Planta (1997) 203: 275–282

Planta

Chalcone synthase activity and polyphenolic compounds of shoot tissues from adult and rejuvenated walnut trees

Anne-Catherine Claudot[1], Dieter Ernst[2], Heinrich Sandermann[2], Alain Drouet[1]

[1]Laboratory of Plant Physiology, University of Orléans, BP 6749, F-45067 Orléans Cedex 2, France
[2]GSF-National Research Center for Environment and Health, Institute of Biochemical Plant Pathology, D-85764 Neuherberg, Germany

Received: 30 September 1996 / Accepted: 17 March 1997

Abstract. Changes in the metabolism of naphthoquinone and flavonoids during the growth of half-sib adult and rejuvenated walnut shoots (*Juglans nigra* × *Juglans regia* L.) were studied at the tissue level for two years after pruning. Moreover, the role of chalcone synthase (CHS; EC 2.3.1.74) in the regulation of flavonoid biosynthesis was investigated at the level of enzyme activity. The end products of walnut flavonoid biosynthesis, myricitrin and quercitrin, which accumulated in the bark and phloem at the end of growth, did not inhibit the biosynthetic process at concentrations of up to 100 μM each. There was no evidence of CHS regulation by feedback or similar mechanisms which might modulate enzyme activity. Mathematical correlation of CHS activity and flavonoid accumulation during shoot growth, however, indicated that CHS is the rate-limiting enzyme of the pathway in bark and phloem and that flavonoids seem to be transported from phloem to bark where they accumulated mainly during growth. In defoliated shoots, naphthoquinone metabolism appeared to be a marker of the walnut rejuvenation stage in the medulla, phloem and buds immediately after cutting and thereafter mainly in buds one year after cutting. Chalcone synthase and flavonoid contents appeared to be markers of the adult stage in the phloem.

Key words: Chalcone synthase – Flavonoid – Growth – *Juglans* (rejuvenation) – Naphthoquinone – Rejuvenation

Introduction

During the past few decades, substantial progress has been made in elucidating the biosynthesis of plant phenolics, one major class of which is the flavonoids. Most enzymological data have been based on work with cultured plant cells (Hahlbrock and Grisebach 1979; Hahlbrock et al. 1980; Hahlbrock 1981) or herbaceous plants (Heller et al. 1979; Kreuzaler et al. 1983). Parsley cell-suspension cultures have been extensively studied because dark-grown parsley cells respond to UV irradiation by accumulating flavonoids and by inducing the enzymes involved in the biosynthesis of these compounds (Heller et al. 1979; Hahlbrock 1981). Furthermore, the changes in the activities of L-phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS), the key enzymes of general phenylpropanoid metabolism in plants and of flavonoid biosynthesis, respectively, have been correlated with transient increases in the transcription rates of their corresponding genes (Kreuzaler et al. 1983; Chappel and Hahlbrock 1984).

Chalcone synthase (EC 2.3.1.74), a homodimeric protein (Kreuzaler et al. 1979) catalyses the formation of the aromatic A ring of the flavonoid skeleton by sequential condensation of three malonyl-CoA-derived acetate units with 4-coumaroyl-CoA, yielding 2′,4,4′,6′ tetrahydroxychalcone (Heller and Forkmann 1988). During recent years, several publications have dealt with CHS from different herbaceous plants (Heller and Forkmann 1988 and references therein) and woody plants (Magel et al. 1991; Claudot and Drouet 1992).

Growing shoots of adult and rejuvenated walnut trees provide a system in which to study the biosynthesis of phenolics at the tissue level and its integration into rhizogenesis and development (Claudot 1992). The changes in naphthoquinone and flavonoid contents which represent the major phenolic compounds in walnut (Jay-Allemand and Drouet 1989) and the activities of PAL and CHS have been previously studied during growth of entirely defoliated walnut shoots (Claudot et al. 1993). Rejuvenation, which induces a higher growth rate and enhanced capability for vegetative propagation, results in an increase in both PAL activity and hydrojuglone glucoside (HJG) content. The PAL activity is proportional to the growth rate of shoots but does not directly control the accumulation of

Abbreviations: CHS = chalcone synthase; HJG = hydrojuglone glucoside

Correspondence to: A. Drouet; Fax: 33(2)37 34 56 17;
E-mail: deug28@imaginet.fr

flavonoids while CHS appears to be the rate-limiting enzyme of the pathway (Claudot et al. 1993).

The aim of this work was to study the variations in the main polyphenolic pathways in the different tissues or organs of adult and rejuvenated walnut shoot cuttings. This study took place for two consecutive years, during the growth period (from late May to late August) which has been previously described by Drouet et al. (1989). Here, the rate-limiting role of CHS in flavonoid formation is examined and we report results which suggest that, through changes in the naphthoquinone or flavonoid pathways, specific tissues or organs are implicated in the rejuvenated stage, while others are implicated in the adult stage.

Materials and methods

Plant material. Two clones of half-sib hybrid walnut trees (*Juglans nigra* × *Juglans regia* L.) were grown at the INRA nursery in Orléans, France. For each clone, two physiological stages were studied: (i) pruned trees which produce rejuvenated shoots conducive to propagation, and (ii) adult trees the shoots of which could not be propagated. In the first year, two 12-years-old trees were pruned 50 cm above ground level 20 d after bud burst (late May), and terminal annual shoots were sampled at random. Sprouts that emerged from the stumps of the pruned adult trees were sampled 30 and 60 d after bud burst. In the following year, terminal shoots from half-sib adult trees and stump sprouts from the trees that were pruned in the first year were cut at random 20, 35, 50, 65, 80 and 100 d after bud burst, measured and immediately immersed in liquid nitrogen for subsequent lyophilization. For each sample date, each shoot was separated into five parts: buds, bark plus fibres of sclerenchyma that were easily detached from the lyophilized shoot with a scalpel, phloem: non-lignified whitish tissue (including sieve elements, fibres, parenchyma and cambium), inserted between sclerenchyma fibres and the lignified xylem, which was scraped off the surface of the central cylinder with a scalpel; wood (secondary xylem) and medulla (medullary parenchyma). Each part was stored under vacuum at room temperature in darkness. Just before extraction, each part was weighed and ground for 3 min in a Dangoumau grinder (Prolabo, Paris, France) to obtain a uniformly fine powder.

Analysis of phenolic compounds. Polyphenols were extracted and purified according to methods described by Macheix (1974) and adapted to walnut (Jay-Allemand et al. 1988). Alcoholic extracts (10 µl representing 2.5 mg of dry material) were analysed by HPLC (Beckman, Fullerton, Calif. USA). Analysis conditions were as follows: the column was lichrosphere 5 µm, 100 Ch-18/11 (Merck, Darmstadt, Germany), 250 mm long × 4.6 mm incl.; solvent A was 1% aqueous acetic acid (pH 2.6) (R) and solvent B was methanol: acetonitrile (1:1, v/v) (B); the gradient was 0 to 20 min: 15 to 40% B into A; 20 to 25 min: 40 to 60% B into A. Flow rate was 1 ml · min^{-1}. Polyphenols were monitored at 340 nm and the major flavonol and naphthoquinone compounds were characterized by comparison of their retention times and UV spectra with those of reference compounds (Beckman detector 160; Jay-Allemand et al. 1988). Quantitative values of phenolic compounds were expressed in mg · (gDW)$^{-1}$. Flavanols were measured according to a modified method of Treutter (1989). Alcoholic extract (20 µl) was added to 10 µl of 2% 4-dimethylaminocinnamaldehyde (DMACA) in methanol and 6 N HCl (v/v) and to 970 µl methanol. After 3 h in darkness, absorbance was measured at 637 nm with a recording spectrophotometer (UV 941; Kontron, Milan, Italy). Results are expressed in mg catechin · (gDW)$^{-1}$.

Enzyme extraction. Chalcone synthase was prepared according to the method of Claudot and Drouet (1992). Lyophilized shoot powder mixed with polyvinylpolypyrrolidone (PVPP), and Dowex (Sigma, St. Louis, Mo., USA) 1X2-200 (1:1:1, by weight; Dowex pre-equilibrated with the extraction liquid) was extracted under nitrogen for 20 min at 4 °C. The extraction liquid contained 0.7 M potassium phosphate buffer (pH 8.0), 0.1% (w/v) BSA, 1.5% (w/v) polyethyleneglycol (PEG) 20 000, 0.4 M sucrose, 1 M $CaCl_2$, 0.2 M ascorbic acid, 50 mM EDTA, 50 mM cysteine and 5 mM sodium diethyldithiocarbamate. After filtration on nylon cloth, the filtrate was centrifuged for 20 min at 37 000 *g*. The supernatant was used as crude enzyme extract.

Protein determination and enzyme assay. The original method of Bradford (1976) was modified (Drouet et al. 1989) as follows: the assay mixture contained 50 µl crude extract to 8 ml reactive solution, and the reference was made with the extraction solution. Protein concentration was expressed in mg · (g DW)$^{-1}$. Activity of CHS was measured according to the method of Claudot and Drouet (1992). Aliquots of the crude enzyme extract were incubated with 0.1 M potassium phosphate buffer (pH 8.0) containing 2% (w/v) BSA, 25 µm 4-coumaroyl-CoA and 16 µM [2-^{14}C]malonyl-CoA (8.34 MBq) for 15 min at 35 °C. The reaction was stopped by adding 350 µl ethyl acetate containing naringenin. This resulted in the extraction of the labelled reaction products which were separated from the substrates. After mixing for 3 min, samples were centrifuged and radioactivity as labelled naringenin 50-µl aliquots of the ethyl acetate phase was assessed in a scintillation counter Beckman (LS 6 000). Enzyme activity was expressed in pkat (mg protein^{-1}).

Enzyme activity controls. Enzyme activities were assayed immediately after preparation of the samples. Boiled enzymes and reactions lacking substrates did not show any activity. The presence of specific inhibitors or activators for enzymes was tested as follows: powder from two different stages of growth were mixed and extracted and the resulting enzyme activity was always identical to the calculated mean of each powder activity measured separately. The CHS reaction was linear with time over a period of at least 20 min at a protein concentration of up to 50–150 mg in the reaction mixture. Up to 20% labelled malonyl-CoA was incorporated into naringenin. The enzymatically formed labelled naringenin was co-chromatographed with unlabelled substrates by TLC and identified by UV fluorescence and autoradiography. In the CHS assay, naringenin was the only product formed.

Mathematical treatment. The data for phenolics were analysed statistically (values are mean of three to ten replicates) and the 95% confidence interval (vertical bars on figures) is shown and significant differences ($P < 0.05$) between following sampling dates (Student *t* test of Snedecor and Cochran 1972) are indicated by an asterisk (*). Enzyme data, when they were analysed statistically (values are mean of three to ten replicates) exhibited a variation coefficient which never exceeded 5%. Mathematical correlation between flavonoid accumulation and enzyme activities was done. When the activity of the rate-limiting enzyme of a pathway is not regulated, e.g. by feedback inhibition, and degradation of products is negligible, product accumulation can be described by the integration of the enzyme activity curve using the equation: $P(t) = \int_0^t E(t)\,dt$ (Knogge et al. 1986, and references therein). Measured flavonoids were the total amount (expressed in µmol per shoot) of myricitrin, quercitrin (the two major flavonols in walnut shoots) and flavanols. Least-squares linear regression between calculated flavonoids (by the equation) and measured flavonoids was carried out and both the slope and the correlation coefficient were measured.

Results

Enzyme kinetics. The influence of reaction products and metabolic end products on CHS reaction rate was analysed. The product of the CHS reaction

2′,4,4′,6′-tetrahydroxychalcone, cannot be used in kinetic studies because it is cyclised spontaneously to naringenin under the optimal assay conditions. Fifty percent inhibition was achieved with 200 μM naringenin and 350 μM coenzyme A (data not shown). The two main end products of walnut flavonoid biosynthesis, two glycosylflavonols, i.e. myricitrin and quercitrin and their respective aglycones, i.e. myricetin and quercetin, did not affect the reaction rate of the enzyme at concentrations as measured up to 100 μM each (data not shown).

Product accumulation and CHS activity. Thirty days after bud burst following the cutting of the two adult trees in June, the sprouts that had emerged from the stump (Fig. 1A) exhibited an HJG/myricitrin ratio of 18, higher than that previously found for the shoots of adult trees (Fig. 1B). This was mainly due to higher contents of HJG in the medulla, phloem and buds (Fig. 2) while flavonoid content and CHS activity did not significantly vary (data not shown).

Sixty days after bud burst, the HJG/myricitrin ratio of rejuvenated shoots decreased to 11 (Fig. 1B) due to increased flavonoid contents in buds and bark (data not shown) while HJG contents remained at a steady level in the different shoot compartments (Fig. 2).

During the year after cutting, the HJG/myricitrin ratio remained at about 11 in rejuvenated shoots while, in shoots of non-pruned adult trees, it decreased dramatically from 11 to 3, after 50 d of growth (Fig. 1B);

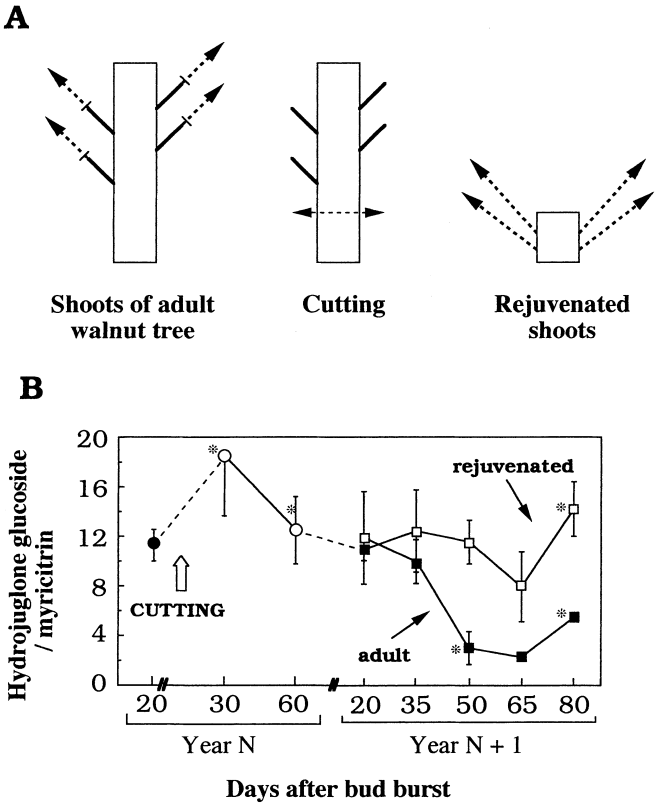


Fig. 1. **A** Scheme for sampling of adult and rejuvenated walnut shoots (indicated as *dotted-line arrows*) **B** Changes in the ratio HJG/myricitrin in annual entire shoots of adult and rejuvenated hybrid walnut trees. Hydrojuglone glucoside and myricitrin contents were measured after purification by HPLC. ●, adult tree shoots sampled year N; ○, rejuvenated shoots sampled year N; ■, adult shoots sampled from control unpruned trees year N+1; □, rejuvenated shoots sampled year N+1. For each physiological situation, significant ($P < 0.05$) differences between following sampling dates are indicated by an asterisk (*)

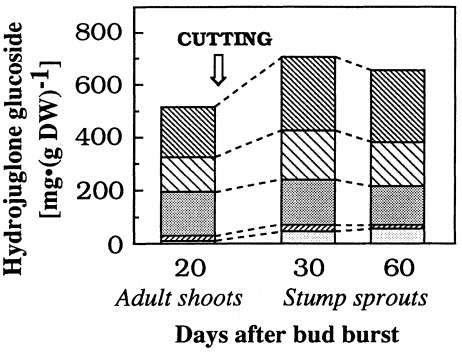


Fig. 2. Effect of rejuvenation on HJG contents in the different tissues of annual shoots. Shoots of adult walnut trees were sampled the day of cutting (20 d after bud burst) and new shoots from the resulting stumps were sampled 30 and 60 d after bud burst. Tissues were separated from the lyophilized shoots. Hydrojuglone glucoside content was measured after purification by HPLC. ▨, medulla; ▨, wood; ▨, phloem; ▨, bark; ▨, buds

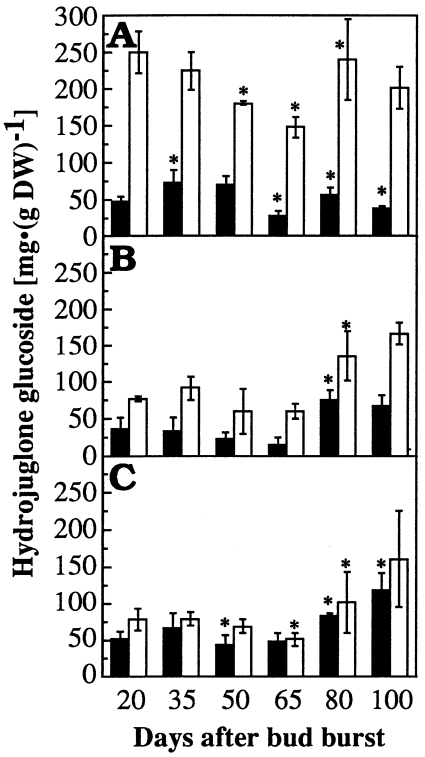


Fig. 3A–C. Changes in HJG content in different tissues during the growth of adult and rejuvenated walnut shoots. Annual shoots were sampled from stumps the year after cutting and from adult trees. Tissues were separated after lyophilization. Hydrojuglone glucoside content was measured after purification by HPLC. **A** Buds; **B** bark; **C** phloem. ■, adult shoots; □, rejuvenated sprouts. For each physiological situation, significant ($P < 0.05$) differences between following sampling dates are indicated by an asterisk (*)

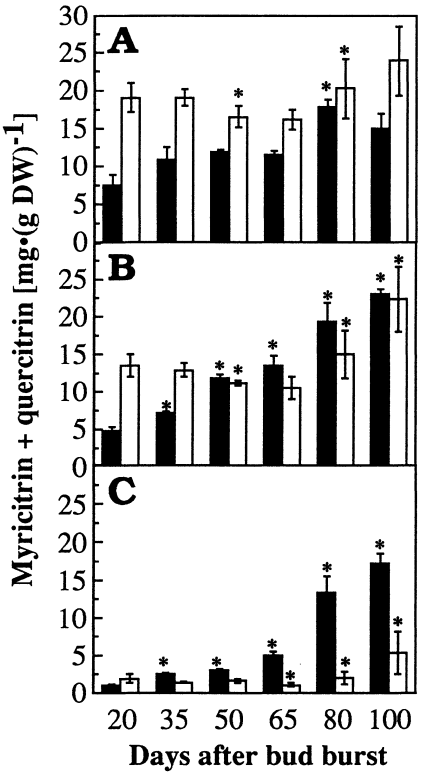
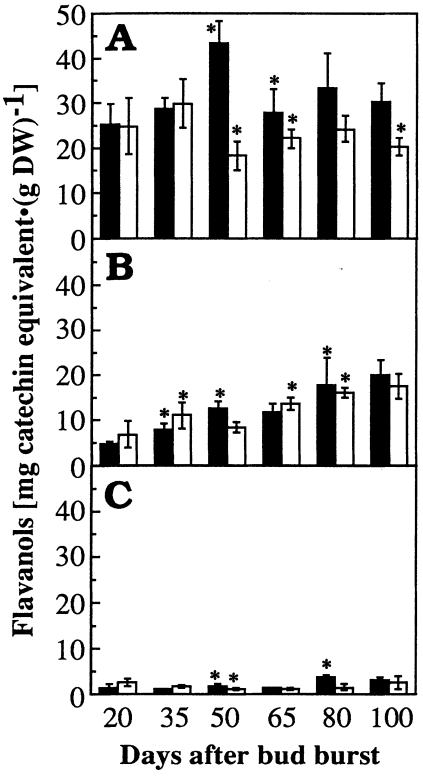


Fig. 4A–C. Changes in flavonol (myricitrin + quercitrin) content in different tissues during the growth of adult and rejuvenated walnut shoots. Annual shoots were sampled from stumps the year after cutting and from adult trees. Tissues were separated after lyophilization. Flavonol content was measured after purification by HPLC. **A** Buds; **B** bark; **C** phloem. ■, adult shoots; □, rejuvenated shoots. For each physiological situation, significant ($P < 0.05$) differences between following sampling dates are indicated by an asterisk (*)



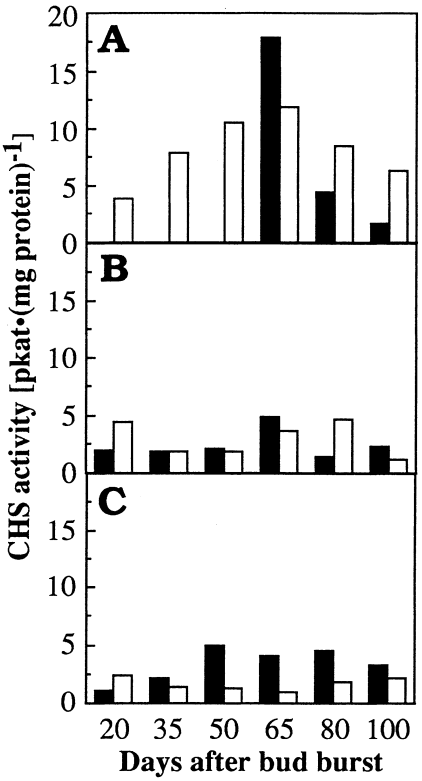


Fig. 6A–C. Changes in CHS activity in different tissues during the growth of adult and rejuvenated walnut shoots. Annual shoots were sampled from stumps the year after cutting and from adult trees. Tissues were separated after lyophilization. Chalcone synthase activity was measured after incubation of the enzyme with 4-coumaroyl CoA and [2-^{14}C]malonyl CoA; reaction products were extracted by ethyl acetate and radioactivity was measured in a scintillation counter. **A** Buds; **B** bark; **C** liber. ■, adult shoots; □, rejuvenated shoots

differences in product accumulation and CHS activity were precisely investigated in the different parts of the shoots of adult and rejuvenated trees (Figs. 3–6).

At the beginning of growth, the HJG content was threefold higher in buds than in bark and phloem of stump sprouts (Fig. 3) and was not detectable in wood and medulla (data not shown). The HJG content in buds and bark (Fig. 3A,B), was higher in stump sprouts than in adult tree shoots while in phloem (Fig. 3C), it did not significantly differ. At the end of growth, the HJG content increased in the bark and phloem of adult tree shoots and rejuvenated shoots (Fig. 3B,C).

At the beginning of growth, the flavonol content (myricitrin, and quercitrin) was higher in buds than in bark and phloem (Fig. 4) and was not detectable in wood

Fig. 5A–C. Changes in flavanol content in different tissues during the growth of adult and rejuvenated walnut shoots. Annual shoots were sampled from stumps the year after cutting and from adult trees. Tissues were separated after lyophilization. Flavanol content was measured after reaction with 4-dimethylaminocinnamaldehyde and 637 nm absorbance recording. **A** Buds; **B** bark; **C** phloem. ■, adult shoots; □, rejuvenated shoots. For each physiological situation, significant ($P < 0.05$) differences between following sampling dates are indicated by an asterisk (*)

and medulla (data not shown). During growth, flavonol content remained at a steady level in buds (Fig. 4A) while it increased at the end of growth in bark and phloem (Fig. 4B,C). At the end of growth, the flavonol content in phloem (Fig. 4C) was threefold higher in adult shoots than in rejuvenated shoots while no significant differences were found in buds and bark (Fig. 4A,B).

At the beginning of growth, the flavanol content was fivefold higher in buds than in bark and phloem (Fig. 5) and was not detectable in wood and medulla (data not shown). During growth, it increased in bark and phloem (Fig. 5B,C) while it remained at a steady level in buds (Fig. 5A). Moreover, no significant differences between adult and rejuvenated shoots were shown in buds, bark and phloem.

For buds of adult tree shoots, at the beginning of growth, CHS activity was not measured because they were too small. The CHS activity was higher in the older buds than in bark and phloem (Fig. 6) and was not detectable in the wood and medulla (data not shown). In buds (Fig. 6A), the CHS activity exhibited a transient maximum in the middle of the growth period. In bark (Fig. 6B), the activity remained at a steady level for adult tree shoots and rejuvenated shoots. In phloem (Fig. 6C), CHS activity exhibited an increase 50 d after bud burst in adult tree shoots while it remained at a steady level in rejuvenated shoots.

Taken together, these results show that the end of growth of annual shoots (80–100 d after bud burst) was characterized by an accumulation of naphthoquinones and flavonoids mainly in bark and phloem while it remained at a steady level in buds. In contrast, the CHS activity reached a transient maximum in the middle of growth (50–65 d after bud burst) mainly in buds and at a lower level in phloem while it remained at a steady level in bark. On the other hand, the adult stage of walnut was mainly marked by a lower content of HJG in buds and bark and higher CHS activity and flavonol content in phloem.

Regulation of flavonoid biosynthesis. Flavonoids are not metabolized during the growth and development of walnut shoots. Furthermore, there is no indication for regulation of CHS by the metabolic end products (see above). Therefore, an attempt was made to correlate mathematically flavonoid accumulation and CHS activity in the different parts of the shoots. For the whole bark + phloem, CHS activity exhibited a peak 65 d after bud burst in adult shoots (Fig. 7A) and was still increasing 80 d after bud burst in rejuvenated shoots (Fig. 7B). Flavonoid amounts increased over the whole period studied. The theoretical curve of calculated flavonoids was below the experimental curve in bark and above it in phloem and buds (data not shown). For the whole bark + phloem, all experimentally derived data on flavonoid accumulation in adult shoots (Fig. 7A) and stump sprouts (Fig. 7B) lay on the theoretical curve. Correlatively, a least-squares linear regression between calculated and measured flavonoids exhibited a slope of 1.107 ± 0.089 for adult shoots with a correlation coefficient $r = 0.990$ and a slope of 1.030 ± 0.023 for rejuvenated shoots with a correlation coefficient $r = 0.999$, showing a good correlation

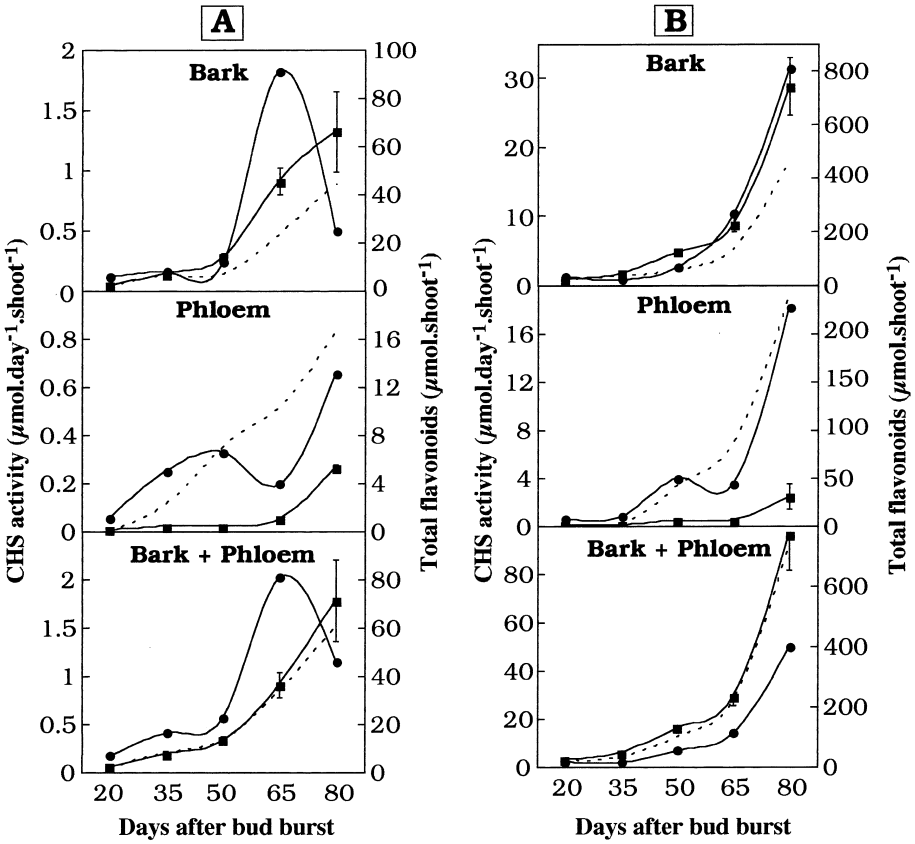


Fig. 7. Mathematical correlation of relative CHS activity and flavonoid accumulation. **A** Adult shoots; **B** rejuvenated shoots. Enzyme activity (●) was measured during shoot development. The curve obtained for relative CHS activity (——) was integrated to yield the product accumulation curve (- - - -) which can be compared with the experimental data for flavonoid accumulation (■). *Vertical bars* indicate 95% confidence intervals to the average

between flavonoid accumulation and CHS activity. These results suggest that CHS is the rate-limiting enzyme of the flavonoid pathway in bark and phloem during walnut shoot growth.

Discussion

Chalcone synthase catalyses the first step in the flavonoid biosynthesis pathway of plants. This key metabolic position makes the enzyme an attractive target for regulatory mechanisms controlling the pathway. The CHS from walnut was inhibited in vitro by coenzyme A, a product of the reaction, as well as by naringenin. This is in close agreement with experiments using a purified enzyme extract from oat leaves (Knogge et al. 1986). Reports on the purified enzyme from parsley and carrot cell-suspension cultures (Kreuzaler and Hahlbrock 1975; Ozeki et al. 1985), as well as on the partially purified enzyme from tulip anthers (Sütfeld et al. 1978), carrot cell cultures (Hinderer and Seitz 1985), oat primary leaves (Knogge et al. 1986), and now walnut tissues, however, do not yield any information on the possible regulation of CHS activity. It can be expected that the products of the reaction inhibit the enzyme, as is the case for coenzyme A (Cleland 1963; Sütfeld et al. 1978). Naringenin, usually applied as a racemate, exerts an inhibitory effect on the reaction possibly because of its partial structural similarity to the chalcone product. Like the CHS from carrot cells (Hinderer and Seitz 1985), and oat primary leaves (Knogge et al. 1986), the non-inhibition of walnut CHS by the walnut flavonols in vitro apparently excludes end-product inhibition in vivo.

Our results allow for a better understanding of the impact of coppicing on the metabolism of phenolics within a shoot. When the trees have just been cut down, a high HJG/myricitrin ratio is due to a high accumulation of HJG first in medulla, phloem and buds and then in buds and bark throughout the growth period one year after cutting. These observations are in close accord with previous results (Jay-Allemand et al. 1988; Jay-Allemand and Drouet 1989; Claudot et al. 1993) and are also associated with the good multiplication rates and propagation of rooted shoots by tissue culture of buds collected the year of cutting (Jay-Allemand 1985).

Naphthoquinones are much more concentrated in walnut roots (Jay-Allemand 1985). After cutting, the increased concentration of HJG in medulla, phloem and buds seems likely to be due to a root effect (Jay-Allemand 1985). Comparisons between coppiced and non-coppiced *Populus* sp. have shown that improvement in water supply due to the increased root/shoot ratio and the reduced shoot competition for moisture leads to the acceleration of growth of decapitated plants (Tschaplinsky and Blake 1989). Consequently, HJG could be transported from roots to buds and then to phloem and bark via the water flow and participate in the acceleration of growth and the increased multiplication rate of new shoots. In addition, when the trees have just been cut down, flavonoid metabolism is not modified and does not seem to be linked to a root effect.

In the years after pruning, the difficulty of propagating the plant material by tissue culture indicates an aging of the stump which can be explained by a decrease in the root effect, which is marked by a smaller content of HJG in buds, phloem and bark at the beginning of growth than in the year of cutting (Figs. 2, 3) and an increase in flavonol content in phloem and bark during growth (Fig. 4). The only suitable propagation period is correlated with the beginning of growth (Claudot et al. 1992) and it is well known that the initiation of root primordia occurs mainly at the proximity of phloem and cambium. Consequently, it appears that decreased content of HJG in a tissue is associated with decreased shoot multiplication rate and that this content is correlated with the root effect which decreases with both growth and the age of the stump, and affects successively medulla, buds, liber and bark. Finally, naphthoquinone metabolism appears to be a marker of rejuvenation in walnut but it remains to be determined if HJG is transported from roots to medulla, buds, phloem and bark or is synthesized in situ, as does the mode of action of HJG on rooting. Moreover, in close accordance with previous results (Claudot et al. 1992), flavonoid metabolism (Myricitrin and quercitrin content and CHS activity), which appears at a higher level in the phloem of adult tree shoots than in the phloem of stump sprouts, can be considered as a marker of the adult stage. Similarly, it has already been shown that, in *Prunus avium*, two other flavonoids, prunin and sakuranin, invariably increased with advancing chronological shoot age (Treutter et al. 1987). In *Hevea*, flavonoids (flavans, flavonols and anthocyanins) are good markers of juvenility and rooting ability (Haffner 1991) and in ivy, maturity is linked to the disappearance of anthocyanins (Murray and Hackett 1991). Nevertheless, little is known in vivo on the regulation of the synthesis, the transport and the mode of action of these compounds.

While little is known about the metabolism of naphthoquinones, it is well known that CHS is the key enzyme of flavonoid metabolism (Heller and Forkmann 1988). An indication for a possible role of CHS as the rate-limiting enzyme of the pathway has already been found in *Petunia hybrida* (Mol et al. 1983), wild carrot (Hinderer et al. 1983), oat primary leaves (Knogge et al. 1986) and walnut shoots (Claudot et al. 1993). It seems likely that CHS could be the key enzyme of flavonoid accumulation in specific tissues of walnut. For the whole bark + phloem from growing adult and rejuvenated tree shoots, mathematical correlation of CHS activity and flavonoid accumulation resulted in the coincidence of experimentally obtained and calculated curves for product formation (Fig. 7). This very close coincidence tends to exclude regulatory mechanisms modulating CHS activity in vivo. Furthermore, this relationship can only be expected for the rate-limiting enzyme of a pathway. Under the particular growth conditions applied to the adult and rejuvenated walnut shoots, therefore, the amount of flavonoids synthesized in bark + phloem appears to be determined mainly by the CHS

activity present in the phloem (Fig. 6). It remains unclear as to how flavonoids can be transported from phloem to bark where they are mainly accumulated. An intercellular transport process of flavonoids has been postulated in anthers of *Petunia* (Koes et al. 1990) and between leaf mesophyll and epidermal layers (Knogge and Weissenböck 1986; Knogge et al. 1986). In contrast to this report, the simultaneous occurrence of CHS in both epidermal layers as well as in the mesophyll of rye and maize correlated with the accumulation of flavonoid products in these tissues, indicating tissue autonomy of flavonoid biosynthesis (Jähne et al. 1993) and needs further investigation.

Furthermore, the presence of flavonoids in response to environmental stimuli and at specific developmental stages is mediated by regulated CHS expression (Wingender et al. 1989). Transcriptional activation or repression of CHS genes is a major factor regulating enzyme levels and hence flavonoid accumulation (Chappel and Hahlbrock 1984; Murray et al. 1994). Moreover, translational regulation of CHS mRNA has also been shown (Knogge et al. 1986; Frenken et al. 1991). To elucidate the regulation of CHS gene expression in adult and rejuvenated walnut tissues, molecular biological studies will be carried out in the future.

Financial support from Eurosilva (MRT No. 88R0765) and Conseil Régional du Centre (grant to AC Claudot) is gratefully acknowledged.

References

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein using the principle of protein-dye binding. Anal Biochem 72: 248–254

Chappell J, Hahlbrock K (1984) Transcription of plant defence genes in response to UV light or fungal elicitor. Nature 311: 76–78

Claudot AC (1992) Rôle de la chalcone synthase dans la biosynthèse des flavonoides au cours de la croissance annuelle des pousses de noyers hybrides (*Juglans nigra* × *Juglans regia*) adultes et rajeunis. Applications à l'enracinement. PhD thesis University Orléans

Claudot AC, Drouet A (1992) Preparation and assay of chalcone synthase from walnut tree. Phytochemistry 31: 3377–3380

Claudot AC, Drouet A, Jay-Allemand C (1992) Tissue distribution of phenolic compounds in annual shoots from adult and rejuvenated walnut trees. Plant Physiol Biochem 30: 61–68

Claudot AC, Jay-Allemand C, Magel EA, Drouet A (1993) Phenylalanine ammonia-lyase, chalcone synthase and polyphenolic compounds in adult and rejuvenated walnut trees. Trees 7: 92–97

Cleland WW (1963) The kinetics of enzyme-catalysed reactions with two or more substrates or products. II. Inhibition: nomenclature and theory. Biochim Biophys Acta 67: 173–196

Drouet A, Weiswald N, Jay-Allemand C, Cornu D (1989) Pentose phosphate pathway and glutamate dehydrogenase activities in adult and rejuvenated hybrid walnut trees. Plant Physiol Biochem 27: 259–267

Frenken P, Niesbach-Klössgen U, Weydemann U, Marechal-Drouard L, Saedler H, Wienand U (1991) The duplicated chalcone synthase genes C2 and Whp (white pollen) of *Zea mays* are independently regulated; evidence for translation control of Whp expression by the anthocyanin intensifying gene. EMBO J 10: 2605–2612

Haffner V (1991) Recherche de marqueurs de la juvénilité / maturité et de la réactivité in vitro lors du microbouturage chez *Hevea brasiliensis*. Relation entre la juvénilité et al réactivité in vitro. PhD thesis University Paris VI

Hahlbrock K (1981) Flavonoids. In: Stumpf PK, Conn EE (eds) The biochemistry of plants, vol 7. Academic Press, New York, pp 425–156

Hahlbrock K, Grisebach H (1979) Enzymic controls in the biosynthesis of lignin and flavonoids. Annu Rev Plant Physiol 30: 105–130

Hahlbrock K, Schröder J, Vieregge J (1980) Enzyme regulation in parsley and soybean cell cultures. In: Fiechter A (ed) Plant cell cultures, vol 2. Springer, Berlin, pp 39–60

Heller W, Forkmann G (1988) Biosynthesis. Advances in research since 1980. In: Harborne JB (ed) The flavonoids. Chapman and Hall, London, pp 399–425

Heller W, Egin-Bühler B, Gardiner SE, Knobloch KH, Matern U, Ebel J, Hahlbrock K (1979) Enzymes of general phenylpropanoid metabolism and of flavonoid glycoside biosynthesis in parsley. Plant Physiol 64: 371–373

Hinderer W, Seitz HU (1985) Chalcone synthase from cell suspension cultures of *Daucus carota* L. Arch Biochem Biophys 240: 265–272

Hinderer W, Noe W, Seitz HU (1983) Differentiation of metabolite pathways in the umbel of *Daucus carota*. Phytochemistry 22: 2417–2420

Jähne A, Fritzen C, Weissenböck G (1993) Chalcone synthase and flavonoid products in primary-leaf tissues of rye and maize. Planta 189: 39–46

Jay-Allemand C (1985) Les marqueurs biochimiques de la juvenilite chez le noyer (*Juglans nigra* × *Juglans regia*). PhD thesis University Montpellier

Jay-Allemand C, Drouet A (1989) Polyphenole und Enzyme als biochemische Marker der Verjüngung bei Walnuss. Erwerbsobstbau 31: 63–69

Jay-Allemand C, Cornu D, Macheix JJ (1988) Biochemical attributes associated with rejuvenation of walnut tree. Plant Physiol Biochem 26: 139–144

Knogge W, Weissenböck G (1986) Tissue distribution of secondary phenolic biosynthesis in developing primary leaves of *Avena sativa* L. Planta 167: 196–205

Knogge W, Schmelzer E, Weissenböck G (1986) The role of chalcone synthase in the regulation of flavonoid biosynthesis in developing oat primary leaves. Arch Biochem Biophys 250: 364–372

Koes RE, van Blockland R, Quattrochio F, van Tunen AJ, Mol JNM (1990) Chalcone synthase promoters in *Petunia* are active in pigmented and unpigmented cell types. Plant Cell 2: 379–392

Kreuzaler F, Hahlbrock K (1975) Enzymatic synthesis of an aromatic ring from acetate units. Partial purification and some properties of flavanone synthase from cell-suspension culture of *Petroselinum hortense*. Eur J Biochem 56: 205–213

Kreuzaler F, Ragg H, Heller W, Tesch R, Witt L, Hammer D, Hahlbrock K (1979) Flavanone synthase from *Petroselinum hortense*. Molecular weight, subunit composition, size of messenger RNA and absence of pantetheinyl residue. Eur J Biochem 99: 89–96

Kreuzaler F, Ragg H, Fautz E, Kuhn DN, Hahlbrock K (1983) UV-induction of chalcone synthase mRNA in cell suspension cultures of *Petroselinum hortense*. Proc Natl Acad Sci USA 80: 2591–2593

Macheix JJ (1974) Les esters hydroxycinnamiques de la pomme. Identification, variations au cours de la croissance du fruit et métabolisme. Thèse Doct Etat University Paris VI

Magel EA, Drouet A, Claudot AC, Ziegler H (1991) Formation of heartwood substances in the stem of *Robinia pseudoaccacia* L. Distribution of phenylalanine ammonium lyase and chalcone synthase across the trunk. Trees 5: 203–207

Mol JNM, Schram AW, De Vlaming P, Gerats AGM, Kreuzaler F, Hahlbrock K, Reif HJ, Veltkamp E (1983) Regulation of flavonoid gene expression in *Petunia hybrida*: description and

partial characterization of a conditional mutant in CHS gene expression. Mol Gen Genet 192: 424–429

Murray JR, Hackett WP (1991) Dihydroflavonol reductase activity in relation to differential anthocyanin accumulation in juvenile and mature phase of *Hedera helix* L. Plant Physiol 97: 343–351

Murray JR, Smith AG, Hackett WP (1994) Differential dihydroflavonol reductase transcription and anthocyanin pigmentation in the juvenile and mature phases of ivy (*Hedera helix* L.). Planta 194: 102–109

Ozeki Y, Sakano K, K Komamine A, Tanaka Y, Noguchi H, Sankawa U, Suzuki T (1985) Purification and some properties of chalcone synthase from a carrot suspension culture induced for anthocyanin synthesis and preparation of its specific antiserum. J Biochem 98: 9–17

Snedecor GW, Cochran WG (1972) Statistical methods applied to experiments in agriculture and biology. Toura State College Press, Ames

Sütfeld R, Kehrel B, Wiermann R (1978) Characterization, development and localization of "flavanone synthase" in tulip anthers. Z Naturforsch 33: 841–846

Treutter D (1989) Chemical reaction detection of catechins and proanthocyanidins with 4-dimethylaminocinnamaldehyde. J Chromatogr 467: 185–193

Treutter D, Feucht W, Schmid P (1987) Aging-dependent responses of phloem flavonoids of *Prunus avium* graftings: flavanone and isoflavanone glucosides. Sci Hortic 32: 183–193

Tschaplinsky TJ, Blake TJ (1989) Photosynthetic reinvigoration of leaves following shoot decapitation and accelerated growth of coppice shoots. Physiol Plant 75: 157–165

Wingender R, Röhnig H, Höricke C, Wing D, Schell J (1989) Differential regulation of soybean chalcone synthase genes in plant defence, symbiosis and upon environmental stimuli. Mol Gen Genet 218: 315–322