is shown.

*Plant transpiration rate vs. light variations and ABA concentration*

After the acclimation period all the excised branches had reached a constant level of transpiration.

In the light experiment E significantly dropped following the reduction of irradiance from 1100 to 10 μmol m<sup>-2</sup> s<sup>-1</sup> (Fig. 2a); the response was almost immediate, not showing significant differences in behaviour among the different groups of branches. The reduction of E was ranging from 10% to 46%. (Fig. 2b) (t-test: p < 0.05 in all the four groups).

In the static ABA experiment a significant reduction of the transpiration rate was showed by all branches treated with ABA, starting already from the lower concentration of 0.005 mM

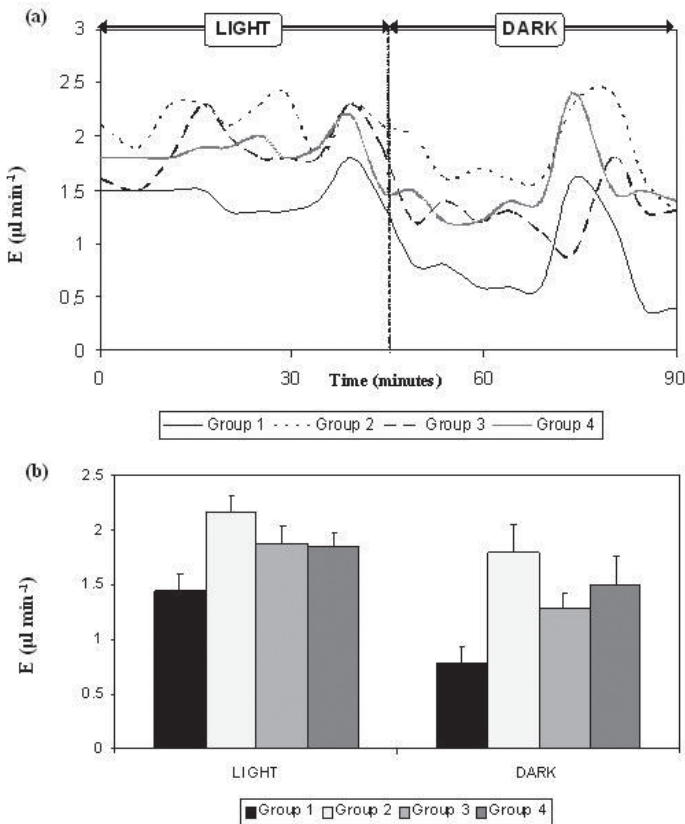


Fig. 2. (a): Trends in plant transpiration rate as a function of time and illumination in four groups of branches. Average values for each group at each moment of measurement are shown; (b): Average values for each group of branches, respectively in conditions of light and darkness.

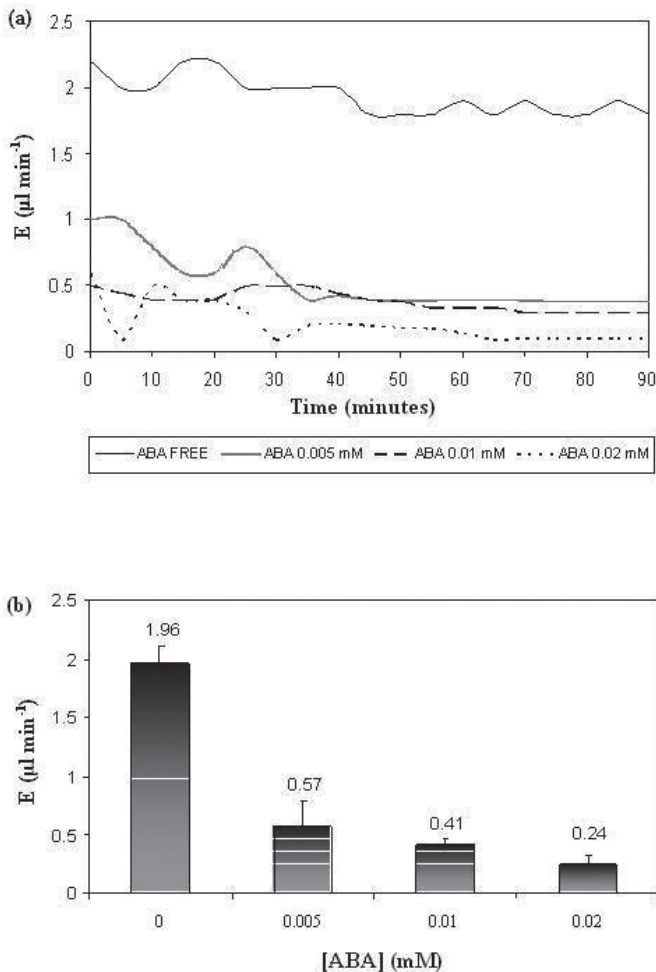


Fig. 3. (a): Trends in plant transpiration rate as a function of time, in four groups of branches treated with different concentrations of ABA. Average values for each group at each moment of measurement are shown; (b): Average values of transpiration  $\pm$  s.d. at the four ABA concentrations.

( $p = 0.014$ ) and this pattern lasted during all the time of the trial (Fig. 3a). ABA treated branches did not show significant ulterior drops of E during the treatment period (Fig. 3a) and showed slightly significant differences between groups ( $p < 0.05$ ) (Fig. 3b).

In the dynamic ABA experiment the level of transpiration rate immediately started to drop following the increase of ABA concentration from 0 to 0.02 mM (Fig. 4a), and got stable 20 min. after (65 min. from the beginning of the trial). When ABA was excluded again from the nutrient solution, E did not increase (Figs 4a, b). The drop of E from non-ABA to ABA conditions was on average of 57% ( $p = 0.015$ ) (Fig. 4b).

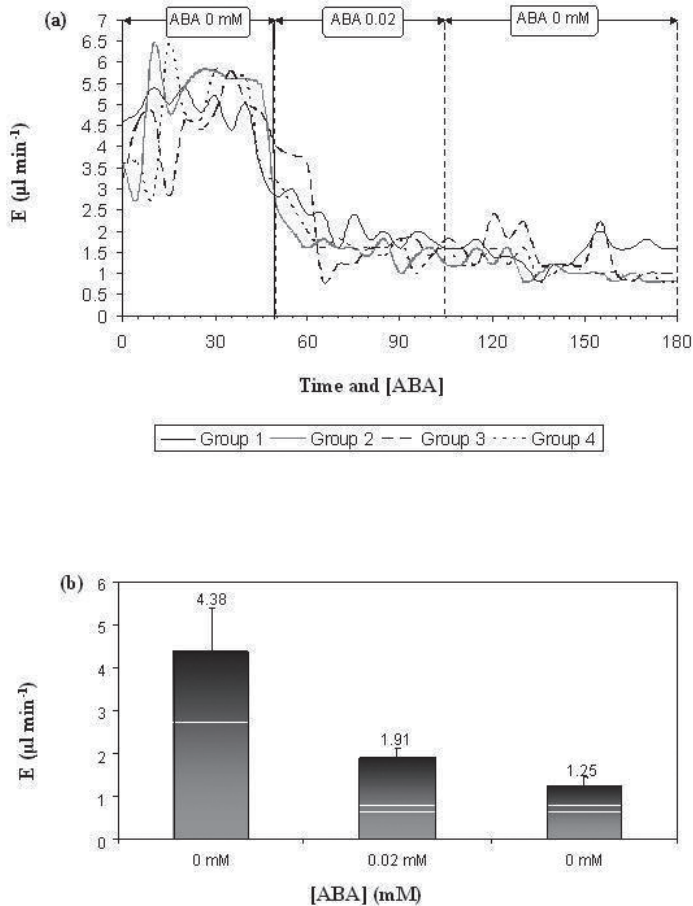


Fig. 4. (a): Trends in plant transpiration rate as a function of time and of changes in ABA concentration for four groups of branches. Average values for each group at each moment of measurement are shown; (b): Average values of transpiration  $\pm$  s.d. at the three consecutive ABA concentrations. Average values are calculated as the mean of the means of the four groups of branches.

#### Water stress experiment

**ABA concentration in the xylem sap** showed a constant and progressive increase in all the plants exposed to drought stress (Fig. 5). This increase already started to be manifest at day 3 of treatment, when the **soil humidity** (calculated as indicated in the water stress experiment paragraph) had decreased just from 74 to 70% in pots that were not irrigated (Fig. 6); this value was still very close to the field capacity represented by the initial conditions of the experiment. The difference between stressed and non stressed plant became significant from day 9 ( $p = 0.026$ ), when soil water content had dropped to 62% in non irrigated pots. At day 15, end of the trial, xylem ABA concentration in stressed plants had become 34 times higher than in non stressed plants.



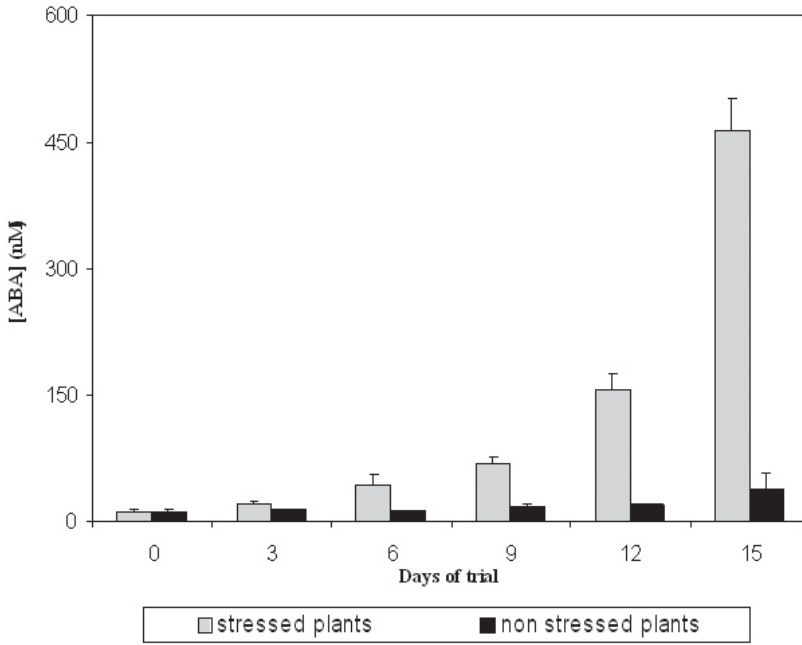


Fig. 5. Average values  $\pm$  s.d. of xylem ABA concentration in stressed and non stressed plants.

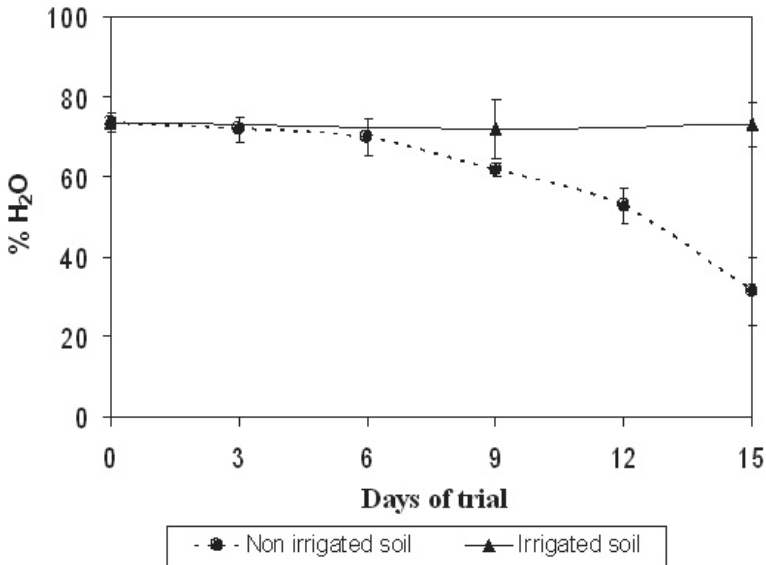


Fig. 6. Trends of H<sub>2</sub>O % in the soil, expressed as weight %, in the cases of irrigated and non irrigated soil during the 15 days of trial.

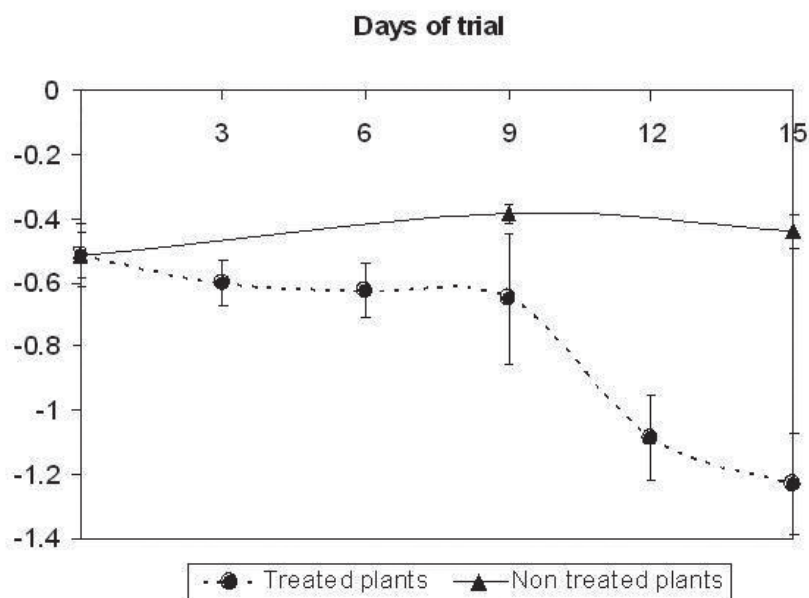


Fig. 7. Trends of leaf water potential of stressed and non stressed plants during the 15 days of the trial.

**Leaf water potential** became significantly lower in treated plants already from day 3 ( $p = 0.018$ ) (Fig. 7), showing a more prompt response to increasing drought conditions than xylem ABA concentration. The decrease was then constant and not very sharp from day 3 to day 9, but at day 12 an ulterior dramatic drop was registered, bringing  $\psi_w$  of stressed plants to values three times lower than control plants. Stressed plants then showed an ulterior but milder decrease at day 15, at the end of the trial.

**Stomatal conductance** significantly decreased in stressed plants starting from day 6 ( $p < 0.01$ ) (Fig. 8), at which a severe drop was registered, while still at day 3  $g_s$  of treated and non treated plants were practically the same. A second severe drop between days 6 and 9 brought  $g_s$  of treated plants to values that were about 100 times lower than control plants, and that dropped almost to zero at days 12 and 15.

**Xylem ABA concentration** showed a significant difference between the first branch and the two lower ones when measured in drought stressed plants at day 15 ( $p = 0.021$ ) (Fig. 9); no significant differences were noticed, in the same plants, between branch 2 and 3 ( $p > 0.05$ ), despite of the fact that all the stressed plants showed a decreasing gradient of (ABA) from the upper to the lower branches. (ABA) in the upper branches was from two to three times higher than in the lower ones. No gradient was detected between the branches of control plants at day 15, nor in all the plants when measured at day 0, before the differentiation of treatments.

**Leaf water potential**, as measured at h 8:00, 10:00 and 13:00 of day 15, showed a different trend depending on the treatment applied, remaining quite constant during the morning in

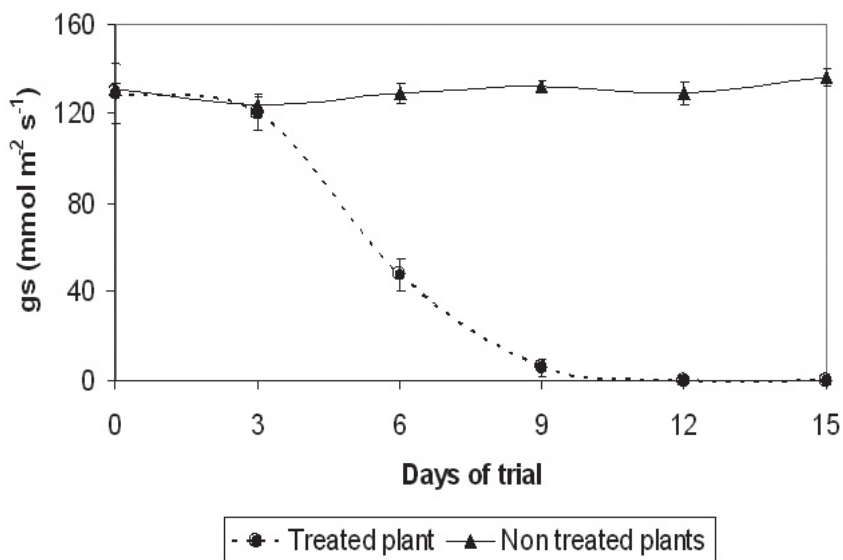


Fig. 8. Trends of stomatal conductance of stressed and non stressed plants during the 15 days of the trial.

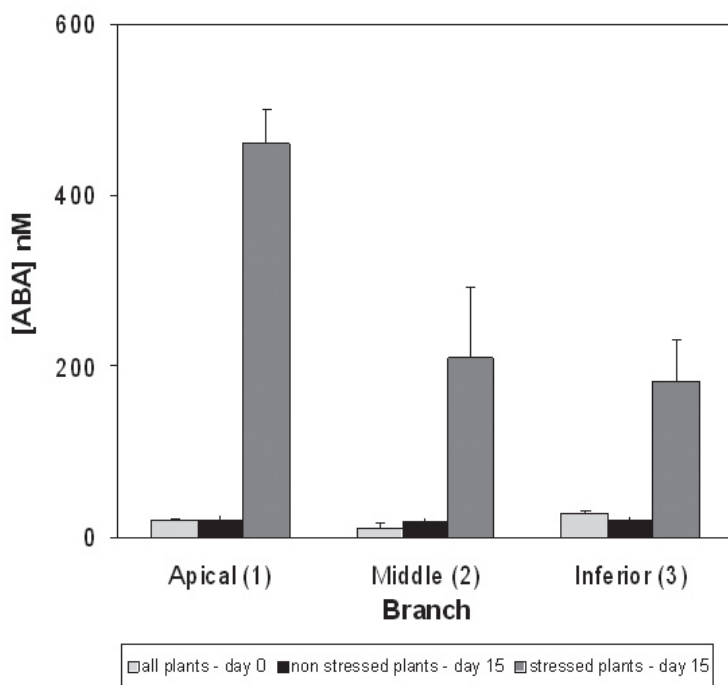


Fig. 9. Average values  $\pm$  s.d. of (ABA) on the three top branches of stressed and non stressed plants, measured at day 0 and day 15 of the trial.

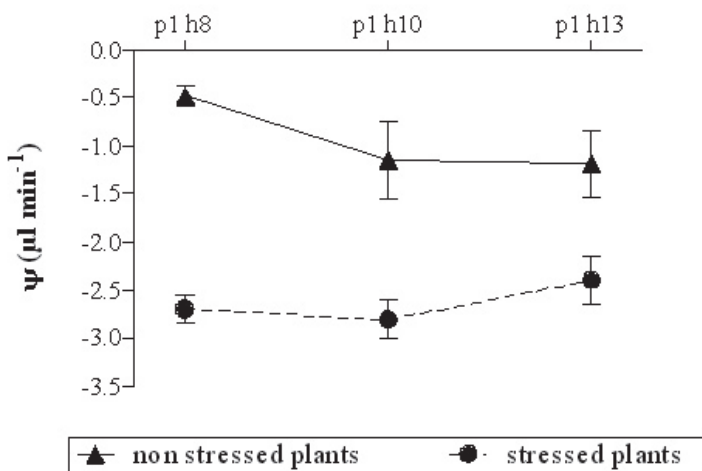


Fig. 10. Trends of leaf water potential in irrigated and non irrigated plants measured at different hours of the morning on the last day of trial.

stressed plants and significantly decreasing with time in non stressed ones (Fig. 10). Control plants showed the maximum value of  $\psi_w$  at h 8:00, moment at which the black plastic sheet simulating the predawn was removed, and subsequently decreased from -0.495 MPa to -1.375 during the morning hours.  $\psi_w$  of stressed plants was on average significantly lower (-2.795 at h 8:00), but it remained more stable, even increasing of 0.315 from h 10:00 to 13:00.

## Discussion

The almost instantaneous response of  $E$  to variations of VPD, with a direct proportionality, shows the high sensitivity of plants, in terms of gas exchanges, to variations of the water status of the surrounding environment, as shown by Turner et al. (1985). The low values of the omega factor (Jarvis, 1985) show a high degree of coupling between plants and the atmosphere, being the transpiration driven by canopy-to-air saturation deficit. Transpiration rate ( $E$ ) is obviously dependent also on the level of irradiance, but the VPD appears to have a more prominent effect on it. The progressive decline of soil moisture leads to a closed connection between VPD and plant transpiration; a lower rate of transpiration is achieved by plants by partial closure of the stomata (Cornic, 2000): hence the close relation between  $g_s$  and water percentage of the soil is here shown.

The increase of photosynthetic activity induced by higher  $CO_2$  concentrations can enhance drought avoidance, thus weakening the effect of drought stress (Xiao et al., 2005); this can be achieved, according to Allen et al. (1998) through improved water relations, since the plant with increased photosynthesis can perform a better osmotic adjustment.

The transpiration rate experiment shows how the response of *Pinus pinaster* to variations of light intensity is particularly fast, and that there was no risk of formation of embolus in the sap flow that could affect the subsequent experiments. Both the static and dynamic experiments about E vs. the concentration of exogenous ABA demonstrated the fundamental role of abscisic acid on stomatal activity (Collins, Kerrigan, 1974; Davies, Zhang, 1991; Freundl et al., 2000; Hose et al., 2000), even if ABA has been demonstrated to not be the only plant hormone responsible for stomatal closure (Veselova et al., 2005). The complexity of the hormonal regulation of stomatal closure in the whole plant organism is also demonstrated by the different results obtained in the experiments conducted with entire plants and with excised branches. Stomatal closure in excised branches was induced at lower concentrations of ABA than the ones necessary in plants grown in pots, and this can be because of a more direct effect and a higher relative concentration of the hormone in the tissues of the excised parts.

The increase of xylem ABA concentration induced by drought, already showed by other Authors (Jiang, Zhang, 2002; Koshita, Takahara, 2004) can suggest a role of ABA in stimulating leaf growth as already theorized by Fricke et al. (2004), contrasting with the general idea of ABA as an inhibitor of growth (Thompson et al., 1997; Cramer et al., 1998).

The decreasing gradient of (ABA) from the apical to the lower branch has been observed also in other plants (Tucker, 1979; Kojima, 2002), and has been put in relation with apical dominance; Speirs et al. (2004) showed how the correlation between ABA and gs vertical gradients in *Vitis vinifera* suggested that the ABA gradient was to be explained at least partially by means of local synthesis. These data can represent, when considered together, a further evidence of a possible role of ABA in growth enhancement.

The quick response of leaf water potential to drought stress had been already shown in literature (Hand et al., 1982; Shisanya, 2004); the fact that this response is even quicker than the one of xylem ABA concentration highlights the close connection between  $\psi_w$  and % H<sub>2</sub>O of the soil (Siddique et al., 2000). If this fact is analyzed jointly with the almost immediate effect of VPD variations on E (as seen in the preliminary experiments), it brings us to argue a very important hydraulic regulation of stomatal activity, through which the plant can face the water stress at least during the first phases of its manifestation (Gucci et al., 1997). The **isohydric** behaviour showed by stressed plants on the morning of day 15 is a further proof of this assumption, since this kind of behavior indicates a better control of the water potential performed by the stomata (Tardieu, Simonneau, 1998) and is usually associated to plants with a better tolerance to drought stress conditions (Fisher et al., 2006).

The reduction of stomatal conductance induced by drought stress is known as “chemical signalling from root to shoot” (Oden, Dunberg, 1984; Comstock, 2002). gs responded to drought conditions more quickly than ABA; the earliest plant response to water reduction was the drop of leaf water potential. The different patterns of response during time of the parameters analyzed can be explained hypothesizing a combined response of the plant, in terms of stomatal closure – reduction of transpiration, to drought conditions (Jones, 1998; Whitehead, 1998; Schulze, 1993). The closure of the stomata is therefore mediated by an hydraulic (through variations of  $\psi_w$ ) and a chemical (through variations of (ABA)<sub>xyl</sub>) way, which become active at different moments.

The hydraulic mechanism of  $\psi_w$  reduction, which is the most easily reversible (Luo, Strain, 1992) is the first one to be put in action, as demonstrated by the fact that  $\psi_w$  responded already at day 3. It can act as an emergency system to face mild or brief drought conditions, inducing a quick but reversible closure of the stomata. If the stress conditions persist the plant increases ABA production, which determines a longer period response of the plant to drought. This is also demonstrated by the fact that in the dynamic ABA – transpiration experiment, the transpiration level remained low even after removing ABA from the nutrient solution. This is in accordance with Trejo and Davies (1991), which observed how in *Phaseolus vulgaris* drought stress caused a closure of the stomata prior to increases of xylem ABA.

In conclusion, plants of *Pinus pinaster* appear to show a combined response, hydraulic and chemical, to drought stress, being the former a short term strategy and the latter a longer term one. The hydraulic response is connected with changes in VPD subsequent to reduction of % H<sub>2</sub>O in the soil; the chemical one is mediated by production of ABA and its concentration in the xylem sap.

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