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Effect of hormone treatment on root formation and endogenous indole-3-acetic acid and polyamine levels of *Glycine max* cultivated in vitro

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Abstract. Accumulation of endogenous indole-3-acetic acid (IAA) in soybean hypocotyl explants was found during adventitious root formation in naphthaleneacetic acid (NAA) and indole-3-butyric acid (IBA) treatments. The auxin-induced root formation was accompanied by increasing levels of putrescine. We suggest that the exogenously applied auxin (IBA and NAA) may act on polyamine synthase and IAA oxidase activity.

Keywords: Adventitious root; *Glycine max*; Indole-3-acetic acid; Polyamine.

Abbreviations: IAA, indole-3-acetic acid; **IBA,** indole-3-butyric acid; **NAA,** naphthaleneacetic acid.

Introduction

It is well known that root formation involves intensive mitotic activity and metabolic changes accompanied by changes of polyamine levels (Friedman et al., 1985; Jarvis et al., 1985; Burtin et al., 1990). Polyamines are considered important in cell division because they stimulate DNA synthesis (Kaur-Sawhney et al., 1980). Polyamine biosynthetic enzyme activity and polyamine levels increase before DNA replication (Cohen et al., 1984). Moreover, auxin seems to be an universal inducer of adventitious roots. Several reports have indicated a positive correlation between endogenous indole-3-acetic acid (IAA) levels in cuttings and the number of adventitious roots produced per cutting (Weigel et al., 1984; Alvarez et al., 1989). Past publications on the role of auxin in adventitious root induction were mainly based on experiments with exogenously applied synthetic auxin. However, there have been far fewer investigations into the effect of exogenously applied auxin on endogenous IAA and polyamine levels in plant tissue. In this study, we examine the effect of two synthetic auxins, naphthaleneacetic acid (NAA) and indole-3-butyric acid (IBA) on adventitious root induction using soybean hypocotyl explants.

Materials and Methods

Plant Tissue Culture

Soybean seeds (*Glycine max*) strain Tk5 were obtained from the Asia Vegetable Research and Development Center in Taiwan. Seeds were surface-sterilized for 1 min in 70% ethanol and soaked in 1% sodium hypochlorite for 10 min. Seeds were rinsed three times in sterile distilled water. Seedlings were grown in darkness at 27°C, and relative humidity (RH) was maintained between 70 to 80%. Young seedlings (5-days old) about 5 cm in height were used for tissue culture. Five mm hypocotyl were cut and cultured on test medium. The basal medium (MSB medium) consisted of inorganic nutrients as found in Murashige-Skoog medium (Murashige and Skoog, 1962), and vitamins of Gamborg's B5 medium (Gamborg et al., 1968), plus 250 mg/L casein hydrolysate supplemented with 3% sucrose. Root formation without intermediate growth of callus was promoted by the addition of 2 mg/L indole-3-butyric acid (IBA) or 2 mg/L naphthaleneacetic acid (NAA) to the culture medium.

Analysis of Indole-3-Acetic Acid in Soybean Hypocotyl Explants

Soybean hypocotyl explants were cultured on three different media and grown in the incubator as described in darkness. Hypocotyl explants (adventitious roots were removed), were extracted after culture for certain days according to the experiments. Portions of explant tissue including the basal part that might be affected by the hormone treatment, usually 5 to 8 g fresh weight, were homogenized in 60 mL of 80% methanol containing sodium ascorbate and Butylated hydroxytoluene (BHT) (Dunlap and Guinn, 1989). The ground tissues were filtrated with suction through Whatman No.1 paper. Methanol in the filtrate was removed by rotary flash evaporation (RFE) at 35°C. The pH of the aqueous residues was adjusted to 8.0 and partitioned twice against an equal volume of ethyl acetate to remove phenolic compounds and other impurities. The pH of the aqueous fraction was adjusted to 2.5, then

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partitioned twice against the same volume of ethyl acetate. The acidic ethyl acetate fraction in methanol was obtained for thin layer chromatography (TLC) purification. TLC was performed on analytical Kieselgel 60 (20 × 20 cm, Schichtdicke, 0.2 mm, Merck). Up to 1.0 mL of extract was loaded on a plate, and the chromatogram was developed for about 1 h with the following solvent system: benzene:acetone:acetic acid (13:6:1 v/v). Identification of the IAA-containing bands was done by comparison with the Rfs of the standard (IAA) running at the same time. After TLC development, the IAA bands were scraped, redissolved in methanol, and quantified by HPLC. Analysis of the TLC plates had to be performed immediately because of the rapid fading or degrading after TLC (Crozier et al., 1980).

High Performance Liquid Chromatography

Partially purified samples were loaded on a reverse phase C_{18} column (5 × 250 mm, TSK gel column, ODS-80TM, Tosoh, Japan). Eluates were monitored with a fluorescence spectrophotometer (Model F-1050, Hitachi). The mobile phases used were 35% methanol in 20 mM ammonium acetate buffer (pH 3.5) for IAA separation. The flow rate was kept at 0.8 mL/min during chromatographing. For detecting IAA, an excitation wavelength of 280 nm with an emission wavelength of 360 nm was used. Eluant Peaks with their retention time and areas were recorded by an attached integrator.

Analysis of Polyamine in Hypocotyl Explants

Tissues were homogenized in 5% cold perchloric acid ($HClO_4$) at a ratio of 100 mg/mL $HClO_4$, according to the methods of Flores and Galston (1982). After extraction for 1 h in an ice bath, samples were pelleted at 40,000 *g* for 20 min, and the supernatant phase, containing the polyamine fraction, was stored frozen at -20°C. Extracts were stable for polyamine analysis by HPLC for more than 6 months under this condition (Martin-Tanguy et al., 1988). HPLC in combination with fluorescence spectrophotometry was used to separate and quantify polyamines (putrescine, spermidine, and spermine) prepared as their dansyl derivative from plant tissue. The polyamines were derivatized according to the methods of Flores and Galston (1982). Fifty aliquots of the supernatant were added to 200 µL of saturated sodium carbonate and 400 µL of dansyl chloride in acetone (7.5 mg/mL) in a 5 mL reaction vial. After vortexing, the mixture was incubated in a thermal reaction block at 60°C for 1 h in darkness. Excess dansyl reagent was removed by reaction with 100 µL of added proline (100 mg/mL) and incubated for 0.5 h. Dansyl polyamines were extracted with 1.0 mL toluene with vigorous vortexing for 30 sec. The mixture separated into an aqueous and an organic phase. The organic phase, containing the polamines, was completely dried under nitrogen (Smith and Davies, 1985). The polyamine residues were dissolved in 1 mL of methanol and were either assayed immediately or stored (no more than 1 week) at -20°C. Standards were processed in the same way, and for each standard 20 nmol polyamine (Sigma, U.S.A.) was dansylated. High performance liquid chromatography (HPLC) of dansylated polyamines was performed according to the method of Smith and Davis (1985). The dansylated samples were eluted from a reverse phase C_{18} column (LiChrosorb RP-18, 10 × 250 mm, Merck, Germany) with 90% methanol at a flow rate of 4.5 mL/min. Samples eluated from the column were detected by an attached fluorescence spectrophotometer (Model F-1050, Hitachi). For dansyl-polyamines an excitation wavelength of 365 nm with an emission wavelength of 510 nm was used. Eluant peaks with their retention time and area were recorded by an attached integrator. All results are the measurement of triplicate experiments.

Results

Effect of IBA and NAA on Adventitious Roots Formation

Preliminary experiments indicated that adventitious roots were induced optimally at 1.0–5.0 mg mL^{-1} of IBA and NAA in MSB medium (data not shown), therefore the concentration of IBA and NAA used in this study was 2 mg/mL. As shown in Table 1, adventitious roots were induced in all treatments, including the soybean hypocotyls grown in hormone-free MSB medium during 12-day culture. Adventitious roots formation was particularly enhanced by MSB + IBA treatment on the 6th day. A similar stimulation occurred in hypocotyls with MSB + NAA treatment on the 12th day during the culture. In terms of numbers of adventitious roots formed on each hypocotyl explant, IBA promoted rooting more than NAA did. Before the formation of adventitious roots, the fresh weight per hypocotyl explant increased significantly on the 3rd day in hypocotyl explant of MSB + IBA and MSB + NAA treatments (Figure 1).

Changes of Polyamine Content during Adventitious Root Formation

The dansyl-polyamine standards were eluated through HPLC with a typical chromatogram of standards run at 90% methanol. All peaks for dansyl polyamines could be fully resolved in 15 min (data not shown). As shown in Figure 2, the accumulation of putrescine was enhanced by both IBA and NAA treatment. The levels of putrescine in soybean hypocotyl explants increased significantly on the 3rd day, and declined on the 6th day in both MSB + IBA and MSB + NAA treatments. In MSB treatment, the increase of putrescine contents in hypocotyl explants occurred 3 days later, on the 6th day during culture.

Table 1. Numbers of adventitious roots induced on each soybean hypocotyl explant cultured on MSB medium containing IBA or NAA. Values are means ± SE of triplicate treatments.

Days	MSB	MSB + IBA	MSB + NAA
6	1.7 ± 0.2	4.4 ± 0.2	2.0 ± 0.2
12	1.7 ± 0.2	7.8 ± 0.4	5.0 ± 0.7

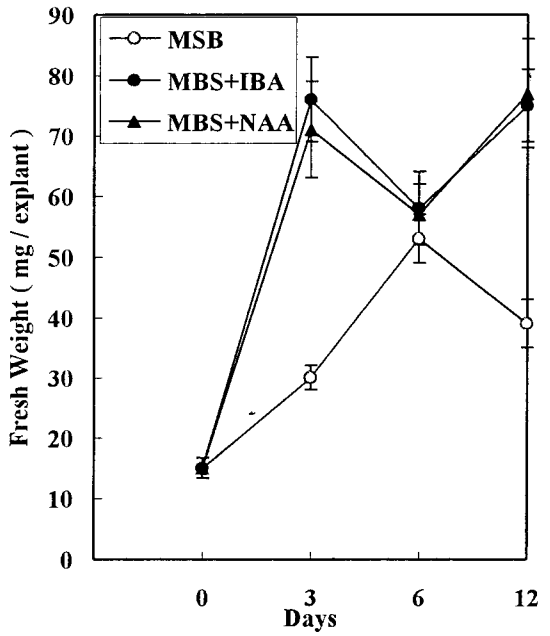

Figure 1. Fresh weight changes during root formation using soybean hypocotyl as explants cultured on MSB medium containing IBA or NAA.

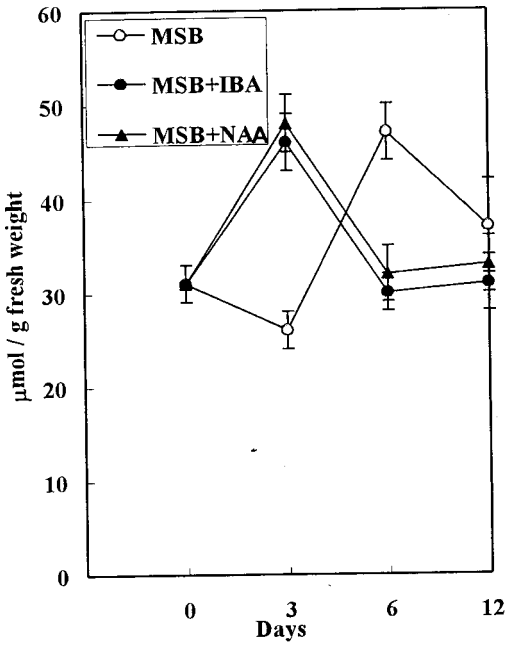

Figure 2. Changes in putrescine levels in hypocotyl explants during root formation cultured on MSB medium containing IBA or NAA.

Soybean hypocotyl explants, cultured in MSB medium with IBA and NAA, accumulated slightly higher levels of spermidine than that of MSB treatment on the 6th day during culture (Figure 3). The levels of spermine in tissues grown in MSB, MSB + IBA and MSB + NAA mediums did not change much on the 6th and 12th days during root formation (Figure 4).

Changes of Endogenous IAA in Soybean Hypocotyl

In our investigation, 68% of IAA standard was recovered during extraction and TLC purification (data not shown) if the chromatograms after TLC were immediately transferred to methanol. The IAA was eluated and analyzed through reverse phase HPLC. A chromatogram of a IAA-containing sample run at 35% methanol in 20

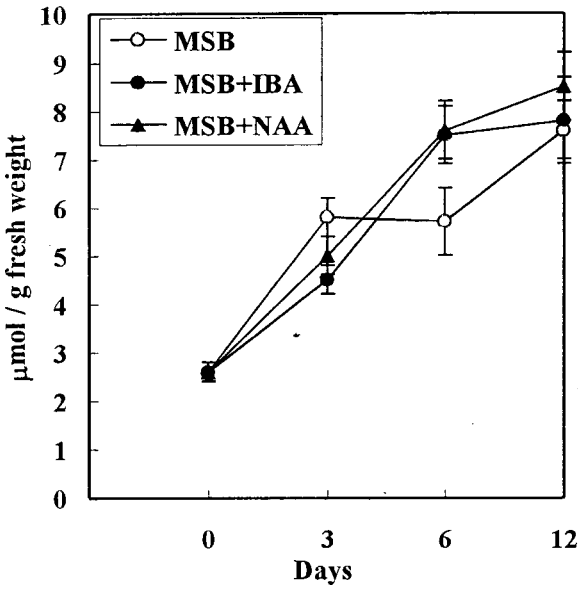

Figure 3. Changes in spermidine levels in hypocotyl explants during root formation cultured on MSB medium containing IBA or NAA.

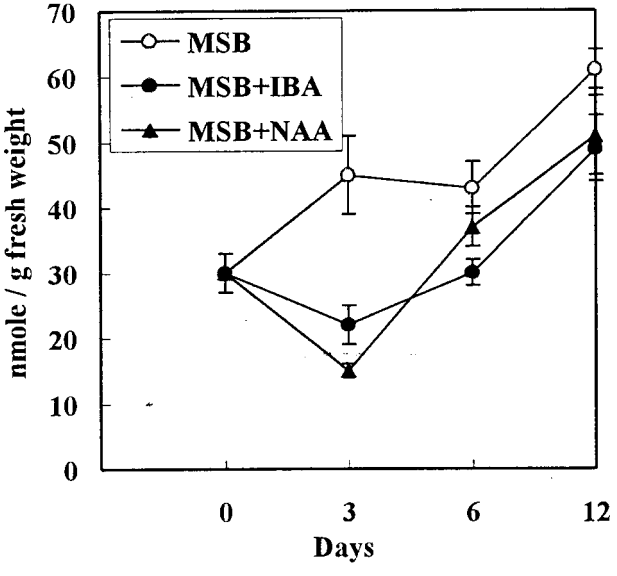

Figure 4. Changes in spermine levels in hypocotyl explants during root formation cultured on MSB medium containing IBA or NAA.

mM ammonium acetate buffer (pH 3.5) is shown in Figure 5. The extract from soybean hypocotyl explants after steps of purification was still far from pure. The endogenous IAA is eluated and separated from those impurities. In order to make sure that this peak was IAA rather than an impurity with a similar retention time, another extract spiked with standard IAA was also run. It showed only one peak with identical retention time (data not shown). The endogenous IAA was detected in the soybean hypocotyl explants after 6 day culture on MSB medium with IBA. The quantity of endogenous IAA was estimated to be 1.5 ng per g fresh weight in soybean hypocotyl explants grown in MSB medium with IBA. No IAA was detected in hypocotyl explants grown either in MSB medium or MSB medium with NAA for 6 days. As the culture was prolonged to the 12th day, endogenous IAA was detected in hypocotyl explants of both MSB + IBA and MSB + NAA treatments. The quantity of IAA was estimated to be 0.29 ng per g fresh weight of hypocotyl explants in MSB + IBA treatment and 0.27 ng per g fresh weight of hypocotyl explants in MSB + NAA treatment (Table 2).

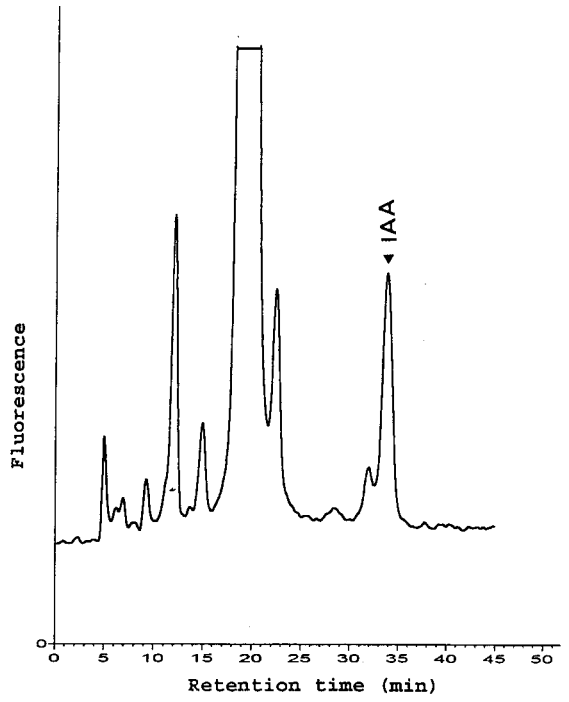

Figure 5. HPLC chromatogram of extracts from hypocotyl explants grown in MSB ± IBA medium for IAA determination.

Table 2. Change in IAA levels (ng/g fresh wt.) in hypocotyl explants during root formation. Values are means ± SE of triplicate treatments.

Days	MSB	MSB + IBA	MSB + NAA
6	–[a]	1.5 ± 0.2	–
12	–	0.29 ± 0.03	0.27 ± 0.02

[a]Not detected.

Discussion

It is known that adventitious root induction can be stimulated by exogenously applied auxins and polyamines, but the mechanism of this physiological response has not yet been elucidated. In this investigation, we examine the change of endogenous IAA and polyamine content during the formation of adventitious roots. The increase in fresh weight per soybean hypocotyl explant on the third day, before the appearance of adventitious roots, is correlated with the increasing levels of putrescine in hypocotyl explants of both MSB + IBA and MSB + NAA treatments (Figures 1 and 2, Table 1). Our data confirmed the results published by Burtin (1990), which indicated that auxin-induced root formation was accompanied by increasing levels of putrescine. An increase of putrescine level in the early inductive phase of rooting has also been found in other plants (Altamura et al., 1991; Baraldi et al., 1995; Hausman et al., 1995). A putrescine peak preceding the IAA peak has also been demonstrated on poplar shoots in vitro (Hausman et al., 1995). Torrigiani et al. (1987) also reported that the first synchronous cell cycle was induced by auxin in Heliantus tuber slices as well as by polyamine metabolic enzymes such as ornithine decarboxylase (ORNdc) and arginine decarboxylase (ARGdc). In addition, polyamine titer increased before and during the S phase. The activity of ORNdc before the accumulation of significant IAA within hypocotyl explants on the third day is enhanced to a certain extent by the absorption of exogenously applied synthetic auxins, IBA and NAA (Figure 2, Table 2). In addition to the increasing activity of ORNdc, the activity of the spermidine synthase (SPDsyn) could also be enhanced by IBA and NAA, resulting in the higher levels of spermidine in hypocotyl explants of the MSB + IBA and MSB + NAA treatments (Figure 3).

Accumulation of endogenous IAA in soybean hypocotyl explants was significant during adventitious root formation in NAA and IBA treatments (Table 2). Our data confirmed the results published by Alvarez et al. (1989), who reported that a high level of endogenous IAA enhanced root formation in apple rootstocks. Accumulation of endogenous IAA may be due to the inhibition of IAA oxidase activity by IBA and NAA. Liu et al. (1996) reported that the endogenous IAA content increased with a decrease of IAA oxidase activity during the formation of adventitous roots in soybean hypocotyls. The conversion of absorbed IBA to IAA may have resulted in a high IAA content in hypocotyl explants treated with MSB + IBA (Ludwig-Muller and Epstein, 1991). IBA has been shown to be converted to IAA by several plant species (Epstein and Lavee, 1984; Baraldi et al., 1993). As far as the relationship between levels of endogenous IAA and polyamine and adventitious root formation is concerned, we suggest that the exogenously applied auxin (IBA or NAA) acts on polyamine synthase (ORNdc and SPDsyn) and IAA oxidase at the gene level (Theologis et al., 1985; McClure et al., 1989; Dietz et al., 1990) or through enzyme regulation.

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