

# Effect of naphthaleneacetic acid on endogenous indole-3-acetic acid, peroxidase and auxin oxidase in hypocotyl cuttings of soybean during root formation

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**Abstract.** The effect of exogenous naphthaleneacetic acid (NAA) on the internal levels of indole-3-acetic acid (IAA) in rooting hypocotyls of *Glycine max* was studied. The hypocotyls of NAA-treated cuttings grew significantly higher numbers of adventitious roots with an increase in endogenous IAA levels that corresponded with a decrease in IAA oxidase activity (32 kDa) examined. Moreover, a decline of peroxidase activity was accompanied by a decrease of lignin content during root formation. Caffeic acid and ferulic acid, two critical phenolic compounds for lignin synthesis, accumulated in NAA-treated tissues. Consequently, the increased IAA levels with a decrease of IAA oxidase activity accompanied a lower lignin content and a reduced peroxidase activity in NAA-treated tissues suggests that the induction of adventitious roots by NAA in soybean cuttings may be due to the higher IAA levels accumulated in tissues.

**Keywords:** Adventitious rooting; Indole-3-acetic acid; IAA oxidase; Naphthaleneacetic acid; Peroxidase; *Glycine max*.

**Abbreviations:** HPLC, high performance liquid chromatography; IAA, indole-3-acetic acid; NAA, naphthaleneacetic acid; TLC, thin layer chromatography.

## Introduction

It is known that adventitious root formation can be stimulated by exogenously applied auxins, yet the mechanism of this physiological response is still disputed. Conflicting results have been reported about the endogenous indole-3-acetic acid (IAA) levels in rooting tissues. In most cases, high levels of IAA are associated with the promotion of adventitious rooting (Weigel et al., 1984; Moncousin et al., 1989; Liu and Reid, 1992; Sagee et al., 1992). In some cases, the levels of endogenous IAA declined during root formation (Hausman, 1993). Furthermore, some reports have indicated that root initiation occurred without any changes of IAA levels in the root regenerating zone (Nordstrom and Eliasson, 1991). The regulation of plant development by IAA may depend on the amount of free IAA present in plant tissues. Free IAA can be modulated via several pathways, including IAA metabolism, synthesis and transport, and the breakdown of conjugated IAA. One well known IAA metabolic pathway consists of the oxidative decarboxylation of the side chain of IAA by IAA oxidase, leading to the formation of either indole-3-methanol or 3-methyleneoxindole (Grambow and Langenbeck-Schwich, 1983; Beffa et al., 1990). Moreover, numerous papers have reported that

auxin-induced changes in peroxidase and IAA oxidase occur during the rooting processes (Gaspar et al., 1985; Mato et al., 1988; Fett-Neto et al., 1992). Yet, little is known about the correlation between IAA levels and IAA oxidase activity during the processes of rooting. One aim of this investigation is to examine the relationship between the change of IAA content and IAA oxidase activity during root formation.

The formation of adventitious roots involves the process of redifferentiation, in which predetermined cells switch from their morphogenetic path to act as mother cells for the root primordia (Friedman et al., 1979; Aeschbacher et al., 1994). Among these changes, the process of lignification in the cell wall, catalyzed by a particular peroxidase, may occur during the rooting (Fukuda and Komamine, 1982; Church and Galston, 1988; Bruce and West, 1989; McDougall, 1992; Sato et al., 1993). In addition to examining the changes in IAA content and IAA oxidase activity, the effect of NAA on lignification during the rooting processes is also investigated.

## Materials and Methods

### *Plant Materials and Rooting Experiments*

Soybean (*Glycine max*) seedlings were germinated and grown in vermiculite at  $27 \pm 0.5^\circ\text{C}$  under 16 h/8 h light/

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dark period and illuminated with white fluorescent light at an irradiance of  $16.7 \text{ W m}^{-2}$ . Cuttings were prepared from 6-day old seedlings each consisting of an apical bud, a pair of primary leaves, an epicotyl, and the uppermost 3.0 cm of a hypocotyl. For rooting experiments, twelve cuttings, four cuttings per vial, were used per treatment, and all experiments were performed four times. Freshly prepared hypocotyl cuttings were introduced to vials containing sufficient deionized water or  $50 \mu\text{M}$  NAA to cover the entire hypocotyl of cuttings for one day. Cuttings were then transferred to water for four days. The number of visible roots was determined four days after transfer. In order to collect sufficient tissues for biochemical assay, freshly prepared hypocotyl, 80 cuttings per pot, were introduced to a 3 liter pot containing water or  $50 \mu\text{M}$  NAA solution.

### *Analysis of Indole-3-Acetic Acid*

*Extraction and partial purification* — Indole-3-acetic acid (IAA) was extracted by grinding 5.0 g fresh weight of plant material in 100 mL 80% methanol (HPLC grade) containing  $0.25 \text{ mg mL}^{-1}$  butylated hydroxytoluene and  $0.5 \text{ mg mL}^{-1}$  sodium ascorbate (Dunlap and Guinn, 1989) using mortar and pestle. The ground tissue was filtered with suction through Whatman No.1 paper. The residue on the filter paper was rinsed three times with extracting solution. Methanol in the filtrate was removed by rotary flash evaporation (RFE) at  $35^\circ\text{C}$ , and the pH of the aqueous residue (about 15 mL) was adjusted to 8.0 with 0.2 N KOH and partitioned twice with an equal volume of ethyl acetate (HPLC grade) to remove some impurities. The ethyl acetate fraction was discarded. Residual ethyl acetate in the aqueous phase was removed by RFE. The pH of the aqueous fraction was adjusted to 2.5 with 0.2 N HCl, then partitioned twice with the same volume of ethyl acetate. The residual water in the acidic ethyl acetate fraction was removed by addition of anhydrous sodium sulfate. The acidic ethyl acetate fraction was evaporated to dryness by RFE. The residue was immediately dissolved in 1.0 mL methanol.

*Thin layer chromatography (TLC)* — The acidic ethyl acetate fraction in methanol was purified first by thin layer chromatography (TLC). TLC was performed on analytical Kieselgel 60 ( $20 \times 20 \text{ cm}$ , Schichtdicke, 0.2 mm, Merk). TLC plates were run with 100% methanol for cleaning and activation before loading. Up to 1.0 mL of extracts were loaded, and the chromatogram was developed for about 1 h with the following solvent system, benzene : acetone : acetic acid (10 : 4 : 1, v/v) (Rademacher and Graebe, 1984). Identification of the unknown was done by comparison with the Rf's of the standard (IAA) running at the same time. After TLC development, the IAA band was scraped immediately and resuspended in 20 mL methanol. The supernatant was evaporated to dryness by RFE. The residue was immediately dissolved in 1.0 mL methanol and then quantified by HPLC. The IAA standard recovered after partition and TLC was 72%.

*High performance liquid chromatography (HPLC)* — Sample (20  $\mu\text{l}$ ), purified by TLC, was analyzed on a reverse phase C-18 column ( $5 \times 250 \text{ mm}$ , TSK gel column, ODS-80 TM, Tosoh, Japan). Elutes were monitored with a fluorescence spectrophotometer (Model F-1050, Hitachi), and the mobile phase was 35% methanol in 20 mM ammonium acetate buffer (pH 3.5) with a flow of  $0.7 \text{ mL min}^{-1}$ . For detecting IAA, an excitation wavelength of 280 nm with an emission wavelength of 360 nm was used. Eluant peak with their retention time and areas were recorded by an attached integrator (Model D-2500, Hitachi). Estimation of IAA was made by fluorimetric detection and by comparing the peak areas with known amounts of IAA (Crozier et al., 1980).

### *Enzyme Preparation and Assay*

*Extraction and partial purification of enzymes* — Tissue was ground in mortar with 10 mL per g fresh weight of 50 mM potassium-phosphate (pH 6.0). The homogenate was centrifuged for 20 min at 20,000 g. After centrifugation, the pellet was discarded and the supernatant was treated with cold acetone added to a final concentration of 70% and centrifuged at 27,000 g for 15 min. The precipitate was resuspended in the same buffer as the crude extract for enzyme assay (Vazquez and Mato, 1991). Protein concentrations were determined according to Bradford (1976) and bovine albumin (BSA) was used as the standard. Standard proteins were used to estimate the molecular mass of enzyme assayed. For the purification, the crude extracts were passed through a Sephacryl S-100 (Pharmacia, Sweden) column ( $1.0 \times 90 \text{ cm}$ ) previously equilibrated with 20 mM potassium-phosphate buffer (pH 6.0). The flow rate was kept at about  $0.25 \text{ mL min}^{-1}$ . Fractions were collected every 4 min and assayed for peroxidase and IAA oxidase activity.

*IAA oxidase assay* — Reaction mixtures contained 0.2 mL of enzyme extracts, 0.78 mL of 50 mM potassium-phosphate buffer (pH 6.0), 0.01 mL of 5 mM  $\text{MnCl}_2$ , 0.01 mL of 5 mM 2,4-dichlorophenol (DCP), and 0.02 mL of  $2.5 \text{ g L}^{-1}$  IAA. Assays were conducted at  $25 \pm 0.5^\circ\text{C}$  for 30 min. The Salkowski reagent (2 mL) was then added and the destruction of IAA was determined by measuring the absorbance at 535 nm after 30 min (Beffa et al., 1990). Each value was the mean of three replicates.

*Peroxidase assay* — The reaction mixture contained 0.1 mL enzyme extract, 0.01 mL 20 mM guaiacol, 0.1 mL 50 mM  $\text{H}_2\text{O}_2$ , and 0.76 mL 2.5 mM 3,3-dimethylglutaric acid (3,3-DGA)-NaOH at pH 6.0. Peroxidase activity was determined spectrophotometrically by monitoring the formation of tetraguaiacol at 470 nm after 10 min incubation at  $30 \pm 0.5^\circ\text{C}$  (Beffa et al., 1990). Each value was the mean of three replicates.

*Measurement of lignin content and phenolic compounds* — One gram of tissues were homogenized in 10 mL ethanol, and centrifuged at 10,000 g for 15 min. The residue was transferred to a glass petri dish in hood for drying (24 h at room temperature). The resulting alcohol insoluble

residue (AIR) was used for lignin determination. Glass tubes contained 50 mg of AIR, 5 mL of 2 N HCl and 0.5 mL of thioglycolic acid. The test tubes were placed in a boiling water bath and shaken initially to hydrate the AIR for 4 h at  $100 \pm 0.5^\circ\text{C}$ . The tubes were cooled and the contents were transferred to polypropylene tubes, centrifuged at 10,000 g for 15 min at room temperature. The pellets were washed once with 5 mL  $\text{H}_2\text{O}$  and resuspended in 5 mL 0.5 N NaOH. The extract was agitated gently at  $25 \pm 0.5^\circ\text{C}$  for 18 h to extract the lignin thioglycolate. The extract was centrifuged (10,000 g, 15 min) again, and the supernatant was transferred to test tubes. One mL of concentrated HCl was added to each test tube, and the lignin thioglycolate was allowed to precipitate at  $4^\circ\text{C}$  for 4 h. Following centrifugation in centrifuge tubes at 10,000 g for 15 min, the orange-brown pellets were dissolved in 10 mL 0.5 N NaOH. The absorbance of the solution at 280 nm was measured (Bruce and West, 1989).

Phenolics were extracted by homogenizing 5.0 g tissues in 100 mL 80% methanol. The ground tissues were filtered with suction through Whatman No.1 paper. Methanol in the filtrate was removed by rotary flash evaporation (RFE) at  $35 \pm 0.5^\circ\text{C}$ , and the pH of the aqueous residues was adjusted to 2.6 with 0.2 N HCl, then partitioned twice with the same volume of ethyl ether. The ethyl ether fraction was collected and evaporated to dryness by RFE. The residue was redissolved in 0.5 mL 100% methanol. Samples of phenolic extracts were injected into a reverse phase C-18 column ( $5 \times 250$  mm, TSK gel column, ODS-80TM, Tosoh, Japan), and eluted in 35% methanol in 20 mM sodium acetate buffer (pH 5.0) for 30 min. Ferulic acid and caffeic acid were quantified using a UV spectrophotometer at 280 nm. The identity of these compounds in extracts was confirmed by their co-elution with authentic standards purchased from Sigma under different HPLC conditions (Yalpani et al., 1993).

## Results

### Effect of NAA on Root Formation

Preliminary experiments indicated that adventitious roots were induced optimally at  $50 \mu\text{M}$  NAA solution (data not shown); therefore the concentration of NAA used in this investigation was at  $50 \mu\text{M}$ . Adventitious roots around the top 2.0 cm of the hypocotyls of soybean cuttings were induced on the 4th day after treatment with NAA (Table 1). Some adventitious roots were also formed near the basal cutting area in both NAA-treated and control tissue.

**Table 1.** Effect of applied NAA on the formation of adventitious roots in various hypocotyl segment of soybean cuttings. Values are means  $\pm$  SE of triplicate treatments.

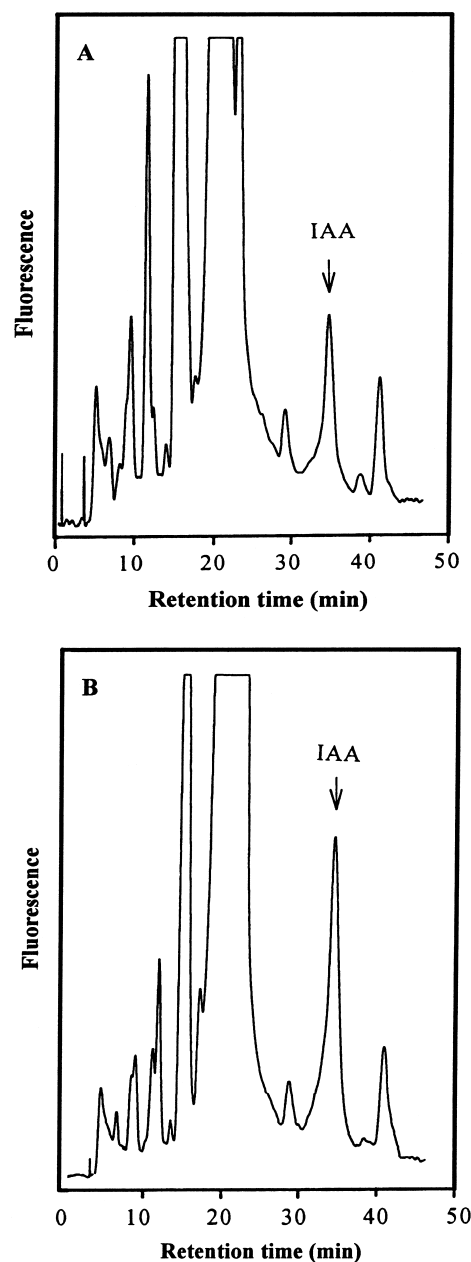
Segment distance from cut base	Number of roots per cutting <sup>a</sup>	
	Control	NAA
0–1 cm	$3.5 \pm 0.6$	$6.4 \pm 0.5$
1–3 cm	0	$4.5 \pm 0.8$

<sup>a</sup>Rooting was scored four days after the initiation of treatment.

In NAA-treated tissue, about five roots were induced in the uppermost 2.0 cm of the hypocotyl per cutting; however, no rooting occurred at all in the uppermost 2.0 cm of control hypocotyls. The formation of adventitious roots promoted by NAA are supported by a great deal of experimental evidence (Geneve and Kester, 1991; Hausman, 1993).

### Changes in Endogenous IAA in Soybean Hypocotyl

HPLC facilitated the separation of IAA from its conjugated forms and other impurities. As shown in Figure 1A and 1B, the extracts from tissues after steps of purification were still far from pure. The endogenous IAA was detected in hypocotyls of soybean cuttings treated with



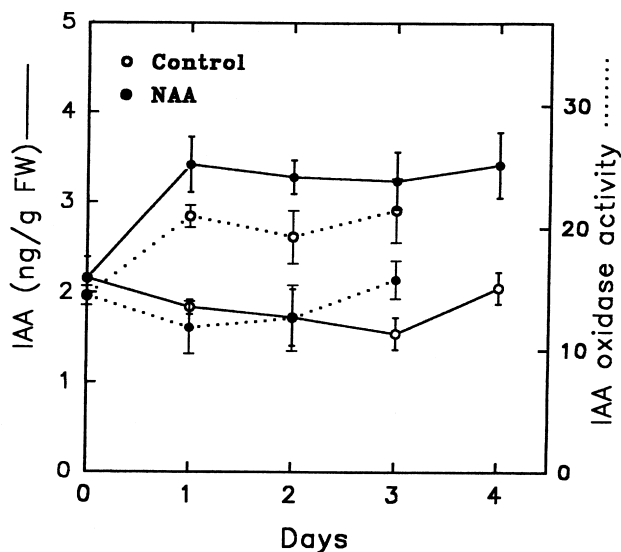
**Figure 1.** HPLC chromatogram of extract from hypocotyl of soybean cuttings treated with water (A) and  $50 \mu\text{M}$  NAA (B).

NAA for one day. 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). Higher levels of endogenous IAA were found in the rooting hypocotyls of cuttings treated with NAA (Figure 2). After one day of treatment, the endogenous IAA content in the NAA-treated tissues was about 1.7-fold higher than that of control. The IAA levels in NAA-treated tissues remained constant during the examination period.

#### IAA Oxidase and Peroxidase Activity

The IAA oxidase activity in crude extracts of NAA treatment was generally lower than that of control (Figure 2). At the end of the first day, the IAA oxidase of soybean cuttings treated with NAA was about a half that of the control. The IAA oxidase activity of NAA-treated cuttings increased slightly as the incubation was prolonged. These results confirmed the data published by Barazi and Schwabe (1984) and Vazquez and Mato (1991) indicating that the reduction of IAA oxidase activity accompanies adventitious root formation. The decrease of IAA oxidase activity in NAA-treated cuttings occurred three days before the appearance of new adventitious roots.

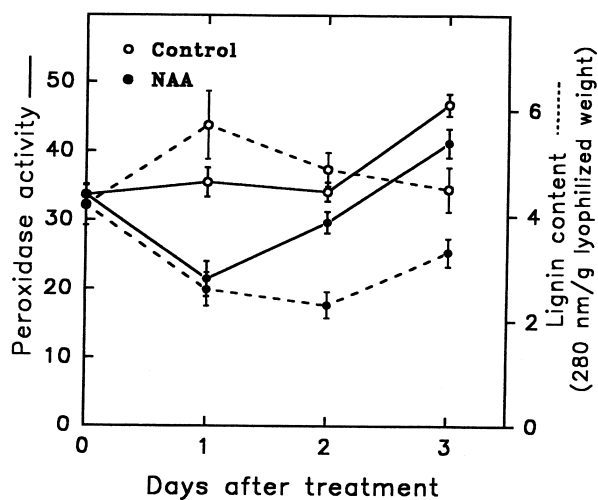
The peroxidase activity in crude extracts of cuttings treated with NAA was also lower than that of control during the test periods. On the first day particularly, there was a significant decline in peroxidase activity of NAA-treated tissues (Figure 3). The peroxidase activity was about 60 per cent that of the control. As the incubation time was prolonged, peroxidase activity in both NAA-treated and control cuttings increased, and the difference between NAA-treated and control tissues became less significant.



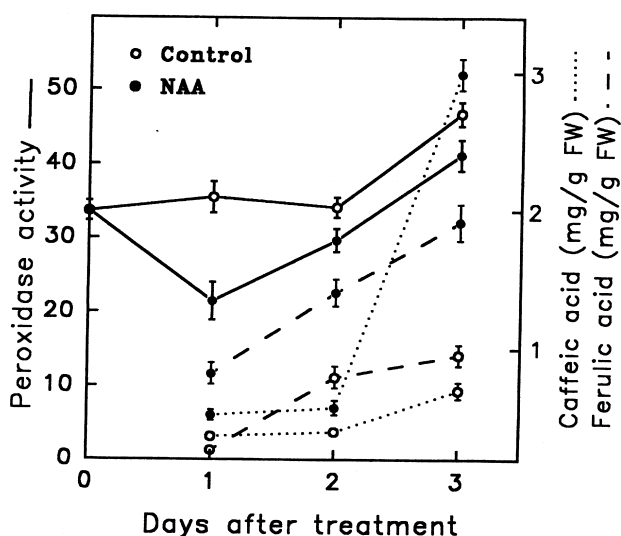
**Figure 2.** Changes of IAA oxidase activity and levels of IAA in hypocotyl extracts of soybean cuttings treated with or without NAA. One unit of IAA oxidase activity is equivalent to a  $\Delta A_{535}$  of 1.0 for 1 mg of protein in 30 min. Values are means  $\pm$  SE.

#### Effect of NAA on Soluble Phenolics and Lignin Content

To investigate lignification, lignin content was measured during root formation. As shown in Figure 3, the lignin content decreased significantly one day after NAA treatment; meanwhile, the soluble phenolics increased (data not shown). The decrease of lignin content in NAA-treated tissues was correlated with the reduction of peroxidase activity indicated in Figure 3. As the culture time was prolonged to the third day, the lignin content of NAA-treated tissues was close to the control. In soluble phenolics, it was also observed that the amount of caffeic acid



**Figure 3.** Changes of peroxidase activity and lignin content in hypocotyl of soybean cuttings treated with or without NAA. One unit of peroxidase activity corresponds to a  $\Delta A_{470}$  of 1.0 for 1 mg of protein in 10 min. Values are means  $\pm$  SE.

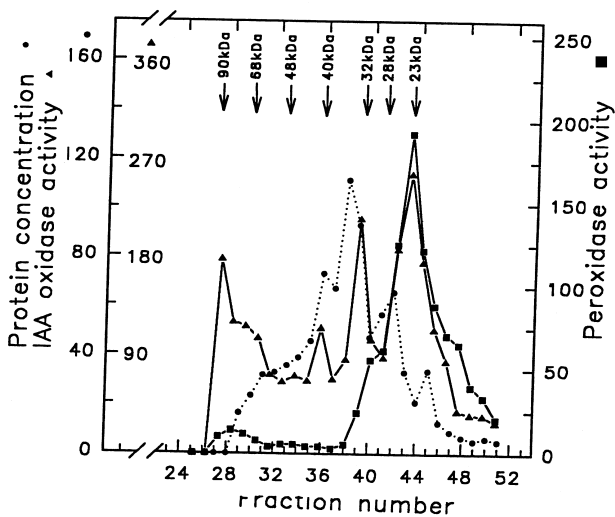


**Figure 4.** Changes of peroxidase activity, contents of caffeic acid and ferulic acid in hypocotyl of soybean cuttings treated with or without NAA. Values are means  $\pm$  SE.

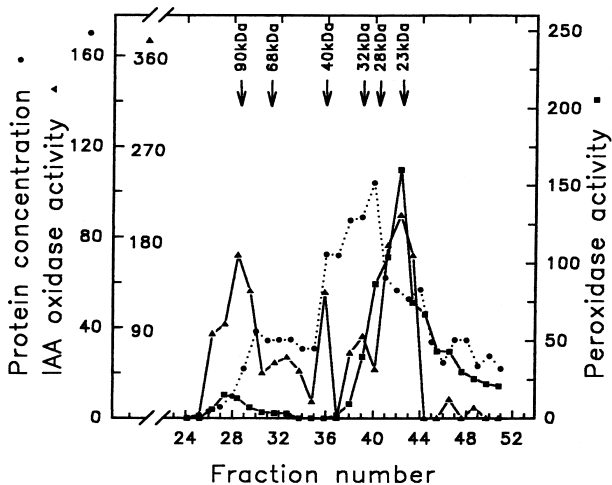
and ferulic acid, two precursors for lignin synthesis, accumulated to a much greater amount in NAA-treated tissues than in the control (Figure 4 and Figure 7).

### Fractionation of Enzymes by Gel Filtration Chromatography

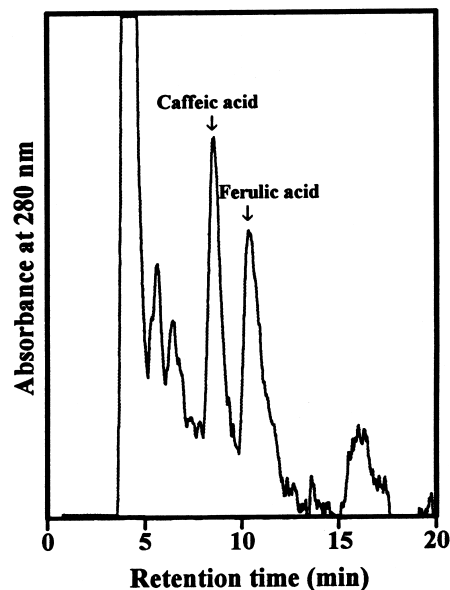
The changes of IAA levels, IAA oxidase, and peroxidase of NAA-treated tissues occurred on the first day after treatment. Therefore, for enzymes fractionation, the soybean cuttings treated with NAA for one day were used for extraction. The crude extract of control tissues eluted from Sephacryl S-100 column is given in Figure 5. Two peaks of peroxidase activity with molecular mass estimated to be 90 kDa and 23 kDa were observed, and four peaks of IAA oxidase activity with molecular mass estimated to be 90 kDa, 40 kDa, 32 kDa and 23 kDa, respectively were



**Figure 5.** Elution profile of protein extract from hypocotyl of soybean cuttings (control) on Sephacryl S-100 column. Unit of protein concentration is given in  $\mu\text{g mL}^{-1}$ .



**Figure 6.** Elution profile of protein extract from hypocotyl of soybean cuttings treated with NAA on Sephacryl S-100 column.



**Figure 7.** HPLC spectrum of phenolic compounds in soybean hypocotyl treated with NAA. Column:  $5 \times 259$  mm, TSK gel; Mobile phase: 35% methanol in 20 mM sodium acetate buffer, pH 5.0; Flow rate:  $0.7 \text{ mL min}^{-1}$ .

obtained. In NAA treatment, a similar elution profile was obtained (Figure 6). Among the IAA oxidase isozymes, in particular, the activity of IAA oxidase (32 kDa) in NAA-treated tissues was reduced significantly, to about 40 per cent that of the control. Peroxidase activity (23 kDa) in NAA-treated tissues was also lower than that of control. There was a correlation between the accumulation of endogenous IAA levels and the decrease of IAA oxidase activity in soybean cuttings after treatment with NAA (Figure 2).

### Discussion

In this investigation, NAA induced considerable adventitious rooting in soybean hypocotyl, which confirms previous published experimental evidence. In order to clarify the conflicting results concerning endogenous IAA levels during the processes of rooting, the levels of IAA and IAA oxidase activity were measured. As far as the correlation between endogenous IAA levels and rooting is concerned, the NAA-treated soybean cuttings not only grew a significant number of adventitious roots around the uppermost 2.0 cm regenerating zone (Table 1), but also showed an increase in endogenous IAA levels (Figure 2). These data confirm previous reports (Moncousin et al., 1989; Liu and Reid, 1992). During the induction of adventitious roots, levels of IAA increased three days before the appearance of new adventitious roots, indicating that the biochemical changes in the processes of rooting caused by exogenous NAA may occur one day after NAA treatment. In addition to IAA content, we also found that the activity of IAA oxidase with M. M. estimated to be

about 32 kDa reduced remarkably. The drop in IAA oxidase activity might correspond to the rise in endogenous IAA levels, confirming previous results (Barazi and Schwabe, 1984; Vazquez and Mato, 1991). This suggests that the reduction of IAA oxidase, particularly the 32 kDa one, may be necessary for adventitious root formation. Mato et al. (1985) did not find any changes of IAA oxidase in NAA-treated cuttings. Their failure might be due to the lack of resolution of IAA oxidases isozymes by gel filtration chromatography (Sephacryl S-100) used in this investigation.

Even though the peroxidase isozymes are not definitively involved in specific metabolic pathways, their tissue levels have been negatively correlated to internal IAA levels (Hausman, 1993) and positively to lignin synthesis (Lewis and Yamamoto, 1990; McDougall, 1992). Peroxidases involved in the lignification processes are known to be localized both in the cell wall (Sato et al., 1993) and in the free space of the cell wall (Imberty et al., 1985). Moreover, the decrease of peroxidase activity is accompanied by a decline of lignin content in NAA treated tissue (Figure 3) indicating that the synthesis of lignin by peroxidase (Fukuda and Komamine, 1982; Sato et al., 1993) may be inhibited, to some extent, by NAA, causing the soluble phenolics, particularly caffeic acid and ferulic acid (Figure 4), to accumulate in NAA-treated tissues.

In conclusion, the increased IAA levels with a fall of IAA oxidase activity, which is accompanied by the lower lignin content caused by a reduced peroxidase activity in NAA-treated tissues, suggest that the induction of adventitious roots by NAA in soybean cuttings may be due to the higher IAA levels accumulated. However, levels of NAA absorbed by treated tissues were very low (Hausman, 1993). It is possible that NAA may act independently on the gene activation level much like indole-3-butyric acid (IBA) as proposed by Kantharaj et al. (1979) and Jarvis et al. (1985), and on the IAA oxidase and peroxidase examined in our studies. Among the IAA oxidase isozymes examined, the one with M. M. estimated to be about 32 kDa could be the main IAA oxidase (32.5 kDa) involved in the oxidative decarboxylation of IAA separated from peroxidases reported by Beffa et al. (1990). Further purification of IAA oxidases (peroxidases) is needed to elucidate the molecular mechanism of how NAA affects or regulates these enzymes during the processes of rooting.

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## 不定根形成時 NAA 對大豆下胚軸生長素內含量，過氧化氫酶素及生長素氧化酶素之影響

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本研究以 naphthaleneacetic acid (NAA) 處理大豆 (*Glycine max*) 莖插，觀察下胚軸不定根之形成與組織內 indoleacetic acid (IAA) 含量及其代謝酶素—生長素氧化酶素 (IAA oxidase) 活性與過氧化氫酶素活性以及木質素含量之變化之關係。NAA 的處理明顯的誘發不定根的形成，伴隨著生長素氧化酶素活性 (32 kDa) 顯著的降低，IAA 含量約增強兩倍；此外，木質素的含量隨著過氧化氫酶素活性的下降而減少，咖啡酸 (caffeic acid) 和阿魏酸 (ferulic acid) (與木質素合成有關的酚類) 亦累積於組織內。結果顯示，處理 NAA 可能引發組織內生長素氧化酶素活性的降低，而累積較多的 IAA。NAA 處理之組織內含較高的 IAA 可能為大豆莖插誘發不定根形成的重要因素之一。

**關鍵詞：**大豆；不定根形成；IAA；生長素氧化酶素；NAA；過氧化氫酶素。