Quantitative variations of indolyl compounds including IAA, IAA-aspartate and serotonin in walnut microcuttings during root induction

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Abstract

Shoots of the hybrid walnut *Juglans nigra* × *Juglans regia* contained serotonin in the micromole range and indole-3-acetic acid (IAA) in the nanomole range. The serotonin level fell by 40 % in 12 h in auxin (IBA) treated whole shoots and then reincreased to a maximum (50 % over the control) after 36 h. The same pattern was followed in the top portions of the shoot but in the shoot bases, serotonin always remained under the control level. The early decrease of serotonin was correlated with an increase in IAA-aspartate. The early decrease and peaking of the serotonin level preceded and corresponded to the increase and peaking of free IAA in the shoot bases. The initial serotonin pool in treated-to-root shoots might thus suffice for the biosynthesis of IAA and IAA-conjugated compounds. Because of its auxin-like properties, the early serotonin peak might be taken into consideration as an endogenous auxin signal for rooting in the present material. If this turns out to be so, the rooting signal for the shoot bases necessarily should come from the apices.

Additional key words: auxins, *Juglans nigra* × *J. regia*, micropropagation, rooting.

Introduction

An early and temporary increase in the endogenous level of indole-3-acetic acid (IAA) is the major event of the inductive phase of adventitious rooting since it appears necessary for the reactivation of cell divisions (Blakesley 1994, Gaspar *et al.*

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Abbreviations: IAA - indole-3-acetic acid; IAAasp - indole-3-acetylaspartic acid; IBA - indole-3-butyric acid; 5-OHT - 5-hydroxytryptamine.
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1994). Such a peaking of auxin has been observed during induction phase of rooting of shoots of *Juglans regia* raised *in vitro* (Heloir *et al.* 1996). Serotonin (5-OH-tryptamine, 5-OHT) is biosynthetized in walnut (Regula *et al.* 1989), where it may serve as a precursor for IAA (Duroux 1993). Serotonin may in itself have auxin-like properties (Umrath and Thaler 1981) and thus act as an auxin signal for the rooting of cotyledon fragments of hybrid walnut (Duroux 1993). With these considerations as starting point we have examined the variations in the level of serotonin in hybrid walnut shoots directly after transfer from an *in vitro* multiplication medium to a rooting medium. In parallel, analyses of free IAA and its conjugate IAA-aspartate were also performed.

Materials and methods

Plants and culture: *In vitro* cultures of *Juglans nigra* × *Juglans regia* (clone A35) were provided by Dr. C. Jay-Allemand from the Institut National de la Recherche Agronomique (INRA, Orléans, France). Axillary proliferation was maintained through regular subcultures (every three weeks) on a G60 medium (Gruselle *et al.* 1995) with 2.5 g dm^{-3} gelrite (*Phytagel, Sigma*), 15×10^{-9} M indolebutyric acid (IBA) and 5×10^{-6} M benzylaminopurine (BAP). These cultures were kept in 600 cm^3 cylindrical jars (12 shoots on 100 cm^3 medium) covered by a glass lid that was held with a sheet of transparent plastic film and exposed to a 16-h photoperiod (*Sylvania Grolux* fluorescent lamps, 17 W m^{-2}), at temperature 28 °C (day) and 25 °C (night). Almost 100 % rooting of the isolated shoots was achieved through the use of two successive rooting media: an inductive medium (ORL) composed of Driver base (Driver and Kuniyuki 1984) diluted 4 times with 2.5×10^{-5} M IBA, 2.5 g dm^{-3} gelrite without cytokinins (5 d in darkness at 20 °C), followed by an expressive medium (EXP) composed of Driver base diluted 4 times in a gelrite-vermiculite mixture without auxin in light (Jay-Allemand *et al.* 1992, Ripetti *et al.* 1994).

Extraction and determination of auxins: IAA and IAAasp were determined in whole shoots, in top (upper two thirds) and bottom (lower basal one third) shoot portions sampled every 12 h during a 120 h passage on the IBA-containing rooting medium. Non-rooting shoots cultured on ORL in the absence of IBA were taken as controls. The detailed methods used have been described elsewhere (Nordström and Eliasson 1991, Nordström *et al.* 1991). Fresh shoots (500 mg) were homogenized in liquid nitrogen. The powder was extracted with 10 cm^3 5 mM phosphate buffer (pH 5.6) containing [1-^{14}C]indole-3-acetic acid as internal standard and butylated hydroxytoluene as antioxidant. After 1 h in darkness, the extract was filtered through a glass fibre filter, which was rinsed with 5 cm^3 of the extraction buffer. The samples were passed through *Bond-Elut C18* columns activated and conditioned to pH 6.5. After washing the columns, the eluate was acidified to pH 2.5 with 2.8 M phosphoric acid. This eluate was applied to a second C18 column, activated and conditioned to pH 2.5. The second column was washed with 2 cm^3 distilled water and 2 cm^3 acidic ethanol (ethanol:glacial acetic acid:water, 20:2:78, v/v). The washings were

combined and applied to a third C18 column, pH 2.5, and eluted as described below. Auxins were eluted twice with 0.6 cm^3 aliquots of methanol (80 %) for each of the second and the third C18 columns (pH 2.5). The methanolic extracts (1.2 cm^3) were injected in a fully automated *Merck-Hitachi* (Darmstadt, Germany) system. The HPLC column was a *Lichrospher 100-RP18*, 12.5 cm × 4 mm internal diameter, 5 μm particle size; column and solvent at 30 °C; flow 1 cm^3 min^{-1}; mobile phase acetonitrile:glacial acetic acid:water (10:2:88, v/v); detection by fluorescence detector (absorbance 292 nm, emission 360 nm).

Extraction and determination of serotonin: Serotonin was determined in the shoot top and bottom portions sampled each 12 h during 120 h passage on the auxin rooting medium. Non-rooting shoots cultured on ORL in the absence of IBA were taken as controls. The methods used were described by Engström and Lundgren (1992). Fresh material (500 mg) was homogenized in 80 % ethanol containing β-^{14}C-5-hydroxy-tryptamine (generous gift of C. Jay-Allemand) as internal standard. The homogenate was filtered on glass fiber, which was rinsed with 5 cm^3 95 % ethanol and the combined eluates were evaporated *in vacuo*. The residue was homogenized with 3 cm^3 water. The sample was washed with 2 × 5 cm^3 petrol-ether and the water residue evaporated *in vacuo*. The residue was dissolved with 1.5 cm^3 methanol:water (50:50, v/v). 0.1 cm^3 of the methanolic extracts were injected. The HPLC column was a *Nucleosil 120-5 C 18*, 10.5 cm × 4 mm internal diameter; column and solvent at 30 °C; flow 1.2 cm^3 min^{-1}, mobile phase 0.01 M phosphate buffer, pH 2.8, and acetonitrile (90:10, v/v); detection by fluorescence detector (absorbance 280 nm, emission 334 nm).

All results are the mean of measurements from at least three separate experiments.

Results and discussion

Variations of endogenous free IAA: In rooting shoots, the IAA level increased to a plateau (in fact a series of peaks) of about 40 nmol g^{-1}(FM) between 36 and 96 h and then decreased towards the initial value (Fig. 1*A*). No corresponding plateau was observed in non-rooting shoots. The level of IAA of the basal shoot portions varied in the same way as found in the whole shoots (Fig. 1*B*). Top portions of rooting shoots (Fig. 1*B,D*) and bases of non-rooting shoots (Fig. 1*C*) did not show any increase in IAA. A similar increase in the IAA level between the 24th and 96th hour was described by Heloir *et al.* (1996) in whole shoots of *Juglans regia* under the same conditions as used here, and regarded as characteristic of the inductive phase of rooting (Norcini *et al.* 1985, Gaspar *et al.* 1994). Generally one single peak of IAA was registered in herbaceous species as a response to an exogenous application of auxin (Blakesley *et al.* 1991). The multiple peaks that constitute the IAA increase in a woody species have also been observed in *Sequoiadendron* with peroxidase as a marker of rooting (Berthon *et al.* 1989). They were interpreted by the existence, among the walnut shoots necessarily arising from different axillary positions, of different populations of shoots reaching successively the rooting induction phase.

Variations of endogenous IAAasp: Whole shoots of the hybrid walnut on the rooting medium accumulated IAAasp (Fig. 2*A*) while the level of this compound remained quite stable in the non-rooting shoots. This increase in IAAasp concerned mainly the

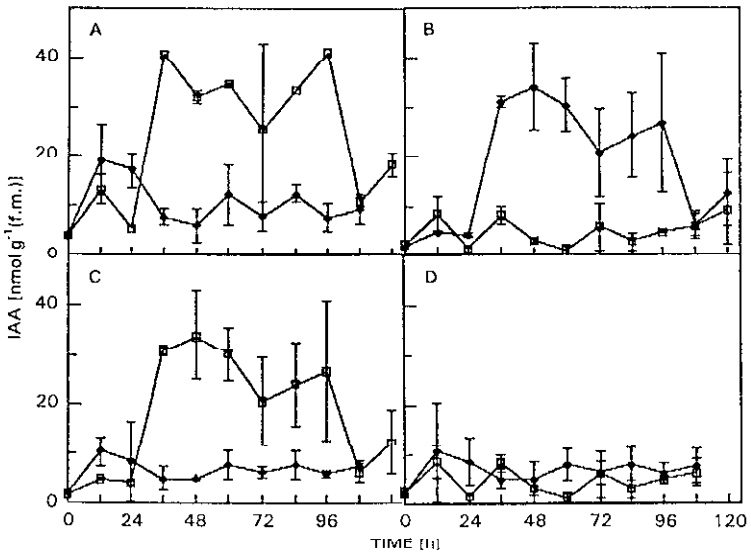

Fig. 1. Level of free IAA in hybrid walnut: *A* - whole shoots of microcuttings on rooting medium (*open squares* - ORL rooting medium, *closed triangles* - control); *B* - apices and bases of shoots on ORL medium (*open squares* - apex, *closed triangles* - base); *C* - bases (*open squares* - ORL rooting medium, *closed triangles* - control); *D* - apices (*open squares* - ORL rooting medium, *closed triangles* - control).

top portions of the rooting shoots (Fig. 2*B,C*). Early accumulation of IAAasp in rooting shoots has also been observed by others (Moncousin *et al.* 1988, Moncousin 1991, Hausman 1993). Since IAAasp mainly accumulates in the apices, it has been proposed to be the mobile factor emanating from the apex and necessary for rhizogenesis (Moncousin 1991). The rooting effect of IAAsp *per se* has never been shown (Nordstrom *et al.* 1991) but the transport and conversion of IAAsp into IAA are quite plausible (Bandurski *et al.* 1995).

Variations in endogenous serotonin: Serotonin was contained in the walnut shoots in the micromole range whereas IAA occurred in the nanomole range. The level of serotonin in whole shoots on the non-rooting medium decreased slowly until the 72th hour (Fig. 3*A*). In shoots on the rooting medium, the level fell to a minimum after 24 h, and then increased to a peak at the 36th hour. In the shoot bases (Fig. 3*B*), the level of serotonin was constant whatever the culture conditions. The variations of serotonin in the shoot top portions (Fig. 3C) paralleled those measured in the whole shoots. Because there are reasons to think that serotonin is converted into IAA (Pilet 1964, Duroux 1993), the decrease of its level in shoots transferred to the rooting medium might be interpreted as a partial utilisation to contribute to the formation of

the IAA bulk. Because the typical serotonin variation occurred in the shoot top portions, it might also be suggested that it is first converted to IAAasp which is then transported to the base where it delivers IAA. In addition the increase of the serotonin level between 24 and 36 h, concomitantly with that of IAA, might suggest

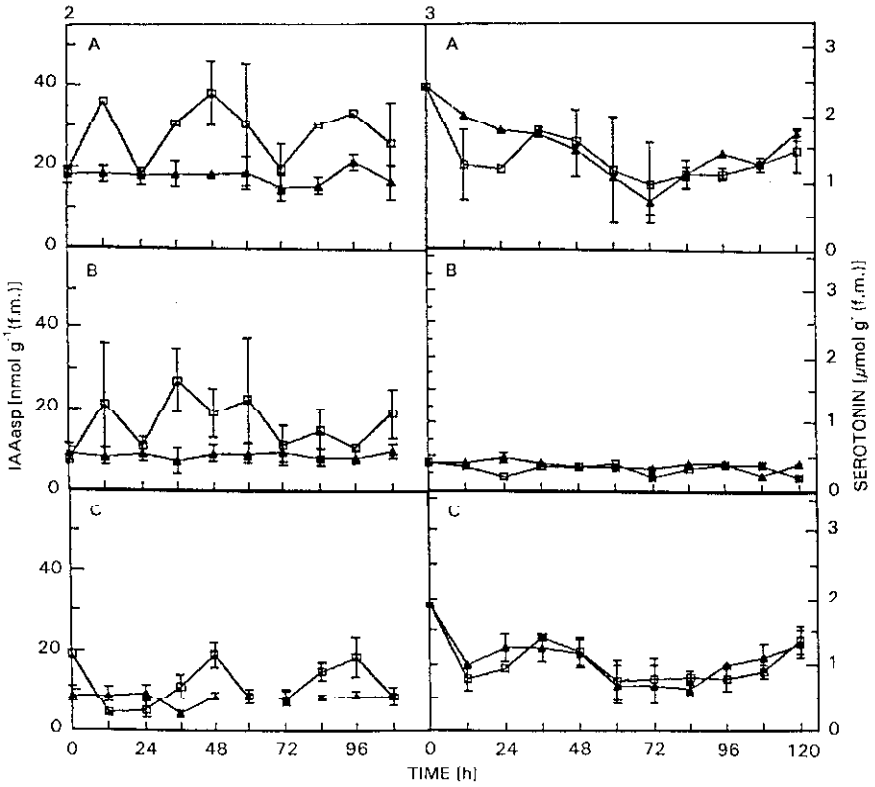

Fig. 2. Level of IAAasp in whole shoots (*A*), in apices (*B*) and in bases (*C*) of microcuttings of hybrid walnut on rooting medium (*open squares* - ORL rooting medium, *closed triangles* - control).

Fig. 3. Level of serotonin in whole shoots (*A*), in bases (*B*) and in apices (*C*) of microcuttings of hybrid walnut on rooting medium. (*open squares* - ORL rooting medium, *closed triangles* - control).

that serotonin (through its own auxin-like properties) participates directly, together with IAA, to the signal for root induction. It has been suggested that serotonin plays this role in cotyledon fragments, where IAA could not be detected (Duroux 1993). The auxin-like activity of serotonin had been shown by Pilet (1964). More recent results by Regula *et al.* (1989) showed that serotonin influenced rhizogenesis in aspen leaves cultivated *in vitro* to the same extent as IAA. These results finally confirm earlier findings that the rooting signal for the bases would partially come from the apices. Further investigations are untertaken in order to evaluate the role of serotonin more precisely than possible for the moment, namely through the application of radiolabelled serotonin or precursors. Because of the 1000-fold

magnitude of serotonin concentration compared to IAA, the relative auxinic activity of serotonin in walnut should also be evaluated.

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