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Uptake and metabolism of indolebutyric acid during root formation on *Malus* microcuttings

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SUMMARY

In this study, the content of active internal indolebutyric acid (IBA) and of IBA-derived indoleacetic acid (IAA) was related to adventitious root formation on the stem base of microcuttings of apple. Maximum root formation was obtained after an incubation of 3 days on medium supplemented with 32 μM IBA or 5 days on medium containing 3·2 or 10 μM of the hormone. Two to three weeks after the start of the auxin treatment, the maximum root number was attained. The absorbed IBA-^{3}H accumulated predominantly in the bottom 1 mm of the stem, i.e. the location where the roots emerge. The metabolism of [^{3}H]-IBA was examined in this part of the apple shoots in relation to adventitious root formation *in vitro*.

The internal content of free IBA (IBA^{int}) corresponded to about 60% of the IBA concentration in the medium from 6 h to 3 days of incubation. The internal content of IBA-derived IAA (IAA^{int}) was about 15% of the IBA concentration in the medium. At incubation periods of 2 and 3 days the shorter period required a higher IBA^{int} and IAA^{int} content to produce the same amount of roots. This observation indicates that in those cases, the mode of action of IBA in root formation resembles a dose effect of the active auxin components in the stem base. Over the whole range of experimental conditions (6–120 h, 0·1–32 μM), however, the product of incubation time and exogenous concentration or internal content of auxins was not related to the number of roots.

The percentage of label recovered in the IBA^{int} and IAA^{int} fractions was the same at suboptimal and optimal IBA concentrations for root formation, and did not change between 0·7 and 3 days after auxin addition. Therefore, root formation was not related to a specific type of auxin metabolism.

Key-words: indolebutyric acid, *Malus*, micropropagation, regeneration, root formation, tissue culture.

INTRODUCTION

During the last decade, micropropagation has become an important tool for the multiplication of fruit trees. *In vitro* multiplication starts with the formation of shoots from surface-sterilized top meristems (Jones *et al.* 1977). Addition of cytokinin leads to the outgrowth of axillary meristems into new shoots. These shoots can either be used for

further multiplication, or adventitious roots can be induced with auxin (Jones *et al.* 1977; Zimmerman & Broome 1981; James 1983). The auxin most commonly used for root formation is indolebutyric acid (IBA). In apple and many other woody species, however, some cultivars produce roots at a very low frequency or not at all, form a root system of poor quality, and/or form roots asynchronously and irreproducibly.

Most studies about the role of auxins in root regeneration deal with the relationship between the auxin concentration in the medium and rooting (Welander 1983; Collet & Le 1987). More insight into the mode of action of auxin is obtained when the level of the physiologically active auxin component inside the tissue is related to the root regeneration process. It is well known that after uptake indoleacetic acid (IAA) is inactivated by conjugation with amino acids (Hangarter & Good 1981), sugars or sugar alcohols (Bandurski *et al.* 1977). IAA can also be converted by decarboxylation (Cohen & Bandurski 1982). These inactivation processes are enzyme-catalysed (Higgins & Barnett 1976; Cohen & Bandurski 1982) and all amino acids can be conjugated (Feung *et al.* 1975). The metabolites formed from IBA are similar to those from IAA (Cohen & Bandurski 1982; Pythoud & Buchala 1989). In addition, IBA can be converted into IAA via β-oxidation as described in grape vine and olive cuttings (Epstein & Lavee 1984), and in apple (Alvarez *et al.* 1989a and b).

Quantitatively, the auxin can exert its action in two ways. The regeneration process may require a threshold concentration of the active auxin in the tissue for a certain period of time (concentration effect). Alternatively, regeneration may depend on the combination of the content of active auxin in the tissue and the incubation time (dose effect). We addressed this question by measurement of the uptake and metabolism of IBA with respect to root formation *in vitro* on apple.

MATERIAL AND METHODS

Culture in vitro

The experimental material of choice was a clone of the apple cultivar M.9-Jork. This clone was propagated for more than 6 years and was kindly provided by B. Kunneman (Center for Plant Tissue Culture Research, Lisse, The Netherlands). Multiplication of the shoots was on MS Medium (Murashige & Skoog 1962) with 165 mM sorbitol, 4·4 μM benzyladenine and 0·6% (w/v) agar (BBL), at 20°C, 16 h light (PAR 80 μmol $m^{-2} s^{-1}$) and 80% RH. Rooting experiments were performed according to Quoirin *et al.* (1977) with 88 mM sucrose, 2·7 μM riboflavin and 0·6% (w/v) agar. IBA was added before autoclaving at various concentrations as indicated with the experiments. Shoots were transferred to rooting medium and after 24–120 h in the dark at 20°C transferred to the light (PAR 80 μmol $m^{-2} s^{-1}$) at 20°C. After exposure to light IBA is rapidly degraded (Gorst *et al.* 1983; Van der Krieken *et al.* 1992a and b). The number of roots that emerged was counted after different time intervals. In the experiments 74 kBq [4-^{3}H] IBA (specific activity 0·6 TBq $mmol^{-1}$) was included to quantify the uptake and conversion of IBA.

Extraction and analysis of IBA and its conjugates

Radioactive IBA and its metabolites were extracted overnight at −80°C in MeOH plus 0·5 mM butylhydroxy toluene from groups of 10 1-mm basal sections of the stem base according to Smulders *et al.* (1990). At the beginning of the extraction procedure, 50 nmol of standard IBA and 50 nmol of standard IAA were added to increase the extraction efficiency and to diminish photo-degradation. The methanol fraction was dried at 40°C under nitrogen. The residue was dissolved in 75% EtOH and chromatographed

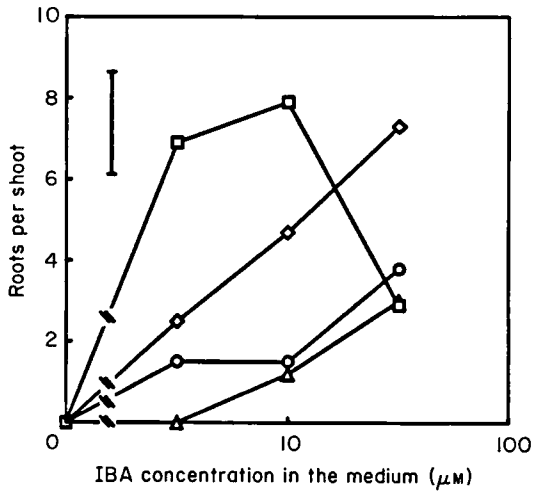

Fig. 1. Root formation after 24 (△), 48 (○), 72 (◇) or 120 h (□) incubation on medium with various IBA concentrations. The average number of roots formed per shoot was counted after 29 days. Values are means of 20 shoots. The vertical bar represents the LSD at $P=0{\cdot}05$.

unidirectionally on silica gel 60 PF_{254} plates in two different solvents. The first solvent was $CHCl_3$:HAc (95:5, v/v) and after drying, the plate was developed in $CHCl_3$:MeOH:HAc (75:20:5, v/v/v). To quantify radioactivity on the plates, the UV-absorbing spots of IBA, IAA and the remaining part of the chromatogram (1 cm sections) were scraped off. All TLC fractions were mixed with 5 ml Ultima Gold scintillation liquid (Beckman) and counted in a liquid scintillation counter. The purity of the IBA and IAA fractions was checked by reversed phase HPLC and by two-dimensional TLC. Unidirectional TLC proved to be sufficient for accurate assessment of the quantity of IBA and IAA.

RESULTS

IBA-mediated root formation

The number of roots formed on the apple shoots depended on both the IBA concentration in the medium and the incubation period on IBA-containing medium (Fig. 1). In general, an increase in either of these factors led to the formation of more roots. Maximum root formation (*c.* eight per shoot) occurred after 5 days of incubation on medium with 3·2 or 10 μM IBA or 3 days with 32 μM. In these treatments more than 90% of the shoots formed roots without visible intervening callus formation. Five days incubation on 32 μM IBA led to a great amount of callus formation and rooting occurred on only 45% of the shoots. Incubation for 3 days on medium with 32 μM IBA resulted in a high number of roots. However, many of these roots emerged via callus. Incubations shorter than 3 days led to suboptimal root formation and also reduced the rooting frequency to about 50% of the microcuttings. Incubation (1–5 days) on medium containing 0·1 μM IBA did not result in any root formation (Fig. 2).

Kinetics of root formation

The time needed for the roots to emerge depended on the IBA concentration in the medium (Fig. 2). At the optimal treatment, roots became visible after *c.* 5 days. At an IBA concentration of 3·2 μM, about 50% of the eventual number of roots was formed within 8

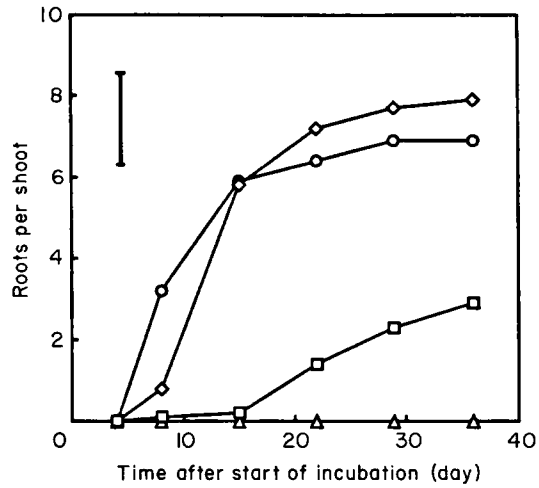

Fig. 2. Kinetics of root formation. After a 4-day incubation period on medium supplemented with 0·1 (△), 3·2 (○), 10 (◇) or 32 (□) μM IBA, roots were counted at the times indicated. Values are means of 20 shoots. The vertical bar represents the LSD at $P=0{\cdot}05$.

days after the start of the auxin treatment. The remaining roots emerged between 8 and 15 days. After incubation on medium with 10 μM IBA roots were formed between 8 and 15 days. On medium with an IBA concentration of 32 μM, root formation was asynchronous and increased continuously between 8 and 36 days.

Kinetics of IBA uptake

The accumulation of IBA-^{3}H was measured at various periods during root regeneration in the stem base (the location where the roots emerge) after incubation on medium containing various IBA concentrations (Fig. 3). The content (μmol per kg f wt.) of radioactive IBA and its metabolites increased with an increase in the medium IBA concentration. The rate of IBA accumulation was especially high during the first 6 h of incubation. After 6 h, the rate of IBA-^{3}H accumulation was approximately constant at the time intervals that were measured.

Internal free IBA and IAA resulting from IBA uptake

Apple shootlets were incubated for various periods of time on medium containing various concentrations of radioactive IBA (Fig. 4). The labelled internal free acids of IBA and IAA (IBA^{int} and IAA^{int}, respectively) were extracted from the stem base, separated on TLC and quantified using liquid scintillation analysis. The content of IBA^{int} increased during the first 6 h of incubation, and fluctuated but did not systematically increase thereafter. On average, the IBA^{int} content was *c.* 60% of the medium IBA concentration. The IAA^{int} content resulting from IBA conversion was *c.* four-fold lower than that of IBA^{int}. The IAA^{int} content, though fluctuating, was basically constant after 6 h incubation at 1–32 μM IBA. No systematic changes occurred in the distribution of absorbed IBA-^{3}H over IBA^{int} and IAA^{int} during the first 3 days on IBA medium.

Distribution of IBA in the shoot

The content of IBA-^{3}H taken up from the medium, and of IBA^{int} and IAA^{int} was determined in the stem base, the rest of the stem and in the leaf/top area of the shoot after

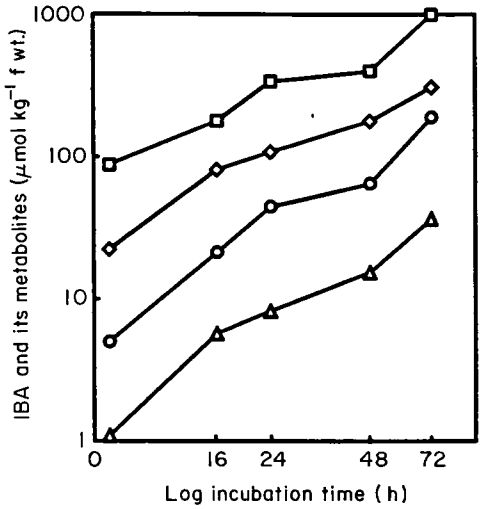

Fig. 3. Kinetics of accumulation of IBA-^{3}H by stem bases. The content of IBA and its metabolites was determined in the stem base after incubation during different times on medium with 1 (△), 3·2 (○), 10 (◇) or 32 (□) μM IBA. For each value groups of five stem bases were pooled. Values are means of triplicate experiments. All SEM values were less than 18% of the average value.

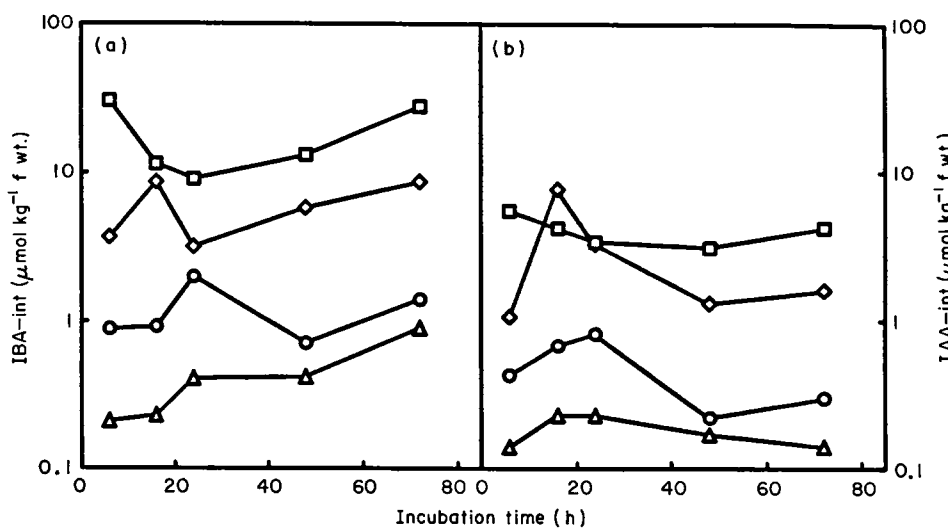

Fig. 4. Content of IBA^{int} (a) or IAA^{int} (b) in the stem base after the same incubations described with Fig. 3. Value are means of triplicate experiments. All SEM values were less than 20% of the average value.

1, 3 and 5 days on 10 μM IBA (Figs 5 and 6). The vast majority (*c.* 80%) of the absorbed IBA-^{3}H accumulated in the stem base (Fig. 5). The content of labelled IBA and its metabolites was, on average, at least one decade higher in the stem base than in the rest of the shoot. The leaf/top area contained about five times more IBA-^{3}H than the higher part of the stem.

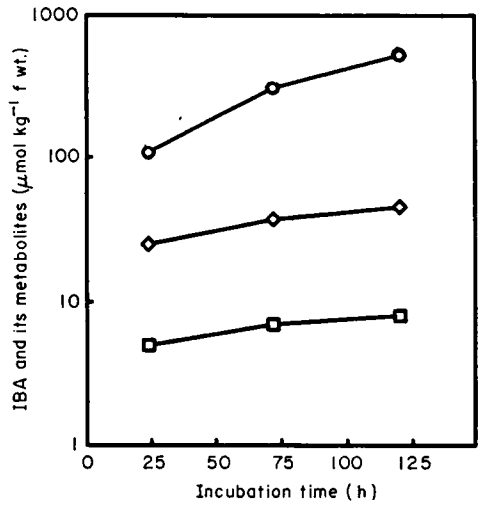

Fig. 5. Distribution of IBA and its metabolites after incubation during different times on medium with 10 μM IBA. Total radioactivity in the stem base (○), the rest of the stem (□) and the leaf/top area (◇). For each value groups of five shoot sections were pooled. The values are means of triplicate experiments. All SEM values were less than 17% of the average value.

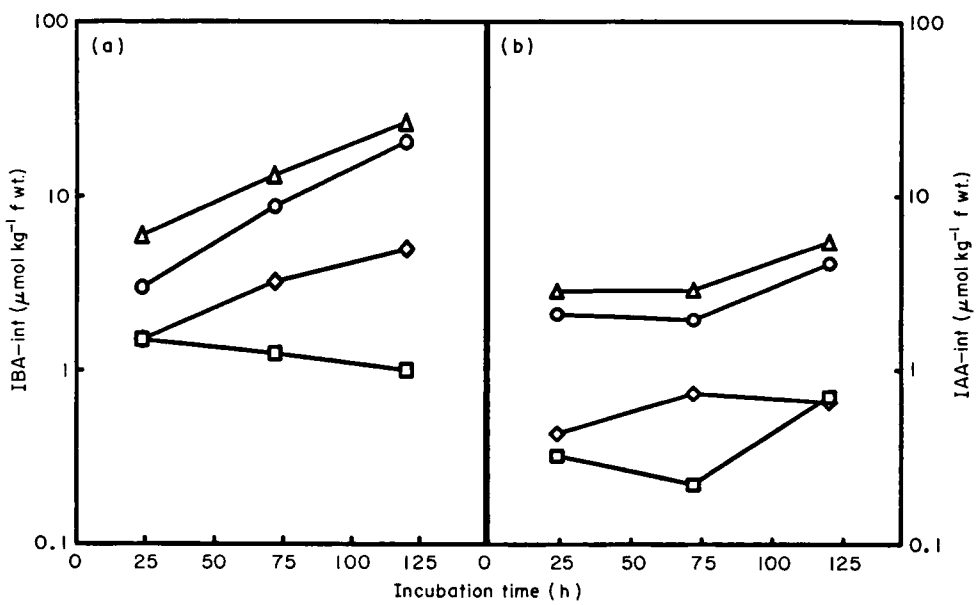

Fig. 6. Distribution of IBA^{int} (a) or IAA^{int} (b) after the same incubations described with Fig. 5. For each value groups of five stem bases were pooled. Values are means of triplicate experiments. All SEM values were less than 20% of the average value.

IBA^{int} was about four times more abundant than IAA^{int} in all shoot parts examined (Fig. 6). The content of both IBA^{int} and IAA^{int} was highest in the stem base. The content of IBA^{int} and IAA^{int} was *c.* five times higher in the stem base than in the leaf/top area. The

difference between the stem base and the rest of the stem was even higher. It holds for IBA^{int} and IAA^{int} also that their presence in the stem base represents more than 70% of these components in the whole microcutting. The ratio of IBA^{int} or IAA^{int} to total IBA-^{3}H was higher in the leaf/top and stem than in the stem base.

DISCUSSION

For optimum root formation, many roots of good quality (e.g. physiologically active and well-structured) should synchronously emerge on all shootlets without a major intervening callus phase. Two factors are of key importance for this process: The IBA concentration in the medium and the time the shoots are cultured on the auxin-containing medium. Both factors were investigated (Figs 1 and 2).

IBA induced the highest number of roots after an incubation of 72 h on medium containing 32 μM IBA or after 120 h with 3·2 or 10 μM IBA. Incubation for 24 and 48 h resulted in suboptimal root numbers. Five days incubation with 32 μM led to callus production and a lower number of roots, that were of poor quality (short and thick). The latter type of root was also found after incubation of shoot explants of eucalyptus on medium with a high auxin concentration (Gorst *et al.* 1983). In apple, the outgrowth of roots was inhibited by prolonged incubation on auxin-containing medium (De Klerk *et al.* 1990).

IBA-mediated root formation *in vitro* was studied on the stem bases of apple shoots. This is the location where the roots emerge and where by far the highest contents of IBA^{int} and IAA^{int} (the active auxin components) were reached (Fig. 6). Relatively few reports deal with the metabolism of IBA. This hormone was found to be conjugated in an identical manner as IAA (Pythoud & Buchala 1989; Wiesman *et al.* 1989). The conversion of IBA into IAA (based on several techniques like GC-MS, TLC, HPLC and GLC) was found in olive and grape vine cuttings (Epstein & Lavee 1984) and in apple (Alvarez *et al.* 1989a). The vast majority of the accumulated IBA-^{3}H, IBA^{int} and IAA^{int} is located in the stem base (Figs 5 and 6). Thus, the stem base is an attractive tissue for studying the relationship between IBA metabolism and root formation. The uptake of IBA from the culture medium was proportional to both the medium concentration and the incubation time (Fig. 3). In contrast, during the first 3 days of incubation, the contents of IBA^{int} and IAA^{int} did not increase with the incubation time but formed a fraction of about 60 and 15% of the medium concentration of IBA, respectively (Fig. 4). The IBA^{int} content always exceeded that of IAA^{int} by four- to six-fold (Fig. 6).

In general, it was not possible to relate the number of regenerated roots to the dose of the active auxin components (Figs 1 and 4). Only within a small range of the medium IBA concentration and the incubation time, the number of roots formed did depend on the dose (incubation time × internal content) of IBA^{int} and IAA^{int} in the tissue. An incubation for 3 days on medium with 10 μM IBA and 2 days on medium with 32 μM IBA led to about the same number of roots (Fig. 1). The IBA^{int} and IAA^{int} levels after incubation of 3 days on medium with 10 μM IBA were 20 and 6·2 μmol × day kg^{-1} f wt., respectively. After 2 days on medium with 32 μM IBA these doses were similar [22 and 7·2 μmol × day kg^{-1} f wt., respectively (Fig. 4)]. Other combinations of incubation time and IBA^{int} and IAA^{int} contents that led to the same number of roots formed, however, resulted in active auxin doses that differed three-fold or more. These findings support the idea that tissue sensitivity varies with time and is at least as important as variation in the internal hormone concentration (Guern 1987).

There was no shift with time in the partition of label absorbed from IBA over the various metabolites including IBA^{int} and IAA^{int}. This indicates that there is no specific auxin metabolism related to root formation.

The regulation of root formation by auxin was studied by measurements of the uptake and metabolism of IBA in the stem base. However, the effect of exogenously applied IBA on the endogenous synthesis of IAA and/or IBA may also influence the process of adventitious root formation. This is currently under investigation in our laboratory.

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