

Effect of hormone treatment on callus formation and endogenous indole-acetic acid and polyamine contents of soybean hypocotyl cultivated in vitro

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Abstract. Callus was formed and grew well when explants were cultured on medium containing both NAA and kinetin. The endogenous IAA in hypocotyls was accumulated on the medium with both hormones, showing the suppression effect of kinetin on IAA degradation. Besides, the accumulation of NAA in hypocotyl explants was also markedly enhanced by kinetin in the culture medium containing both hormones. In the MS+NAA+Kinetin treatment, the accumulation of spermidine and spermine occurred on the sixth day and was accompanied by rapid callus growth. Increasing putrescine levels were found during the first 3 days in culture when cell multiplication was rapid. The accumulation of endogenous IAA, exogenous NAA, spermidine, and spermine are in proportion to the callus growth in soybean hypocotyl explants.

Keywords: Callus; *Glycine max*; Indole-3-acetic acid; Polyamines.

Introduction

Cytokinin and auxin are known to promote callus formation in tissue culture (Skoog and Armstrong, 1970; Letham, 1974; Akiyoshi et al., 1983). Auxin has a wide variety of effects on plant growth and morphogenesis. Indole-3-acetic acid (IAA), a natural auxin of higher plants, is involved in regulating cell elongation, cell division and differentiation (Dietz et al., 1990). Cytokinins can promote cell enlargement in certain tissues (Rayle et al., 1982; Ross and Rayle, 1982). Moreover, polyamines, another class of growth regulating substance, are reported to be involved in several aspects of plant development (Berlin, 1981; Galston, 1983; Smith, 1985). Numerous investigations have correlated an increase in polyamine levels with cell division and a drop in polyamines during any subsequent lessening metabolic activity (Heimer and Mizrahi, 1982; Walker et al., 1985). Little is known about the change of endogenous IAA and polyamines affected by the exogenous application of auxin and cytokinin in hormone-treated tissue culture. In the present study, the effect of naphthaleneacetic acid (NAA), a synthetic auxin, and kinetin on the levels of endogenous IAA and polyamines in soybean hypocotyl explants during the formation of callus was investigated.

Materials and Methods

Plant Tissue Culture

Soybean seeds (*Glycine max*) strain Tk5 were obtained from the Asia Vegetable Research and Development Center in Taiwan. Soybean seeds were collected and washed in tap water followed by one rinse in sterile distilled water. Soybean seeds were surface sterilized for 1 min in 70% ethanol, soaked in 1% sodium hypochlorite plus a drop of a liquid detergent (Tween-20), and then shaken gently (150 rpm) for 20 min, followed by three rinses in sterile distilled water. Seedlings were grown from seeds in culture tubes (diameter 2 cm) containing 10 mL B5 medium (Gamborg et al., 1968). Soybean seedlings were placed in a growth chamber under the following conditions: 27°C, in darkness, RH 70 to 80% after 7 days, the young seedlings were about 5 cm tall for tissue culture.

Hypocotyl segments (5 mm in length) were cut from the connection of two cotyledons of soybean plants after 7 days of growth in four different media. The basal medium (hormone-free) consisted of inorganic nutrients as found in Murashige-Skoog medium (Murashige and Skoog, 1962) and vitamins of Gamborg's medium, B5 medium, (Gamborg et al., 1968) plus 250 mg/L casein hydrolysate supplemented with 3% sucrose (MS medium). Plant growth regulators, naphthaleneacetic acid (NAA), 2 mg/L, or kinetin, 1 mg/L, or both were added for different treatments (MS+NAA, MS+Kinetin, MS+NAA+Kinetin). The pH of the medium was adjusted to 5.7 prior to addition of 9% (w/v) agar and sterilized by autoclaving

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at 121°C, 1.2 atm for 20 min. The cultures were maintained in test tubes (20 × 110 mm) containing 10 mL medium under controlled conditions: 27°C in darkness, RH 70 to 80%.

Analysis of IAA and NAA in Hypocotyl Explants

Hypocotyl explants of soybean were cultured on four different media and grown in the incubator as described. Callus with explants in all treatments was extracted after a 6-day culture. Portions of explant tissue, usually 2 to 3 g fresh weight, were placed in 125 mL flasks with 30 mL of 80% methanol containing sodium ascorbate and Butylated Hydroxytoluene (BHT) (Dunlap and Guinn, 1989). The samples were homogenized in chilled mortar with pestles in extracting solvent. The ground tissues were filtered with suction through Whatman No. 1 paper. Methanol in filtrates was removed by rotary flash evaporation (RFE) at 35°C, and the aqueous residues (about 15 mL) were slurried with insoluble polyvinylpyrrolidone (PVP). The pH of the aqueous phase 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 extracted twice by partitioning 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 Kiesel gel 60 (20 × 20 cm, Schichtdicke, 0.2 mm, Merck). Up to 1.0 mL of hormonal extract were loaded on a plate, and the chromatogram was developed for about 1 h with the following solvent systems: benzene:acetone:acetic acid (13:6:1 v/v). Identification of the unknowns was done by comparison with the R_fs of standards (IAA and NAA) running at the same time. After TLC development, the IAA and NAA bands were scraped, redissolved in methanol, and concentrated. The residues were filtered, concentrated, and dissolved in 1.0 mL 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; Liu et al., 1996).

High Performance Liquid Chromatography

Partially purified after TLC samples were introduced on a reverse phase C₁₈ 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 50% and 60% methanol in 20 mM ammonium acetate buffer (pH 3.5) for IAA and NAA separation. The flow rate was kept at 0.8 mL/min during chromatographing. For detecting IAA and NAA, 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 Polyamines

Tissues were usually homogenized in chilled mortar with pestles in 5% cold perchloric acid (HClO₄) at a ratio

of 100 mg/L HClO₄, according to the methods of Flores and Galston (1982). After extraction for 1 h in an ice bath, samples were pelleted at 48,000 g for 20 min, and the supernatant phase, containing the polyamine fraction, was stored frozen at -20°C, HClO₄. Extracts were stable for polyamine analysis by HPLC for more than 6 months under these conditions (Martin-Tanguy et al., 1988). HPLC in combination with fluorescence spectrophotometry was used to separate and quantitate polyamines (putrescine, spermidine, and spermine) prepared as their dansyl derivative from plant tissue. The procedure gave sensitive and consistent results for polyamine determinations in plant tissue. In a standard mixture, the minimal detection level is 1 pmol.

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 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 two phases, aqueous and organic. The organic phase, containing the polyamines, 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) in glass vials at -20°C. Standards were processed in the same way, and 20 nmol for each standard polyamine (Sigma, U.S.A.) was dansylated. HPLC of dansylated polyamines was performed according to the method of Smith and Davies (1985). The dansylated samples were eluted from the C₁₈ column with 80% or 90% methanol at a flow rate of 0.8 mL/min. Samples eluted 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.

Results

Effect of NAA and Kinetin on Callus Growth

As shown in Figure 1, the callus growth was remarkably affected by hormone treatment. Callus induction was initiated on the third day. After a 6-day culture, the medium with both NAA and kinetin (MS+NAA+Kinetin) gave the highest callus growth. Somewhat less callus was induced in the medium with NAA (MS+NAA) or kinetin (MS+Kinetin) alone. Little callus was formed in hypocotyl explants cultured in the hormone-free medium (MS).

Effect of Applied Hormones on The Levels of IAA and NAA

The extracts from soybean explants after steps of purification by partition and TLC were still far from pure, yet

the endogenous IAA and absorbed NAA in tissues were separable in reverse phase HPLC using various kinds of mobile phase. In order to make sure that the peak represented IAA, not the other similar compounds, another run of extract spiked with standard IAA showed that there was only one peak with the identical retention time (data not shown). The level of IAA was estimated to be 17.0 ng per g fresh weight in callus growing explants on the medium with NAA and kinetin together, which was much higher than that of the treatments with NAA or kinetin alone (Table 1). The absorbed NAA in explant tissue was detected in the NAA treatment and NAA + kinetin treatment after a 6-day culture. Hypocotyl explants cultured on the medium with both NAA and kinetin on the sixth day accumulated considerable NAA, 16.8 ng per g fresh weight tissue. The level of absorbed NAA in the MS+NAA treatment was 2.8 ng per g fresh weight (Table 1).

Table 1. The accumulation of IAA and NAA contents in hypocotyl explants of soybean after 6-day culture in varying treatments.

| | IAA (ng/g) | NAA (ng/g) |
|----------------|------------|------------|
| MS+Kinetin | 5.7±0.7 | — |
| MS+NAA | 4.2±0.3 | 2.8±0.4 |
| MS+Kinetin+NAA | 17.0±1.2 | 16.8±1.4 |

±, Denotes the standard error, n=3.

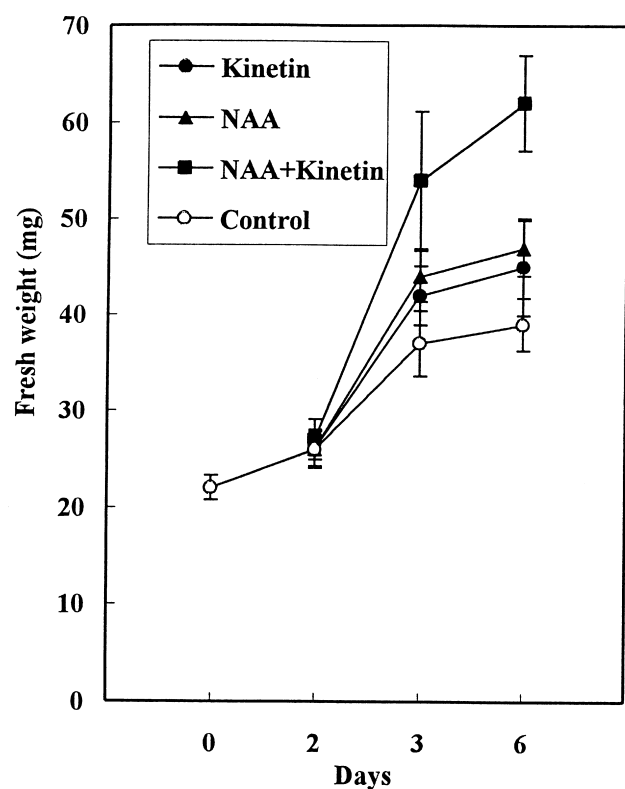


Figure 1. Changes in fresh weight of soybean hypocotyl explants cultured in four different media. Values are means ± SE.

Polyamine Content

The dansyl-polyamines are separable under a variety of conditions through reverse phase HPLC. A typical chromatogram of standards runs at 80% and 90% methanol, and all peaks for dansyl-polyamines could be resolved in less than 15 min (data not shown). The naturally occurring polyamines detected in the extract of soybean hypocotyl explants were putrescine, spermidine, and spermine.

As shown in Figure 2, the accumulation of putrescine was enhanced by either NAA or kinetin. The levels of putrescine increased considerably on the third day and dropped on the sixth day in all hormone treatments. The amounts of putrescine in the tissue did not change much in hormone-free medium (MS) during a 6-day culture. The levels of spermidine in hypocotyl explants in hormone-treated media increased on the third day and reached the maximum levels on the sixth day during culture (Figure 3). Hypocotyl explants, cultured in MS medium with NAA and kinetin (MS+NAA+Kinetin), accumulated the highest levels of spermidine among the treatments (Figure 3). As shown in Figure 4, the amounts of spermine in hypocotyl explants did not increase before the sixth day during culture. The levels of spermine in tissue grown in MS medium with NAA and kinetin accumulated the most among the hormone treatments. The levels of spermine in tissue in hormone-free MS medium dropped on the second day, increased on the third day, and then kept constant during culture period.

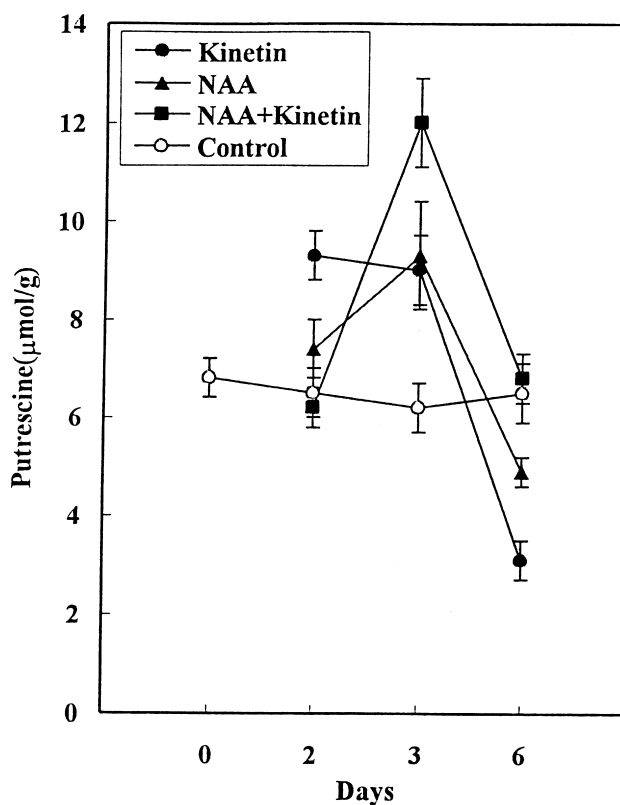


Figure 2. Changes in levels of putrescine in soybean hypocotyl explants cultured in four different media. Values are means ± SE.

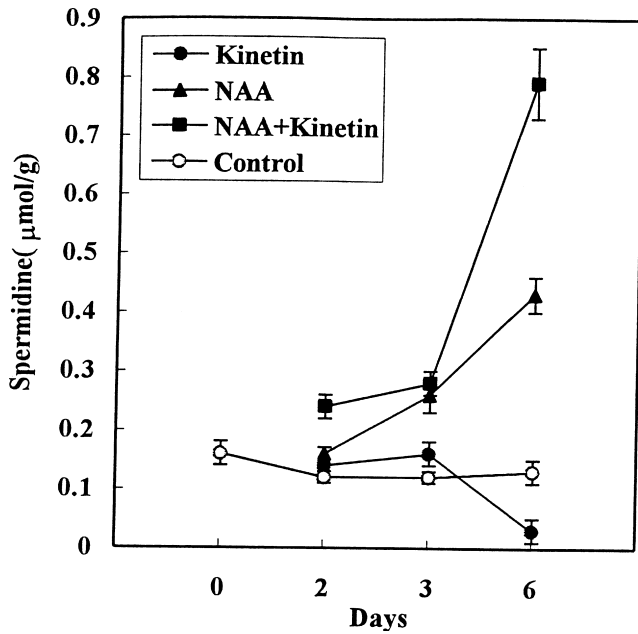


Figure 3. Changes in levels of spermidine in soybean hypocotyl explants cultured in four different media. Values are means \pm SE.

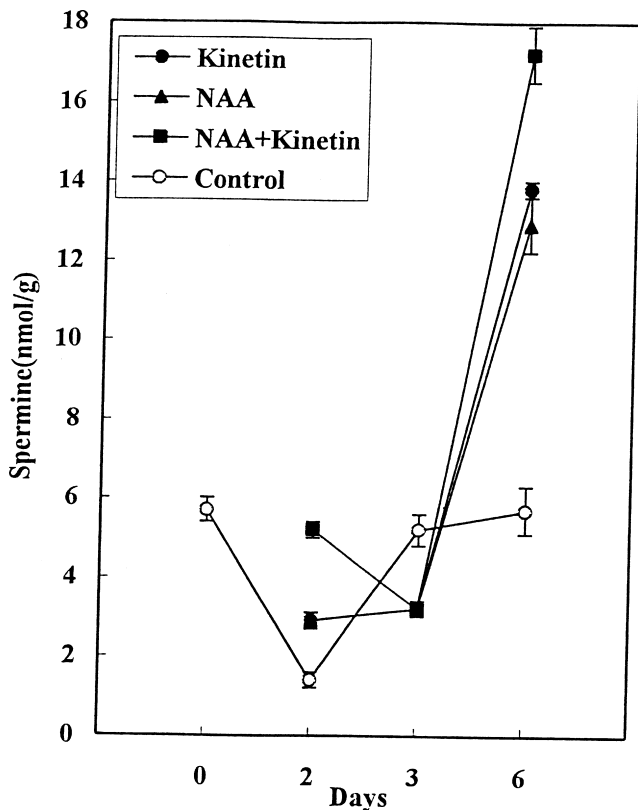


Figure 4. Changes in levels of spermine in soybean hypocotyl explants cultured in four different media. Values are means \pm SE.

Discussion

The endogenous IAA in callus from hypocotyl explants has markedly accumulated in MS+NAA+Kinetin medium on the sixth day (Table 1). This accumulation is probably due to the inhibition of IAA conversion by kinetin or NAA during the 6-day culture. Beffa et al. (1990) reported that several synthetic auxins (2,4-D and NAA) inhibited the auxin-oxidase activity of extract from *Zea mays* L. cv LGll apical root segments. Liu et al. (1996) also reported that the exogenous NAA caused the accumulation of endogenous IAA through reducing the activity of IAA oxidase. Moreover, the increase of endogenous IAA, is accompanied by the accumulation of the exogenously applied NAA in culture medium. The accumulation of NAA in the tissue enhanced by kinetin in the callus grown on MS+NAA+Kinetin medium might be due to promotive absorption. Our results suggest that the promotion of the callus growth in MS medium with NAA and kinetin may be due to the high levels of IAA and NAA accumulated in cultured hypocotyl explants.

During callus growth in soybean hypocotyl explants, higher levels of putrescine, spermidine, and spermine are accumulated in tissue grown on MS medium with NAA, or kinetin, or both hormones. Endogenous levels of putrescine and spermidine, and spermine in hypocotyl explants are markedly enhanced on the third day when the callus is induced to proliferate (Figures 2, 3, 4). That polyamine biosynthesis is associated with cell division and changes in free polyamine levels during morphogenic processes is well known (Desai and Mehta, 1985; Walker et al., 1985). Martin-Tanguy et al. (1988) reported that the application of 2,4-dichlorophenoxyacetic acid (2,4-D) and benzyladenine (BA) to *Nicotiana tabacum* tissue accelerates growth and activates polyamine biosynthesis. The polyamines' metabolic enzymes, such as spermidine synthase (SPDsyn) and spermine synthase (SPMsyn), may be regulated by the exogenously applied synthetic hormones (NAA or kinetin) in this study. Among these enzymes, SPDsyn activity was estimated to be enhanced about 3-fold by the addition of NAA and kinetin in callus induction using hypocotyl as explants (Figure 4). The increase of SPDsyn activity could also be caused by the drop of the levels of putrescine and the rapid rise of the amounts of spermidine in tissue on the sixth day during culture in MS medium with NAA and kinetin. Heimer and Mizrahi (1982) demonstrated a significant level of polyamine metabolic enzymes activity correlated with cell division in tobacco suspension culture and tomato ovaries. Torrigian et al. (1987) also reported that the first synchronous cell cycle was induced by auxin in *Helianthus* tuber slices, and polyamines' metabolic enzymes (such as ornithine decarboxylase, ODC, arginine decarboxylase, and ADC) and that the polyamine titer was increased before and during the S phase. In our investigation, we further determine that the callus growth enhanced by NAA and kinetin is accompanied by the accumulation of endogenous IAA and polyamines (putrescine, spermidine, and spermine) in soybean hypocotyl explants. NAA or kine-

tin might regulate the polyamine metabolic enzymes through activation of the genes coded for these enzymes (McClure et al., 1989; McClure and Guilfoyle, 1989; Theologis et al., 1985), or through regulating these enzymes' activities.

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