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# Do trees use reserve or newly assimilated carbon for their defense reactions? A $^{13}\text{C}$ labeling approach with young Scots pines inoculated with a bark-beetle-associated fungus (*Ophiostoma brunneo ciliatum*)

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**Abstract** – Three-year-old saplings of *Pinus sylvestris* L. were labeled with  $^{13}\text{CO}_2$  prior to inoculating the trunk with *Ophiostoma brunneo ciliatum*, a blue-staining fungus usually associated to *Ips sexdentatus*. During incubation, half the trees were submitted to a severe drought that decreased photosynthesis and natural  $^{13}\text{C}$  content in non-labeled saplings. A large  $^{13}\text{C}$ -excess was obtained in wood and phloem, especially in the fractions of soluble proteins, starch and soluble sugars of labeled saplings. Drought increased  $^{13}\text{C}$ -excess, due to reduced photosynthesis and smaller dilution of  $^{13}\text{C}$  by the addition of newly assimilated  $^{12}\text{C}$ . The induced-reaction zones in inoculated saplings displayed large total C (58 g 100 g<sup>-1</sup>) because of the accumulation of secondary metabolites. They also showed much larger  $^{13}\text{C}$ -excess than any other compartment: the contribution of stored C to the reaction zones was much higher than that of currently assimilated C. Moreover, drought lowered the contribution of the latter, as shown by the increase of  $^{13}\text{C}$  in the reaction zones. We conclude that stored C was readily mobilized for the construction of reaction tissues, and that the contribution of currently assimilated C was only minor.

*Ophiostoma brunneo ciliatum* / bark beetles / *Ips sexdentatus* /  $^{13}\text{C}$  labeling / storage compounds

**Résumé** – Les arbres utilisent-ils du carbone de réserve ou du carbone récemment assimilé pour la construction des zones de réaction dans la tige? Une étude de marquage au  $^{13}\text{C}$  de jeunes pins sylvestres inoculés avec un champignon (*Ophiostoma brunneo ciliatum*) associé aux scolytes. De jeunes pins sylvestres (*Pinus sylvestris* L.) âgés de trois ans ont été marqués avec du  $^{13}\text{CO}_2$  puis inoculés dans le tronc avec *Ophiostoma brunneo ciliatum*, un champignon habituellement associé au scolyte *Ips sexdentatus*. Pendant l’incubation, la moitié des arbres a été soumise à une sécheresse sévère qui a fortement réduit la photosynthèse et l’abondance naturelle en  $^{13}\text{C}$  des individus non marqués. Un fort excès en  $^{13}\text{C}$  a été détecté dans le bois et le phloème ainsi que dans les protéines solubles, l’amidon et les sucres solubles des individus marqués. La sécheresse a amplifié cet excès, du fait d’une photosynthèse réduite et donc d’une moindre dilution du  $^{13}\text{C}$  par du  $^{12}\text{C}$  récemment assimilé. Les zones de réaction induite autour des points d’inoculation présentaient de fortes teneurs en C (58 g 100 g<sup>-1</sup>), du fait de l’accumulation massive de métabolites secondaires. Elles présentaient également un excès de  $^{13}\text{C}$  plus marqué que n’importe quel autre tissu : ces zones de réaction étaient donc essentiellement constituées à partir de C provenant des réserves avec une faible contribution de C récemment assimilé. De plus, la sécheresse a augmenté la contribution du C de réserve, comme le montre l’augmentation de l’excès de  $^{13}\text{C}$  dans les zones de réaction.

*Ophiostoma brunneo ciliatum* / scolyte / *Ips sexdentatus* /  $^{13}\text{C}$  marquage / composés de stockage

## 1. INTRODUCTION

Conifers are frequently attacked by bark beetles that carry hyphae of associated blue-staining fungi (*Ophiostomatales*, [28]). The beetles dig galleries into bark and phloem, and simultaneously inoculate the fungus. The association between the bark beetle and the fungus is mutualistic, the fungus contributing to the installation of the insect into the tree. Bark beetles and their associated fungi are a severe threat to conifers, and epidemic population outbreaks may result in severe decline and mortality of trees. Conifers are able to contain the two aggressors with defense systems limiting insect activity and fungal development. Two major defense mechanisms are involved: (1) *preformed defense*, which consists in a

flow of pre-existing resin promoted by mechanical disruption due to insect foraging, (2) *induced defense* [1, 7, 39], which is a non-specific reaction extending rapidly through inner bark and sapwood [2, 22, 35, 41, 50]. It consists of: (i) an active accumulation of secondary metabolites around attack zones, that limits the progression of the aggressor; and (ii) the build-up of a wound periderm that isolates the reaction zone from the rest of the tree [6, 32, 35, 39, 42, 50]. Induced defense is an essential component of tree resistance to bark beetles and associated fungi [1, 7, 31, 39]. It is very efficient against bark-beetles building longitudinal maternal galleries like *Ips typographus* in Spruce [6, 7], *I. sexdentatus* and *Tomicus piniperda* in Scots pines [35, 37] and various *Dendroctonus* species in American pines [9, 40, 42].

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The capacity of a tree to contain attacks depends on the rapidity with which it synthesizes large amounts of secondary metabolites, which, at least partly, depends on its ability to mobilize carbon around the points of attack [7]. Synthesis of secondary metabolites is a very costly process in terms of energy and depends on the availability of carbohydrates close to the attack points [12, 49].

It has been suggested the carbon used to build the induced-reaction zones originates directly from current assimilates [7]. Stored compounds accumulated in various tissues, such as inner bark around the induced-reaction zones or other tissues, may also be mobilized. Indeed, a decrease of soluble sugars and lipids in the phloem was observed as a consequence of construction of the induced-reaction zones [44]. The ability of trees to stop bark beetle attacks may be correlated with the level of soluble carbohydrates around attack points [5, 42]. Carbon used to build-up the induced-reaction zones may also originate from starch hydrolysis around the attack points [42, 44]. In fact, starch decreased in the phloem of *Picea abies* after mass inoculation with *Ceratocystis polonica*, but no correlation was found between starch concentration in the phloem and tree resistance [5]. During mass attacks, available carbohydrates may be consumed rapidly and subsequent transport of soluble sugars from needles is required [5].

It is difficult to infer from this evidence which is the main source of carbon (photosynthesis vs. storage) used to build-up induced-reaction zones in conifers, despite the widely accepted view that the capacity of a tree to contain attacks might be less influenced by starch reserves than by assimilates produced in the needles [4, 5, 7, 18, 39]. A large contribution of newly assimilated carbon to reaction zones would lead to an easy explanation of the interactions between tree resistance to attacks, and environment: any factor reducing photosynthetic assimilation would rapidly lead to a decreased resistance [7]. Various abiotic factors, such as drought stress, air pollution and temperature stress, as well as attacks by biotic agents, may alter the resources available for defense to such a degree that even relatively resistant genotypes would become susceptible [23]. Drought for instance is thought to increase the susceptibility of trees to bark beetles/fungi attacks [11, 17, 43]. Drought can also change the balance between newly assimilated and stored C in supplying the reaction zones of attacked conifers [23].

Labeling trees with a stable carbon isotope ( $^{13}\text{C}$ ) is a powerful tool to follow dynamics of newly assimilated and of stored C [3, 19]. We report here on an experiment aiming at quantifying the relative contribution of the two available sources of carbon (assimilation, storage) in supplying the induced-reaction zones of three-year-old Scots pines. Pines were inoculated into the trunk with *Ophiostoma brunneo-ciliatum*. Prior to inoculation, the saplings were subjected to a long-term  $^{13}\text{C}$  labeling of their reserves. Specifically, we examined (1) if the source of carbon used in the induced-reaction zones derived from storage or from new assimilates and (2) if a severe drought applied during the development of the induced-reaction zones modulated the relative contribution of the two available sources of carbon.

## 2. MATERIAL AND METHODS

### 2.1. Plant material

Eighteen three-year-old saplings of Scots pine (*Pinus sylvestris* L., Provenance: Forest of Haguenau, Eastern France), produced in a nursery at Orléans (France), were planted in 10 L plastic pots filled with a sand-peat mixture (2:1, v/v) and grown for 7 months (from April to October) in a greenhouse (temperature: 12–25 °C, relative humidity: 50–95%; transmitted irradiance: two thirds of outside irradiance with a maximum photon flux density of 1 200  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ). at Champenoux (INRA Nancy, France). All saplings were watered with an automated drip irrigation, and supplied with a slow release fertilizer (Nutricote® 100 N/P/K 13/13/13 + oligo-elements; 4  $\text{g}\cdot\text{L}_{\text{soil}}^{-1}$  = 40  $\text{g}\cdot\text{pot}^{-1}$ ).

### 2.2. Labeling procedure

Twelve individuals were randomly sampled in this population, and submitted to a  $^{13}\text{C}$  labeling procedure for one month during July–September (Fig. 1). The six remaining saplings were not labeled and left in the greenhouse.

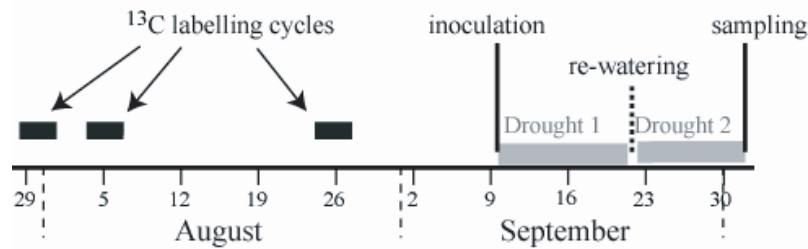
The twelve saplings were placed in a controlled environment chamber (VTPH 5/1 000, Vötsch Industrie-technik GmbH, Reiskirchen-Lindenstruth, Germany) operating as a semi-closed system designed for  $^{13}\text{C}$  labeling [47], and exposed during three 24 h-long cycles to  $^{13}\text{CO}_2$ -enriched air (4 atom%  $^{13}\text{C}$ ) at a constant  $\text{CO}_2$  concentration of 380  $\mu\text{mol}\cdot\text{mol}^{-1}$  air. This was achieved by continuously mixing a small flow of  $^{13}\text{CO}_2$  diluted in  $\text{N}_2$  (cylinder 1, 11 atom%  $^{13}\text{C}$ , Eurisotop, CEA, France) with a flow of industrial  $\text{CO}_2$  (Cylinder 2, 1.08 atom%  $^{13}\text{C}$ ). Chamber temperature was  $20 \pm 1$  °C and relative humidity was 77%. Three high-pressure SONT sodium vapor discharge lamps (Philips Electronics N.V., Amsterdam, The Netherlands) provided a photosynthetic photon flux density of approx. 400  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  at plant level. Between the three labeling cycles, saplings were returned to the glasshouse.

### 2.3. Inoculation

The eighteen saplings (12 labeled and 6 unlabeled) were inoculated during September. Mycelia strains of *Ophiostoma brunneo-ciliatum* (Ophiostomatales, associated usually to the bark beetle *Ips acuminatus*, Scolytidae) were collected from blue sapwood of attacked pine saplings. Monospore cultures of the fungus were used after incubation on a malt agar medium for three weeks. Culture plugs (5 mm) were inoculated into the cambial zone of the trunk. The hole was plugged again with the removed bark disk. Five inoculation points were made per sapling, at 5 cm intervals on the two-year-old segment of the stem, yielding a local density of about 400 inoculations per  $\text{m}^2$  of stem surface.

### 2.4. Drought treatment and monitoring of drought stress

The 18 saplings were kept in the greenhouse during the 3 weeks of incubation, and half of them (6 labeled and 3 unlabeled) were randomly selected and submitted to two cycles of drought (11 and 10 days) by withholding irrigation (Fig. 1). Every second day, predawn needle water potential ( $\Psi_{\text{wp}}$ ) was measured with a Scholander pressure chamber, and gas exchange of a current year twig with a 4L portable photosynthesis chamber LiCor 6 200 (LiCor, Lincoln, Nebraska, USA), around midday (between 12 h 30 and 14 h 00 local



**Figure 1.** Flow diagram presenting the schedule of the experiment, with three periods of  $^{13}\text{C}$  labeling followed by an inoculation with *Ophiostoma brunneo-ciliatum*, two successive drought cycles, and sampling of the Scot pine saplings at the end of the experiment.

time). Net  $\text{CO}_2$  assimilation rate ( $A$ ,  $\mu\text{mol m}^{-2}\text{s}^{-1}$ ) and stomatal conductance to water vapor ( $g_s$ ,  $\mu\text{mol m}^{-2}\text{s}^{-1}$ ) were computed as in [48]. At the end of the experiment, saplings were harvested and the projected needle area was measured with a leaf area meter (Delta-T Devices, Cambridge, UK). Once  $\Psi_{\text{wp}}$  had reached a threshold of around  $-2$  MPa (after approx. 10 days), saplings were watered to field capacity and left to dehydrate freely again for a second drought cycle. Saplings were sampled at the end of this second cycle.

## 2.5. Sampling

Three weeks after inoculation (October 2), areas of induced reaction zones in the phloem were measured in all saplings as described in [25]. An aliquot of healthy and reaction tissues (phloem, sapwood), and of needles was collected, frozen in liquid nitrogen, freeze-dried then weighed and ground to a fine homogeneous powder with a Cyclotec 1093 laboratory mill (Tecator AB, Höganäs, Sweden) prior to biochemical analyses. Needles, stem, branches and roots of saplings were dried in an oven (36 h at  $60^\circ\text{C}$ ) and weighed.

## 2.6. Extraction and purification of C and N metabolites from sapwood and phloem

Starch, soluble proteins, soluble sugars and amino-acids were extracted and purified according to [8, 14]. 200 mg of lyophilized powder was suspended with 5 mL of a ternary mixture (methanol/chloroform/water; 12/5/3) for 30 min at ambient temperature, centrifuged for 10 min at 2000  $g$  (Jouan MR 22i, France). The procedure was repeated on the pellet until a colorless supernatant was obtained. Starch was extracted from the pellet by solubilization in HCl 6N, vacuum-dried (Maxi-Dry plus, Heto-model DW1, 0-110, Heto-Holten A/S Allerød, Denmark) and weighed for further isotopic analyses. The supernatants were combined and vacuum-dried overnight. The dried samples were solubilized in distilled water and filtered through C18 (Waters, USA), cationic (Dowex-50W 8X-400, Sigma-Aldrich, USA) and anionic (Amberlite IRA-416, Fluka chemical, Switzerland) columns to separate soluble sugars from other biochemical compounds. The sugar fraction was eluted with distilled water, and vacuum dried. Cationic columns were rinsed with  $\text{NH}_4\text{OH}$  4N to elute amino acids. The amino acid fraction was vacuum-dried and weighed for isotopic analyses.

Extraction of soluble proteins was performed on 200 mg of lyophilized powder suspended with 2 mL of phosphate buffer (0.05 M pH 7.2), and stirred over night at ambient temperature. The solution was centrifuged 10 min at 12000  $g$  and the supernatant was collected. This procedure was repeated 2 times. Then 0.2 mL HCl 6N was added to the liquid phase. Solution was boiled at  $100^\circ\text{C}$  for one

hour and cooled at  $4^\circ\text{C}$  overnight to precipitate soluble proteins. The precipitate was centrifuged for 10 min at 10000  $g$  and the pellet was vacuum-dried and weighted for isotopic analyses.

## 2.7. Isotopic analyses

After lyophilization, purified metabolites were transferred to tin capsules (Courtaige Analyze Service, Mont Saint-Aignan, France) for isotope analysis. Isotopic analyses (samples of 0.4 mg C) were done with an elementary analyzer (NA 1500, Carlo Erba, Italie) coupled to an isotopic ratio mass spectrometer (IRMS, Delta S Finnigan MAT). Values of isotopic ratio ( $^{13}\text{C}/^{12}\text{C}$ ) were automatically corrected with the PDB standard to obtain  $\delta^{13}\text{C}$ :

$$\delta^{13}\text{C}(\text{‰}) = (R_s/R_{\text{PDB}} - 1) \times 10^3,$$

where  $R_s$  and  $R_{\text{PDB}}$  are isotopic ratios ( $^{13}\text{C}/^{12}\text{C}$ ) of sample and standard, respectively.

## 2.8. Statistical analyses

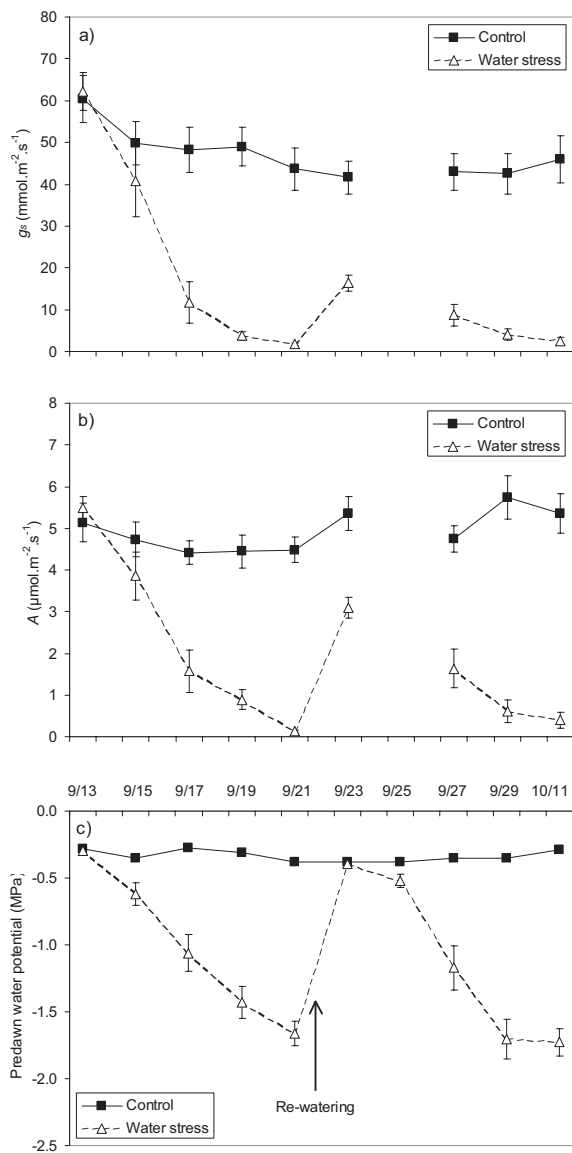
Normalized variance analyses were made using the general linear model (GLM) procedure of SAS (SAS Institute, Cary, NC) followed by Scheffe's multiple comparison test (or least significant difference (LSD) when  $n < 5$ ) at a significance level of 0.05. Mean values  $\pm$  SE at  $p = 0.05$  were shown in figures.

## 3. RESULTS

### 3.1. Water relations after inoculation

Stomatal conductance ( $g_s$ ) and net  $\text{CO}_2$  assimilation ( $A$ ) were close to  $50 \text{ mmol.m}^{-2}.\text{s}^{-1}$  and  $5 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ , respectively, in well-watered controls (Figs. 2a and 2b). Daily water use was about  $0.45 \text{ L day}^{-1}$  from an available soil water reserve of about 2 L. Predawn needle water potential ( $\Psi_{\text{wp}}$ ) fluctuated around  $-0.34$  MPa throughout the experiment (Fig. 2c).

The first drought cycle (Fig. 1) induced after 8 days, severe decreases of  $\Psi_{\text{wp}}$  down to  $-1.7$  MPa, and of  $g_s$  and  $A$  (Fig. 2). Re-watering during day 9 allowed a recovery of  $\Psi_{\text{wp}}$  to values close to controls. The second drought cycle resulted in similarly severe responses. Drought stress was short but severe, and saplings displayed suppressed photosynthesis and transpiration during peak stress. However, shoot and root biomass did not display any detectable effect of drought stress and reached  $203 \pm 19 \text{ g}$  and  $120 \pm 22 \text{ g}$  (means  $\pm$  C.I.), respectively, at the end of the experiment.

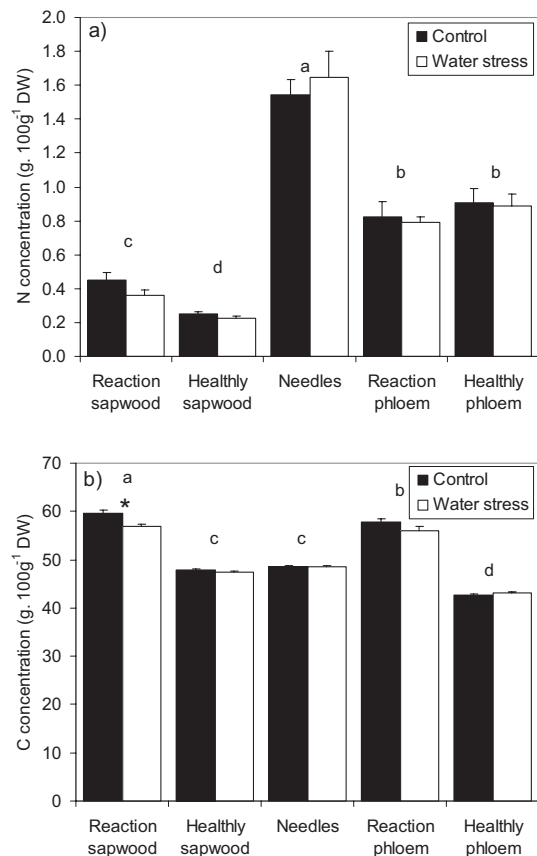


**Figure 2.** Time course of stomatal conductance ( $g_s$ , a), net  $\text{CO}_2$  assimilation ( $A$ , b), and predawn needle water potential (c) of control and water-stressed Scots pine saplings during the course of two drought cycles separated by a phase of re-watering to field capacity. Means  $\pm$  SE ( $n = 9$ ).

The induced-reaction zones were readily built up after inoculation with *Ophiostoma brunneo-ciliatum* and drought decreased significantly their area from  $50.1 \text{ mm}^2$  in well-watered controls to  $42.7 \text{ mm}^2$  in stressed saplings ( $p = 0.0251$ ).

### 3.2. Nitrogen and carbon concentration in healthy tissues and in induced-reaction zones

Nitrogen concentration was around 1.6, 0.8 and  $0.3 \text{ g } 100 \text{ g}^{-1}$  in needles, phloem and sapwood, respectively (Fig. 3a). N concentration was very close in healthy and reaction phloem. Reaction sapwood displayed a higher N

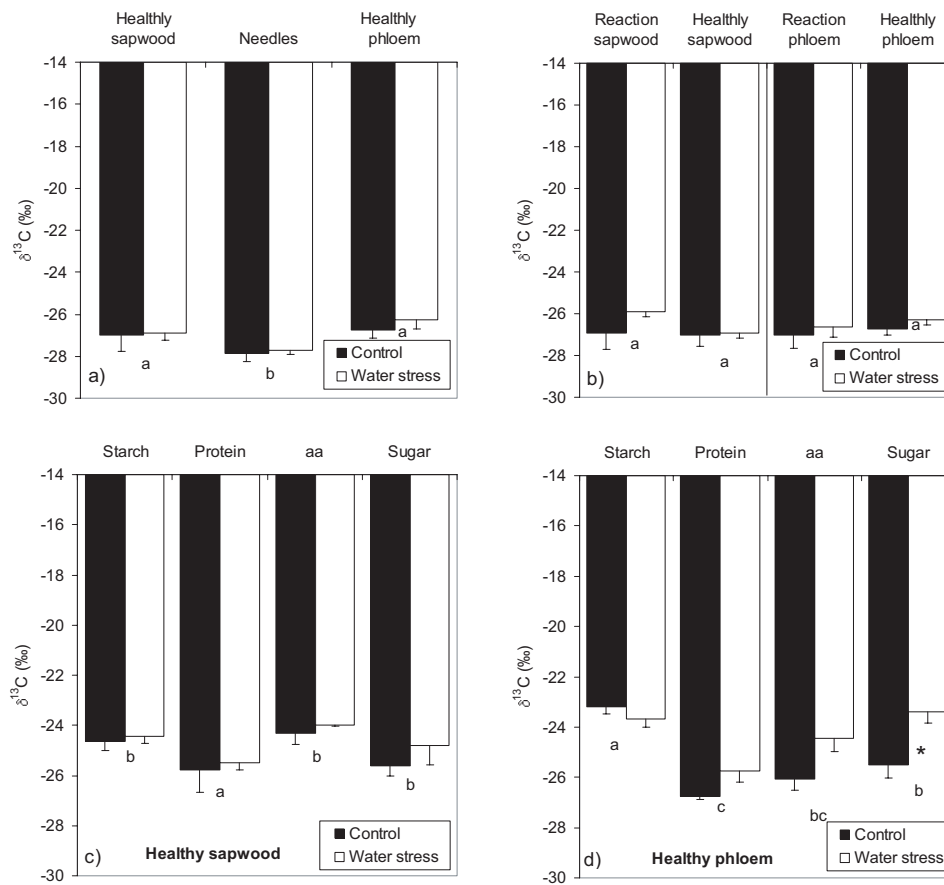


**Figure 3.** Nitrogen and carbon concentrations (a, b) in the shoots of control and water-stressed Scots pine saplings inoculated or not with *Ophiostoma brunneo-ciliatum*. Tested tissues included needles, healthy and reaction tissues (sapwood, phloem). Means  $\pm$  SE ( $n = 9$ ). Different letters indicate significant differences among tissues. Stars indicate a significant drought effect;  $p < 0.05$ .

concentration ( $0.4$ ) than healthy sapwood ( $0.2$ ). C concentration was lower in healthy phloem than in needles and healthy sapwood (Fig. 3b). The reaction zones displayed much higher C concentrations than their healthy counterparts ( $58$  vs.  $48 \text{ g } 100 \text{ g}^{-1}$ ). No drought effect was observed on C and N, with the exception of a slight decrease of C concentration in the reaction sapwood of drought stressed saplings.

### 3.3. Effect of inoculation on $\delta^{13}\text{C}$ in healthy and reaction tissues of unlabeled saplings

$\delta^{13}\text{C}$  was about  $-26.9\text{‰}$  in phloem and sapwood and  $-27.8\text{‰}$  in needles of unlabeled saplings ( $p = 0.0047$ ; Fig. 4a). Drought did not alter these values.  $\delta^{13}\text{C}$  of reaction tissues was very close to that of their healthy counterpart (Fig. 4b), showing that the synthesis of defense compounds did not result in a detectable C isotope discrimination. More pronounced differences were detected between biochemical compounds extracted from sapwood and phloem (Figs. 4c and 4d). In sapwood,  $\delta^{13}\text{C}$  varied between  $-24\text{‰}$  (amino acids and starch) and  $-26\text{‰}$  (soluble sugars and soluble proteins),



**Figure 4.** Isotopic composition ( $\delta^{13}\text{C}$ ) of shoot tissues (a, b), and of some N and C-based non structural compounds of healthy sapwood (c) and healthy phloem (d), in unlabeled, control and water-stressed Scots pines submitted or not to inoculations with *Ophiostoma brunneo-ciliatum*. Means  $\pm$  SE ( $n = 3$ ). Stars indicate a significant drought effect;  $p < 0.05$ .

with no detectable effect of drought (Fig. 4c). In phloem tissues, the situation was more contrasted, with significant differences among compounds (Fig. 4d). Proteins displayed the lowest ( $-28\text{‰}$ ) and starch the highest values (around  $-23\text{‰}$ ). Soluble sugars and amino-acids ranked in between these two extremes. Drought ended to a marked increase of  $\delta^{13}\text{C}$  in both amino acids and soluble sugars of the phloem (Fig. 4d), which reflects the expected drought-induced decrease of discrimination during photosynthesis [21].

### 3.4. Effect of inoculation on $\delta^{13}\text{C}$ in healthy and reaction tissues of labeled saplings

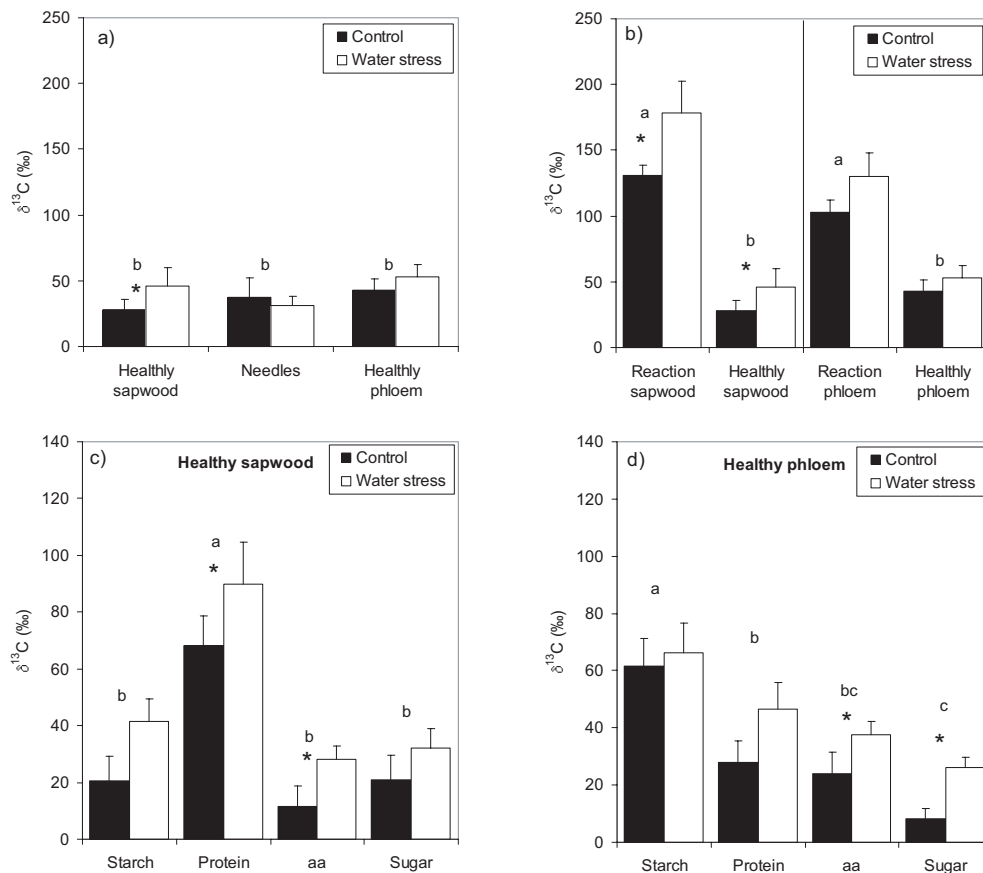
During October, all tissues of labeled trees showed increased  $\delta^{13}\text{C}$  with respect to unlabeled ones (Figs. 4 and 5).  $\delta^{13}\text{C}$  varied from  $+30$  to  $+50\text{‰}$  in the different tissues, and was increased by drought in sapwood and phloem tissues (Fig. 5a).  $\delta^{13}\text{C}$  was much larger in reaction than in healthy tissues ( $140$  vs.  $50\text{‰}$  in phloem and  $180$  vs.  $50\text{‰}$  in sapwood; Fig. 5b). Moreover, drought had a visible impact on these tissues and induced large increases of  $\delta^{13}\text{C}$  (up to  $+180\text{‰}$ ).

Delta  $^{13}\text{C}$  of biochemical compounds extracted from healthy sapwood and phloem of irrigated controls varied with tissue and drought treatment (Figs. 5c and 5d). In sapwood

(Fig. 5c), the highest  $\delta^{13}\text{C}$  was measured in soluble proteins ( $70$ – $110\text{‰}$ ), while starch, amino acids and sugars were much less labeled ( $10$ – $40\text{‰}$ ). In the phloem (Fig. 5d), highest  $\delta^{13}\text{C}$  was found in starch and soluble proteins ( $+60\text{‰}$ ) and lowest  $\delta^{13}\text{C}$  in amino acids and soluble sugars ( $10$  to  $40\text{‰}$ ). Drought markedly increased  $\delta^{13}\text{C}$  of many of these compounds; this increase was significant for proteins and amino acids in the sapwood (Fig. 5c), and for the amino acids and soluble sugars in the phloem (Fig. 5d). Nonetheless, none of these compounds reached the levels of  $\delta^{13}\text{C}$  in the reaction tissues.

## 4. DISCUSSION

Inoculation of *Ophiostoma brunneo ciliatum* into the trunk of well-watered Scot pine saplings induced the build-up of well delimited reaction zones such as described earlier [10,11]. An inoculation density of  $400\text{ m}^{-2}$  induced enough defense reactions for biochemical analyses, but remained below the threshold inoculation density ( $900\text{ m}^{-2}$ ) needed to kill vigorous young Scots pines [25]. The severe drought which was imposed immediately after inoculation, resulted in a drop of predawn needle water potential  $\Psi_{wp}$ , a severe stomatal closure and a large decline of net  $\text{CO}_2$  assimilation. A reduction of the area of the induced-reaction



**Figure 5.** Isotopic composition ( $\delta^{13}\text{C}$ ) of shoot tissues (a, b), and of some N and C-based non structural compounds of healthy sapwood (c) and healthy phloem (d), in labelled, control and water-stressed Scots pines submitted or not to inoculations with *Ophiostoma brunneo-ciliatum*. Means  $\pm$  SE ( $n = 6$ ). Stars indicate a significant drought effect;  $p < 0.05$  (aa = amino acids).

zones was also noted. As a consequence, C availability was reduced and the defense ability of the Scot pines against fungus development may have been significantly decreased. However, such a treatment was not drastic or long enough to significantly reduce tree biomass or to cause enhanced senescence of old needles.

The induced-reaction zones showed increased C concentrations compared to healthy tissues, which reflects accumulation of secondary metabolites with low oxygen content, such as phenols, terpenes and tannins in the reaction zones [11, 15, 20]. Moreover, reaction sapwood displayed higher N concentrations than the healthy one, probably in relation with an increase of protein-based chemical defenses [23]. No drought effect was observed on C and N concentration of healthy and injured tissues, with the exception of a slight decrease of C concentration in the reaction sapwood of drought stressed saplings. This result indicates that metabolic changes occurred in this tissue in response to drought. Decreases of the size of induced-reactions and small changes in the phenolic composition of injured tissues were also recorded in severely water-stressed Scots pine trees [11].

Values of  $\delta^{13}\text{C}$  of tissues of unlabeled Scots pine saplings were typical of the isotopic signature of  $\text{C}_3$  plants [21, 26].

Isotopic discrimination by key enzymes generates measurable isotopic gradients in pools of metabolic intermediates, resulting in end-products with different isotopic compositions [24, 45]. Drought induced a marked increase of  $\delta^{13}\text{C}$  in both amino acids and soluble sugars of healthy phloem. This  $\delta^{13}\text{C}$  increase reflects the expected decrease in  $^{13}\text{C}$  discrimination during C assimilation in water-stressed plants [21].

The  $^{13}\text{C}$  labeling-technique allowed to label C stored during August after cessation of shoot growth and early wood formation [27, 46]. Our results show that three weeks after inoculation, sapwood and phloem tissues of saplings were highly enriched in  $^{13}\text{C}$  as compared to unlabeled ones. As expected, the non-structural C compounds susceptible to be C suppliers for the construction of reaction zones (soluble sugars, starch, amino acids, ...) were much more enriched than the bulk tissues. The most enriched compounds were soluble proteins in healthy sapwood, which  $\delta^{13}\text{C}$  was additionally increased by drought (from +70 to +120‰). During the formation of the induced-reaction zones, two sources of carbon were available: (1) newly assimilated C, with a negative  $\delta^{13}\text{C}$  (−23 to −29‰) and (2) stored C with a positive  $\delta^{13}\text{C}$  (+30 to +120‰). Basing on a two source model, the isotopic signature of induced-reaction zones should be between these extreme

values and the computation of a mixing coefficient should produce an estimate of the relative contribution of each source. The isotopic analyses revealed that the induced-reaction zones were very strongly labeled, implying they were to a large extent built from stored C. This conclusion is in agreement with [42] and [44] who suggested that induced-reaction zones were built from carbon reserves by starch hydrolysis around reaction zones. The fact that reaction zones were even more intensely labeled than the metabolites of surrounding tissues, both in well-watered and water-stressed saplings, was a surprise. One line of explanation for this apparent discrepancy is related to the very fast construction of the induced-reaction zones [35, 42, 50] implying a rapid consumption of heavily labeled C reserves, before  $^{13}\text{C}$  was diluted by accumulation of newly assimilated C. Another line of explanation, non exclusive of the first one, could be a preferential remobilization of C assimilated (and labeled) during August with respect to older, unlabelled C that would be less easily accessed. In fact, one has to take into account that storage compounds were probably not uniformly labeled, and that recently stored (and also more readily available compounds) were probably more labeled than what was measured from bulk products. This can be particularly true for C mobilization from starch granules that display a layered structure (the oldest being accessible for hydrolysis only after the newest ones were digested by alpha-amylases) [13].

All tissues of water-stressed Scots pine saplings were significantly more enriched in  $^{13}\text{C}$  than their counterparts from well-watered saplings. This can only be explained by the fact that after labeling,  $^{13}\text{C}$  in stored carbon was diluted by newly assimilated carbon in controls, but much less in stressed saplings where carbon assimilation was severely depressed. A similar effect was observed in the reaction zones. It is not possible, on the basis of our data, to produce a quantitative model for the contribution of different compartments to the C in reaction zones, but the fact that drought induced a similar shift in compounds from healthy tissues as well as in reaction zones, comes in support of a predominant contribution of stored carbon to the reaction zones.

Induced-defense results generally in decreases in sugar and starch concentrations in inner bark [5, 7, 42, 44]. However, the amount of reserves available around the attack points may become critical due to changes in source-sink relationships, as influenced by the environment and biotic stresses [18]. At that stage, the capacity of the tree to respond the fungal spread may rely more on the availability of current assimilates from the foliage [5]. Abiotic factors, such as nutrient supply and water relations, have the potential to modify the plant-insect-fungus interaction. During beetle aggregation, anything that contributes to the depletion of the host tree's ability to synthesize secondary metabolites increases the probability of successful beetle mass attacks [28, 31]. Extreme water deficits must lead to a collapse of the carbon budget, declining photosynthesis and concomitant decreases in secondary metabolism [38]. Inducible responses result from changes in gene expression, that influence the biochemical regulation of secondary metabolism [38]. However, the physiological and nutrient status of host trees is also important and

susceptible to modulate production of carbon-based defenses such as phenolics [30]. The impact of internal C resources on responses to massive attacks by *Ophiostoma brunneo ciliatum* requires further attention, particularly in situations of limiting resource availability.

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