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Comparing carbon isotope composition of bulk wood and holocellulose from *Quercus cerris*, *Fraxinus ornus* and *Pinus radiata* tree rings

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Abstract: Tree-ring $\delta^{13}C$ is widely employed in ecophysiological studies, because it represents an integra-ted proxy of the ratio between photosynthesis (*A*) and stomatal conductance (*g*), which expresses the intrin-sic water use efficiency (*iWUE*), strongly affected by the environmental conditions experienced by the plant during its life span. Tree-ring $\delta^{13}C$ also reflects long term variations of atmospheric CO_2 concentration and of its carbon isotope composition, partly due to increasing anthropogenic emissions. Carbon isotope abundan-ces in tree rings can be assessed on bulk wood as well as on wood biochemical components, wich show different $\delta^{13}C$ values because of secondary discrimination during biosynthesis. We present the results of a comparison between $\delta^{13}C$ values of bulk wood and holocellulose samples obtained from the last three (1999, 2000 and 2001) annual growth rings of two hardwood (*Quercus cerris* L. and *Fraxinus ornus* L.) and one conifer (*Pinus radiata* D. Don) species. We found that $\delta^{13}C$ values differed significantly among tree species, both in the case of holocellulose and bulk wood, but only in the case of *P. radiata* bulk wood samples tend to provide more negative $\delta^{13}C$ values than holocellulose, as reported in the literature. We suggest that, at least for the two hardwood species studied, bulk wood is a suitable material to work with for $\delta^{13}C$ assessment, whilst in *P. radiata* holocellulose could provide a more stable and reliable index, when studying plant ecophysiological responses to changing environmental conditions.

Introduction

Tree-ring carbon stable isotope ratio ($^{13}C/^{12}C$) depends on discrimination against the heavier isotope, ^{13}C, during photosynthesis (CO_2 diffusion through the stomata and carbon fixation in the chloroplast) and during secondary biochemical reactions (translocation and differentiation of photosynthates).

According to the model by Farquhar et al. (1982), in C_3 plants carbon isotope discrimination strongly depends on CO_2 concentration in leaf intercellular spaces (c_i), which is further regulated by stomatal conductance (*g*) and by photosynthetic efficiency (*A*). Several environmental factors affect stomatal conductance and, therefore, plant carbon stable isotope composition.

Tree-ring $\delta^{13}C$ is widely employed in ecophysiological studies, because it represents an integrated proxy of the ratio between photosynthesis (*A*) and stomatal conductance (*g*), which expresses the intrinsic water use efficiency (*iWUE*) strongly affected by the environmental conditions experienced by the plant during time. Tree-ring $\delta^{13}C$ also reflects long term variations of atmospheric CO_2 concentration and of its carbon isotope composition, partly due to increasing anthropogenic emissions (Stuiver & Braziunas 1987, Feng & Epstein 1995).

Carbon stable isotope composition in tree rings can be assessed on bulk wood as well as on wood biochemical components, such as lignin, holocellulose and α-cellulose. Different wood components show

different δ¹³C values: for instance, cellulose is ¹³C enriched by 1-2 ‰ relative to whole wood, while lignin is depleted by 2-6 ‰ relative to whole wood and by 4-7 ‰ relative to cellulose (Benner et al. 1987, Brugnoli & Farqhuar 1999). Depletion observed for secondary plant products, especially lignins, aromatic compounds, flavonoids originating from the pathway of shikimic acid, is due to the kinetic isotope effect of the pyruvate dehydrogenase reaction (Melzer & Schmidt 1987, Gleixner et al. 1993).

For assessing tree ring δ¹³C, analysis of cellulose extracts could theoretically be preferred because *i*) cellulose does not migrate from the tree ring in which it was formed and *ii*) less reactions (and fractionations) occur during its synthesis from photosynthates, in comparison with biosynthesis of lignin and other wood compounds. Bulk wood analyses may lead to δ¹³C values more affected by lignin, resins and highly mobile carbon reserves, such as carbohydrates, which tend to migrate between adjacent tree rings and whose amount may vary widely between sapwood and heartwood (Tans et al. 1978, Leavitt & Danzer 1993, Sheu & Chiu 1995). Furthermore, the ratio of lignin and cellulose concentrations is quite variable in bulk wood, thus, when measuring its δ¹³C, it would represent the mixing ratio of lignin and cellulose and not the water use efficiency of the plant studied (Wilson & Grinsted 1977). On the other hand, cellulose extraction is a time-consuming process, requiring the employment of several reagents and may represent a bottle-neck when trying to analyse long-time tree ring series.

Borella et al. (1998) provided an in-depth analysis of the uncertainties arising when measuring tree ring δ¹³C and, in particular, tried answering the question if cellulose extraction is needed for δ¹³C assessment; they showed good correlations ($r^2 > 0.8$) between δ¹³C of α-cellulose and bulk wood for hardwood species (oak and beech), concluding that 'wood δ¹³C is as good climate proxy as cellulose'. On the other hand, they warned that extractives should at least be removed from conifer wood, as it can have variable and high resin content.

In this paper we present the results of a comparison between δ¹³C values of bulk wood and holocellulose obtained from the last three (1999, 2000 and 2001) annual growth rings of two hardwood (*Quercus cerris* L, *Fraxinus ornus* L.) and one conifer (*Pinus radiata* D.Don) species, growing in the same forest.

In the literature many methods for cellulose extraction have been described (see among others Seifort 1960, Green 1963, Tans & Mook 1980, Brenninkmei-

jer 1983, Pereira 1988, Loader et al. 1997, McFarlane et al. 1999, Brendel et al. 2000), but the "Jaime-Wise" method (Green 1963) and its subsequent modifications (Leavitt & Danzer 1993, Sheu & Chiu 1995, Livingstone & Spittlehouse 1996) are used in most cases.

The main goal of the present paper is to test for differences between δ¹³C values of bulk wood and holocellulose, in order to assess which one can be a more suitable proxy of water use efficiency of trees and, therefore, of short term changing environmental conditions.

Materials and Methods

Plant material was collected in December 2001, on *Q. cerris* (n = 44), *F. ornus* (n = 14) and *P. radiata* (n = 6) trees growing in the Stio municipality (Southern Italy, province of Salerno, 40°18'32" N, 15°15'07" E, 700 m a.s.l.), at an altitude of about 700 m a.s.l. Climate is Mediterranean, humid type; mean temperature is 13 °C and annual precipitation 1200 mm.

The studied forest is a 22-24 year old *P. radiata* D. Don plantation wich extends over 106 ha. Within the forest two stands have been selected, homogeneous according to all environmental conditions but for light regime. The stands have been identified as follows: lightly thinned stand (one over three rows of trees) with natural regeneration of *Q. cerris* (*n* = 16 in S2); strongly thinned stand (one over two rows of trees) with natural regeneration of *Q. cerris* (*n* = 15 in S3) and *F. ornus* (*n* = 14 in S3). As a control a coppice of native *Quercus* species (*n* = 13 in S1) has been selected too, growing in the same area.

In the case of hardwoods, trees were cut at their base and a 3 cm thick wood disc was sawn off from the lowermost stem portion; in the case of pine, a wood core was extracted by means of an increment borer from the NW facing side of the stem at breast height. *Q. cerris* and *F. ornus* were 6-7 years old saplings, while *P. radiata* 22-24 years old trees. Tree-ring width was quite regular in each studied species for all the rings sampled.

Taken soon to the laboratory, samples were oven dried at 60 °C for 24 hours. Afterwards, thin shavings were obtained with a razor blade from each of the last three (1999, 2000 and 2001) annual growth rings (whole ring for hardwoods and NW facing section of the ring for the conifer); material was further reduced to 0.2 mm particles using a micro-gear miller and a ceramic mortar. Of each wood sample: *i*) one half underwent direct analysis of carbon isotope abundances into a Finnigan Delta Plus (Finnigan, Bremen, Germany) mass spectrometer (bulk wood

samples, *WS*); *ii*) the other half underwent holocellulose (hereafter indicated as cellulose) extraction according to the Jaime-Wise method (Green 1963), modified by Leavitt & Danzer (1993); afterwards, holocellulose was analysed into the same Finnigan Delta Plus mass spectrometer (cellulose samples, *CS*). Cellulose extraction was carried out in two steps: *i*) removal of sugars and lipids by means of a soxhlet apparatus, keeping the samples 48 hours in a 2:1 solution of toluene and ethanol and 48 hours in pure ethanol, *ii*) removal of lignin, keeping the samples 4 days in a solution of sodium chlorite ($NaClO_2$) and glacial acetic acid (CH_3COOH) at 70 °C (bleaching). Before bleaching, samples were taken out from the soxhlet to dry and boiled in a beaker with

Tab. 1 - Differences among adjacent tree ring $\delta^{13}C$ values according to one-way ANOVA.

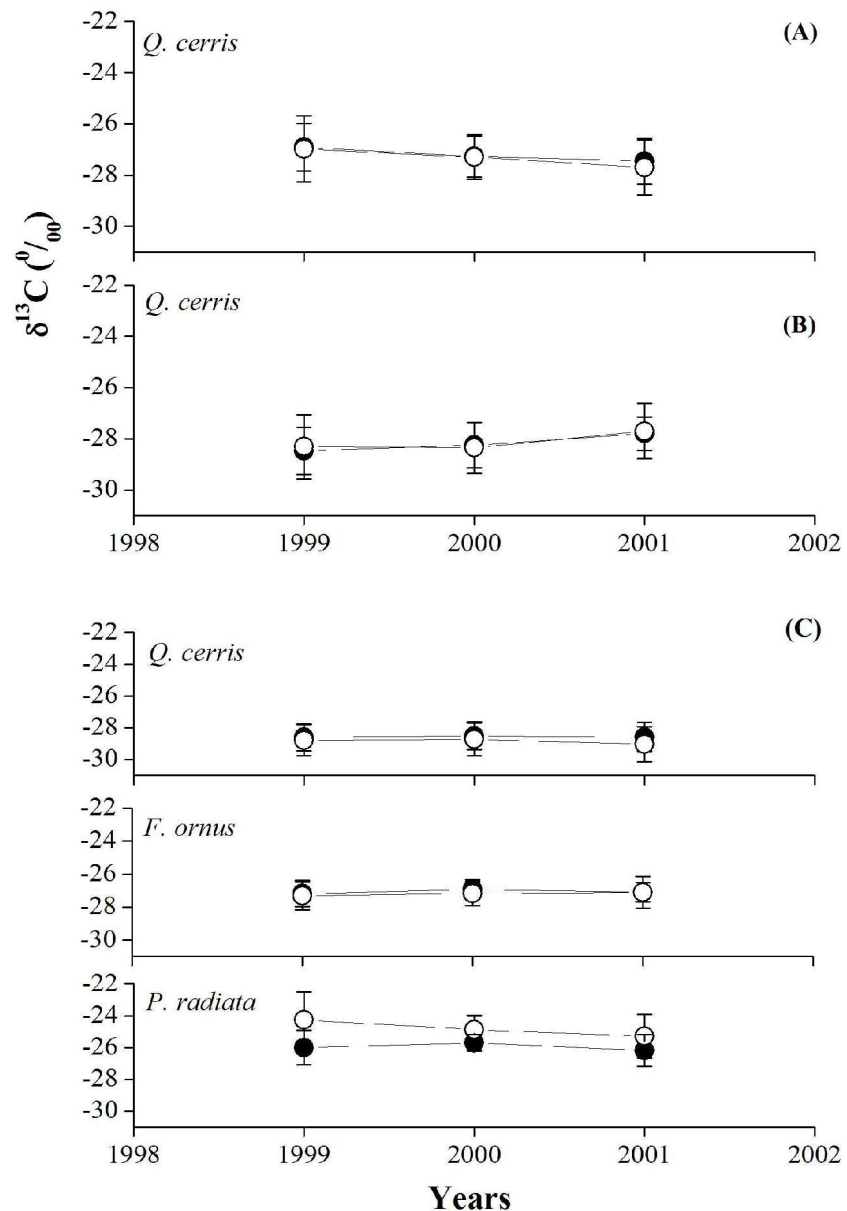| Species	Bulk wood			Holocellulose		
	F	*df*	*P*	*F*	*df*	*P*
Quercus cerris	0.381	131	0.684	0.056	143	0.945
Fraxinus ornus	0.307	47	0.737	0.129	49	0.879
Pinus radiata	0.616	17	0.501	0.707	15	0.513

Fig. 1 - Tree ring $\delta^{13}C$ values of bulk wood (•) and holocellulose (○) according to year in S1 (A), S2 (B) and S3 (C) for all the studied species.
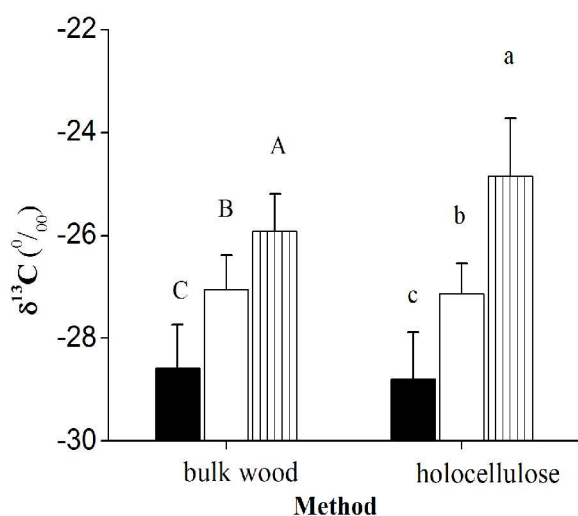
Fig. 2 - Comparison between species; $\delta^{13}C$ values (mean ± SD) of bulk wood and cellulose of *Q. cerris* (left column), *F. ornus* (central column) and *P. radiata* (right column). Different capital (bulk wood) and lower case (holocellulose) letters indicate significant differences ($P < 0.05$) among species according to S-N-K multiple comparison test; $\delta^{13}C$ values from different growth rings in the same plant have been considered as replicates.

deionised water for 6 hours to remove hydro-soluble sugars. The level of the solution during bleaching was kept constant refilling the beaker when needed; at the beginning of each day of bleaching samples were accurately rinsed in deionised water and the solution was replaced by a newly prepared one.

All statistics were carried out using the SPSS statistical package (SPSS Inc. 1989-1999).

Results and Discussion

According to one-way ANOVA, tree-ring carbon isotope composition ($\delta^{13}C$) did not differ among annual growth rings in any of the studied tree species, as well as holocellulose (*CS*) or bulk wood samples (*WS*) were considered (tab. 1).

In the present case of study, site climatic conditions did not show significant differences among years (data not shown): mean annual temperature was

slightly above the average value and water was not a limiting factor since precipitation amounts were larger than the average value. Also, light environment in each stand did not change significantly during the short study period. Therefore, the absence of significant variations in $\delta^{13}C$ among years could be attributed to steady climatic and environmental conditions experienced by the trees during the different years, as showed also by ring widths (data not shown). In fact, yearly tree ring $\delta^{13}C$ values did not differ among them both in the case of *BW* and *CS* (fig. 1). $\delta^{13}C$ values of adjacent tree rings from the same plant may thus be averaged in case of short term physiological studies, according to previous findings (Borella et al 1998), for reducing the effect of lignin migration and of translocation of previous year photosynthates during earlywood construction.

Values of $\delta^{13}C$ differed significantly among tree

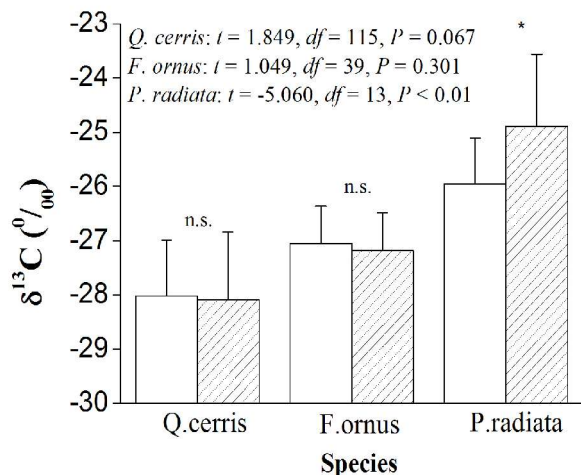**Fig. 3** - Comparison between methods; $\delta^{13}C$ values (mean ± SD) of bulk wood (left column) and holocellulose (right column) of *Q. cerris*, *F. ornus* and *P. radiata*. n.s. means not significant whilst * indicates significant differences, according to paired sample *t*-test; $\delta^{13}C$ values from different growth rings in the same plant have been considered as replicates.

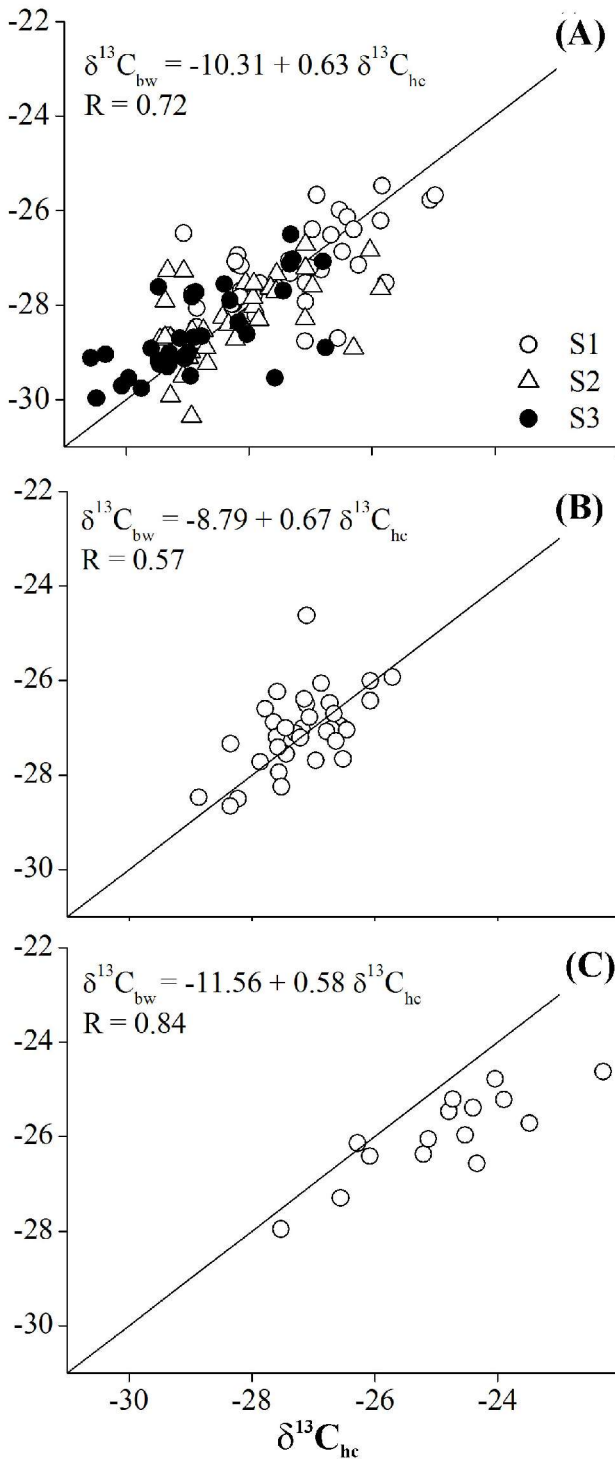**Fig. 5** - Scatter of bulk wood $\delta^{13}C$ residuals *vs.* holocellulose $\delta^{13}C$ relative to the zero value.

(Ehleringer 1991) and the observed differences in isotopic composition could likely be attributed to contrasting ecophysiological characteristics between the studied species (Tans et al. 1978, D'Alessandro et al. in preparation) as well as to varying lignin to cellulose ratios among years and species. In fact, the two species show different stomatal behaviours as discussed in the literature: *Q. cerris* exhibits a lower stomatal control than *F. ornus* (Tretiach 1993) when experiencing water stress (Nardini et al. 1999).

At least in part, the $\delta^{13}C$ values in *Q. cerris* and *F. ornus* saplings may also be affected by a 'juvenile effect' (Francey & Farquhar 1982, Bert et al 1997), which consists of low and/or steadily increasing values of wood and cellulose $\delta^{13}C$ during the early stages of tree growth. This "juvenile effect" has been attributed to a different microenvironment during the phase of seedling establishment, to uptake of ^{13}C depleted respired CO_2 from the canopy floor and from the soil, or to age-related physiological factors (Freyer 1979, Francey 1981, Marshall & Monserud 1996, Bert et al. 1997).

In both *Q. cerris* and *F. ornus* no significant difference in $\delta^{13}C$ was found between *CS* and *WS* (according to paired-samples *t*-test), despite holocellulose was found to be depleted in ^{13}C by 0.04 ‰ in oak and by 0.09 ‰ in ash, relative to bulk wood (fig. 3). Holocellulose $\delta^{13}C$ could have been affected by hemicellulose, whose content is greater in juvenile wood (Rowell et al 2000) and which is depleted in ^{13}C with respect to cellulose (Benner et al. 1987, McFarlane et al. 1999). On the other hand, in *P. radiata* $\delta^{13}C$ values were significantly more negative in *WS* than in *CS*.

Indeed, looking at the relationships between individual $\delta^{13}C$ values in *CS* and *WS* samples, it can be

Fig. 4 - Relationship between $\delta^{13}C$ values of bulk wood (y-axis) and holocellulose (x-axis) in *Q. cerris* (A), *F. ornus* (B) and *P. radiata* (C); the line represents the 1:1 relationship.

species, both in the case of *CS* and *WS*. In particular, *Q. cerris* showed the lowest (more negative) isotopic composition (fig. 2); carbon isotope discrimination is considered a good proxy of water-use efficiency
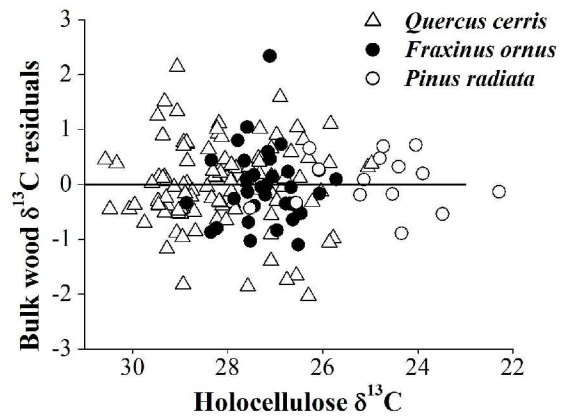
observed that in *Q. cerris* and *F. ornus* data points are rather well scattered around the one-to-one line, with no apparent systematic deviation (fig. 4A and B), whilst in the case of *P. radiata* WS tend to provide more negative $\delta^{13}C$ values (fig. 4C). Besides, the large range of variation of $\delta^{13}C$ values in *Q. cerris* for both CS (5.6‰) and WS (4.8‰) depends on the fact that trees were sampled in three different stands with changing light conditions; therefore $\delta^{13}C$ values varied among stands depending on the characteristics of the above canopy cover (pine versus native oak coppice, data not shown). In fact, according to one-way ANOVA, $\delta^{13}C$ values in *Q. cerris* differed significantly among stands, for both *WS* ($F = 26.936$, $df = 129$, $P < 0.00001$) and *CS* ($F = 18.247$, $df = 111$, $P < 0.00001$), with S1 showing higher $\delta^{13}C$ values relative to S2 and S3.

With respect to the relationship reported by Borella et al. (1998) (fig. 4A) for oak wood, we have found a lower correlation coefficient between $\delta^{13}C$ of *CS* and *WS*, due to larger scatter in our data, but a closer agreement to the one-to-one relationship. The slope of regression between holocellulose and bulk wood is always smaller than 1, showing that the isotopic variations in *CS* are larger than the variations in *WS*. This result could indicate that some interesting climatic or environmental signal may be preserved in $\delta^{13}C$ of holocellulose while it can be partially lost or masked in $\delta^{13}C$ of bulk wood. This can be due to the fact that, unlike wood lipids and other compounds, holocellulose derives directly from the primary photosynthates (Gleixner et al. 1993); thus, its carbon isotope composition could better reflect the climatic and environmental conditions which affected the photosynthetic pathway.

On the other hand, it is worth noting that regression residuals are unevenly distributed, *i.e.* irregularly scattered, around the zero value (fig. 5), thus suggesting caution in the use of a linear regression model.

We may conclude that in *Q. cerris* and *F. ornus* , wood carbon isotopic composition is mainly affected by cellulose $\delta^{13}C$ and bulk wood seems a suitable material to work with when studying plant ecophysiological response to changing environmental conditions. Only under conditions which may favour rapid polysaccharides degradation, as may occur with wood material suitable for paleoclimatic studies, extraction of lignin may be recommended (Schleser 1999). As for *P. radiata*, our results suggest that $\delta^{13}C$ of bulk wood could deviate significantly from $\delta^{13}C$ of holocellulose, possibly as an effect of secondary compounds, such as oleoresins, whose amount can vary from year to year and lignin, which tend to migrate among as well as within tree rings. In this case, cellulose $\delta^{13}C$ could represent a more stable and reliable index compared to bulk wood.

References

Benner R, Fogel ML, Sprague EK, Hodson RE (1987). Depletion of ^{13}C in lignin and its implication for stable carbon isotope studies. Nature 329: 708-710.

Bert D, Leavitt SW, Dupouey JL (1997). Variations of wood $\delta^{13}C$ and water-use efficiency of Abies alba during the last century. Ecology 78 (5): 1588-1596.

Borella S, Leuenberger M, Saurer M, Siegwolf R (1998). Reducing uncertainties in $\delta^{13}C$ analysis of tree rings: pooling, milling and cellulose extraction. Journal of Geophysical Research 103 D16: 19519-19526.

Brendel O, Iannetta PPM, Steward D (2000). A rapid and simple method to isolate pure α-cellulose. Phytochemical Analysis 11: 7-10.

Brenninkmeijer CAM 1983. Deuterium, oxygen-18 and carbon-13 in tree rings and peat deposits in relation to climate, Ph.D. Thesis, Rijksuniv. Groningen, Groningen, Netherlands.

Brugnoli E, Farqhuar GD (1999). Photosynthetic fractionation of carbon isotopes. In: Photosynthesis: Physiology and Metabolism (Leegood RC, Sharkey TD, von Caemmerer S eds.), Kluver Academic Publisher, Boston, pp. 399-434.

D'Alessandro CM, Saracino A, Borghetti M (in preparation). Comparison of tree-ring bulk wood and cellulose $\delta^{13}C$ efficiency in the study of the ecophysiology of the natural regeneration of hardwood species (Quercus cerris L. and Fraxinus ornus L.) experiencing different light regimes in Pinus radiata D. Don plantations.

Ehleringer JR (1991). $^{13}C/^{12}C$ fractionation and its utility in terrestrial plant studies. In: Carbon Isotope Techniques (Coleman DC, Fry B eds.), Academic Press, New York, pp. 187-200.

Farquhar GD, O'Leary MH, Berry JA (1982). On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. Aus-

tralian Journal of Plant Physiology 9: 121-137.

Feng X, Epstein S (1995). Carbon isotopes of trees from arid environments and implications for reconstructing atmospheric CO_2 concentration. Geochimica et Cosmochimica Acta 59 (12): 2599-2608.

Francey RJ (1981). Tasmanian tree rings belie suggested anthropogenic $^{13}C/^{12}C$ trends. Nature 290: 232-235.

Francey RJ, Farquhar GD (1982). An explanation of $^{13}C/^{12}C$ variations in tree rings. Nature 297: 28-31.

Freyer HD (1979). On the ^{13}C record in tree rings. Part I. ^{13}C variations in northern hemispheric trees during the last 150 years. Tellus 31: 124-137.

Gleixner G, Danier HG, Werner AR, Schmidt HL (1993). Correlation between the ^{13}C content of primary and secondary products in different cell compartments and that in decomposing basidiomycetes. Plant Physiology 102: 1287-1290.

Green JW (1963). Wood cellulose. In: Methods in carbohydrate chemistry (Whistler RL ed), Academic Press, New York, pp. 9-21.

Leavitt SW, Danzer SB (1993). Method for batch processing small wood samples to holocellulose for stable carbon isotope analysis. Analytical Chemistry 65: 87-89.

Livingstone NJ, Spittlehouse DL (1996). Carbon isotope fractionation in tree ring early and late wood in relation to intra-growing season water balance. Plant Cell and Environment 19: 768-774.

Loader NJ, Robertson I, Barker AC, Switsur VR, Waterhouse JS (1997). An improved technique for batch processing of small whole wood samples to α-cellulose. Chemical Geology 136: 313-317.

Marshall JD, Monserud RA (1996). Homeostatic gas-exchange parameters inferred from $^{13}C/^{12}C$ in tree rings of conifers. Oecologia 105: 13-21.

Melzer E, Schmidt HL (1987). Carbon isotope effects on the pyruvate dehydrogenase reaction and their importance for relative carbon-13 depletion in lipids. Journal of Biological Chemistry 262: 8159-8164.

McFarlane C, Warren RC, White DA, Adams MA (1999). A rapid and simple method for processing wood to crude cellulose for analysis of stable carbon isotopes in tree rings. Tree Physiology 19: 831-835.

Nardini A, Lo Gullo MA, Salleo S (1999). Competitive strategies for water availability in two Mediterranean *Quercus* species. Plant, Cell and Environment 22: 109-116

Pereira H (1988). Variability in the chemical composition of plantation eucalyptus (Eucalyptus globulus Labill.). Wood Fibre Science 20: 82-90.

Rowell RW, Han JS, Rowell JS (2000). *Characterization and factors effecting fiber properties*. In: Natural Polymers and Agrofibers Composites (Frollini E, Leão AL, Mattoso LHC eds), IQSC/USP, UNESP and Embrapa Instrumentação Agropecuária. Sãn Carlos – Brazil, pp. 115-134.

Schleser GH, Frielingsdorf J, Blair A (1999). Carbon isotope behaviour in wood and cellulose during artificial aging. Chemical Geology 158: 121-130.

Seifort K (1960). Zur Frage der Cellulose-Schnellbestimmung nach der Acetylacetone-Methode. Das Papier 14: 104-106.

Sheu DD, Chiu CH (1995). Evaluation of cellulose extraction procedure for stable carbon isotope measurements in tree ring research. International Journal of Environmental Analytical Chemistry 59: 59-67.

Stuiver M, TF Braziunas (1987). Tree cellulose $^{13}C/^{12}C$ isotope ratios and climatic change. Nature 328:58-60.

Tans PP, Mook WG (1980). Past atmospheric CO_2 levels and the $^{13}C/^{12}C$ ratios in tree rings. Tellus 32:268-283.

Tans PP, de Jong AFM, Mook WG (1978). Chemical pretreatment and radial flow of ^{14}C in tree rings. Nature 271: 234-235.

Tretiach M (1993). Photosynthesis and transpiration of evergreen Mediterranean and deciduous trees in an ecotone during a growing season. Acta Oecologica 14: 341-360.

Wilson AT, Grinsted MJ (1977). $^{13}C/^{12}C$ in cellulose and lignin as palaeothermometers. Nature 265: 133-135.