Molecules **2007**, *12*, 1614-1622

molecules

ISSN 1420-3049

www.mdpi.org/molecules

Full Paper

Characterization of Phenolic Compounds in *Pinus laricio* Needles and Their Responses to Prescribed Burnings

Magali Cannac [1], Vanina Pasqualini [1,*], Stéphane Greff [2], Catherine Fernandez [2] and Lila Ferrat [1]

[1] Laboratoire Systèmes Physiques de l'Environnement, U.M.R. C.N.R.S. 6134, Université de Corse, 20250 Corte, France; E-mails: cannac@univ-corse.fr (Magali Cannac), ferrat@univ-corse.fr (Lila Ferrat)

[2] Institut Méditerranéen d'Ecologie et Paléoécologie, U.M.R. C.N.R.S. 6116, Aix-Marseille Université, Centre de St. Jérôme, Case 421, 13397 Marseille, Cedex 20, France; E-mails: stephane.greff@univ-provence.fr (Stéphane Greff), catherine.fernandez@univ-provence.fr (Catherine Fernandez)

* Author to whom correspondence should be addressed; E-mail: pasquali@univ-corse.fr

Received: 30 May 2007; in revised form: 19 July 2007 / Accepted: 19 July 2007 / Published: 30 July 2007

Abstract: Fire is a dominant ecological factor in Mediterranean-type ecosystems. Management strategies include prescribed (controlled) burning, which has been used in the management of several species, such as *Pinus nigra* ssp *laricio* var. *Corsicana*, a pine endemic to Corsica of great ecological and economic importance. The effects of prescribed burning on *Pinus laricio* have been little studied. The first aim of this study was to characterize total and simple phenolic compounds in *Pinus laricio*. The second aim was to understand: i) the short term (one to three months) and medium term (three years) effects of prescribed burning, and ii) the effects of periodic prescribed burning on the production of phenolic compounds in *Pinus laricio*. The first result of this study is the presence of total and simple phenolic compounds in the needles of *Pinus laricio*. 3-Vanillyl propanol is the major compound. After a prescribed burning, the synthesis of total phenolic compounds increases in *Pinus laricio* for a period of three months. Total phenolic compounds could be used as bioindicators for the short-term response of *Pinus laricio* needles to prescribed burning. Simple phenolic compounds do not seem to be good indicators of the impact of prescribed burning because prescribed burnings are low in intensity.

Keywords: Total phenolics, simple phenolics, prescribed burning, *Pinus laricio*

Introduction

Phenolic compounds are present in terrestrial and aquatic higher plants and are extremely diverse [1]. They are of particular interest because of their involvement in the response of the plant to environmental stress, such as pollutants [2-7] or temperature variations [8-11]. Fire is a constant and periodic threat in Mediterranean forests. The Mediterranean region suffers some 50,000 fires/year, which burn 600,000 ha of forest annually [12]. These fires are a real ecological hazard, and also a threat to people. Management strategies include many preventive tools such as mechanical treatment and prescribed burning [13]. Prescribed burning allows burning vegetation under pine stands: i) to reduce fuel loads and fire risks, ii) to recreate natural disturbance dynamics, and iii) restore the ecosystem by means of fire [14,15].

In the mountains of Corsica (France – Mediterranean island), the dominant tree is the Corsican Pine *(Pinus nigra* ssp *laricio* (Poir.) Maire var. *corsicana* (Loud.) Hyl.) [16]. This tree is a variety endemic to Corsica and is exploited for timber production under the control of the "*Office National des Forêts"*. Only the composition and chemical variability of its oleoresin had previously been investigated [17]. The effects of prescribed burning on *Pinus laricio* have also been little studied [18]. A few studies have examined the effects of prescribed burning on the production of phenolic compounds in *Pinus pinaster* Ait [9]. The levels of total and simple phenolic compounds were found to be bioindicators of thermal stress because they vary after heat damage to stems and crowns.

The first aim of this study was to characterize total and simple phenolic compounds in *Pinus laricio*. The second aim was to understand i) the effects of prescribed burning in the short term (one to three months) and medium term (three years) and ii) the effects of periodic prescribed burnings on the production of phenolic compounds in *Pinus laricio*. Our investigation is intended to clarify whether these parameters could be used as bioindicators for the impact of prescribed burning.

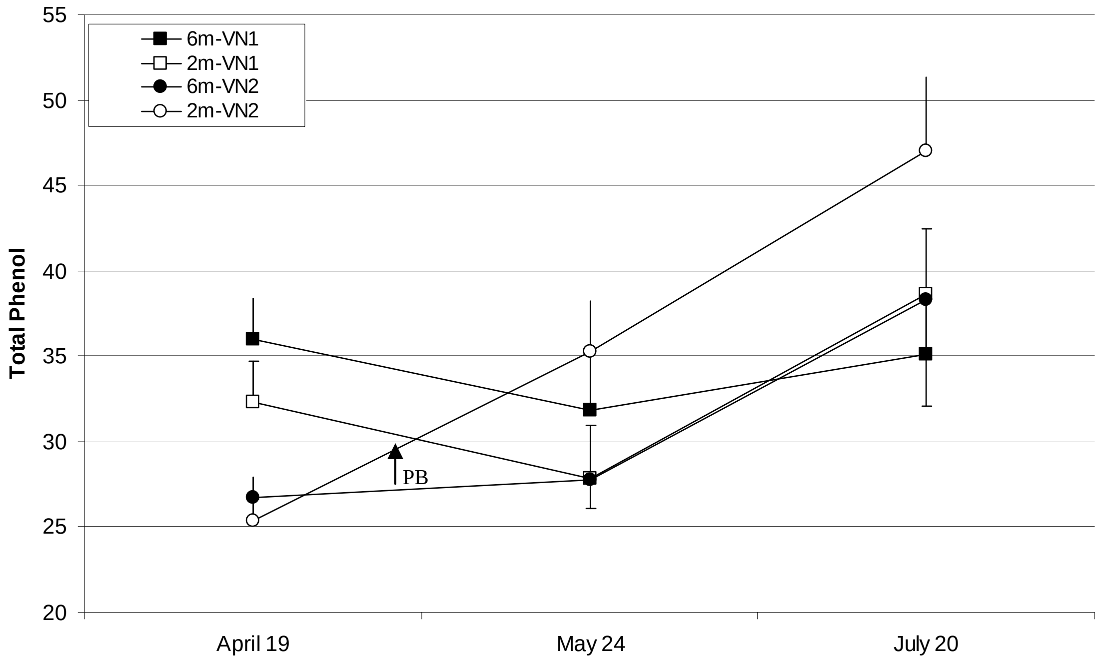
Results and Discussion

Total Phenolic Compounds

The first result of this study is the presence of total phenolic compounds in *Pinus laricio*. The mean content of total phenolic compounds in the needles varied from 25.6±2.6 to 47.0±4.4 mg of gallic acid/g dry weight (Figure 1), which is of the same order as the levels found in the literature [7,9,19]. Before the prescribed burning, total phenolic compound levels were higher at VN1 (unburned station) than at VN2 (burned station) (Anova, $p<0.05$; Figure 1). One month after the prescribed burning, from April to May, at 2 m height, the phenolic compound levels at the burned station increased (Anova, $p<0.05$; Figure 1). There was no difference between April and May at the unburned station (Anova, $p>0.05$). After three months, phenolic compound levels were still higher at the burned station at 2 m height (Anova, $p<0.05$; Figure 1); indeed the levels increased from May to July (Anova, $p<0.05$).

While this increase in level at the burned station was detected at 2 m height (Anova, $p<0.05$), there was no significant difference from May to July at 6 m height (Anova, $p>0.05$).

Figure 1. Total phenolic compounds contents (mg of gallic acid/g dry weight ± standard error) in needles of *Pinus laricio* in Valdu-Niellu forest, before and after (1 and 3 months) a prescribed burning (PB) at an unburned station (VN1) and a burned station (VN2), at 2 m and 6 m height.



The prescribed burning may activate a secondary metabolism to produce defence compounds, such as phenolic compounds [9]. Our results essentially show an increase in total phenolic compounds, only at 2 m height, two months after prescribed burning. This increase in phenolic compounds could be explained by the action of a pigment [20], which increases at the same time [18].

The carbon/nutrient balance hypothesis argues that, under conditions of nutrient limitation, plants accumulate large reserves of carbohydrates [21]. Variation in phenolic concentrations has been documented in plants as a by-product of the internal resource balance [21] and in response to nutrient stress [21]. When nitrogen is present in sufficient quantities, carbon is allocated to growth rather than the production of secondary metabolites [22]. The increase in the nutrient content of the soil after prescribed burning [23] cannot directly explain the increase in total phenolic compound contents. Another explanation for the variation in phenolic compounds could be the activity of Phenylalanine Ammonia-Lyase (PAL). PAL is considered to be the principal enzyme in the phenylpropanoid pathway, catalysing the transformation of phenylalanine into trans-cinnamic acid, which is the prime intermediary in the biosynthesis of phenolic compounds [24]. PAL is a key enzyme in the synthetic pathway of phenolic compounds and increases under the influence of temperature [25]. Rivero [25] shows a correlation between PAL and increases in phenolic compounds in tomatoes (*Lycopersicon esculentum*) following thermal stress. The increase in phenolic compounds may therefore be correlated with an increase in PAL when *Pinus laricio* undergoes thermal stress, such as a prescribed burning.

Simple phenolic compounds

Levels of simple phenolic compounds were of the same order as those found in other species, such as *Betula pubescens* Ehrh. [4], *Pinus pinaster* Ait [9], *Pinus halepensis* Mill. [6] and *Pinus sylvestris* L. [26]. Fifteen simple phenolic compounds were identified and quantified (Table 1). 3-Vanillyl propanol (Figure 2) was the major compound found in the needles of *Pinus laricio*, with an average of 568.3 µg/g of dry matter at the control station. Phenylethyl alcohol, benzoic acid, vanillylacetone, vanillic acid and isoferulic acid are the other most abundant compounds with a mean concentration higher than 100 µg/g of dry matter at the control station (Table 1).

Figure 2. Structure of 3-vanillyl propanol.

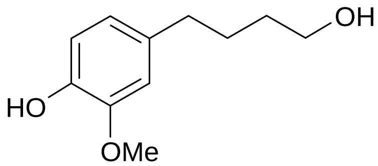


Table 1. Mean concentrations (µg/g of dry matter ± standard error) of simple phenolic compounds in the needles of *Pinus laricio* in Ospedale forest (a,b: significant difference between stations, Anova, $p<0.05$); RI= Kovats Retention Index; CAS N°: Chemical Abstract Service Number.

RI	Common Name of phenolic compounds	CAS N°	OSP1	OSP2	OSP3	OSP4
1232	Phenylethyl alcohol	60-12-8	116.8 ± 43.0	130.8 ± 79.7	169.6 ± 40.8	63.6 ± 8.0
1251	Benzoic acid	65-85-0	70.5 ± 12.0	73.9 ± 20.2	158.5 ± 40.3	112.0 ± 49.6
1303	Benzeneacetic acid	103-82-2	112.4 ± 50.2	57.1 ± 19.7	63.7 ± 24.3	55.6 ± 8.0
1479	Eugenol	97-53-0	7.9 ± 4.9	7.6 ± 3.7	21.3 ± 5.1	24.2 ± 10.1
1522	Salicylic acid	69-72-7	9.7 ± 2.4 (a)	6.6 ± 1.5 (a,b)	3.0 ± 3.0 (a,b)	1.8 ± 1.8 (b)
1542	Vanillin	121-33-5	65.2 ± 9.7 (a,b)	63.7 ± 7.5 (a,b)	94.0 ± 3.0 (a)	60.1 ± 15.8 (b)
1692	Dihydro p-coumaryl alcohol	10210-17-0	24.2 ± 2.2 (a,b)	31.8 ± 1.8 (a,b)	59.2 ± 28.2 (a)	15.6 ± 3.6 (b)
1764	Vanillylacetone	122-48-5	141.7 ± 31.8	145.4 ± 18.3	132.5 ± 11.0	95.3 ± 13.4
1778	Vanillic acid	121-34-6	166.8 ± 32.0 (a,b)	198.4 ± 31.6 (a)	164.5± 5.0 (a,b)	108.5 ± 1.3 (b)
1833	3-Vanillyl propanol	3579-91-7	140.1 ± 160.6	550.8 ± 188.3	568.3 ± 100.7	317.0 ± 31.4
1861	Dihydroferulic acid	1135-23-5	9.4 ± 3.3	7.8 ± 3.0	14.9 ± 2.3	8.0 ± 2.7
1909	Syringic acid	530-57-4	26.4 ± 15.6	30.0 ± 17.5	5.9 ± 5.9	24.6 ± 12.8
1937	Ferulic acid	1135-24-6	56.4 ± 12.6	71.6 ± 1.9	79.8 ± 6.4	53.8 ± 10.9
1951	p-Coumaric acid	7400-08-0	40.1 ± 16.9	53.5 ± 8.6	55.8 ± 15.1	28.5 ± 5.2
2075	Isoferulic acid	537-73-5	95.6 ± 19.0 (a)	167.1 ± 24.9 (b)	169.8 ± 25.6 (b)	96.2 ± 19.1 (a,b)

Differences were found for some simple phenolic compounds before and after the burning treatments: salicylic acid, vanillin, dihydro-*p*-coumaryl alcohol, vanillic acid and isoferulic acid (Anova, $p<0.05$; Table 1). Some of these differences could not be explained by the impact of prescribed burnings. Only vanillin and dihydro-*p*-coumaryl alcohol seemed to show differences between the control station (OSP3) and the station burned in April 2006 (OSP 4; Table 1) which could be due to the impact of recent prescribed burning.

Other authors have observed variations in the concentrations of some simple phenolic compounds and no variations for others following environmental stress such as severe pollution caused by a nickel-copper smelter [4] or air pollution [6]. Following a thermal stress event, Alonso [9] highlights variations in levels of esterified syringic acid, which may be due to crown scorching, but these variations change with additional trunk heating levels. So, burning intensity seems to be very important and could be a consequence of variations in phenolic compounds. When damage exceeded a determined level, the same authors found that the tree no longer reacted by activating the production of defense compounds, and their levels decreased. Our experiments were carried out at stations subjected to low intensity prescribed burnings, which did not seem to have an impact on simple phenolic compounds.

The first result of this study is the determination of the levels of total and simple phenolic compounds in the needles of *Pinus laricio*. After a prescribed burning, *Pinus laricio* increased the synthesis of total phenolic compounds for a period of three months. Total phenolic compounds could be used as bioindicators for the short-term response of *Pinus laricio* needles to prescribed burning. Simple phenolic compounds do not seem to be a means of assessing the impact of prescribed burning because these operations are low in intensity.

Experimental Section

General

Two study sites on the island of Corsica were selected. The first site, a natural regeneration of *Pinus laricio*, is located in the Valdu-Niellu forest in northwest Corsica. The second site is located in Ospedale forest, southeast Corsica, on a *Pinus laricio* plantation. The general characteristics of the study sites and *Pinus laricio* stands are presented in Table 2.

At both sites, stations were established adjacent to each other with three replicates in each station and were selected to be similar in structure and composition. The prescribed burnings were conducted in 2003 by forest firefighters and in 2006 by the *Office National des Forêts* (Table 3). A pruning operation was performed in February 2006 up to 4 m high over the entire Ospedale forest site.

1-year-old needles (their growth began in spring 2005) were collected from six dominant trees at each station. In the Valdu-Niellu forest, in order to study the short-term impact of prescribed burning, samples were taken on April 19, 2006, before the prescribed burning and on May 24 and July 20, 2006, 1 and 3 months after the treatment. Needles were collected from the pines at two heights: 2 m and 6 m above ground level. A total of 72 needle samples were collected for total phenolic compounds. In the Ospedale forest, in order to study the short and medium-term impact of prescribed burning

(3 years) and the impact of periodic prescribed burning, needles were sampled on June 11, 2006 in the middle of the canopy. A total of 24 needle samples were collected for simple phenolic compounds.

Table 2. General characteristics of study sites and *Pinus laricio* stands (mean per site); Dbh: Diameter at breast height).

Forest	**Valdu-Niellu**	**Ospedale**
Geographical coordinates	42°17’03’’N 8°55’22’’E	41°41’37’’N 9°12’36’’E
Elevation (m)	1070	950
Slope (%)	5 to 10	5 to 10
Substrate	Granitic	Granitic
Station type	Mesophylous	Mesophylous
Vegetation cover	60%	40%
Age stand (years)	13	22
Dbh (cm)	13	13
Total height (m)	6.6	6.7
Basal area (m^2/ha)	21.3	14.1

Table 3. The study stations and data concerning the prescribed burnings in the *Pinus laricio* stands.

Forest	Valdu-Niellu (VN)		Ospedale (OSP)			
Stations	VN1	VN2	OSP1	OSP2	OSP3	OSP4
Surface	0.1 ha	0.1 ha	0.05 ha	0.05 ha	0.05 ha	0.05 ha
Burning	No	April 24, 2006	April 27, 2003	April 27, 2003 April 4, 2006	No	April 4, 2006

Total Phenolic Compounds

The method of extraction of the phenolic compounds was based on the work of Penuelas [3]. One-half gram (dry weight) of needles per sample was extracted with a 70% (v/v) aqueous methanol solution (20 mL) acidified with a few drops of 1 M HCl. The mixture was left at ambient temperature for 1.5 h with shaking, and then filtered. Quantification of the total phenolic compounds was carried out by colorimetric reaction using the Folin-Ciocalteu reagent. After 1 h, the reaction was completed and measured at 720 nm on a spectrophotometer (UV-1201, Shimadzu). The quantitative results are expressed with reference to gallic acid.

Simple Phenolic Compounds

The extraction protocol and analysis of simple phenols were developed by the *Institut Méditerranéen d'Ecologie et Paléoécologie* in Marseilles. Ultrapure water (20 mL) containing 100µL of internal standard (500 mg of Protochualdehyde in 50 mL methanol), were added to dry *Pinus laricio* needle powder (5 g). After being shaken for 1 hr at room temperature and filtered on Whatman paper, an aliquot of solution (2 mL) was extracted three times with methylene chloride (1 mL). These different fractions were then gathered, conserved for 10 min in a freezer in darkness and then lyophilized (freeze-dryer Lyovac GT2, Stéris®; 1 hr). Acetonitrile (400 µL) and BSTFA+1%TMCS (N,O-bis(trimethylsilyl)trifluoroacetamide + 1 % trimethylchlorosilane - Sigma-Aldrich, 100 µL) were added and shaken for 1 hr at 70°C to form silylated derivatives. Analyses were performed using a Hewlett-Packard GC6890 (coupled to a 5973N Mass Spectrometer Detector) equipped with a HP5MS capillary column (30 m x 0.25 mm x 0.25 µm - J&W Scientific). Sample volumes were injected in pulsed splitless mode (20 psi) for 1 min with an ALS 7683 Automatic Injector at 250°C. The purge flow was set to 30 mL/min. Helium (99.995%) was used as carrier gas. A constant flow mode (1ml/min) was set throughout the run. The oven temperature was initially programmed at 70 °C, ramped to 270 °C at a rate of 5 °C /min and maintained at this temperature for 10min.

The EI mode mass spectrometer parameters were as follows: ion source, 230 °C; MS quadrupole, 150 °C; electron energy, 70eV; Electron Multiplier Energy, 1100-1200V; data were acquired in scan mode from 40 to 500 amu.

Simple phenols were qualified by comparison of mass spectra with those from a personal library. The Kovats retention index was determined by co-injection of Wisconsin Diesel Range hydrocarbons (C10 to C20 from AccuStandard, Inc).

Statistical Analysis

Analyses of variances (Anova) were used when the conditions of application were satisfied (variance homogeneity and normality of stand). The Statistical Graphics Corporation's "Statgraphics for Windows®" software was used for these various tests.

Acknowledgements

We thank the Office National des Forêts and the forest firefighters for carrying out the prescribed burnings on our study sites, and G. Syx and E. Voron for their participation in field missions. This work benefited from the financial support of a national research program (Contrat de plan Etat-Région).

References

1. Harborne, J.B. In *Secondary plant products. Encyclopaedia of Plant Physiology;* Bell, E.A., Charlwood, B.V., Eds.; Springer-Verlag: Berlin-Heidelberg, **1980**; vol. 8, pp. 329-402.
2. Giertych, M.J.; Karolewski, P. Changes in phenolic compounds content in needles of Scots pine (*Pinus sylvestris* L.) seedlings following short term exposition to sulphur dioxide. *Arbor. Korn. Rocz*. **1993**, *38*, 43-51.

3. Penuelas, J.; Estiarte, M.; Kimball, B.A.; Idso, S.B.; Pinter, P.J; Wall, G.W.; Garcia, R.L.; Hansaker, D.J.; Lamortz R.L.; Hendrix, D.L. Variety of responses of plant phenolic concentration to CO2 enrichment. *J. Exp. Bot*. **1996**, *47*, 1463-1467.
4. Loponen, J.; Lempa, K.; Ossipov, V.; Kozlov, M.V.; Girs, A.; Hangasmaa, K.; Haukioja, E.; Pihlaja, K. Patterns in content of phenolic compounds in leaves of mountain birches along a strong pollution gradient. *Chemosphere* **2001**, *45*, 291-301.
5. Ferrat, L.; Pergent-Martini, C.; Romeo, M. Assessment of the use of biomarkers in aquatic plants for the evaluation of environmental quality: application to seagrass. *Aquat. Toxicol.* **2003**, *65*, 187-204.
6. Pasqualini, V.; Robles, C.; Garzino, S.; Greff, S.; Bousquet-Melou, A.; Bonin, G. Phenolic compounds content in *Pinus halepensis* Mill. needles: a bioindicator of air pollution. *Chemosphere* **2003**, *52*, 239-248.
7. Robles, C.; Greff, S.; Pasqualini, V.; Garzino, S.; Bousquet-Melou, A.; Fernandez, C.; Korboulewski, N.; Bonin, G. Phenols and flavonoids in *Pinus halepensis* Mill. needles as bioindicators of air pollution. *J. Environ. Qual.* **2003**, *32*, 2265-2271.
8. Chalker-Scott, L.; Fuchigami, L.H. In *Low temperature stress physiology in crops*; Li, P.H. Ed.; CRC Press: Florida, **1989**, 67-79.
9. Alonso, M.; Rozados, M.J.; Vega, J.A.; Perez-Gorostiaga, P.; Cuinas, P.; Fonturbel, M.T.; Fernandez, C. Biochemical responses of *Pinus pinaster* trees to fire-induced trunk girdling and crown scorch: secondary metabolites and pigments as needle chemical indicators. *J. Chem. Ecol*. **2002**, *28*, 687-700.
10. Dela, G.; Etti, O.; Rinat, O.; Nissim-Levi, A.; Weiss, D.; Oren-Shamir, M. Changes in anthocyanidin concentration and composition in “Jaguar” rose flowers due to transient high-temperature conditions. *Plant Sci.* **2003**, *164*, 333-340.
11. Nacif de Abreu, I.; Mazzafera, P. Effect of water and temperature stress on the content of active constituents of Hypericum brasiliense Choisy. *Plant Physiol. Bioch.* **2005**, *43*, 241-248.
12. Quézel, P.; Médail, F. *Ecologie et biogéographie des forêts du bassin méditerranéen*. Elsevier, Ed., Lavoisier Publ. Paris, **2003.**
13. Fernandes, P.M.; Rigolot, E. The fire ecology and management of maritime pine (*Pinus pinaster* Ait.). *For. Ecol. Manag.* **2007**, *241*, 1-13.
14. Weber, M.G.; Taylor, S.W. The use of prescribed fire in the management of Canada's forested lands. *For. Chron.* **1992**, *68*, 324-334.
15. Graham, R.T.; McCaffrey, S.; Jain, T.B. Science basis for changing forest structure to modify wildfire behavior and severity. *Gen. Tech. Rep. RMRS-120*. Rocky Mountain Research Station, USDA Forest Service, **2004**..
16. Gamisans, J.; Marzocchi, J.F. *La Flore Endémique de la Corse*. Edisud Ed.: Aix-en-Provence, France, **1996**.
17. Rezzi, S.; Bighelli, A.; Castola, V.; Casanova, J. Composition and chemical variability of the oleoresin of *Pinus nigra* ssp. laricio from corsicana. *Ind. Crop. Prod.* **2005**, *21*, 71-79.
18. Cannac, M.; Ferrat, L.; Morandini, F; Chiaramonti, N.; Santoni, P.A.; Pasqualini, V. Bioindicators for the short-term response of *Pinus laricio* needles to thermal pruning. In *4th International Wildland Fire Conference*; Seville, Spain, 13-17 May **2007**.

19. Musika, R.M. Terpenes and phenolics in response to nitrogen fertilization: a test of the carbon/nutrient balance hypothesis. *Chemoecology* **1993**, *4*, 3-7.
20. Rama, R.S.; Pellisier, F; Prasat, M.N.V. In *Plant Physiology*; Prasad, M.N.V., Ed.; John Wiley and Sons: New York, **1997**; pp. 253-303.
21. Bryant, J.P.; Chapin, F.S.; Klein, D.R. Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. *Oikos* **1983**, *40*, 357-368.
22. Coley, P.D.; Bryant, J.P.; Chapin III, FS. Resource availability and plant antiherbivore defense. *Science* **1985**, *230*, 895-899.
23. Monleon, V.J.; Chromack, K.; Landsberg, J.D. Short- and long-term effects of prescribed underburning on nitrogen availability in ponderosa pine stands in central Oregon. *Can. J. For. Res.* **1997**, *27*, 369-378.
24. Rösler, J.; Krefel, F.; Amrhein, N.; Sohmid, I. Maize phenylalanina ammonia-lyase activity. *Plant Physiol.* **1997**, *113*, 175-179.
25. Rivero, R.M.; Ruiz, J.M.; Garcia, P.C.; Lopez-Lefebre, L.R.; Sanchez, E.; Romero, L. Resistance to cold and heat stress : accumulation of phenolic compounds in tomato and watermelon plants. *Plant Sci.* **2001**, *160*, 315-321.
26. Roitto, M.; Markkola, A.; Julkunen-Tiitto, R.; Sarjala, T.; Rautio, P.; Kuikka, K.; Tuomi, J. Defoliation-induced responses in peroxidases, phenolics, and polyamines in scots pine (*Pinus sylvestris* L.) needles. *J. Chem. Ecol.* **2003**, *29*, 1905-1918.

Sample Availability: Samples of the compounds are available from authors.