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Variations in abscisic acid, indole-3-acetic acid and zeatin riboside concentrations in two Mediterranean shrubs subjected to water stress

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Abstract

Water stress induced an increase in endogenous concentrations of ABA in *Lavandula stoechas* L. plants to 13100 pmol ABA g^{-1} FW, which may contribute to the maintenance of water relations between the second and the third day of water stress treatment. After the third day, a sharp decrease in ABA levels was observed to 2630 pmol ABA g^{-1} FW, together with a decrease in water content and water potential and a loss of plant response to water stress. Water deficit did not induce an increase in endogenous ABA concentration, which remained at 514 pmol ABA g^{-1} FW in *Rosmarinus officinalis* L., which is more sclerophyllous than *L. stoechas*. Nevertheless, the relative water content of *Rosmarinus officinalis* L. after seven days of water stress decreased more than 40% and ψ reached values of −3.2 MPa. *R. officinalis* showed lower levels of ABA, but significantly higher levels of IAA and ZR than *L. stoechas* (4 times and 6 times respectively in well watered-plants). The increase in ABA levels is not a common mechanism in these two Mediterranean shrubs which survive under water stress conditions.

Abbreviations: ABA – abscisic acid; d – days of water stress treatment; DW – dry weight; FW – fresh weight; IAA – indole-3-acetic acid; RP – Reversed Phase; RWC – relative water content; TW – turgid weight; WC – water content; ZR – zeatin riboside; ψ, water potential

1. Introduction

Lavender (*Lavandula stoechas* L.) and rosemary (*Rosmarinus officinalis* L.) are two native Mediterranean evergreen half-shrubs well adapted to survive under water stress conditions by mechanisms of drought tolerance or avoidance. The semi-sclerophyllous to sclerophyllous leaf consistency is a common characteristic of plants growing in Mediterranean climate and is important in avoiding dehydration [7]. Nevertheless, few biochemical studies in xeromorphic leaves have been reported; the high level of secondary metabolites hinders the purification of plant extracts, so more research effort is needed in this field [13]. The knowledge of variations in phytohormone concentrations in Mediterranean shrubs adapted to recurrent drying cycles will provide valuable information about the physiological responses of those plants to water stress.

The functional role of the plant hormone abscisic acid (ABA) in the adaptation of a variety of plants to water stress has been well documented and reviewed [21, 2, 10]. Variations in ABA levels induced by stress do not completely account for the physiological responses of the plant to drought, and an interaction with other chemical signals such as auxins (IAA) and cytokinins requires careful study [11]. Abiotic stresses alter the levels of plant hormones in general [14] and these variations could act as modulators of plant responses to water stress. Furthermore, each hormone

can influence the levels of at least one of the other types of hormone [6] and it is suggested that the variations in endogenous concentrations of different plant growth regulators may modulate plant water balance [3]. Nevertheless, there is scarce and conflicting evidence on the variations of indole-3-acetic acid levels in water-stressed plants [18, 8, 9]. A decrease in the cytokinin content of droughted roots and their possible function as root-to-shoot signals have been described [2, 1]. However, indole-3-acetic acid and cytokinins do not necessarily decline during the adaptation of plants to water stress [22]. At present, there are still few studies using the same crude plant extract for phytohormone determination in plants subjected to water stress. Nevertheless, such studies may clarify the function of hormones as modulators of plant responses to water stress.

L. stoechas and *R. officinalis* differ in leaf morphology: *L. stoechas* is less xeromorphic than *R. officinalis* [15], and therefore these species could present a different behaviour to water stress. The aim of this work was to study the endogenous concentrations of abscisic acid, indole-3-acetic acid and zeatin riboside in these two shrubs subjected to water stress.

2. Materials and methods

2.1 *Plant material and growth conditions*

Two-year-old plants of *R. officinalis* and *L. stoechas* obtained from seeds were cultivated in controlled conditions (12 h photoperiod, at a photon flux of 400 μmol m^{-2} s^{-1}, air temperature 25/20 °C day/night and 49% relative humidity). Plants were grown in 17 cm diameter plastic pots filled with a calcic Luvisol (FAO). Plants were watered daily to container capacity: well watered plants. The stress treatment was imposed by lack of watering. Samples were taken early in the morning when ψ was −2.6 MPa for stressed plants and experiments were continued until stressed plants reached values of −3.2 MPa; this meant witholding water for two, three, and four days in *L. stoechas* and for three, four, five and six days in *R. officinalis*. For the studies of water relations and endogenous ABA, IAA and ZR, homogenous apical non-woody shoots ten cm long were selected. The values are means of three samples (collected from separate experiments) ± SE.

2.2 *Water relations*

Leaf relative water content (RWC) was calculated as:

$$RWC = (FW - DW/TW\text{-}DW) \times 100,$$

where FW = fresh weight in the field; DW = Dry weight, after drying samples in an oven at 80 °C to constant weight; TW = turgid weight, after rehydrating fresh shoots for 24 h.

Water content of shoots (g g^{-1} DW) was estimated from the fresh and dry weight.

Leaf water potential (ψ) was obtained from 10 cm long shoots using a pressure chamber (ARIMAD-2) containing a wet filter paper at the bottom of the chamber.

2.3 *Analysis of endogenous ABA, IAA and ZR*

After determination of fresh weight, leaves were immediately frozen in liquid nitrogen and stored at −20 °C until hormone analysis. IAA, ABA and ZR were purified from the same plant extract, as described by Prinsen et al. (1995). After grinding in liquid nitrogen in a mortar and pestle, leaf samples were extracted overnight at −20 °C in 80% MeOH (9 ml g^{-1} FW), to which DL-cis, trans-[G-^{3}H]-ABA (300 Bq, 2.26 TBq/mmol, Amersham), [$^2H_6$]-ABA (200 ng, for preparation see Milborrow, 1971), (3-[5(n)-^{3}H]-indole-acetic acid (300Bq, 788GBq/mmol, Amersham), indole-[2,4,5,6,7-$^2H_5$]-3-acetic acid (200 ng, Aldrich) and [^{3}H]-trans-zeatin-riboside dialcohol (200 Bq, 1.4 TBq $mmol^{-1}$, Amersham) were initially added as tracers for localization (^{3}H) and isotope dilution (^{2}H) purposes. After centrifugation (24,000 *g*, 15 min, 4 °C) the extract was purified on a Reversed Phase (RP)-C_{18} cartridge (500 mg, Varian). The effluent was evaporated *in vacuo* and the sample was redissolved in 15 ml 0.04 M ammonium acetate buffer pH 6.5. The samples were purified using a DEAE-Sephadex A25 (4 ml) cartridge to which a RP-C_{18} (Varian) cartridge was coupled underneath.

Cytokinins were eluted from the RP-C_{18} cartridge with 4 ml 80% methanol, evaporated in a speed-vac (VR1 HETO Lab. Eq.), redissolved in 500 μl PBS and analyzed by RIA [17] using zeatin-riboside specific polyclonal chicken egg yolk antibodies. Standards and samples were tested in triplicate. The results were expressed as pmol cytokinin-riboside equivalents per gram fresh weight (pmol ZR-equiv. g^{-1} FW).

ABA and IAA were eluted with 6% formic acid from DEAE-Sephadex A25 and concentrated on a RP

C_{18} (Varian) cartridge with 5 ml diethyl ether and evaporated *in vacuo*. A preparative ion supression RP-HPLC (50/49.5/0.5, v/v/v, methanol/H_2O/acetic acid; Alltech Econospere C_{18}, 100 × 4.6 mm, 3μm; 0.5 ml/min) was coupled to a fluorimetric detector (Schimadzu RF530, λ_{ex} 285 nm, λ_{em} 360 nm). 1 minute fractions were collected during the time interval corresponding to the retention times of the radioactivity of a ^{14}C-IAA (3-indolyl-(1-^{14}C)-acetic acid, 100 Bq, 2.18 GBq/mmol, Amersham)-and ^{3}H-ABA (133 Bq) reference respectively. 1/10 aliquot of the collected fractions were counted for ^{14}C and ^{3}H of the internal tracer (Packard TRICARB 1500 Liquid Scintillation Analyser).

The ABA samples were methylated with diazomethane [20]; Me-ABA was analyzed by GC-MS separative chemical ionization in selective ion mode (CI-SIM) HP 5890 Series II, coupled to a VG TRIO 2000 quadrupole mass spectrometer (column 25 m, 0.25 mm ID; gas phase He, temperature gradient from 120–240 °C, 15°/min, 250 °C injection temperature, CI gas amonium; retention time of Me-ABA 5.59 min [19]). For the detection of Me-ABA and Me-$^{2}H_6$-ABA, 278 and 284 were used as selective diagnostic ions, respectively [5]. The integrated peak areas corresponding to these diagnostic ions were used for calculations. After methylation, the IAA containing fractions were further derived to IAA-Me-HFB [16]. IAA-Me-HFB was analyzed by GC.MS positive electron impact in selective ion mode (EI^+-SIM) HP 5890 Series II, coupled to a VG TRIO 2000 quadrupole mass spectrometer (column 25 m, 0.25 mm ID; gas phase He, temperature gradient from 0–6 min: 25 °C/min, 6–8 min 50 °C/min; 8–10 min 300 °C, 250 °C injection temperature; retention time IAA-Me-HFB 8.9 min). For the detection of IAA-Me-HFB and $^{2}H_5$-IAA-Me-HFB, 326 and 329 were used as selective diagnostic ions, respectively.

The results were expressed as pmol ABA g^{-1} FW or pmol IAA g^{-1} FW and were analyzed using a paired "t" test.

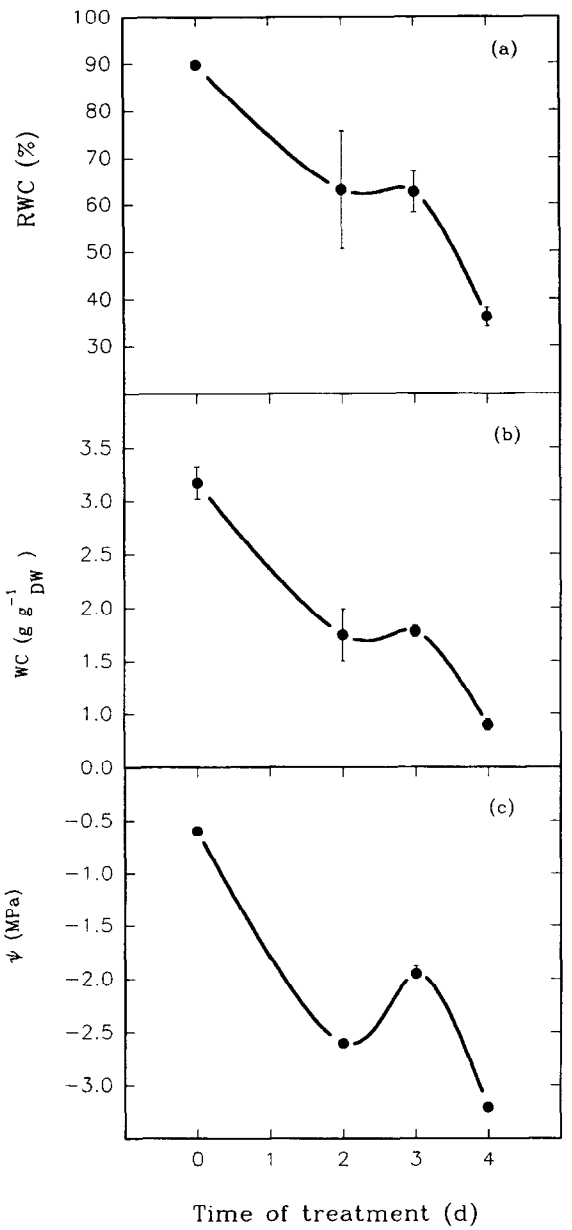

Figure 1. Effect of water stress on (a) relative water content (RWC), (b) water content (WC) and (c) water potential (ψ) of *Lavandula stoechas* shoots. The vertical bars represent ± standard error (n = 3).

3. Results

3.1 *Water relations*

Water status in *L. stoechas* shoots is shown in Figure 1. After two days of water stress a decline of 30% in RWC and of 45% in water content (WC) was observed. ψ decreased from −0.6 to −2.6 MPa. There were practically no differences in plant water relations between two and three days of water stress. After four days of water deficit, RWC and WC declined again: RWC became 60% lower than in well watered plants, WC decreased to 0.94 g g^{-1} DW and ψ fell to −3.2 MPa. Thereafter, the experiments were discontinued, since plants appeared completely wilted.

Figure 2 show water relations in *R. officinalis*. The RWC (82.6%) of rosemary well watered plants was lower than that of lavender well watered plants (89%). After three days of water stress a decrease of 25% in RWC and of 45% in WC was seen in rosemary; the ψ decreased from -1.3 MPa to -2.75 MPa and afterwards a further slight decline in plant water content and ψ was seen. After six days of water stress, no signs of wilting were observed in rosemary plants.

3.2 *ABA, IAA and ZR concentrations*

The ABA concentration in *L. stoechas* leaves after three days of water stress (13100 ± 400 pmol ABA g^{-1} FW) was significantly higher than those of well watered plants (900 ± 700 pmol ABA g^{-1} FW). Afterwards, the ABA content decreased to a similar value to that of the well watered plants (Figure 3a).

L. stoechas showed no significant differences in endogenous ZR concentration throughout water stress treatment (Figure 3a). A slight increase was observed after 2 days, followed by a decrease in the ZR content.

The free IAA concentration in *L. stoechas* increased over the first two days of water stress, reaching a peak of 189 ± 6 pmol IAA g^{-1} FW by the second day; then, it decreased to 63 ± 16 pmol IAA g^{-1} FW at three days and rose again on the fourth day of water stress (Figure 3b).

The ABA concentration in *R. officinalis* leaves (Figure 4b) from well watered plants was lower than that in leaves of *L. stoechas*. There were no significant differences in endogenous ABA levels throughout the treatment.

The ZR content of *R. officinalis* leaves (Figure 4a) increased during the three days of treatment (29 ± 6 nmol ZR equiv. g^{-1} FW) and then slowly decreased during the remaining experimental period (reaching values of 10.8 ± 2.5 nmol ZR equiv. g^{-1} FW). The ZR concentration observed in *R. officinalis* was always higher than that of *L. stoechas*.

The free IAA concentration in *R. officinalis* was significantly higher than IAA concentration in *L. stoechas*, reaching a peak (1740 ± 260 pmol IAA g^{-1} FW) after three days of water stress. Then, it showed a gradual decline during the course of the experiment to 300 ± 100 pmol IAA g^{-1} FW by the last day. There were no significant differences between well watered plants and plants subjected to four, five or six days of water stress (Figure 4b).

During the water stress treatment the ratio ZR/ABA decreased from 3.1 in well watered plants to 0.5 after 3 days. On the fourth day a significant decrease in ABA concentration and a tendency to decrease in ZR levels were observed.

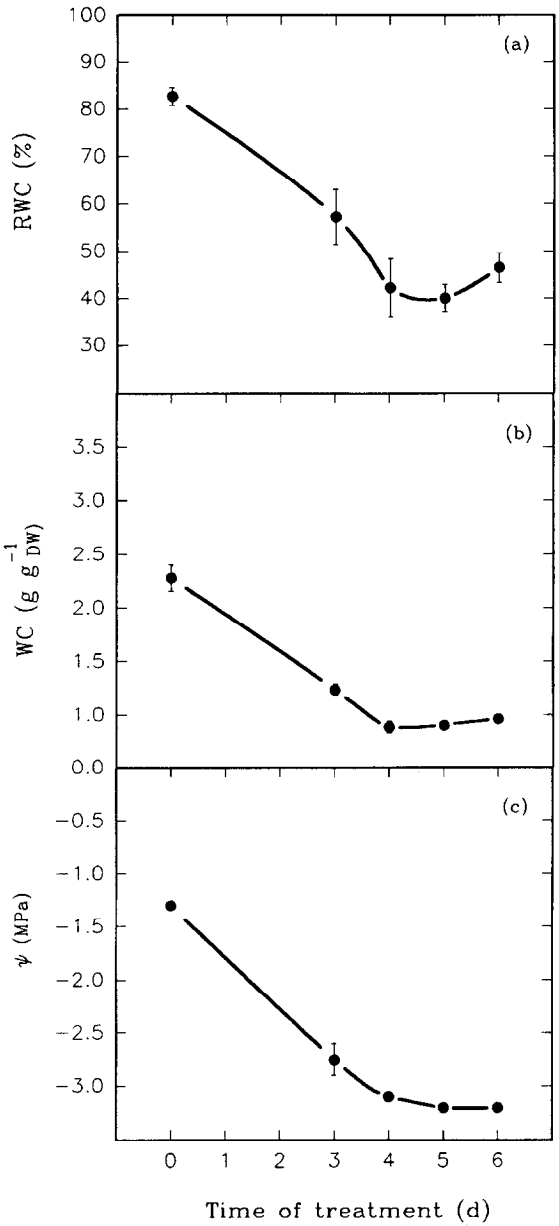

Figure 2. Effect of water stress on (a) relative water content (RWC), (b) water content (WC) and (c) water potential (ψ) of *Rosmarinus officinalis* shooots. The vertical bars represent ± standard error (n = 3).

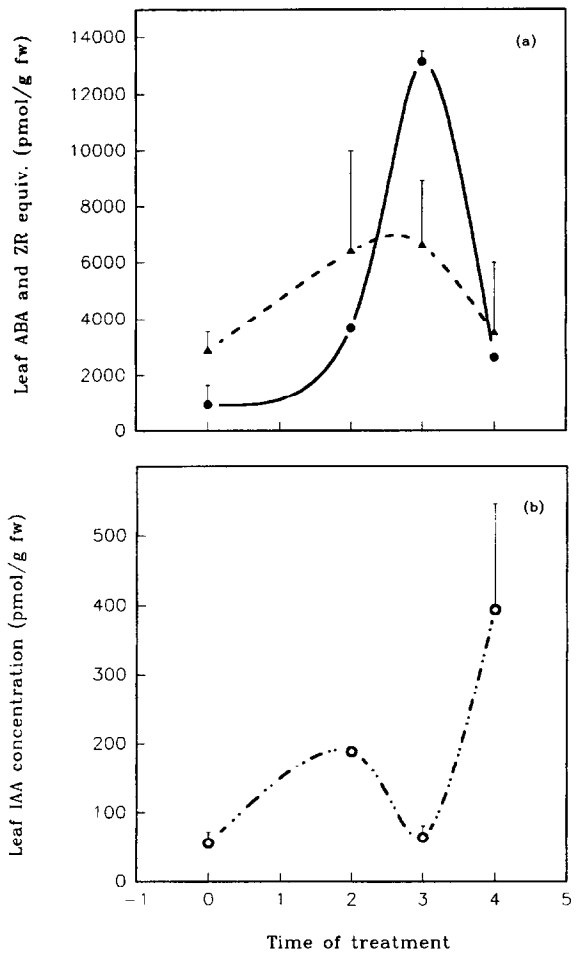

Figure 3. Effect of water stress on endogenous concentration of (a) ZR equiv. and ABA, (b) IAA, in *Lavandula stoechas* leaves. (▲) ZR, (●) ABA, (○) IAA. The vertical bars represent ± standard error (n = 3).

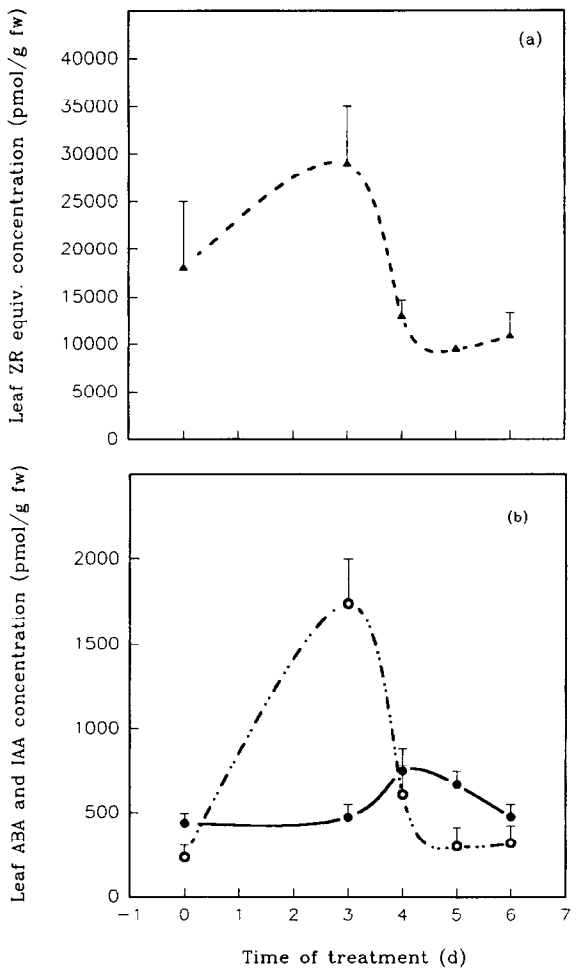

Figure 4. Effect of water stress on endogenous concentration of (a) ZR equiv., (b) ABA and IAA, in *Rosmarinus officinalis* leaves. (▲) ZR, (●) ABA, (○) IAA. The vertical bars represent ± standard error (n = 3).

4. Discussion

In both species WC declined more rapidly than RWC indicating that adaptation to water stress was taking place.

The increase observed in the ABA level in *L. stoechas* after 2 days may explain the lack of differences in WC, RWC and ψ between 2 and 3 days of water stress treatment. After 3 days, the sharp decline in ABA could be related to a loss of the plant capacity to respond to water stress [8, 9].

R. officinalis is more sclerophyllous than *L. stoechas* and transpiration rates are always lower than those of *L. stoechas*. Although ψ reached −3.2 MPa, no signs of stress were observed in these *R. officinalis* plants; after an initial decrease in water content and ψ, practically no differences in these parameters from 3 to 6 d were observed. Leaf xeromorphy enables plants to reduce water loss and maintain RWC.

In *L. stoechas* the increase in ABA content appeared to be correlated with drought resistance in plants and a sharp decline in ABA levels observed at severe water stress conditions could be related with the loss of capacity of plant to withdraw stress. In well watered plants the bulk leaf ABA concentration was about 16 times higher than that of IAA. After 2 days the endogenous IAA rose approximately three fold with respect to those of well watered plants. Similar results have been reported for aralia plants subjected to stress [9]. It was hypothesized [14] that, under stress condi-

tions which impair plant growth, the cytokinin activity decreases, while the ABA acitivity increases. Nevertheless, no significant differences in zeatin riboside levels were recognized in our study. Our results agree with those of Zholkevich and Pustovoitova (1993), who found that IAA and cytokinins do not necessarily decline during the adaptation to water stress.

R. officinalis showed no significant changes in ABA content, which could be related to the lack of differences observed in water relations between 3 and 6 d. In comparison to *L. stoechas*, the endogenous ABA concentration in *R. officinalis* well watered plants was 3 times lower. ABA endogenous concentration of water stressed *R. officinalis* plants was found to be up to 30 times lower than those observed in *L. stoechas* water stressed plants. Levitt (1980) stated that drought avoidance is largely morphological-anatomical in nature, thus it could explain the lack of the common response to water stress such as an increase in the endogenous ABA concentration. Nevertheless, the levels of IAA and ZR were significantly higher than those observed in *L. stoechas*. In well watered plants of *R. officinalis* endogenous ZR concentration was 6 fold higher than in *L. stoechas* and it remained high throughout the water stress treatment. Similar behaviour was observed for endogenous concentrations of IAA. Hormonal interactions may affect hormonal levels in plant tissues. High levels of cytokinins increase the levels of IAA and reduce the levels of ABA [6]. The ability of one hormone to affect the level of another is probably of great significance in the regulation of plant growth and plant responses to water stress, and merits more critical assessment. The complexity of these interactions introduces a new dimension to the mechanism of hormone action. The high concentrations of ZR and IAA observed in *R. officinalis* could be related with the delay in leaf senescence. Meanwhile, higher levels of ABA observed in *L. stoechas* contribute to the acceleration of leaf senescence [4].

The increase in endogenous ABA concentration in response to water stress may not be a general mechanism in these two autochthonous Mediterranean shrubs adapted to water stress avoidance. Anatomical and ultrastructural characteristics, and also hormonal interactions, should be taken into account.

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