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ORIGINAL PAPER

Changes of secondary metabolites in *Pinus sylvestris* L. needles under increasing soil water deficit

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Abstract
· **Key message** **A multiphasic response to water deficit was found in Scots pine primary and secondary metabolism. First, an increase of terpenoids coincided with the stomatal closure. Second, an accumulation of proline, ABA, and shikimic acid was detected when photosynthesis was negligible.**
· **Context** Drought-induced mortality is characterized by a major needle yellowing followed by severe defoliation and whole branch death. Before these external visual symptoms of drought stress take place, different alterations occur in plant metabolism.

· **Aims** This study aims to detect changes in primary and secondary metabolism of *Pinus sylvestris* L. in response to a decrease in soil water availability.
· **Methods** We analyzed needle water potential, photosynthetic characteristics, and concentrations of proline, terpenoids, shikimic acid, total polyphenols, and abscisic acid (ABA) in *P. sylvestris* through a 55-day soil water deficit period.
· **Results** Concentrations of most metabolites varied with the decrease in soil water availability, but changes in different compounds were triggered at different times, highlighting a multiphasic response. Increases in monoterpene and sesquiterpenoid content at moderate water deficit coincided with stomatal closure which preceded the accumulation of proline, ABA, and shikimic acid under severe water deficit when net photosynthesis was negligible.
· **Conclusion** This work confirms that most of the secondary metabolites under investigation in *Pinus sylvestris* did not increase until a moderate to severe water deficit was experienced, when photosynthesis was limited by stomatal closure.

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1 Introduction

Tree populations are facing new and rapidly changing selective pressures such as more frequent and more severe droughts, threatening the conservation of forests and related ecosystem services (Lindner et al. 2010; Martínez-Vilalta et al. 2012). Northern hemisphere conifer *Pinus sylvestris* L. has an exceptionally wide range of dispersal from subarctic to close to Mediterranean and warm temperate ecosystems, extending to its southern distribution limit to different mountain ranges of the Iberian Peninsula (Matías and Jump 2012). In these southernmost habitats, this species appears sensitive to an increase in the aridity, as indicated by major tree deaths during severe drought episodes (Galiano et al. 2010; Peguero-Pina et al. 2011; Sanz et al. 2014). The onset of drought-dependent mortality is visible by general needle yellowing followed by severe defoliation and whole branch death in a large fraction of trees (Sanz et al. 2014).

Before these external visual symptoms of water deficit take place, numerous alterations in plant metabolism occur, including photosynthesis limitation, alteration of carbon allocation (Teskey et al. 1987), changes in nutrient uptake, and variations in the levels of soluble sugars, inorganic ions, and amino acids (Schulze 1991; Turtola et al. 2003). Most recent studies on water deficit effects have focused on photosynthetic metabolism (e.g., Niinemets and Keenan 2014); however, water deficit also leads to modifications in secondary metabolism, and these changes often have remained hidden, but might provide a better insight into species and genotype variations in drought responses and into fine-tuning of plant functioning at a given level of primary metabolism (Niinemets 2016).

As key changes under water deficit, several authors have reported accumulation of proline, which has been considered as a mechanism of osmotic adjustment helping plants to extract water from drier soils (Xiao et al. 2008; Verbruggen and Hermans 2008; Vilagrosa et al. 2010). However, changes in secondary metabolism should not be directly related to adjustments to water deficit, but to other co-occurring stresses that can threaten the weakened plants, including adjustment to potentially more severe herbivory and pathogen infections. In conifers, it has been demonstrated that water deficit can modulate the composition of the oleoresin, changing the terpenoid profiles (monoterpenes, sesquiterpenoids, and diterpenoids, in particular, resin acids) (Lluisà and Peñuelas 1998; Turtola et al. 2003). Moderate water deficit typically results in increased woody and needle terpene concentrations (Lluisà and Peñuelas 1998; Turtola et al. 2003), but severe water deficit can reduce terpene contents (Yani et al. 1993). The role of terpenoids in the resistance or susceptibility of trees to attacks by diseases, insects, and animals and the influence of water deficit on such attacks

have been studied (Lluisà and Peñuelas 1998; Pureswaran et al. 2004; Moreira et al. 2009; Karanikas et al. 2010), but the effects of water deficit severity on terpenoid content and composition and the corresponding effects on plant-insect and plant-pathogen interactions are poorly understood. Apart from terpenoids, phenolics and lignin are also involved in plant defense (Gamir et al. 2014). These compounds derived from shikimic acid (Schafellner et al. 1999; Gamir et al. 2014), the contents of which also seem to increase under water deficit (Becerra-Moreno et al. 2015). In addition, the accumulation of other compounds such as abscisic acid (ABA) has also been related to water deficit (Munné-Bosch et al. 2009; Corcuera et al. 2012). Increase of ABA in water-deficient plants induces stomatal closure and reduces leaf growth (Tardieu et al. 1992; Brodribb and McAdam 2013), allowing to fine-tune the stomatal responses to water deficit.

Although changes in all of the abovementioned compounds have been related to water deficit, in most of the studies, only two situations have been considered—well-watered and plants subjected to water deficit—and they fail to investigate the metabolic transition through the development of the stress treatment. Moreover, the apparent contradictory results found in different papers about the effect of water deficit on the profile of some secondary compounds may be explained by the lack of a comparable experimental framework. In such a case, the interaction between water conservation and net CO_2 uptake as water deficit increases recommends the simultaneous measurement of physiological parameters and secondary metabolites in order to establish a correspondence between them. For these reasons, the main objective of this study was to monitor changes in content of all the key metabolites through mild to severe soil water deficit together with primary plant physiological characteristics—water potential, stomatal conductance, and net CO_2 uptake. More specifically, we have analyzed the contents of proline, terpenoids (monoterpenes, sesquiterpenoids, and resin acids), shikimic acid, total polyphenols, and ABA during a soil water deficit period in *P. sylvestris*, a species particularly threatened by global change. We hypothesized that the plant response as soil water deficit increases may reflect a timetable of coordinated events between primary and secondary metabolism, which could reveal the physiological thresholds behind major changes in secondary metabolism. Understanding such coordinated changes will provide a framework to diagnosing the deficit situation in plants before external visual symptoms of drought stress become perceptible.

2 Materials and methods

2.1 Plant material and experimental conditions

Scots pine (*P. sylvestris* L.) seeds from "Sistema Ibérico Septentrional" provenance (41°54′N, 02°53′W; Soria, Spain)

were sown and cultivated in 2000 (mixture of 80% substratum and 20% perlite in 500-mL containers) inside a greenhouse. After the first growth cycle, seedlings were transplanted to 10-L containers, and after 2 years, they were again transplanted to 50-L containers (80% substratum, 20% perlite) and cultivated in a common garden (41°39′N, 0°52′W, Zaragoza, Spain; mean annual temperature 15.4 °C, total annual precipitation 298 mm). All plants were grown under the same environmental conditions and irrigated every 3 days.

On August 2012, eight trees (12 years old) were moved from outside into a greenhouse (mean daily temperature 18.1 ± 0.5 °C, mean daily relative humidity 77.6 ± 1.1%). Once there, trees were watered every second day to field capacity until September 12, 2012. At this date, watering of five experimental trees was stopped, while the other three trees were kept well-watered during the experiment as control trees. Soil water deficit was imposed for 55 days, and during the treatment, measurements of water potential and gas exchange were conducted periodically as the severity of water deficit increased. Measurements of water potentials were conducted at predawn while measurements of needle gas exchange were made at 8:00 h solar time (early morning). In addition, current-year needle samples for metabolite measurements in experimental and control trees were collected at predawn and stored at −80 °C for sample preservation until analysis.

2.2 Water potential and gas exchange measurements

Predawn water potential (Ψ_{pd}, MPa) was measured in shoots of *P. sylvestris* with a Scholander pressure chamber following the methodological procedure described by Turner (1988). Net CO_2 uptake (A, μmol CO_2 m^{-2} s^{-1}) and stomatal conductance (g_s, mmol H_2O m^{-2} s^{-1}) were measured in needles with a portable gas exchange system (CIRAS-2, PP Systems, Herts, UK). Measurements were performed at controlled cuvette CO_2 concentration (C_a) of 400 μmol mol^{-1}, photosynthetic quantum flux density incident on the leaf surface of 1300 μmol photons m^{-2} s^{-1}, and ambient relative humidity.

2.3 Analysis of monoterpenes and sesquiterpenoids and resin acid contents

For monoterpene and sesquiterpenoid analysis, frozen needles were cut into small pieces, introduced in vial tubes, and extracted twice with *n*-hexane at room temperature for 2 h. The extracts were filtered (PTFE 0.20 μm), and 1 μL was injected in a gas chromatography-mass spectrometer (GC-MS) (Agilent Technologies GC 6890 Series, MSD 5973N). 1-Chlorooctane was added as an internal standard.

Resin acids were extracted from freeze-dried and powdered needles, basically following the methodology of Gref and Ericsson (1985). One hundred milligrams was extracted with diethyl ether/petroleum ether (1:1, *v/v*) and twice with diethyl

ether. Supernatants were combined and evaporated to dryness under a stream of nitrogen. The residue was redissolved in 1 mL of diethyl ether and filtered (0.22 μm Nylon filter). Heptadecanoic acid was used as an internal standard. An aliquot of extract was dried again before derivatization that was performed with *N,O*-bis-(trimethylsilyl)-trifluoroacetamide with trimethylchlorosilane (BSTFA-TMS)/acetonitrile (1:1; *v/v*) (90 min, 75 °C). One microliter of the solution was injected in the GC-MS.

Separation of the compounds was carried out with a HP-5MS column (30 m, 0.25 mm, 0.25 μm). The temperature program for monoterpenes and sesquiterpenoids was 60 °C isothermal 1 min, increased to 246 °C at 3 °C min^{-1}. For resin acids, the temperature program was 60 °C isothermal 2 min, increased to 270 °C at 5 °C min^{-1}. Helium was used as a carrier gas. Injector temperature was 220 °C in split mode (ratio 1:10). The transfer line temperature was 240 °C. The mass selective detector was operated in electron impact mode at 70 eV. MS source and MS quad temperatures were 230 and 150 °C, respectively, for monoterpenes and sesquiterpenoids and 250 and 200 °C, respectively, for resin acids. An MSD ChemStation (ver. E.02.00) workstation was used for data capture and processing with Scan and the selected ion monitoring (SIM) techniques. The identification of the chemical constituents was based on comparisons of their relative retention times and mass spectra with those obtained from authentic standards and/or mass spectra in NIST MS search 2.0 and Wiley 275 libraries. Further, the Kovats index (KI) was calculated using a homologous series on *n*-alkanes (Adams 2009).

2.4 Abscisic acid determination

Fifty milligrams of lyophilized tissue was extracted twice with 3 mL of acetone/water/formic acid (80:19:1, *v/v/v*) (30 min, 2000 rpm) and centrifuged (15 min, 3000 rpm, 4 °C). The acetone was evaporated under a nitrogen stream, and the remaining aqueous extract was adjusted to 1.2 mL with Milli-Q water. The extract was partitioned twice with diethyl ether, dried under nitrogen, and redissolved in 500 μL acetonitrile/water (30:70, *v/v*) containing 0.1% formic acid. The extract was filtered (0.45-μm Nylon filter) and diluted (1:5) before the UPLC system injection (ACQUITY, Waters). One nanograms per microliter of [$^2H_6$]-ABA (Gómez-Cadenas et al. 2002) was added as an internal standard.

An Excel 2 C18-AR column (50 × 2.1 mm, ACE, UK) was used, and the temperature was set at 36 °C. The mobile phase was composed of methanol 70% (solvent A) and acetonitrile 90% (solvent B) which contain 0.1% formic acid. The solvent gradient was programmed to change linearly: 0–1 min, 100% A; 1–2.5 min, 100–50% A; 2.5–2.8 min, 50% A; and 2.8–3 min, 100% A, for 2 min equilibration prior to the next injection. The solvent flow rate and injection volume were set at 0.15 mL min^{-1} and 20 μL, respectively.

Mass spectrometric analyses were performed by an ACQUITY Waters triple quadrupole mass spectrometer. The electrospray conditions were as follows: Polarity ES, capillary voltage 3.0 kV, source temperature 120 °C, desolvation gas temperature 350 °C, cone gas flow 90 L h^{-1}, and desolvation gas flow 900 L h^{-1}. High-purity nitrogen was used as the nebulizer and auxiliary gas, and argon was used as the collision gas. Analysis of ABA was based on appropriate multiple reaction monitoring (MRM) mode: [^{2}H$_6$]ABA and ABA were monitored at m/z transitions of 269 → 159, 225, and 263 → 153, 219, respectively. The cone voltage (V) and collision energies (eV) were optimized to maximize the transition signals: [^{2}H$_6$]ABA 20 V and 12 eV, and ABA 15 V, 10 eV, and 12 eV. The raw data were acquired and processed with a MassLynx 4.1 software. Quantification was performed using calibration curves based on the ABA/[^{2}H$_6$]ABA ratio of standard solutions.

2.5 Quantification of proline, shikimic acid, and total polyphenols

Needle proline contents were determined following the procedure of Bates et al. (1973). Fifty milligrams of lyophilized tissue was processed with 3% (w/v) sulfosalicylic acid, and an aliquot of the supernatant was colored by the addition of ninhydrin. The heated ninhydrin-proline complex (100 °C, 1 h) was mixed with 4 mL of toluene. The condensation product was measured spectrophotometrically at 520 nm (Shimadzu UV-1700). The proline concentration was calculated using a L-proline standard curve.

Shikimic acid was determined together with resin acids via the GC-MS analyses. Total polyphenol content was estimated in an aliquot of the extracts obtained to determine ABA. Two hundred milliliters was diluted with Milli-Q water and mixed with Folin-Ciocalteau reagent. The absorbance was measured at 725 nm (Velioglu et al. 1998). Results were expressed as milligrams of gallic acid equivalents (GAEs) per gram dry weight.

2.6 Determination of physiological phases

Three physiological phases (I, II, and III) were established to analyze the metabolic compounds extracted from the needles. To define the limits of each phase, we took the points of physiological change of the relationships between Ψ_{pd} and gas exchange (g_s and A). The first limit ($\Psi_{pd,1}$), between phases I and II, was defined as the change in the slope of the relationship between Ψ_{pd} and g_s. $\Psi_{pd,1}$ was calculated as the join-point of a two-linear segmented model. This model is a non-linear model that fits a curve compound of two linear models with different slopes. The point at which the switch between the two functions occurs is generally called a joint-point, which can easily be associated with a change in the trend of the studied variable (Schabenberger and Pierce

2002). The second limit ($\Psi_{pd,2}$) was defined as the point where A reached first time a value of zero since the application of the water deficit treatment. $\Psi_{pd,2}$ was calculated from a linear model adjusted to the relationship between Ψ_{pd} and A.

2.7 Statistical analyses

While the contents of proline, shikimic acid, and ABA were related directly to Ψ_{pd}, terpenoid contents were grouped taking into account the delineation of the three phases. One-way ANOVAs were performed to compare the contents of the metabolic compounds between the different physiological phases (I, II, and III). Multiple comparisons were carried out among the different phases using the post hoc Tukey's honestly significant difference test. For a more concise view, a principal component analysis (PCA) was made with those metabolic compounds that changed statistically along the soil water deficit period. Data was expressed as means ± standard error. Statistical analyses were carried out using SAS version 8.0 (SAS, Cary, NC, USA), and all statistical analyses were considered significant at $P < 0.05$.

3 Results

3.1 Physiological phases of water deficit development

The relationship between predawn water potential (Ψ_{pd}) and stomatal conductance (g_s), fitted by a two-linear segmented model ($R^2 = 0.92$, $P < 0.001$), had a joint-point at ca. −0.6 MPa (Fig. 1a). For the Ψ_{pd} range of 0 to −0.6 MPa, g_s steeply decreased from maximum values of 500 mmol H$_2$O m^{-2} s^{-1} to values around 150 mmol H$_2$O m^{-2} s^{-1}. For Ψ_{pd} values lower than −0.6 MPa, g_s decreased with a smaller slope towards values of 50–20 mmol H$_2$O m^{-2} s^{-1}. The linear relationship between net CO$_2$ uptake (A) and Ψ_{pd} ($R^2 = 0.87$, $P < 0.001$) (Fig. 1b) showed a constant decrease in A from maximum values around 12 μmol CO$_2$ m^{-2} s^{-1} to zero value at ca. −1.8 MPa. Thus, Ψ_{pd} and gas exchange characteristics suggested the existence of two key physiological modifications along the decrease in soil water availability in *P. sylvestris*. The first one was at $\Psi_{pd,1}$ = −0.6 MPa, at which point the rate of decrease in g_s with Ψ_{pd} changed, and the second one was at $\Psi_{pd,2}$ = −1.8 MPa, where photosynthesis reached values of zero. These two criteria were used to define the three physiological phases of soil water deficit development: phase I ($\Psi_{pd} > −0.6$ MPa), denoting a mild water deficit; phase II (−0.6 > Ψ_{pd} > −1.8 MPa), denoting a moderate water deficit with significantly reduced values of g_s and A; and phase III (−1.8 > Ψ_{pd} > −2.4 MPa), denoting a severe water deficit with completely suppressed photosynthesis. For the control trees, the average (± SE) values of Ψ_{pd}, g_s, and A observed through the experiment were −0.39 ± 0.01 MPa,
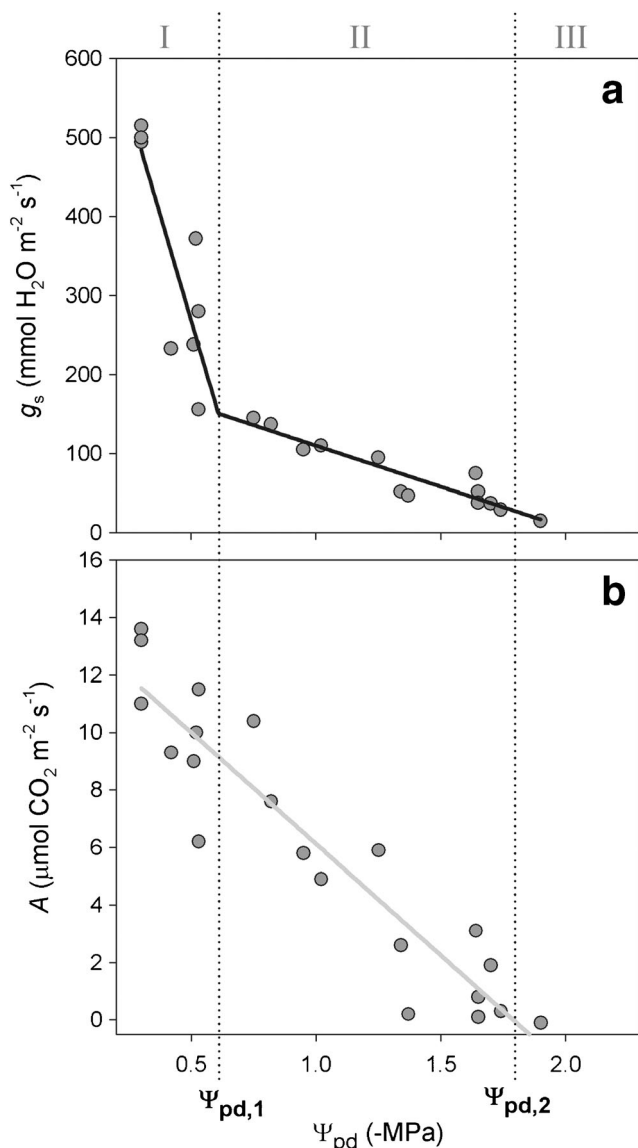
Fig. 1 Relationships of predawn shoot water potential (Ψ_{pd}) with stomatal conductance (g_s) (**a**) and with net CO_2 uptake (A) (**b**) measured in five Scots pines along a water deficit period. *I–III*: physiological phases considered. $\Psi_{pd,1}$: limit between phases I and II defined as the change in the slope of the relationship between Ψ_{pd} and g_s. $\Psi_{pd,2}$: limit between phases II and III defined as the point where A is zero

397 ± 15 mmol H_2O m^{-2} s^{-1}, and 11.1 ± 0.2 µmol CO_2 m^{-2} s^{-1}, respectively.

3.2 Modification of needle secondary metabolism through the water deficit phases

Average contents of most monoterpenes and sesquiterpenoids increased with increasing the severity of water deficit (Table 1), except for a few compounds (e.g., sabinene or γ-muurolene) that did not vary significantly among the phases (Table 1). The statistical analysis revealed significant

differences in the content of volatile terpenes (e.g., α-pinene, limonene, or β-caryophyllene) already between phases I and II ($P < 0.05$). In contrast, the content of resin acids tended to decrease throughout the phases (Table 1). This apparent decrease was statistically significant ($P < 0.05$) for three of the seven resin acids analyzed (palustric, levopimaric, and neoabietic acids). For all terpenoids, mean values of control trees did not differ significantly from the averages of the water-deficient trees at phase I (Table 1).

During water deficit phases I and II, proline and shikimic acid remained practically constant with mean values around 0.35 µmol g^{-1} and 0.82 mg g^{-1}, respectively. In phase III, needle proline and shikimic acid content rose up to mean values of 1.24 ± 0.06 µmol g^{-1} and 2.40 ± 0.12 mg g^{-1} respectively, i.e., ca. threefold higher than the mean values for phases I and II (Fig. 2a, b). There was no significant effect of soil water deficit treatment on total polyphenol content (Fig. 2b). Abscisic acid (ABA) content was statistically invariable (around 1.0 µg g^{-1}) for a Ψ_{pd} range of 0 to -1.3 MPa. Beyond -1.3 MPa, ABA content started to increase exponentially (Fig. 2c). ABA content reached at the end of phase II 2.8 ± 0.5 µg g^{-1} and in phase III 11.7 ± 0.0 µg g^{-1} (both statistically different from the initial values, $P < 0.05$). Control trees had mean values of proline, shikimic acid, and ABA of 0.38 ± 0.02 µmol g^{-1}, 0.61 ± 0.07 mg g^{-1}, and 0.73 ± 0.15 µg g^{-1}, respectively, through the experimental period. These control values were not statistically different from the mean values of water-deficient trees at phases I and II, but they were significantly lower than those at phase III ($P < 0.05$).

A PCA based on those compounds that changed statistically along the water deficit period (monoterpenes and sesquiterpenoids, proline, shikimic acid, and ABA) indicated that the first and second axes accounted for 56 and 29% of the total variation, respectively (Fig. 3). While we detected a segregation between the three physiological phases established (I–III), values of control trees were not segregated from the values of phase I (Fig. 3).

4 Discussion

4.1 Soil water deficit severity in *Pinus sylvestris*: mild water deficit primarily alters photosynthetic characteristics

Modifications in needle predawn water potential and gas exchange characteristics defined two important physiological thresholds during the water deficit period of *P. sylvestris*. The first threshold was obtained at -0.6 MPa and about 70% of stomatal closure (Fig. 1a). The second threshold was observed at -1.8 MPa, where plants reached a zero net CO_2 uptake. The first, mild water deficit phase (Ψ_{pd} range from 0 to -0.6 MPa) was characterized by significant reductions in stomatal conductance (from 500 to 150 mmol

Table 1   Average (± SE) concentrations (µg g^{-1}) of monoterpenes, sesquiterpenoids, and resin acids of three control trees and five water deficiency-treated trees during three physiological phases (I–III)

|	Terpene	Control trees	Water-deficient trees		
			I $\Psi_{pd} > -0.6$	II $-0.6 > \Psi_{pd} > -1.8$	III $-1.8 > \Psi_{pd} > -2.4$
Monoterpenes (µg g^{-1})	Tricyclene	21.4 ± 1.5a	23.3 ± 2.3a	35.3 ± 3.9b	44.9 ± 6.4b
	α-Thujene	1.10 ± 0.09a	1.19 ± 0.12a	1.83 ± 0.17b	2.41 ± 0.31c
	α-Pinene	275 ± 18a	266 ± 24a	456 ± 67b	528 ± 98b
	Camphene	49.8 ± 3.9a	53.9 ± 5.6a	76.8 ± 69.0b	94.4 ± 13.0b
	Sabinene	4.7 ± 0.5a	4.6 ± 0.6a	5.78 ± 0.72a	6.84 ± 1.02a
	β-Pinene	29.0 ± 2.1a	47.1 ± 7.4ab	62.3 ± 8.6b	66.3 ± 14.6b
	Myrcene	19.9 ± 1.8a	19.2 ± 2.2a	30.3 ± 3.4b	39.7 ± 5.5b
	α-Phellandrene	0.49 ± 0.04a	0.44 ± 0.06a	0.84 ± 0.11b	1.15 ± 0.21b
	Limonene	5.22 ± 0.50a	5.63 ± 0.71a	11.1 ± 1.52b	11.2 ± 1.92b
	(Z)-β-Ocimene	0.57 ± 0.05a	0.55 ± 0.08a	1.23 ± 0.29b	1.07 ± 0.17b
	(E)-β-Ocimene	5.66 ± 0.53a	7.55 ± 1.40ab	11.12 ± 1.52b	11.94 ± 2.25b
	Terpinolene	0.39 ± 0.04a	0.42 ± 0.05a	1.23 ± 0.29b	1.29 ± 0.46b
Sesquiterpenoids (µg g^{-1})	β-Elemene	0.93 ± 0.08a	1.01 ± 0.11ab	1.46 ± 0.19bc	1.68 ± 0.28c
	β-Caryophyllene	11.7 ± 0.5a	8.8 ± 0.6a	14.0 ± 1.9b	17.1 ± 2.5b
	β-Copaene	0.078 ± 0.007a	0.072 ± 0.009a	0.20 ± 0.06b	0.15 ± 0.103ab
	Aromadendrene	0.047 ± 0.005a	0.057 ± 0.015a	0.18 ± 0.04b	0.26 ± 0.081b
	α-Humulene	5.01 ± 0.22a	4.25 ± 0.23a	7.23 ± 1.06b	8.70 ± 1.485b
	γ-Muurolene	0.54 ± 0.05a	0.48 ± 0.06a	1.63 ± 0.53a	1.47 ± 0.95a
	Germacrene D	20.7 ± 1.6a	16.0 ± 1.9a	26.2 ± 5.9a	20.7 ± 9.3a
	β-Selinene	0.28 ± 0.02a	0.37 ± 0.09a	0.77 ± 0.14b	1.03 ± 0.283b
	Bicyclogermacrene	0.25 ± 0.02a	0.23 ± 0.02a	0.51 ± 0.11b	0.60 ± 0.22b
	α-Muurolene	0.72 ± 0.05a	0.71 ± 0.08a	0.99 ± 0.10b	1.04 ± 0.155b
	γ-Cadinene	0.64 ± 0.05a	0.73 ± 0.09a	1.73 ± 0.40b	1.97 ± 0.678b
	δ-Cadinene	0.42 ± 0.03a	0.54 ± 0.06a	1.26 ± 0.30b	1.54 ± 0.58b
	Germacrene D-4-ol	0.54 ± 0.08a	0.62 ± 0.10a	0.95 ± 0.13b	0.94 ± 0.180b
	α-Cadinol	0.15 ± 0.01a	0.25 ± 0.02a	0.42 ± 0.10a	0.43 ± 0.149a
Resin acids (µg g^{-1})	Pimaric acid	81 ± 8a	100 ± 11a	91 ± 10a	89 ± 17a
	Isopimaric acid	22 ± 2a	24 ± 4a	22 ± 3a	23 ± 3a
	Palustric acid	175 ± 18a	195 ± 38a	91 ± 17b	25 ± 8c
	Levopimaric acid	513 ± 34a	606 ± 62a	405 ± 62a	152 ± 35b
	Dehydroabietic acid	667 ± 43a	743 ± 102a	747 ± 77a	730 ± 84a
	Abietic acid	183 ± 12a	205 ± 24a	178 ± 23a	138 ± 22a
	Neoabietic acid	2007 ± 213a	2608 ± 469a	1495 ± 206b	523 ± 143c

Phases are defined according to changes in predawn water potential (Ψ_{pd}), stomatal conductance, and net assimilation rate (Fig. 1). Compounds in each group are arranged according to their retention time, from shortest to longest. Lowercase letters indicate statistically significant differences (Tukey test, $P < 0.05$) in compound contents among the phases

H$_2$O m^{-2} s^{-1}) with moderate changes in photosynthesis rates over a very narrow range of Ψ_{pd} (Fig. 1). These physiological changes would have induced at the end of phase I, a significant reduction of plant transpiration and a higher instantaneous water use efficiency. Additionally, the strong decrease in stomatal conductance over a very narrow range of Ψ_{pd} has been reported previously in *Pinus radiata* and interpreted as an evidence of strongly isohydric stomatal response to water deficit (Brodribb and McAdam 2013).

During the mild phase, the average values of all metabolite contents did not significantly differ from those in control trees.
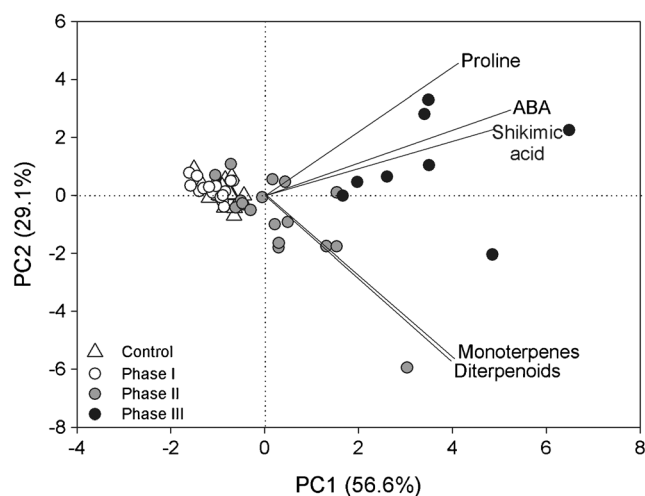
4.2 Moderate soil water deficit induces changes in needle secondary metabolism

Phase II ($-0.6 > \Psi_{pd} > -1.8$ MPa) was characterized by strongly reduced stomatal conductance (below 150 mmol H$_2$O m^{-2} s^{-1})
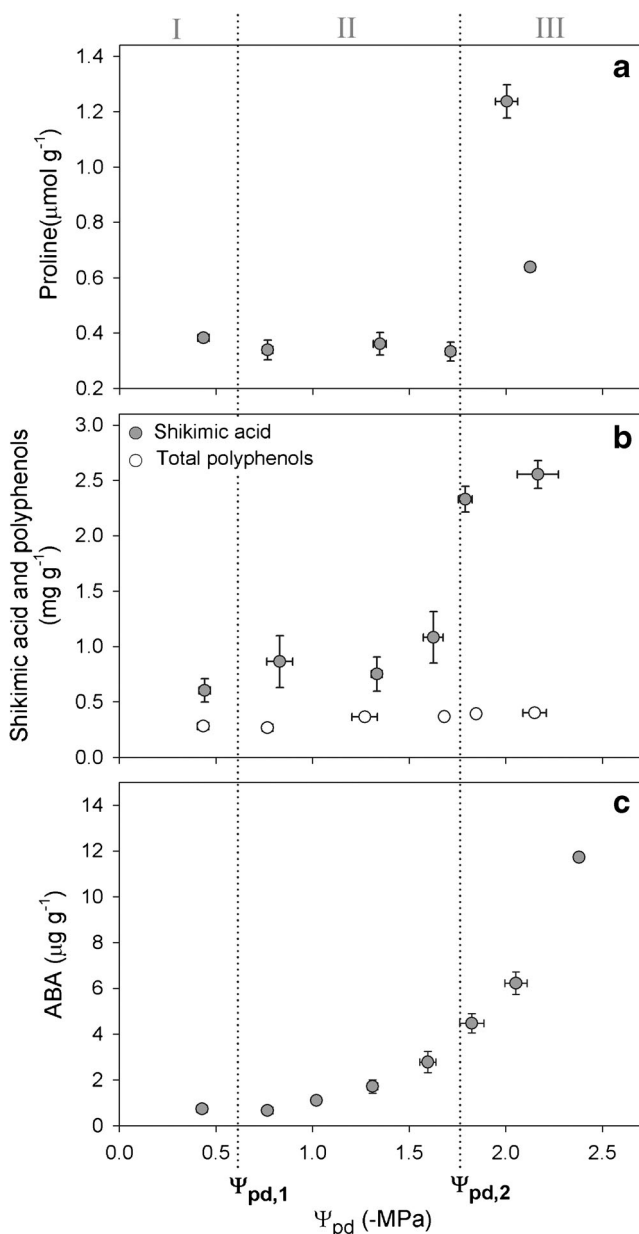
Fig. 2 Relationships between predawn shoot water potential (Ψ_{pd}) and average ($\pm$ SE) **c**ontents of proline (**a**), shikimic acid and total polyphenols (**b**), and abscisic acid (ABA) (**c**) in needles of five water-deficient Scots pines. $\Psi_{pd,1}$, $\Psi_{pd,2}$, and *dotted lines* indicate the limits of the three physiological phases considered (*I*, $\Psi_{pd} > -0.6$ MPa; *II*, $-0.6 > \Psi_{pd} > -1.8$ MPa; and *III*, $-1.8 > \Psi_{pd} > -2.4$ MPa)

Fig. 3 Relationship between the first two principal components (PC1 vs. PC2) of the principal component analysis (PCA) computed on needle metabolic compound variation. *Symbols* correspond to control (*triangle*) or water-deficient trees (*circle*) and are *color-coded* according to each physiological phase: I, *white*; II, *gray*; III, *black*

and net CO_2 uptake rate (from 9 to 0 $\mu mol\ CO_2\ m^{-2}\ s^{-1}$) (Fig. 1). At this stage, trees started to accumulate most of the volatile terpenoids analyzed (monoterpenes and sesquiterpenoids). As stomatal aperture cannot significantly control the emissions of hydrophobic compounds (Niinemets and Reichstein 2003), the accumulation of these volatile terpenes (although mostly stored in the resin ducts) cannot be explained by massive reductions in stomatal closure and rather suggests an increment in their synthesis. Such an increment has been reported in several cases in

plants treated with a water deficiency (Lluisà and Peñuelas 1998) and suggested to reflect changes in the source/sink balance due to inhibition of growth (Lerdau et al. 1994; Constable et al. 1999). Indeed, this level of moderate soil water deficit usually substantially decreases the growth of conifers (e.g., Turtola et al. 2003) and can lead to shifts in the translocation of the carbon fixed in photosynthesis to the formation of secondary compounds (Lorio and Sommers 1986; Turtola et al. 2003; McDowell 2011; Niinemets 2016). From an ecological perspective, greater contents of monoterpenes and sesquiterpenoids could enhance the tree resistance to herbivory and pathogens (Litvak and Monson 1998; Cheng et al. 2007).

Regarding resin acids, two of them, palustric and neoabietic acid, decreased analogously as the observations of Hodges and Lorio (1975) on *Pinus taeda* xylem oleoresin. Other studies had also reported variations in the concentration of wood resin acids with increasing the severity of water deficit (Turtola et al. 2003) or an increase in resin acids in needles after the onset of a water stress decay process (Sanz et al. 2014). However, to the best of our knowledge, no studies have reported a decrease in resin acids in conifer needles during a water deficit period. Such a decrease might reflect reprogramming of the defense metabolite pathway between syntheses of larger (resin acids) and smaller (monoterpenoids) plastidial isoprenoids, but so far, the way such metabolic regulation could occur is not known (Rajabi Memari et al. 2013). Terpenoids are components of constitutive defenses against pests and pathogens with non-volatile resin acids involved in direct defenses (reducing palatability for herbivores and sealing wound sites) (Hodges and Lorio 1975; Wainhouse 2004) and with more volatile monoterpenes and sesquiterpenoids involved in both direct (reducing palatability and serving as herbivore repellents) and indirect defenses

(signals for herbivore enemies) (Lewinsohn et al. 1993; Litvak and Monson 1998). Thus, changes in the composition of terpenoids can importantly modify the needle capacity for direct and indirect defenses.

Finally, the slight increase of ABA observed at the end of phase II (Fig. 2c) indicated the beginning of the contribution of ABA in stomatal closure. In order to reduce water loss in the plant, ABA affects guard cells by inducing osmotic efflux and therefore turgor loss and reduction of stomatal aperture. Stomatal closure detected before ABA concentration rising may respond only to the change in water potential at the leaf level (Buckley 2005; Brodribb and McAdam 2013).

4.3 Severe soil water deficit effects on needle secondary metabolism

Under severe soil water deficit, in phase III ($-1.8 > \Psi_{pd} > -2.4$ MPa) characterized by minimum values of stomatal conductance and zero or close to zero net CO_2 uptake (Fig. 1), there was a sudden increase in the concentration of proline, shikimic acid, and ABA. The accumulation of proline in phase III confirms the suggestion that P. sylvestris was under a severe soil water deficit stress (Vilagrosa et al. 2010). On the other hand, the carbon fixed during phase II under a moderate water deficit could also be used in phase III for synthesis of shikimic acid, one of the key sources of carbon for defense instead of growth (Becerra-Moreno et al. 2015). Regarding the rapid increase in foliar ABA levels, Brodribb and McAdam (2013) suggested that such a major increase results in complete stomatal closure and drastic reduction of water loss. Both strategies, the increase of defense compounds and the prevention of desiccation by stomatal closure, would benefit the tree during a short time period. If water deficit persists, with zero or close to zero photosynthetic carbon uptake and a continuous demand for carbohydrates to produce secondary compounds and to maintain metabolism, the carbohydrate reserves will be depleted, leading eventually to starvation or an inability to cope with biotic attacks (McDowell 2011). However, the time it takes to succumb to water deficit will ultimate depend on the size of the reserve pools (Niinemets 2010).

Nevertheless, contrary to shikimic acid, total polyphenol content was unaffected in the water potential range of our experiment. Given that polyphenols are derived from shikimic acid, and as the shikimic acid content did not change significantly until leaf water potential was less than −1.8 MPa, there is still a possibility that an increase in total needle polyphenol content could occur at more negative water potential values than the ones reached in this study. Alternatively, enhanced shikimic acid contents might reflect increased transformation of shikimic acid into lignin (Becerra-Moreno et al. 2015) as observed in Pinus canariensis (Grill et al. 2004). This could serve as a mechanical barrier against pathogens (Monties and Fukushima 2001). In addition, more strongly lignified cell walls can enhance needle mechanical resistance avoiding collapse under excess water stress (Cochard et al. 2004) and have

a greater change in water potential for a given change in needle water content, thereby allowing for extraction of water from drier soil (Niinemets 2001). Further studies looking into lignin formation and changes in needle water extraction capacity are necessary to test these hypotheses.

Finally in phase III, the concentration of most volatile terpenes that had increased significantly in phase II remained statistically constant, while the concentration of some resin acids (palustric, levopimaric, and neoabietic acid) kept decreasing significantly, which might be a continuation of the reprogramming process of the defense metabolite pathway which started in phase II.

5 Conclusion

This study establishes a direct correspondence between physiological parameters and secondary metabolites through their simultaneous measurements along a soil water deficit period. Our results allowed the distinction of clearly physiological phases in response to water deficit evolution. In this way, a moderate water deficit (phase II) would be associated to increased levels of needle volatile terpenes with respect to phase I, whereas a severe water deficit (phase III) would be associated to an increment in proline, shikimic acid, or ABA. Although there are biochemical processes related to secondary metabolism and plant-pathogen relations that are still poorly understood and deserve further investigations, this work confirms that most of the secondary metabolites did not increase until the plant was under a moderate to severe water deficit.

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