# STUDIES ON THE FORMATION OF ADVENTITIOUS ROOT INITIAL IN MUNG BEAN SEEDING HYPOCOTYL

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**Abstract**: The formation of adventitious roots was studied by using 7-day-old mung bean hypocotyl cuttings. The cuttings presoaked for 24 hrs in NAA at a concentration of $1\times10^{-4}$ M produced more adventitious roots than at any other concentration of NAA used and more than in any other auxin tested. The basal part of the cuttings formed the roots in four rows; these originated from the phloem parenchyma cells. Cell division of these parenchyma cells was evident after 36 hrs of incubation in distilled water. The formation of adventitious roots was inhibited by cycloheximide (5 $\mu$g/ml), and 5-fluorouracil ($1\times10^{-3}$ M) but not by $\alpha$-amanitin (1 $\mu$g/ml). Presence of 5-FU in the beginning of the incubation period in distilled water following 24 hrs presoaking inhibited the cell division and consequently inhibited the formation of root initials. 5-FU also inhibited the formation of adventitious roots even after cell divisions had occurred. Using $^3$H-uridine as a tracer, RNA synthesis was measured before any cell division had occurred in the root initials. NAA stimulated the RNA synthesis nearly 2 fold and this was inhibited by 5-FU. It is concluded that rRNA synthesis is essential before cell division can occur in the formation of root initials. The ultimate formation of adventitious roots from root initials also requires continuous cell division and also continuous synthesis of rRNA.

## INTRODUCTION

Adventitious roots are found widely on all vascular plants and are generally formed on stems, leaves or old roots. The development of adventitious roots through stem cuttings is important in the asexual propagation of plants. Before the initiation of adventitious roots, the cells of the potential rooting region show some biochemical changes. Using RNA synthetic inhibitors Jalouzot reported that the initiation of the adventitious roots of *Cicer arietinum* required RNA synthesis[6]. Fellenberg reported that antimetabolites of RNA synthesis inhibited the root formation in etiolated pea epicotyls[2]. Using Pyronin Y dye and histoautoradiography technique Chang and Chan demonstrated that there was an increase of RNA accumulation and this accumulation persisted through the rooting process until the formation of a root primordia in the potential rooting zone. However no RNA buildup was found in adjacent cortical or phloem ray parenchyma[1]. These reports show that before adventitious roots are formed new RNA synthesis is required. Key *et al.*[8] showed that 2,4-D stimulated RNA synthesis then cell division followed in the nonmeristematic regions of soybean hypocotyl tissues. The increase of RNA by 2,4-D was rRNA, and this was based on the increase of RNA polymerase I activity.[3]

In order to investigate the relationship between adventitious root initiation and RNA synthesis or protein synthesis, the authors used auxins and RNA or protein synthetic inhibitors to observe their effects on adventitious root formation. Also in order to ascertain what kind

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of RNA is initially necessary for adventitious root initiation, studies were made with different kinds of RNA synthetic inhibitors and they were applied before the formation of adventitious root initials.

## MATERIALS AND METHODS

Mung bean seeds (*Phaseolus aureus*, Tainan No. 2) were germinated in moist vermiculite and grown in the growth chamber under 12 hour photoperiod (light intensity 5000-6000 lux) with day and night temperature at 26°C and 20°C for 7 days. Cuttings were prepared by removing cotyledon and cutting off the root system 3 cm below the cotyledon node. These were dipped in the test solution and kept in the test solution or were placed in it for 24 hours and then transfered to distilled water for 6 days. All cuttings were kept in the growth chamber under 12 hour light period as above. The number of adventitious roots were recorded at 7th day. For histological studies, basal 5 mm segments of the cuttings were excised at various periods, fixed in F. A. A. under partial vacuum, dehydrated in an ETOH-TBA series, embedded in paraffin. These paraffin embedded tissues were cut at 11 $\mu$ and stained with iron hematoxylin[7]. For measurement of RNA synthesis, the mung bean cuttings were presoaked in various test solutions for 24 hours, then the hypocotyl segments were cut into 1 cm lengths. 180 sections were incubated in a flask containing 10 ml of incubation medium[13] at 28°C in a water bath shaker for 2 hours. The incubation medium contained $5\times10^{-4}$ M ammonium citrate (pH 6.0), 1% sucrose, 50 $\mu$g/ml chloramphenicol and 1 $\mu$Ci/ml $^3$H-uridine (3.65 mCi/$\mu$mole, New England Neuclear). After incubation, the medium was discarded, the sections were washed with 0.01 M cold uridine. Then, the sections were blotted with tissue paper to remove excess moisture and homogenized in 0.01 M Tris buffer (pH 7.4) for 1 min at setting ♯4-5 with Williams Polytron Model PT-ST (Brinkmann Instrument). The homogenate was filtered through glass wool, the filtrate was centrifuged at 10,000×g for 30 min and the supernatant was collected. To 0.1 ml supernatant 2 ml of 10% ice-cold trichloroacetic acid was added for precipitation. The precipitates were collected on GF/A glass filter discs (Whatmann), washed with cold 5% trichloroacetic acid and 95% ethanol, dried under a heat lamp and counted in a Parkard liquid scintillation spectrometer.

## RESULTS AND DISCUSSION

Adventitious root initials were visible at the base of mung bean hypocotyls 4 days after cuttings were made. They generally appeared in four rows (Fig. 1), two of them were in direct lines below the primary leaves and the other two were in direct lines below the cotyledons. If the cuttings were kept continuously in NAA at a concentration of $1\times10^{-5}$ M, there was no significant promotion of adventitious root initials. When the cuttings were presoaked with NAA at a concentration of $1\times10^{-4}$ M for 24 hours and then transfered to distilled water for 6 days, the number of adventitious root initials increased 3 fold over the controls i.e. over those kept in water (Fig. 2 and 3). Adventitious roots were only formed at the base of the cuttings kept in the water (i.e. the controls), however, the NAA treated hypocotyls formed adventitious roots over their whole length of hypocotyl in addition to the base and these were arranged in four rows (Fig. 1). When the four auxins namely NAA, IAA, IBA and 2,4-D were compared for their ability to stimulate the formation of adventitions roots, the results

ETOH-TBA: ethanol, tert-butanol.
5-FU: 5-fluorouracil.
NAA: naphthalene acetic acid.
IAA: indoleacetic acid.
IBA: indolebutyric acid.
2,4-D: 2,4-dichlorophenoxyacetic acid.
rRNA: ribosomal RNA.
mRNA: messenger RNA

Fig. 1. Photograph of mung bean seedling cuttings.

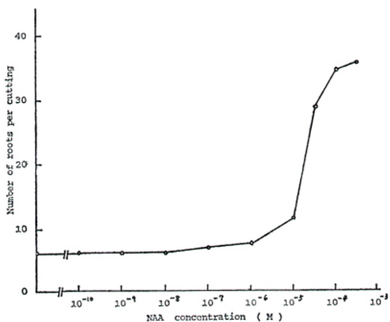


Fig. 2. Promotion of NAA at various concentrations on adventitious root initiation. The mung bean cuttings were presoaked with NAA at the concentrations from $1\times10^{-10}$ M to $1\times10^{-3}$ M for 24 hrs and then transfered to distilled water for 6 days.

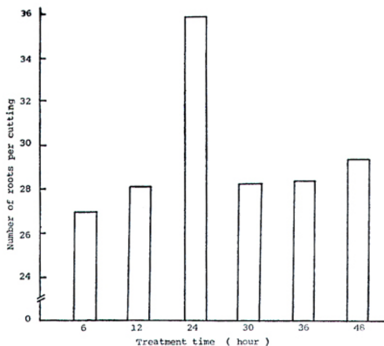


Fig. 3. Effects of the different presoaked periods on adventitious root initiation. The mung bean cuttings were presoaked with NAA at a concentration of $1\times10^{-4}$ M for 6, 12, 24, 30, 36 and 48 hrs and then transfered to distilled water for remaining hrs till 7th day. The number of adventitious roots of control (i.e. kept in water) was 6.0.

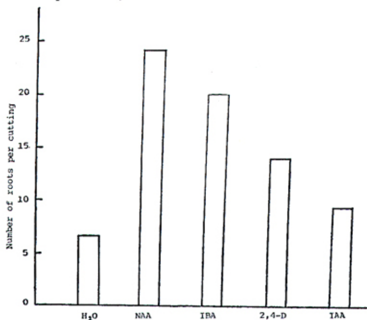


Fig. 4. Comparison of the effects of four auxins on adventitious root initiation. The mung bean cuttings were presoaked with NAA, IBA, 2,4-D and IAA at a concentration of $1\times10^{-4}$ M, $1\times10^{-4}$ M, $1\times10^{-5}$ M and $1\times10^{-4}$ M respectively for 24 hrs and then transfered to distilled water for 6 days. The number of adventitious roots of control (i.e. kept in water) was 7.

showed that NAA was the most effective auxin and this was followed by IBA then 2, 4-D and finally IAA (Fig. 4).

Adventitious roots originated from the phloem parenchyma cells between the vascular bundles as is shown in Fig. 5 (the rectangular enclosed region) and 6. These phloem parenchyma cell began to divide at 36 hours after cutting (Fig. 7). The root primordia were formed 48 hours after cutting (Fig. 8). The growth of the root primordia continued and at 96 hours after cutting they protruded through the epidermis. At this time we were able to see and count the root initials on the outside of the hypocotyls. Transverse sections close to the base of the hypocotyls of the NAA treated hypocotyls showed almost the same anatomical features as the controls which had been kept in water. The only difference was that the cell divisions were more obvious after 36 hours in the NAA treated hypocotyls (Fig. 9).

Cycloheximide at a concentration of 1–5 $\mu$g/ml inhibited not only adventitious root initiation but also killed the hypocotyl cuttings within 48 hours. These hypocotyls began to swell at their upper part and became thin at the lower part before they finally wilted and died. Since cycloheximide is a potent protein synthetic inhibitor of eucaryotic cells, it is suggested that adventitious root initiation requires protein synthesis. The function of cycloheximide is to freeze the movement of ribosomes on mRNA(9). Lin and Key(11) showed it prevented the dissociation of polyribosome to monoribosome and also prevented the completion of nascent polypeptide chains from ribosomes.

$\alpha$-Amanitin up to a concentration of 1 $\mu$g/ml showed no inhibition in adventitious root initiation. It appears that $\alpha$-amanitin at low concentration (0.5 or 1 $\mu$g/ml) stimulates adventitious root initials 2 fold in NAA treated hypocotyls.

5-FU at a concentration of $1 \times 10^{-3}$ M inhibited the formation of root initials. No cell division was observed in the phloem parenchyma cells in the potential root primordia zones within 102 hours after treatment with 5-FU (Fig. 10). When the hypocotyls which had begun to have cell divisions (after 36 hours treatment) were put into 5-FU solution, adventitious root formation was inhibited (Table 1). 5-FU is a rRNA synthetic inhibitor in higher plants(5). Lin and Key reported that 5-FU inhibited cell division but not cell elongation(12). Our results agreed with theirs in that cell division of the parenchyma cells in the potential region was inhibited by 5-FU. It appears rRNA synthesis is important in adventitious root formation.

$^3$H-uridine was used as a tracer to study RNA synthesis before cell divisions had occurred in the formation of root initials. The graph of RNA synthesis for 4 hours incubation when tested with $^3$H-uridine gave a straight line as is seen in Fig. 11. NAA stimulated $^3$H-uridine incorporation nearly 2 fold (Table 2) during the 2 hours of incubation as compared with the water control. 5-FU inhibited $^3$H-uridine incorporation by 60% and it inhibited more $^3$H-uridine incorporation in the NAA treated hypocotyls than in water controls. But $\alpha$-amanitin

Figs. 5-10. Transverse sections of the hypocotyl of mung bean cuttings.

Figs. 5-8. Cuttings were soaked with distilled water and basal 5 mm segments were excised at various periods.

5: O-hr cutting showed the potential rooting zones within the rectangular enclosed regions.

6: O-hr cutting showed the magnification of the potential rooting zones.

7: 36-hr cutting showed the beginning of the cell division with arrow.

8: 48-hr cutting showed the root primordia.

Fig. 9. Cuttings were presoaked with NAA at a concentration of $1 \times 10^{-4}$ M for 24 hrs and then transfered to distilled water for 12 hrs and basal 5 mm segments were excised.

Fig. 10. Cuttings were treated with 5-FU at a concentration of $1 \times 10^{-3}$ M for 102 hrs and basal 5 mm segments were excised.

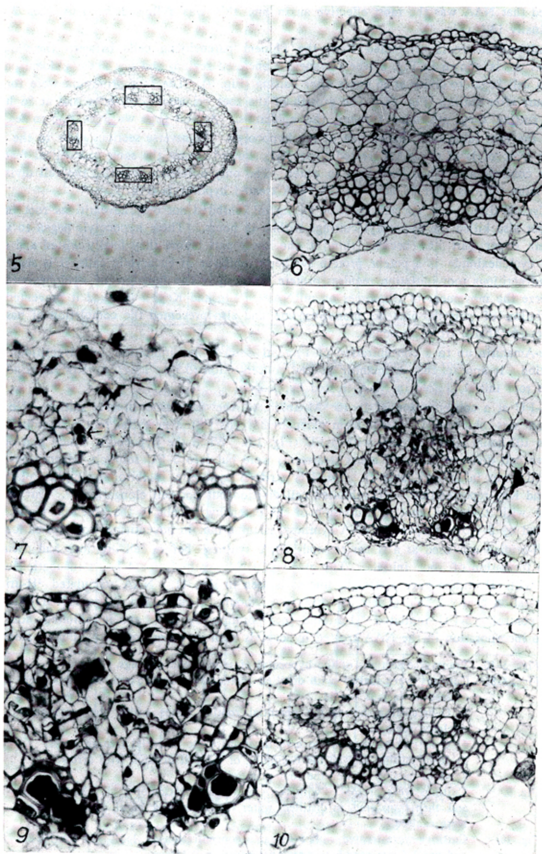
5
6
7
8
9
10

Table 1. The inhibition of 5-FU in adventious root initiation

| treatment / time (hour) | A* No. of root initial | B** No. of root initial |
|---|---|---|
| 24 | 0 | 0 |
| 36 | 0 | 0 |
| 48 | 0 | 0 |
| 57 | 0 | 0 |
| 72 | 6 | 14 |
| 83 | 6 | 27 |
| 93 | 7 | 34 |

* Cuttings were presoaked with distilled water for various periods and then transfered to 5-FU at a concentration of $1\times10^{-3}$ M. Time shows the presoaked period.

** Cuttings were presoaked with NAA at a concentration of $1\times10^{-4}$ M for 24 hrs and then transfered to distilled water. Then, at various periods transfered to 5-FU. Time includes the NAA presoaded 24 hrs and period before transfer.

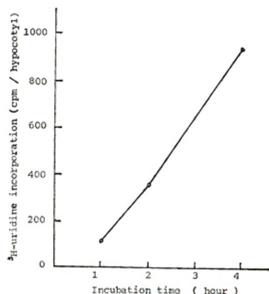


Fig. 11. The linear relationship between the incorporation time of $^3$H-uridine and RNA synthesis. Sections of hypocotyls of water control were incubated at 28°C in a water bath shaker for 1, 2 and 4 hrs. The incubation medium and other steps were as methods.

did not affect $^3$H-uridine incorporation as is seen in Table 2. In eucaryotic cells, RNA polymerase I is catalyzed to synthesize rRNA[4] and it is shown to be insensitive to $\alpha$-amanitin[10].

From these results and others, auxins are seen to have very close relations with adventitious root initiation. Adventitious root initiation requires rRNA synthesis first before division can occur in the phloem parenchyma cells. Auxin promotes massive rRNA accumulation and increases the formation of adventitious root initials. The addition of 5-FU, which inhibited

Table 2. The inhibitions of RNA synthetic inhibitors on RNA synthesis of the mung bean hypocotyls

| treatment | cpm/hypocotyl | | | | |
|---|---|---|---|---|---|
| | control | 5-FU | α-amanitin | 5-FU sensitive | α-amanitin sensitive |
| CONTROL (distilled water) | 2450 | 960 | 2480 | 1490 | 0 |
| NAA | 3900 | 1820 | 3640 | 2080 | 260 |

Cuttings were presoaked with NAA (at a concentration of $1\times10^{-4}$ M) or distilled water for 24 hrs then the hypocotyl segments were cut into 1-cm lengths. Sections were preincubated with inhibitors (5-FU at a concentration of $1\times10^{-3}$ M or 1 μg/ml α-amanitin) for 2 hrs. Then, $^{3}$H-uridine (1 μCi/ml) was added and incubation was continued for another 2 hrs.

rRNA synthesis, inhibited root initiation. Thus the growth of adventitious root primordia were inhibited by 5-FU. It is concluded that continuous synthesis of rRNA is essential for adventitious root formation.

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