Monoterpene responses to interacting effects of drought stress and infection by the fungus *Heterobasidion parviporum* in two clones of Norway spruce (*Picea abies*)☆

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ARTICLE INFO

ABSTRACT

To study simultaneous biotic and abiotic stress effects on monoterpene mediated defence response, four-year-old seedlings of two clones of *Picea abies* (c386: "relatively resistant" c386; c171: "relatively susceptible" clone 171) were inoculated with an isolate of rot fungus *Heterobasidion parviporum* and simultaneously subjected to a drought stress recovery cycle. Fungal growth into the wood was generally higher in well-watered (W) than in drought-stressed (D) plants. As predawn water potential decreased, gas exchange and maximal photochemical efficiency of PSII also declined. Water potential and chlorophyll fluorescence showed differences between clones in D plants, c386 being more tolerant than c171, while stomatal conductance and net photosynthesis differed between clones in W plants; c171 showing higher values than c386. A recovery of physiological functions was observed after re-watering. The clones showed different constitutive monoterpene profiles. The infection generally caused some extensive compositional changes in the relative contents of several monoterpenes and their enantiomers; also, sterile infection (wounded only samples) affected the relative proportions of monoterpenes, although to a lesser extent than reactions elicited by the fungus. The trend in monoterpene response to disease treatments was the same in D and W plants of both clones. In particular, higher proportion of δ-3-carene was found constitutively in c386 in comparison with c171; besides, infection with *H. parviporum* increased the relative contents of δ-3-carene in all the samples, even if proportions of this monoterpene in wounded plus inoculated tissues of c386 were significantly higher than cortical tissues from non-inoculated branches only in W seedlings 44 days after treatment.

Although drought stress decreased the total absolute contents of monoterpenes, total monoterpene concentrations significantly increased in response to infection by *H. parviporum*.

1. Introduction

Regional-scale vegetation mortality events appear to be increasing in a variety of biomes throughout Earth, and are frequently associated with increased temperatures, droughts, and often (but not always) with outbreaks of biotic agents, such as insects and pathogens (Allen et al., 2010; Das et al., 2016; Wong and Daniel, 2017). The physiological mechanisms underlying tree mortality involve the water and carbohydrates storage and transport systems (McDowell et al., 2011). In temperate zones, drought is associated with enhancement of disease development in several tree species by many pathogens that may affect both the tree crown and the root system (Madar et al., 1989). Indeed, the heat wave and the severe drought that Western Europe experienced in 2003 had serious consequences for forest health (Desprez-Loustau et al., 2006).

Based on predictions of future climate change, increased frequency and severity of drought in combination with other disturbances (including fungal diseases) are expected to affect the health of Norway spruce (*Picea abies* (L.) Karst.) (Hanewinkel et al., 2013), the most important commercial conifer in Europe. This forest species spreads from Siberia to the Northern and Central Europe extending the area of its natural range area to the Alps and Balkan Peninsula. In temperate European forests dominated by Norway spruce, recent wind storms and beetle outbreaks have caused widespread damage (Seidl et al., 2011).

The distribution area of Norway spruce fits well with the natural range of the stem and root rot fungus *Heterobasidion annosum sensu lato*. This fungus is considered one of the most harmful pathogens for conifer trees in the Northern hemisphere causing remarkable losses of merchantable wood volumes both in plantations as well in natural forests (Woodward et al., 1998). Norway spruce stands affected by *H. annosum* are more susceptible to bark beetle and are more likely to suffer from windthrow (Korhonen and Stenlid, 1998).

Exposure to drought may also make trees more vulnerable to insects (Gaylord et al., 2013) and pathogens (Niinemets, 2010). Drought stress enhanced the development of *Diplodia pinea* on Italian cypress (Madar et al., 1995), *Fusarium compactum* on *Cupressus sempervirens* (Madar et al., 1996) and *Sphaeropsis sapinea* on *Pinus resinosa* (Blodgett et al., 1997a,b). A combination of mass inoculation (*Leptographium wingfieldii*) and drought stress resulted in a dramatic loss of stem hydraulic conductivity in *Pinus sylvestris* that was paralleled by conspicuous damage to the sapwood (Croisé et al., 2001). Water-stressed plants of *Eucalyptus globulus* were more susceptible to *Neofusicoccum eucalyptorum* than non-stressed ones, drought-primed plants being slightly less susceptible to the attack of this pathogenic fungus than the non-primed ones (Barradas et al., 2018). In addition to drought, other factors triggered by climate change may favour certain pathogens, i.e., warming temperature, pathogen migration and vegetation shift (La Porta et al., 2008).

The influence of drought on *H. annosum*, *Phytophthora cinnamomi*, and *Armillaria* spp. was outlined by several authors (Lindberg and Johansson, 1989; Brasier et al., 1993; Wargo and Harrington, 1991; Puddu et al., 2003). Inoculations experiments with seedlings of Norway spruce showed that drought may affect the rate of growth of *H. parviporum* inside the tree (Lindberg and Johansson, 1992). Biotic stress caused by pathogen infection triggers the expression of defence related genes and induces systemic host responses (Hammerschmidt, 1999). Gori et al. (2013) found that, *H. parviporum* is somewhat more aggressive in adult trees growing at low than high elevation, causing a more rapid decline of tree growth and decreasing the ability of host Norway spruce to cope with drought. Increasing drought has the potential to favour diseases caused by *Heterobasidion* spp., eventually leading to tree mortality (Linares et al., 2010).

In conifers, induced stress responses include changes in anatomy and woody phloem as well as changes in the composition and flow of resins (Sancho-Knapik et al., 2017; Gershenzon and Dudareva, 2007; Phillips and Croteau, 1999). Resins are complex mixtures of different classes of terpenoids containing the concentrations of monoterpenoids and diterpene acids, and small quantities of sesquiterpenoids (Langenheim, 1994). As the largest class of natural compounds, terpenes are implicated in multiple ecological roles; however, many of these compounds serve primarily as defence substances since they show antimicrobial, fungistatic and insecticidal properties (Cobb et al., 1968; Langenheim, 1994).

Heterobasidion infection in Norway spruce is challenged through accumulation of terpenoid classes and phenolic compounds at the site of infection (Arnerup et al., 2011). The formation of this reaction zone can be expensive in terms of carbon (Oliva et al., 2012), thus inducing growth reductions on attacked trees (Oliva et al., 2010). Moreover carbon-expensive barriers may also imply reduced physiological capacity of trees to cope with drought stress. In addition, genotypic differences in Norway spruce can be expressed in growth traits (Ununger et al., 1988) and drought sensitivity (Karlsson et al., 1997; Modrzynski and Erikkson, 2002), as well as in infection frequency and mortality rate following inoculation with *H. annosum* (Swedjemark et al., 2001). Resistance to *Heterobasidion* in Norway spruce can be detected by inoculation tests (Dimitri, 1994; Swedjemark and Stenlid, 1994), measuring the extent of lesions developing in the wood and inner bark (Swedjemark et al., 1997) or with molecular methods (Hietala et al., 2003; Bodles et al., 2006).

The production of Norway spruce clones showing less susceptibility

to the pathogen can be promising in order to reduce losses by this pathogenic fungus (Dimitri, 1994). Among the three species of *Heterobasidion*, *Heterobasidion parviporum* Niemela & Korhonen is the most harmful species attacking with preference Norway spruce (Korhonen, 1978; Niemela and Korhonen, 1998). It causes root and butt rot of established trees. The fungus spread primarily via spores, which settle, germinate and form a mycelium on freshly cut stumps or other recently exposed wood surface. The mycelium then extends into the wood and colonizes the stump or the whole tree, including its roots. It may, then, transfer from infected stumps or trees to healthy trees via contact between roots (Korhonen and Stenlid, 1998).

In order to test the hypothesis that Norway spruce responses to fungal infection differ between clones, and are also affected by environmental conditions, we conducted a controlled environment experiment on seedlings subjected to water deficit (simulating drought stress) and inoculated with an isolate of rot fungus *Heterobasidion parviporum* in conjunction with wounding to stimulate tree defences.

Understanding resistance or susceptibility to pathogens in host genotypes and metabolic changes associated with fungal infection will provide useful information when breeding for resistance against *Heterobasidion* spp. in Norway spruce. The specific objective of this study was to characterize the effects of drought stress treatment and *H. parviporum* infection on defensive monoterpenes in Norway spruce clones, exploring the mechanisms of chemical resistance to the attack of this pathogenic fungus in plants under drought stress conditions.

2. Material and methods

2.1. Plant material

Two clones c171 and c386 of Norway spruce produced at the Forestry Research Institute of Sweden (SkogForsk) were selected for the experiment. According to preliminary tests (B. Karlsson, personal communication), clone c386 was considered "relatively resistant", whereas clone c171 was considered "relatively susceptible" to *H. parviporum* infections. Ten 4-year old ramets of each clone were grown in 4-l pots, filled with 1/3 peat and 2/3 sand, in a greenhouse at the University of Firenze (Italy, 43°46′55.44″ N, 11°13′5.41″ E); the average greenhouse temperature was 20 °C during the day and 10 °C during the night, ramets were exposed to natural light, 11 h day^{-1}. Prior to inoculation all ramets were in good condition and experienced identical light and temperature treatment.

2.2. Experimental treatment

Inoculation by an Italian isolate of *H. parviporum* was performed on two-year-old branches of each seedling (regularly watered to field capacity or drought-stressed). The inoculations were conducted on day 0 (21 October) (0 days after treatment: DAT) on two branches of each seedling by applying a fresh *Heterobasidion* colony plug, grown on malt/agar 1.5%, on a small wound (5 mm long) made in the bark, following to Swedjemark et al. (2001). Other two branches for each seedling, were wounded and inoculated with sterile water-agar.

Seedlings were randomly assigned to one of the two treatment groups: (1) five seedlings for each clone were well-watered (well-watered seedlings: W seedlings) to field capacity each day; (2) starting from a well-watered condition, on day 0 water was withheld from five drought-stressed seedlings (drought-stressed seedlings: D seedlings) for each clone for a 22-day period (until stomata closed). After 22 days from beginning the treatment (day after treatment: DAT), D seedlings were re-irrigated to avoid irreversible damage. Samples from D and W plants were collected 22 DAT, at the peak stress, and 44 DAT, at the end of the experiment.

Seedlings were arranged in a complete randomized block design in the greenhouse and pots were rotated every 3 days throughout the experiment to remove potential microclimate effects.

2.3. Fungal growth

Samples for fungal growth measurements were collected 22 and 44 DAT from W and D seedlings. Fungal wood colonization was measured by dividing the branches into 5 mm pieces from the inoculum point. The branch pieces were put in Petri plates (water/agar substrate) in a constant temperature chamber at $20 \pm 2\,°C$. After 4 days, each piece was observed at the dissecting microscope, looking for conidial form.

2.4. Physiological response

Photosynthetic parameters and water relations were measured to monitor drought stress development (1,4,8,11,15,18 and 23 DAT) and during recovery from drought stress (25, 30 and 44 DAT), on non-inoculated 2-year old branches of five replicate plants for each spruce clone – water treatment combination.

Predawn water potential was determined using a custom-made pressure chamber.

Gas exchange was measured between 9:00 and 12:00 using a portable infra-red gas analysis system (Ciras-1, PP-System, UK) equipped with conifer cuvette with light unit ($1000\,\mu mol\ photons\ m^{-2}\ s^{-1}$); ambient CO_2 was set at 370 ppm and air humidity at 80%. At the end of the experiment, the shoots were harvested and the projected needle areas measured with a Licor-3000 planimeter. Gas exchange measurements were expressed on a projected needle area basis.

Chlorophyll fluorescence was determined on intact needles between 9:00 and 12:00 with a portable PAM 2000 fluorimeter (Walts-Effeltrich Co., Germany), on the same plant material used for gas exchange measurements. Needles were sampled at the end of the dark period (8 h). Minimal (dark) fluorescence (F_0) yield was obtained upon excitation with a weak measuring beam from a pulse light-emitting diode, while maximal fluorescence yield (F_m) was determined after exposure to a 0.8 s saturating pulse of white light. Therefore, the potential photochemical efficiency of PSII (F_v/F_m) was estimated as the ratio of variable (F_v) to maximal fluorescence yield (Schreiber et al., 1994).

2.5. Monoterpenes

Cortical samples were taken from the zones inoculated with *H. parviporum* (I) and sterile water (L) (0.5 cm above and below inoculation point), as well as bark from non-inoculated branches (C) and used for terpene analysis.

Cortical samples were sampled from branches of five ramets of each clone on 0 DAT prior to inoculation, to detect the constitutive monoterpene profiles. Sampling from *H. parviporum* inoculum zone was performed 22 DAT at the peak stress (drought + fungal), from five W and five D seedlings of each clone and repeated at the end of the experimental period (44 DAT). Sampling from sterile water inoculation zone and cortical tissues from non-inoculated branches in D and W seedlings was carried out on the same dates. Approximately 0.04–0.02 g fresh weight per sample was ground and placed in glass vials with, respectively, 1–0.5 ml pentane with tridecane as an internal standard (0.2 g tridecane in 250 ml pentane).

Each vial was sealed with a Teflon septum and crimped with an aluminium cap. The vials were shaken for 24 h and filtered using FP 30/0.45 CA-S filters with 0.2 μm pores (Schleicher and Schuell), and then stored at $-20\,°C$ until analysis.

Monoterpenes were analyzed by gas chromatography-flame ionization detection (GC-FID) with a Perkin-Elmer Autosystem XL GC, and enantiomeric monoterpenes were separated on a 30 m Cyclodex-B capillary column, 0.25-mm-diameter, (J & W Scientific, CA). Analysis was carried out under the following conditions: H_2 (carrier gas) at 69 kPa; injector temperature at 230 °C; detector temperature at 250 °C. The oven temperature programming started at 4 °C (isothermal, 5 min), and increased to 200 °C, at 1.5 °C min^{-1},then to 220 °C, at 20 °C min^{-1}; the final temperature of 220 °C was maintained for 5 min. Monoterpenes

were identified by comparison of retention times with those of standards under the same conditions. Absolute amounts of terpenoids (total concentrations) were determined by comparison with the tridecane internal standard and expressed as mg g^{-1} dry weight (DW). The relative amount (proportions or percentages) of each monoterpene comprising the oleoresin mixtures was expressed as a percentage of total monoterpenes (monoterpene profiles).

2.6. Statistical analysis

Differences among fungal colonization in the wood were determined using an ANOVA and Tuckey post-hoc test.

Ecophysiological traits measured on sampling times were not normally distributed (Kolmogorov–Smirnov one-sample test) and were therefore analysed using the Friedman test, the nonparametric analogue of repeated measures analyses of variance. Analysis of covariance (ANCOVA) was performed to test for equality of correlation coefficients.

Relative contents (percentages or proportions) of monoterpenes (monoterpene profiles) and total monoterpene concentrations in each sample were calculated and compared among disease-injury categories and water-regime conditions of the plants. Normality was not achieved after an arcsine square-root transformation, so tests were performed using the non-parametric Kruskal–Wallis rank-sum test followed by the Mann–Whitney *U* Test for multiple comparisons. Monoterpenes measured on sampling times were not normally distributed (Kolmogorov-Smirnov one-sample test) and were analysed using the Friedman test.

Differences were accepted when significant at the 5% level. Statistical analyses were performed using SYSTAT 12.0 software (Systat Software Inc., USA).

3. Results

3.1. Fungal growth

No dead branches or visible lesions were recorded after inoculation. The length of fungal colonization 44 DAT differed between D and W ramets of both clones. The fungal growth in "relatively susceptible" c171, after six weeks of incubation, ranged between 10 and 40 mm in D plants and 27–72 mm in W ramets; whilst in "relatively resistant" c386 values were 19–27 and 36–70 mm, respectively. Differences between the clones were not significant. Fungal growth into the stem was generally higher in W (from 27 mm to 72 mm) than in D (from 10 mm to 40 mm) plants. Statistically different fungal growth was observed between the treatments 44 DAT, while no difference was observed 22 DAT.

3.2. Physiological response

Predawn water potential (Ψ_{pd}) stomatal conductance (G_s), net photosynthetic rate (P_n), and potential photochemical efficiency of PSII (F_v/F_m) were significantly affected during the course of the drought stress-recovery cycle (Table 1).

Predawn water potential did not differ significantly between clones, in W plants, averaging approximately -0.7 MPa throughout the experiment (Fig. 1A). Predawn water potential decreased progressively in D plants from day 7. Differences in Ψ_{pd} between the two clones (D plants) were significant only 14 ($n = 7$, $\chi^2 = 4.6$, $p < 0.05$) and 23 DAT ($n = 6$, $\chi^2 = 3.9$, $p < 0.05$). The decrease in Ψ_{pd} was stronger in "relatively susceptible" c171 than in "relatively resistant" c386 23 DAT, reaching a minimum value of -3.3 MPa and -1.4 MPa (on average), respectively. However, soon after re-watering (2 days, particularly in "relatively resistant" c386), Ψ_{pd} recovered to values similar to those observed before the application of the drought stress treatment.

In W plants values of G_s and P_n were more often higher in "relatively resistant" c386 (averaging 102 mmol m^{-2} s^{-1} and 9.6 μmol m^{-2} s^{-1},

Table 1

Statistical results of the *Friedman* test examining variation in predawn water potential (Ψ_{pd}), stomatal conductance (G_s), net photosynthetic rate (P_n), relative fluorescence (F_v/F_m) in seedlings of two Norway spruce clones during the course of a drought stress-recovery cycle (degrees of freedom = 8; 9 for ψ_{pd}): χ^2 value; *p*-value where * $p < 0.05$. NS: not significant. Well-watered (W) plants; drought-stressed (D) plants.

Variable	Clone	N. Samples	Water regime	χ^2 (*p*)
ψ_{pd} [MPa]	c171	27	W	12.09 NS
	c171	29	D	16.88*
	c386	27	W	12.69 NS
	c386	28	D	18.15*
G_s [mmol (CO_2) m^{-2} s^{-1}]	c171	41	W	11.67 NS
	c171	36	D	16.00*
	c386	41	W	10.78 NS
	c386	38	D	17.67*
P_n [μmol(CO_2) m^{-2} s^{-1}]	c171	42	W	10.67 NS
	c171	34	D	15.22*
	c386	41	W	13.56 NS
	c386	36	D	14.22*
F_v/F_m	c171	41	W	6.33 NS
	c171	37	D	17.89*
	c386	42	W	10.93 NS
	c386	40	D	17.25*

respectively) than in "relatively susceptible" c171 (averaging 83 mmol m^{-2} s^{-1} and 8.0 μmol m^{-2} s^{-1}, respectively). Gas exchange started decreasing after the imposition of drought stress, following Ψ_{pd} (Fig. 1B, C). After imposing drought stress, G_s and P_n decreased more rapidly in "relatively resistant" c386 than in "relatively susceptible" c171, reaching stable low values after 8–11 days (minimum of 14.0 and 6.9 mmol m^{-2} s^{-1}, and 1.02 and 0.96 μmol m^{-2} s^{-1}, respectively). The recovery of G_s and P_n after re-watering of D plants was slow in both

clones, reaching the values exhibited by the W plants only at the end of the experiment.

The maximal photochemical efficiency of PSII, estimated as F_v/F_m, dropped after imposing drought stress (Fig. 1D), although lagging behind Ψ_{pd} and gas exchange. The decrease of F_v/F_m, after the imposition of drought stress, started later (3 days) in "relatively resistant" c386 than in "relatively susceptible" c171, reaching minimum values of 0.5–0.6. Recovery of F_v/F_m did not differ significantly between the two clones, and it was completed 7 days after re-watering. In W plants, F_v/F_m averaged 0.8 throughout the experimental period.

The variations of G_s and P_n in response to drought stress were similar, resulting in a high correlation between gas exchange parameters (Fig. 2A). Slopes of these relationships revealed similar intrinsic water-use efficiency (evolution of P_n in relation to variation in G_s) in these clones. Likewise, plotting G_s *vs.* Ψ_{pd} did indicate a genotypic effect in the stomatal response to decreasing water availability (Fig. 2B).

3.3. Monoterpenes

The main constituents contained in the bark were, according to their retention time, in increasing order: (−)-α-pinene, (+)-α-pinene, myrcene, sabinene, δ-3-carene, (+)-β-pinene, (−)-β-pinene, (−)-limonene, *p*-cymene, (+)-limonene, γ-terpinene e terpinolene (Fig. 3). The enantiomeric composition of (+)-β-pinene was determined in samples, in which it occurred in sufficient concentrations; nevertheless, only the (−) enantiomer was detected.

3.3.1. Relative contents (percentage or proportions) of monoterpenes (monoterpene profiles)

Mann–Whitney *U* Test results on the proportions of constitutive monoterpenes from plants sampled on 0 DAT prior to inoculation and drought stress treatment, showed significant differences between five
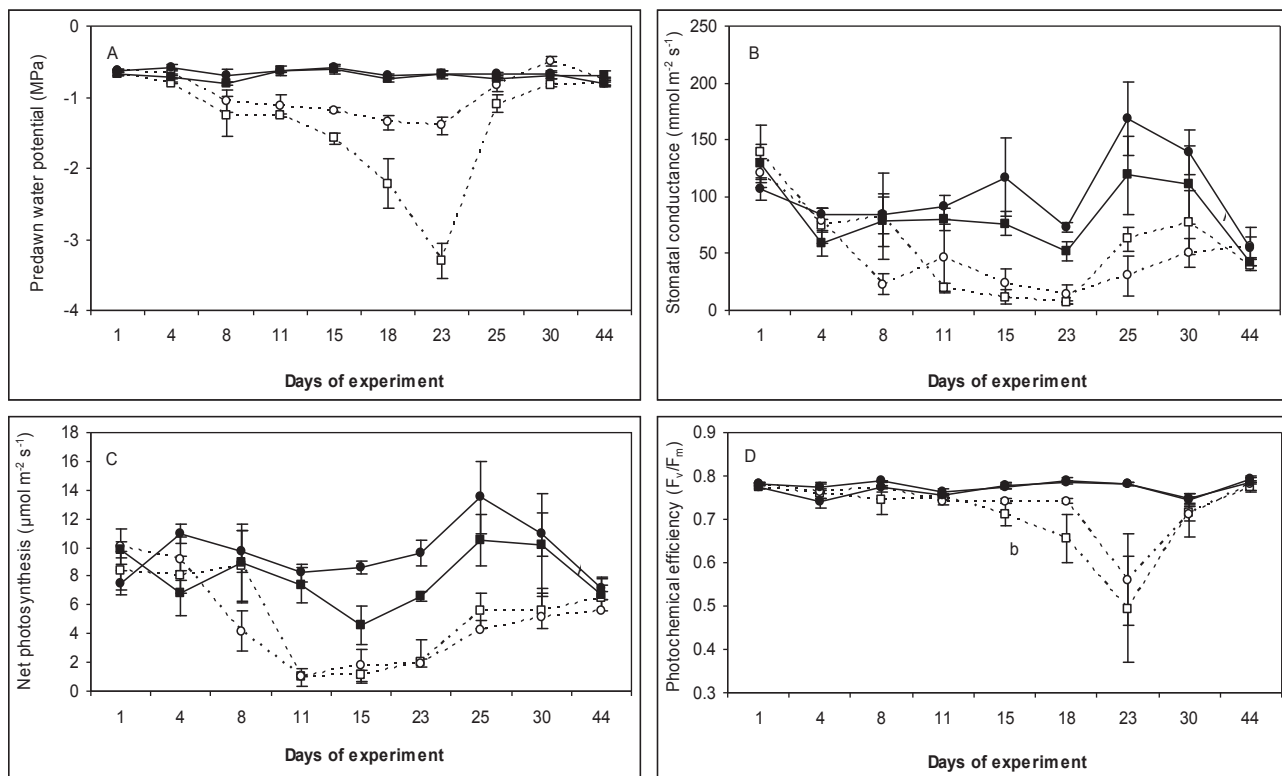
Fig. 1. Physiological responses of two clones of *P. abies* to water stress-recovery cycle. On day 0 all seedlings were watered to field capacity. Seedlings were then either watered daily (W: well-watered) or not watered (D: drought-stressed). Stressed plants were irrigated daily since day 23. Inoculations with *H. parviporum* were made on day 0. Values are means ± SE (n = 3–5). Filled circle and solid line is c386 (W), hollow circle and dashed line is c386 (D), Filled square and solid line is c171 (W), hollow squares and dotted line is c171 (D).
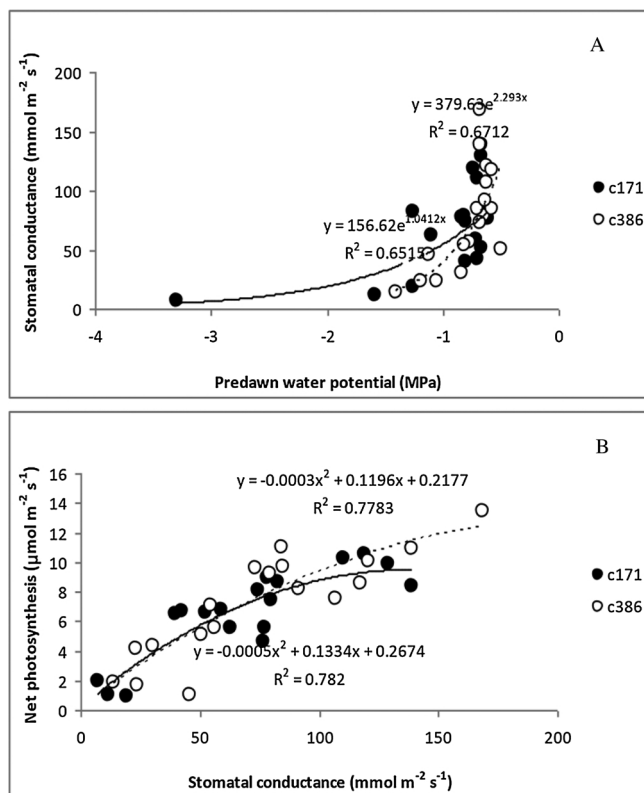
Fig. 2. Regressions between stomatal conductance and (A) predawn shoot water potential, and (B) net photosynthesis of seedlings of Norway spruce exposed to a period of drought stress. The curves were fitted using exponential or non-linear, second order regressions ($p < 0.001$).

c171 ramets and five c386 ramets: c386 characterized by high relative contents of δ-3-carene ($p < 0.01$, $\chi^2 = 5.95$), (−)-limonene ($p < 0.01$, $\chi^2 = 6.05$), γ-terpinene ($p < 0.02$, $\chi^2 = 5.21$), and terpinolene ($p < 0.01$, $\chi^2 = 6.00$); while, high proportions of (−)-α-pinene

($p < 0.01$, $\chi^2 = 6.16$), (+)-α-pinene ($p < 0.01$, $\chi^2 = 6.00$), (−)-β-pinene, ($p < 0.01$, $\chi^2 = 6.35$) and (+)-limonene ($p < 0.01$, $\chi^2 = 6.53$), were observed in c171 (Fig. 3).

Mean relative amounts ± standard error of monoterpenes within spruce clone, disease treatment, sampling time and water status condition are reported in Table 2.

C samples on the same day did not show significant variations between D and W plants, in the relative content of all the monoterpenes, except *p*-cymene in both clones and terpinolene in c171 seedlings 22 DAT (Table 3).

Also, sampling time (0 DAT, 22 DAT and 44 DAT) did not significantly change the relative contents of all the monoterpenes in C samples of W seedlings within the same spruce clone, except for sabinene in "relatively susceptible" c171 as indicated by Friedman test (Supplementary material Table S1).

Kruskal–Wallis ANOVA between disease treatments within the same spruce clone, water regime and sampling time (Supplementary material Table S2) showed that alterations in the relative contents of several monoterpenes were significantly affected by wounding and inoculation with *H. parviporum*.

With specific regard to c171, proportions of δ-3-carene, (−)-limonene and terpinolene increased in all I tissues; the relative contents of myrcene was lower in L samples than in the other treatments in W and D plants sampled respectively 22 DAT and 44 DAT; proportions of sabinene and γ-terpinene were higher in I tissues than in the other treatments of W plants sampled 44 DAT.

The relative contents of (−)-α-pinene and (+)-α-pinene decreased in I tissues in both W and D plants sampled 22 DAT and W plants sampled 44 DAT; inoculation with *H. parviporum* also induced a decrease in proportions of (−)-β-pinene in W plants on both sampling times.

Proportions of myrcene decreased in L samples in W and D plants sampled, respectively, 22 and 44 DAT.

With specific regard to c386, the proportions of (−)-limonene increased in all I tissues; higher relative contents of (+)-α-pinene were detected in I and L tissues than in C samples in D plants 44 DAT; the relative content of myrcene was higher in I samples than in the other treatments in W plants sampled 22 DAT and 44 DAT, while it decreased
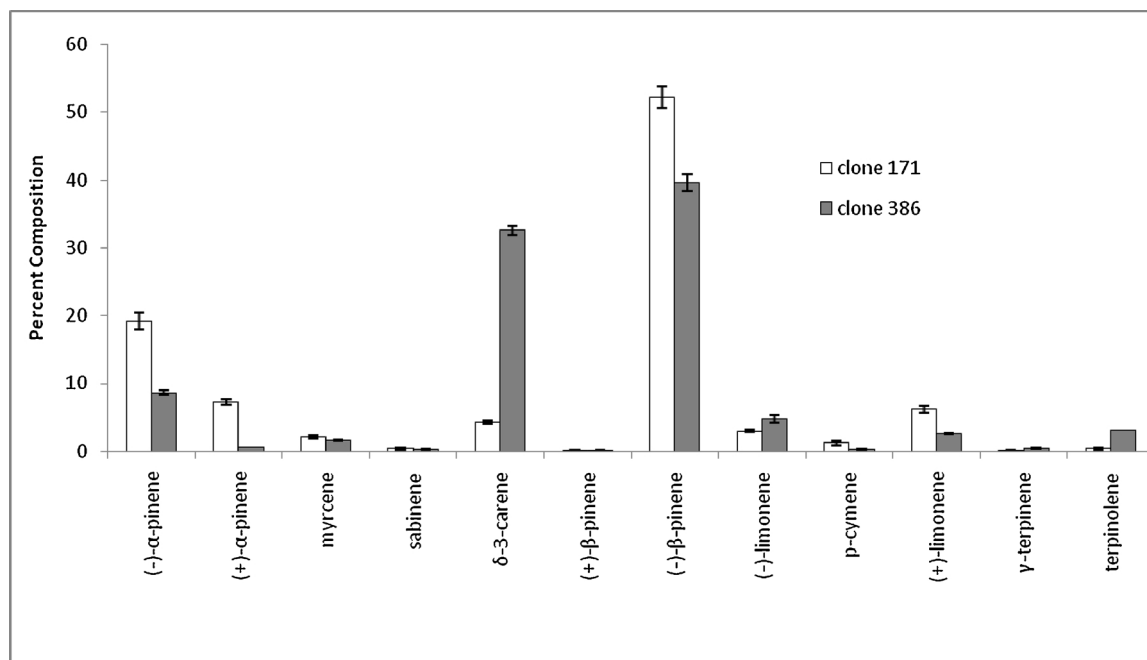

Fig. 3. Monoterpene profiles (bars indicate means ± standard error) obtained from two Norway spruce clones on day 0: "relatively susceptible" clone 171 and "relatively resistant" clone 386 to attack by *Heterobasidion parviporum*.

Table 2

Relative contents of monoterpenes (means ± standard error) of two Norway spruce clones in response to *Heterobasidion annosum*. Treatments: drought-stressed plants (D); well-watered (W); cortical tissues from non-inoculated branches (C); wounded plus inoculated with *H. parviporum* (I); wounded only (L). Different letters indicate significantly different disease treatment means (Mann–Whitney test) within the same Norway spruce clone, water regime, and sampling time. Other monoterpenes (α-thujene, camphene, α-phellandrene, tricyclene, β-phellandrene, cineole, terpineol, α-terpinene, bornylacetate) and an unknown compound representing about 4–8% of total monoterpenes were omitted in the table .

Monoterpene	D			W		
	I	L	C	I	L	C
Clone 171						
Day 22						
(−)-α-pinene	16.30 ± 0.63 a	18.56 ± 0.72 b	19.71 ± 0.24 b	12.75 ± 0.32 a	19.92 ± 0.91 b	19.14 ± 1.22 b
(+)-α-pinene	4.27 ± 0.85 a	8.34 ± 1.22 b	8.59 ± 1.49 b	1.53 ± 0.30 a	6.35 ± 0.98 b	7.22 ± 0.45 b
myrcene	2.31 ± 0.10	2.36 ± 0.56	2.27 ± 0.14	2.75 ± 0.07 b	1.49 ± 0.54 a	2.09 ± 0.22 b
sabinene	0.11 ± 0.07	0.31 ± 0.16	Trace	0.13 ± 0.03	0.10 ± 0.10	0.37 ± 0.09
δ-3-carene	10.58 ± 1.81 b	5.31 ± 1.89 a	4.09 ± 0.66 a	18.82 ± 0.41 b	5.26 ± 0.99 a	4.33 ± 0.23 a
(+)-β-pinene	0.63 ± 0.16	0.98 ± 0.09	0.20 ± 0.20	Trace	0.15 ± 0.15	Trace
(−)-β-pinene	45.77 ± 1.61	48.15 ± 0.38	51.91 ± 1.43	36.95 ± 0.35 a	52.24 ± 2.7 b	52.20 ± 1.6 b
(−)-limonene	7.45 ± 1.55 b	3.55 ± 0.86 ab	2.80 ± 0.46 a	15.32 ± 1.11 b	2.23 ± 0.76 a	2.98 ± 0.14 a
p-cymene	0.74 ± 0.12	0.90 ± 0.54	0.19 ± 0.19	0.60 ± 0.05 a	0.20 ± 0.20 a	1.22 ± 0.28 b
(+)-limonene	6.11 ± 0.41	5.80 ± 0.31	6.39 ± 0.24	5.56 ± 0.23	6.70 ± 0.94	6.17 ± 0.52
γ-terpinene	0.04 ± 0.04	Trace	Trace	0.28 ± 0.07 b	Trace	Trace
terpinolene	0.94 ± 0.17 b	0.35 ± 0.18 b	Trace a	1.81 ± 0.06 b	0.16 ± 0.16 a	0.37 ± 0.10 a
Day 44						
(−)-α-pinene	15.53 ± 0.81 a	19.29 ± 0.85 b	19.17 ± 0.69 b	11.87 ± 0.70 a	17.55 ± 0.82 b	19.77 ± 1.75 b
(+)-α-pinene	3.84 ± 0.88 a	7.22 ± 0.55 b	7.83 ± 0.71 b	1.05 ± 0.12 a	7.43 ± 0.88 b	7.57 ± 2.2 b
myrcene	2.59 ± 0.16 b	1.72 ± 0.29 a	2.17 ± 0.04 b	2.88 ± 0.28	2.19 ± 0.22	2.44 ± 0.44
sabinene	0.17 ± 0.05	0.19 ± 0.11	Trace	0.20 ± 0.02 b	0.18 ± 0.18 b	Trace a
δ-3-carene	12.57 ± 2.16 b	4.66 ± 2.5 a	4.22 ± 1.14 a	22.83 ± 1.79 c	5.1 ± 0.69 b	4.46 ± 0.46 a
(+)-β-pinene	0.63 ± 0.17	1.00 ± 0.05	0.32 ± 0.32	Trace	0.4 ± 0.24	Trace
(−)-β-pinene	43.90 ± 1.50	50.77 ± 2.03	51.26 ± 1.70	27.07 ± 5.16 a	53.50 ± 1.90 b	53.91 ± 3.97 b
(−)-limonene	9.03 ± 1.67 b	2.56 ± 0.21 a	2.87 ± 0.55 a	21.54 ± 1.89 c	4.36 ± 0.32 b	1.10 ± 0.69 a
p-cymene	0.58 ± 0.02	1.47 ± 0.75	0.76 ± 0.41	0.63 ± 0.04	0.23 ± 0.14	0.19 ± 0.19
(+)-limonene	6.51 ± 0.51	4.38 ± 1.46	6.10 ± 0.22	5.42 ± 0.63	6.38 ± 0.60	6.20 ± 1.36
γ-terpinene	0.10 ± 0.04	Trace	Trace	0.27 ± 0.03 b	Trace a	Trace a
terpinolene	1.26 ± 0.23 b	0.24 ± 0.24 a	0.18 ± 0.18 a	2.04 ± 0.23 b	0.14 ± 0.14 a	Trace a
Clone 386						
Day 22						
(−)-α-pinene	8.83 ± 1.53 a	14.6 ± 1.44 b	8.02 ± 0.34 a	7.11 ± 0.16 a	11.44 ± 0.86 b	8.62 ± 0.3 a
(+)-α-pinene	0.33 ± 0.09	0.33 ± 0.20	0.49 ± 0.04	0.43 ± 0.09	0.63 ± 0.33	0.55 ± 0.06
myrcene	1.63 ± 0.43 b	0.79 ± 0.35 a	1.74 ± 0.06 b	2.57 ± 0.11 b	1.49 ± 0.75 a	1.65 ± 0.07 a
sabinene	0.15 ± 0.05 a	0.20 ± 0.14 b	0.48 ± 0.04 b	0.19 ± 0.06	0.16 ± 0.16	0.31 ± 0.08
δ-3-carene	34.18 ± 0.82	35.13 ± 3.71	32.26 ± 0.84	36.00 ± 1.47	28.28 ± 2.36	32.56 ± 0.72
(+)-β-pinene	Trace	Trace	Trace	Trace	Trace	Trace
(−)-β-pinene	28.06 ± 4.23	33.77 ± 3.31	38.17 ± 1.05	21.67 ± 0.76 a	39.98 ± 1.87 c	29.61 ± 1.24 b
(−)-limonene	13.07 ± 2.98 c	2.67 ± 0.71 a	4.86 ± 0.46 b	16.95 ± 1.04 b	6.49 ± 1.61 a	4.78 ± 0.53 a
p-cymene	0.46 ± 0.13 b	0.07 ± 0.07 a	0.57 ± 0.03 b	0.73 ± 0.09	0.91 ± 0.50	0.27 ± 0.11
(+)-limonene	2.74 ± 0.70	3.93 ± 0.5	2.71 ± 0.09	3.39 ± 0.19	3.07 ± 0.29	2.61 ± 0.14
γ-terpinene	0.38 ± 0.09 b	0.09 ± 0.09 a	0.53 ± 0.04 b	0.48 ± 0.01	0.14 ± 0.14	0.44 ± 0.02
terpinolene	3.04 ± 0.78 b	2.04 ± 0.55 a	3.26 ± 0.14 b	3.77 ± 0.16 b	1.73 ± 0.94 a	3.03 ± 0.05 b
Day 44						
(−)-α-pinene	9.65 ± 0.39	11.48 ± 1.13	8.83 ± 0.82	5.72 ± 0.3 a	11.62 ± 0.94 b	8.3 ± 0.83 b
(+)-α-pinene	0.60 ± 0.07 b	0.48 ± 0.16 b	Trace a	0.38 ± 0.05	0.65 ± 0.17	0.33 ± 0.2
myrcene	1.77 ± 0.16	1.11 ± 0.37	1.84 ± 0.48	2.43 ± 0.05 b	1.17 ± 0.34 a	1.45 ± 0.51 a
sabinene	0.22 ± 0.07	0.12 ± 0.12	Trace	0.16 ± 0.07 b	Trace a	Trace a
δ-3-carene	31.49 ± 1.67	28.01 ± 0.64	30.31 ± 1.1	45.06 ± 1.08 b	34.21 ± 1.52 a	28.11 ± 4.68 a
(+)-β-pinene	Trace a	0.57 ± 0.19 b	Trace a	Trace	0.31 ± 0.19	Trace
(−)-β-pinene	34.46 ± 2.21 a	43.11 ± 3.53 b	42.87 ± 2.78b	15.22 ± 1.30 a	38.88 ± 2.91 c	26.77 ± 3.77 b
(−)-limonene	9.26 ± 2.08 b	4.67 ± 0.9 a	4.46 ± 0.58 a	18.99 ± 1.20 b	3.91 ± 1.07 a	6.44 ± 4.24 a
p-cymene	0.30 ± 0.10	0.12 ± 0.12	Trace	0.49 ± 0.03 b	0.07 ± 0.07 a	Trace a
(+)-limonene	3.08 ± 0.16	2.48 ± 0.09	2.03 ± 0.73	2.95 ± 0.08	2.03 ± 0.61	2.26 ± 0.76
γ-terpinene	0.31 ± 0.1	0.21 ± 0.12	Trace	0.57 ± 0.02 b	Trace a	Trace a
terpinolene	2.84 ± 0.26	2.33 ± 0.25	2.29 ± 0.79	4.03 ± 0.39 b	1.83 ± 0.52 a	2.40 ± 0.85 ab

in L samples in D plants sampled 22 DAT. The proportions of sabinene, δ-3-carene, γ-terpinene, and terpinolene increased more in I tissues than in the other treatments in W plants sampled 44 DAT.

The relative contents of (−)-α-pinene increased in all L samples 22 DAT, while it decreased in I tissues in W plants collected 44 DAT.

The relative contents of (−)-β-pinene increased in L samples, while it decreased in I tissues in W plants sampled 22 DAT and 44 DAT.

The Mann–Whitney U Test within the same clone, indicated that disease treatment and sampling time showed significant variations in

the relative content of monoterpenes in response to drought stress (Table 3).

With specific regard to c171, the relative contents of (−)-α-pinene, (+)-α-pinene, (+)-β-pinene, and (−)-β-pinene in all I tissues were higher in D than in W plants, while the proportions of δ-3-carene, (−)-limonene, γ-terpinene, and terpinolene decreased in response to drought stress.

L samples showed lower proportions of (−)-limonene and higher relative contents of p-cymene in D plants than in W plants 44 DAT.
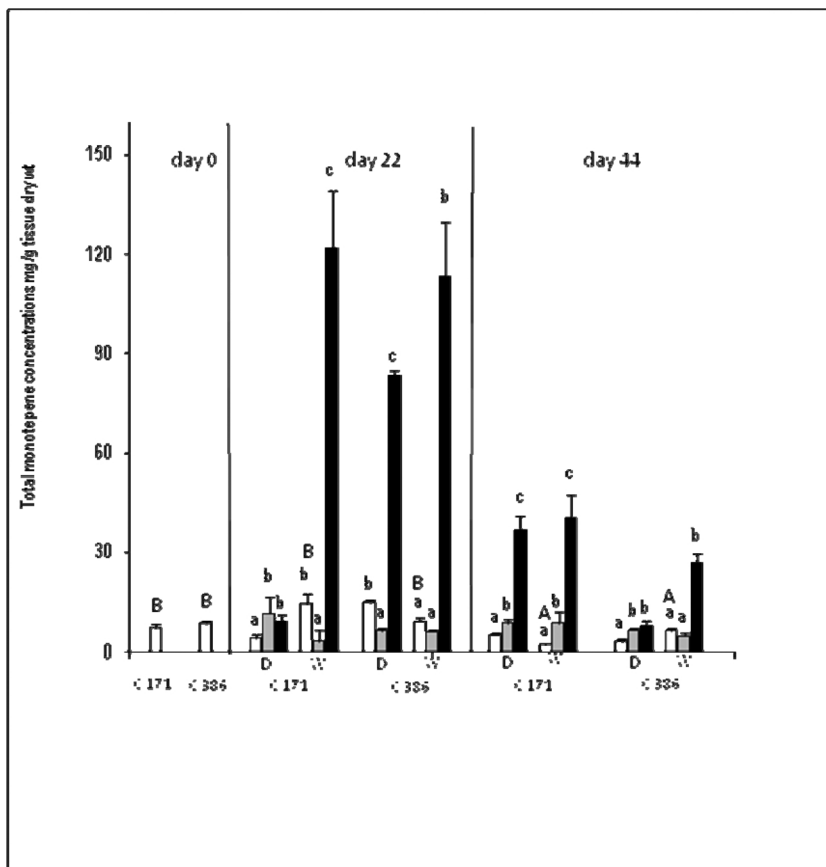
Table 3

Statistical results of the Mann–Whitney U Test examining variations in the relative content of monoterpenes in response to drought stress (d.f. = 1) within the same clone, disease treatments and sampling time. Disease treatments: cortical tissues from non-inoculated branches (C); wounded plus inoculated with *H. parviporum* (I); wounded only (L); *p*-value where * $p < 0.05$, and ** $p < 0.01$. NS: not significant. N° samples: 3–5.

Monoterpene	Clone	χ^2 (p)					
		C		L		I	
		Day 22	Day 43	Day 22	Day 43	Day 22	Day 43
(−)-α-pinene	c171	3.81 NS	0.02 NS	0.50 NS	2.16 NS	6.70**	6.82**
	c386	3.15 NS	0.08 NS	0.02 NS	0.54 NS	0.88 NS	6.00*
(+)-α-pinene	c171	0.27 NS	0.02 NS	1.12 NS	0.15 NS	6.00*	6.1**
	c386	0.27 NS	2.28 NS	0.40 NS	1.83 NS	0.27 NS	3.84*
myrcene	c171	0.53 NS	2.04 NS	0.50 NS	2.94 NS	6.34**	1.32 NS
	c386	0.27 NS	0.78 NS	1.14 NS	0.14 NS	4.80*	6.00*
sabinene	c171	2.81 NS	0.00 NS	1.86 NS	0.34 NS	0.18 NS	0.09 NS
	c386	3.15 NS	0.00 NS	0.03 NS	1.25 NS	0.41 NS	4.86*
δ-3-carene	c171	2.45 NS	3.05 NS	0.52 NS	1.50 NS	5.20*	6.00**
	c386	0.27 NS	0.08 NS	0.02 NS	1.40 NS	0.88 NS	6.00*
(+)-β-pinene	c171	1.00 NS	1.66 NS	4.85 NS	2.23 NS	5.53*	5.54*
	c386	0.00 NS	0.00 NS	0.10 NS	0.80 NS	0.10 NS	0.00 NS
(−)-β-pinene	c171	2.81 NS	0.20 NS	0.23 NS	0.96 NS	5.70*	6.86**
	c386	0.88 NS	0.00 NS	0.56 NS	0.00 NS	2.46 NS	6.00*
(−)-limonene	c171	2.81 NS	1.14 NS	0.50 NS	6.00*	6.20**	6.00**
	c386	0.27 NS	1.33 NS	5.00*	0.06 NS	1.8 NS	6.00*
p-cymene	c171	4.19*	1.42 NS	1.86 NS	6.21*	0.53 NS	0.27 NS
	c386	3.96*	0.00 NS	4.36 *	0.11 NS	3.94*	3.84*
(+)-limonene	c171	0.01 NS	1.82 NS	0.12 NS	2.94 NS	1.32 NS	2.46 NS
	c386	0.88 NS	0.02 NS	2.36 NS	0.06 NS	0.09 NS	1.50 NS
γ-terpinene	c171	0.00 NS	0.00 NS	0.15 NS	0.00 NS	5.12*	6.90**
	c386	3.15 NS	0.00 NS	0.4 NS	2.81 NS	0.10 NS	4.86*
terpinolene	c171	5.53**	1.66 NS	0.34 NS	0.11 NS	6.99**	4.80*
	c386	0.88 NS	0.02 NS	0.20 NS	0.24 NS	0.27 NS	4.00*

With specific regard to clone 386, proportions of (−)-α-pinene, (+)-α-pinene, sabinene, and (−)-β-pinene, were higher in I tissues in D than in W plants 44 DAT.

The percentages of myrcene and *p*-cymene in all I tissues were lower in D than W plants, while the proportions of δ-3-carene, (−)-limonene, γ-terpinene, and terpinolene decreased in response to drought stress only 44 DAT.

The relative content of (−)-limonene in L samples was lower in D than in W plants.

Significant variations of the relative contents of monoterpenes were also observed between the two clones within treatments and water plant status (Supplementary material Table S3).

The Friedman Test within the same spruce clone, disease treatment and water status condition showed significant variations in the relative contents of several monoterpenes in response to sampling time (Supplementary material Table S1).

With specific regard to c171, the proportions of myrcene were higher in I tissues in D plants 44 DAT than 22 DAT.

I tissues in W plants showed higher percentages of (−)-β-pinene and lower proportions of sabinene, δ-3-carene, (−)-limonene 22 DAT than 44 DAT.

L samples in W plants showed lower relative content of (−)-limonene 22 DAT than 44 DAT.

With specific regard to c386, the proportion of δ-3-carene was higher in I tissues in D plants 22 DAT than 44 DAT.

L samples in D plants showed lower percentages of (−)-β-pinene and (−)-limonene 22 DAT than 44 DAT.

I tissues in W plants showed higher proportions of (−)-α-pinene, sabinene, and (−)-β-pinene, and lower percentages of δ-3-carene, and γ-terpinene 22 DAT than 44 DAT.

3.3.2. Total concentrations of monoterpenes (TCM)

Fig. 4 shows variations in total monoterpene concentrations in c171 and c386 to the attack of *Heterobasidion parviporum*.

Kruskal–Wallis ANOVA between treatments within the same spruce

clone, water regime and sampling time (Supplementary material Table S4) showed significant changes in total concentrations of monoterpenes (TCM) expressed as absolute amount (mg g^{-1} dry bark).

TCM were higher in all I tissues than in L samples or cortical tissues from C branches with only two exceptions: there were no differences in TCM between I tissues and L samples in D plants of c171 and c386 respectively 22 DAT and 44 DAT; these plants also showed a lower TCM in cortical tissues from C branches compared with samples from the disease treatments.

Sterile infection caused an increase in TCM in comparison with C branches in all seedlings of "relatively susceptible" c171, except W seedlings 22 DAT that showed a lower TCM when compared with the other treatments.

L samples in D seedlings of c386 44 DAT showed a higher TCM in comparison with C branches, while TCM decreased in L samples in D seedlings collected 22 DAT.

The Mann–Whitney U Test within the same spruce clone, disease treatment and sampling time showed significant variations in TCM in response to drought stress (Table 4).

TCM in I tissues were lower in samples from D plants than in W plants of c171 and c386 collected, respectively, 22 DAT and 44 DAT.

Cortical tissues from C branches showed lower TCM in D plants than in W seedlings of c171 22 DAT, while higher TCM were observed in samples in D plants than in W plants of c386 and c171 22 DAT and 44 DAT, respectively.

Significant variations of TCM were also detected between the two clones within treatments and water plant status (Supplementary material Table S6).

Friedman Test within the same spruce clone, disease treatment and water status (Supplementary material Table S5) showed significant variations in TCM in response to sampling times.

I tissues in D plants of c171 showed lower TCM 22 DAT than 44 DAT, while higher TCM were observed in samples in W plants of c171 and in W and D plants of c386 22 DAT than 44 DAT.

I branches in W plants of c171 and D plants of c386 showed higher

Fig. 4. Changes in total monoterpene concentrations (TCM) in the "relatively susceptible" clone 171 (c171) and the "relatively resistant" clone 386 (c386) to the attack of *Heterobasidion parviporum*. Treatments: drought-stressed plants (D); well-watered (W); cortical tissues from non-inoculated branches (C) (white bar); wounded plus inoculated with *H. parviporum* (black bar); wounded only (grey bar). Bars indicate means + standard errors. Different minor letters on the top of the bars indicate significantly different disease treatment means within the same clone, water regime and sampling time (Mann–Whitney *U* test), while capital letters on the top of the bars indicate significant differences in TCM between different sampling times (day 0, day 22, day 44) in C samples within the same clone (Friedman test).

Table 4

Statistical results of the Mann–Whitney *U* Test examining the variations of the total monoterpene concentrations in response to drought stress (d.f. = 1) within Norway spruce clones, disease treatment and sampling time. Treatments: cortical tissues from non-inoculated branches (C); wounded plus inoculated with *H. parviporum* (I); wounded only (L); *p*-value where * $p < 0.05$. NS: not significant. N° samples: 3–5 for each group.

Variable	Clone	$\chi^2(p)$					
		C		L		I	
		Day 22	Day 44	Day 22	Day 44	Day 22	Day 44
TCM	c171	5.77*	4.81*	3.12 NS	0.54 NS	5.33*	0.54 NS
	c386	4.25*	1.50 NS	0.20 NS	0.30 NS	1.84 NS	5.00*

TCM 22 DAT than 44 DAT.

TCM in C branches in W plants of both clones showed a significant decrease at 44 DAT, while not significant changes in TCM were observed in samples of both clones between 0 DAT and 22 DAT.

4. Discussion

No significant differences in fungal growth were observed between the two clones. Fungal growth was reduced under drought stress treatment (D seedlings). At the end of the experiment, *Heterobasidion* colonization was increased in W seedlings. This result can be related with the intensity of stress and to non-monotonic relationships between water deficit and fungal infection, with an optimum at intermediate values. Lindberg and Johansson (1992) found that, in Norway spruce, resistance to fungal infection was elevated at high water potentials (−0.3 to −0.5 MPa) decreasing, at moderate drought stress (−0.5 to −1.5 MPa) and increasing again at water potentials below −1.5 MPa.

In the present experiment, trees were re-watered when predawn water potential was −3.4 MPa in "relatively susceptible" c171 and −1.3 MPa in c386. It is possible that the inoculated seedlings (D) grown in pots were probably quickly dehydrated when roots were subjected to drought stress, resulting in less carbon accessibility for fungal multiplication (Oliva et al., 2014). Available carbon reserves in W seedlings, conversely, may have facilitated the access of necrotrophic pathogens to essential carbon sources. On the other hand, long-term experiments are needed to clarify whether prolonged drought stress may reduce carbon reserves to an extent that limits the availability of carbohydrates to support defence, predisposing the xylem to attacks by fungal pathogens (La Porta et al., 2008).

As stress progressed, plants showed a reduction in predawn water potential, though with a larger drop in c171 (below −3 MPa) than in c386, which can be related to differences in susceptibility to fungal infection between these Norway spruce clones. A marked decrease in water potential under drought conditions may cause an imbalance between carbon supply and demand, and the decrease in both carbon uptake (source) and growth (sink) in "relatively susceptible" clone may favour disease development, thus accelerating drought-induced tree mortality. Minimum predawn water potentials of about −1.4 MPa may already coincide with the threshold of water potential inducing significant xylem cavitation for this species (Cochard, 1992; Lu et al., 1996). At water potential below −3 MPa, important reductions in xylem conductivity and stomatal conductance can be already detected (Borghetti et al., 1989; Mayr et al., 2003), and conductivity losses of up to 100% were found in Norway spruce with low water potential approaching −4 MPa (Mayr et al., 2002). The decrease in gas exchange preceded the decrease in water potential, reflecting an isohydric strategy (Lyr et al., 1992), reducing stomatal conductance at early stages of soil drought stress.

Reduced stomatal aperture in response to drought stress may have prevented Norway spruce seedlings from xylem dysfunction (Lu et al.,

1996). Because of the threshold-type relationship between stomatal conductance and net photosynthesis in conifers (Teskey et al., 1986), photosynthetic capacity may be sacrificed owing to reduced stomatal conductance. However, only W seedlings showed genotypic discrimination in gas exchange; these differences may be largely attributed to stomatal behaviour, since clone-related differences in predawn water potential were insignificant in W seedlings. Variation in intercellular CO_2 concentration between treatments was unclear (data not shown) and a possible role of mesophyll metabolism in affecting leaf conductance of Norway spruce in long-term responses to drought stress cannot be ruled out. The relative reduction of stomatal conductance and net photosynthesis due to the decline in water availability reported here was comparable to that observed on maturing and mature Norway spruce in the field (Oren et al., 1986; Zimmermann et al., 1988; Lu et al., 1996; Ward et al., 2008). Nevertheless, differences between clones in stomatal conductance and net photosynthesis disappeared under water deficit conditions.

Patterns of net photosynthesis and stomatal conductance were similar, resulting in the close coupling of gas exchange components, without differences in instantaneous water use efficiency between clones (though c171 showed a delayed response of stomatal conductance and net photosynthesis to decreasing water potential in comparison with c386). Stomatal conductance and net photosynthesis were found to be strongly correlated in four-year-old seedlings of Norway spruce subjected to drought stress (Ditmarová et al., 2010). Pronounced sensitivity to drought stress is well known in Norway spruce, although not all physiological parameters display the same degree of sensitivity to dehydration, and may change in concert, enhancing the capability of trees to withstand water stress to some extent (Ditmarová et al., 2010).

The potential photochemical efficiency of PSII also decreased following drought stress, after an initial pronounced decline in gas exchange. Values reached at the peak of stress indicate impairment of photosynthetic machinery ($F_v/F_m < 0.6$ indicates sustained non-radiative energy dissipation), although this was reversible and promptly recovered to pre-stress values after re-watering. As in the case of water potential, chlorophyll fluorescence showed differences between clones in D plants, c386 being more tolerant than c171. Changes in chlorophyll fluorescence in young interior spruce trees during drought stress were found to reflect down-regulation of primary photochemistry and the augmentation of photoprotective mechanisms to avoid over-reduction and photoinhibitory damage (Eastman and Camm, 1995). A similar drop in the potential photochemical efficiency of PSII with drought stress was previously observed in Norway spruce (Pukacki and Kaminska-Rozek, 2005; Ditmarová et al., 2010); although, in contrast to our experiment, values did not recover to pre-stress conditions. Bigras (2005) observed an initial drop in the potential photochemical efficiency of PSII at leaf water potential ranging from −1 to −2 MPa.

Marked differences in the constitutive monoterpene profiles were observed between the two clones; in particular, c386 showed high amounts of δ-3-carene, (−)-limonene, γ-terpinene, and terpinolene, while c171 was characterized by high proportions of (−)-α-pinene, (+)-α-pinene, (−)-β-pinene, and (+)-limonene. Chemosystematic studies, mainly in conifer tree species, have demonstrated that, although the total concentrations of terpenes are clearly influenced by the environment, the constitutive monoterpene profiles in mature organs are stable and little affected by environmental factors; therefore, terpenes have proven to be very useful as biochemical markers in plant genetics to characterize species, provenances, hybrids and clones (Baradat et al., 1991; Hanover, 1992; Elzinga et al., 2015; Barbero and Maffei, 2016).

In Norway spruce, Kotzias et al. (1992) observed that monoterpene percentages including the enantiomers of (±)-α-pinene, remained constant throughout the year, while total concentrations of monoterpenes were strongly reduced during the summer months. In the present study, there were no differences in percentage monoterpene

composition of cortical tissues from non-inoculated branches between drought-stressed and well-watered Norway spruce seedlings. Moreover, monoterpene profiles in cortical tissues from non-inoculated branches did not show significant variations among 0 DAT, 22 DAT and 44 DAT.

These findings support previous research showing that constitutive monoterpene profiles are not affected significantly by abiotic factors and remain stable (Langenheim, 1994; Tognetti et al., 1997; Michelozzi et al., 2004).

Previous experiments showed the discriminating power of terpene markers for studying clonal variation in Norway spruce (Boscherini and Michelozzi, 1993; Silvestrini et al., 2004). These variations in constitutive monoterpene profiles between different chemotype clones can have considerable consequences to plant chemical defence, since monoterpenes provide toxic, deterrent or inhibitory effects to herbivores, insects and microbial pathogens (reviewed by Gershenzon and Croteau, 1991; Langenheim, 1994). Therefore, differences in the constitutive monoterpene-rich resins between c171 and c386 may play an important role in the first line of the plant's defence mechanisms against infection with *H. parviporum*.

Conifer tree species have the potential to quantitatively vary monoterpenes in induced resins as we observed in the present experiment. Indeed, the infection generally caused some extensive compositional changes in monoterpenes. Sterile infection also affected relative concentrations of monoterpenes, but these reactions were less extensive than those elicited by the fungus. In particular, a higher proportion of δ-3-carene was found constitutively in c386 in comparison with c171; moreover, infection with *H. parviporum* increased the relative contents of δ-3-carene in all the samples, even if proportions of this monoterpene in I tissues of c386 were significantly higher than C branches only in W seedlings 44 DAT. δ-3-Carene has been reported to be involved in some conifer-pest interactions (Krupa and Fries, 1971; Croteau et al., 1987; Pasquier-Barre et al., 2001; Fäldt et al., 2003) and also in the defense mechanism of spruce trees against the attack of *Heterobasidion* spp.

Forrest (1982) observed that δ-3-carene, although present in small proportions relative to total monoterpene concentrations, was significantly higher in sitka spruce (*Picea sitchensis*) trees infected by *H. annosum* than uninfected plants. Woodward et al. (2007) found that in Sitka spruce clones less susceptible to *H. annosum*, the relative amount of δ-3-carene was higher in the wounded plus inoculated and wounded only treatments compared to the samples collected 25 cm above the wound.

δ-3-Carene also appears to be involved in the defensive chemistry of Norway spruce against the attack by *H. parviporum*; clones with a relatively high concentration of constitutive δ-3-carene were more resistant to this pathogenic fungus than those with low concentrations (Michelozzi, data not published). In Norway spruce trees, the relative proportion of δ-3-carene was found to increase in response to inoculation with *H. parviporum* (Zamponi et al., 2007).

The same expression pattern of changes in δ-3-carene proportions was evident for terpinolene and these results were consistent with 3-carene synthase that forms stereospecifically (+)-3-carene (78% of total product) together with terpinolene (11% of total product) (Fäldt et al., 2003).

Also, the relative content of (−)-limonene increased significantly following fungal inoculation in all the samples. These data were not in agreement with previous results from Zamponi et al. (2007), who found that proportions of (−)-limonene decreased significantly in 15-year-old Norway spruce trees grown in a field trial and infected by *H. parviporum*. Although this contrasting response could be due to differences in experimental design between studies, i.e., greenhouse pot vs field experiments, sampling time, and different ages of the plants, another possible explanation is that plant chemical defences can vary with plant ontogeny. Under the selective impacts of herbivores, trees during their juvenile-to-mature transition process can promote changes in terpene mixtures to reduce the incidence of pest and diseases (Hanover, 1992; Boege and Marquis, 2005).

In addition, some changes in the relative contents of (−/+)-α-

pinene, myrcene, (−/+)-β-pinene, *p*-cymene and γ-terpinene, did not show a clear trend with disease treatments.

Although water stress induced significant variations in the proportions of several monoterpenes in the plants within the same spruce clone, disease treatment and sampling time, a similar trend in monoterpene responses to wounding plus inoculation and wounding only were observed in W and D plants.

No clear trend was observed in the proportions of several monoterpenes within the same spruce clone and disease treatment between different sampling times.

Finally, the results showing no significant changes in monoterpene profiles from C branches across 0 DAT, 22 DAT and 44 DAT, besides demonstrating the environmental stability of these compounds as mentioned above, strongly suggest that no systemic induction of monoterpenes occurred in Norway spruce seedlings.

Systemic induced resistance (SIR) is a form of defense mechanism that is induced in plant portions distal to localized infections and involving structural and biochemical responses. This mechanism occurs not only in herbaceous plants (Durrant and Dong, 2004), but also in different conifer tree species (Bonello et al., 2001; Bonello and Blodgett, 2003; Luchi et al., 2005; Wallis et al., 2007).

Although systemic induction of traumatic resin ducts was observed in Norway spruce (Krekling et al., 2004; Krokene et al., 2008), our data are in agreement with the study by Zhao et al. (2010) showing that terpene composition did not change in the untreated tissues after treatment and distinct chemical changes were restricted to the pre-treated area of the stem.

It must be pointed out that terpenoid mixtures, either constitutive or induced, with their qualitative and quantitative compositional variations have more defensive values than single compounds. Additive or synergistic actions can enhance the ecological functions of different constituents composing the terpene mixture; moreover, the complexity of the terpenoids occurring in the mixtures, can impede the capacity of the herbivores to develop resistance to plant chemical defences (reviewed by Langenheim, 1994, Cates, 1996).

Therefore, certain combination of monoterpenes can confer greater protection than the content of individual components such as δ-3-carene, limonene, terpinolene, etc., against infection by *H. parviporum* in Norway spruce trees. Our results provide a foundation for future investigations on the relationships between terpene profiles and the attack of this fungus in order to characterize less susceptible Norway spruce chemotypes. Besides, a more comprehensive understanding of the function of antifungal properties of Norway spruce resin, requires knowledge of the defensive roles of sesquiterpenes and diterpenes and other compounds as phenols (Gershenzon and Dudareva, 2007).

An interesting observation of this study was the most copious production of monoterpenes occurring in I tissues. Differential elicitation of monoterpene synthesis is known in pest and pathogen attacks relative to mechanical wounds (Tomlin et al., 2000; Krekling et al., 2004; Woodward et al., 2007). Herbivore attacks yield more accumulation of defensive compounds in comparison with mechanically wounded trees (Cheniclet, 1987; Lieutier, 1993; Schmidt et al., 2005). The increase in monoterpene contents in inoculated tissues is due to defensive functions of these compounds that are dosage dependent (Gershenzon and Croteau, 1991; Langenheim, 1994; Cates, 1996).

Drought stress inhibited the production of monoterpenes in the induced resin of infected plants that did not result in enhanced susceptibility to *H. parviporum*. Indeed, fungal growth was lower in D than W seedlings. Although the majority of studies reported the contrary, some experiments showed that a specific abiotic stress may increase the resistance of plants to pests and diseases (reviewed by Suzuki et al., 2014).

It is worth noting to observe the decrease in TCM in cortical tissues from C branches in W plants of both clones 44DAT. A possible explanation is that the plant has already developed the capability to manage resource allocation and, therefore, the local need of resources

to support defense production at the attack site can be met by translocation from distant sources within the individual tree.

5. Conclusions

In conclusion, the two clones showed different physiological response to drought stress, "relatively resistant" c386 being more tolerant than "relatively susceptible" c171. Overall, the range of physiological response of Norway spruce to different drought stress intensities indicated some degree of plasticity in carbon economy and water relations, though varying with the genotype. Differences in the relative content of constitutive monoterpenes and their enantiomers were observed between the two Norway spruce clones. Alterations in proportionate monoterpene composition and increase in total monoterpene concentrations occurred in response to the attack of *H. parviporum*. Monoterpene responses to mechanical wounds were also observed; however, these reactions were less extensive than responses to infection. In general, drought stress did not affect the monoterpene proportions in cortical tissues from non-inoculated branches. Although drought stress induced variations in the relative contents and decreases in the absolute amounts of monoterpenes, the trend in the relative proportions of monoterpenes in response to infection by *H. parviporum* was similar in D and W plants of both clones.

Our results on the defensive role of δ-3-carene warrant further studies in order to see if this compound in combination with other monoterpenes may be used as biochemical markers for selection of less susceptible Norway spruce trees to infection by *H. parviporum*.

These findings provide a basis for future investigations on the relationships between terpene profiles and the attack by this fungus. A more comprehensive understanding of the function of antifungal properties of Norway spruce resin, requires knowledge of the defensive roles of the other terpene resin constituents such as sesquiterpenes and diterpenes, which can reinforce the biological activity of monoterpenes. A better knowledge of the ecological roles of terpenes may have implications for disturbance ecology and adaptive management of forests.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.envexpbot.2018.03.007.

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