BIOCHEMICAL BASIS OF ADVENTITIOUS ROOT FORMATION ON ETIOLATED STEM SEGMENTS

K. K. NANDA, N. C. BHATTACHARYA AND V. K. KOCHHAR

Department of Botany, Panjab University, Chandigarh-14, India

ABSTRACT

Segments (2.5-cm-long) of **Populus nigra** L. obtained from etiolated axillary branches did not root in water or auxin alone, but rooted in 0.5% ribose, glucose and sucrose and more profusely with 0.1 mg/l indole-acetic acid (IAA) or indole-butyric acid (IBA) added to the medium. 5-fluorodeoxyuridine (FUDR), 5-fluorouracil (FU), actinomycin-D and cycloheximide inhibited rooting. The deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and protein contents of segments cultured in glucose plus IAA were higher than in water or in glucose/IAA solutions containing cycloheximide or actinomycin-D. New isoenzymes of peroxidase and IAA-oxidase developed in solutions containing IAA and glucose, as did two new low-molecular-weight RNAs. New isoenzymes also developed in solutions containing actinomycin-D and cycloheximide. The physiological significance of these facts is discussed, and a biochemical explanation for the root initiation process is proposed.

INTRODUCTION

Nanda *et al.* (1968, 1970), Nanda and Anand (1970) and Nanda (1971) demonstrated that seasonal changes in auxin effects on rooting (which relate to morpho-physiological status) are determined by changes in the levels of endogenous regulatory substances and nutritional status. These in turn are caused by changes in temperature and light conditions prevailing during the annual cycle of plant growth. It has also been shown that a proper balance between auxin and nutritional levels is necessary for optimal production of adventitious roots (Nanda *et al.,* 1971; Nanda and Jain, 1971, 1972) and also for differentiation of callus (Kumar, 1972; Nanda *et al.* 1974). This paper describes the results of some experiments that were undertaken to study the mechanism of action of auxin at the subcellular level, and the role of nutrition on the biochemical basis of adventitious root formation in *Populus nigra* L.

MATERIAL AND METHODS

Fifteen-cm stem cuttings of *Populus nigra* L. taken from trees growing on the University campus were planted in sand in earthenware pots in the dark. The etiolated branches that developed from the axillary buds were cut into 2.5-cm segments and were planted vertically in holes on polythene sheets stretched over 10-cm Petri dishes containing the test solutions. Experiments were carried out in an air-conditioned room

(28 ± 2°C) and all test solutions contained 30 μM chloramphenicol to prevent microbial growth. At periodic intervals observations were recorded of the number of rooted segments and roots per rooted segment. Determinations of DNA, RNA and protein contents were made by the modified methods of Burton (1956), Mejbaum (1939) and Lowry et al. (1951), respectively. In Experiment 4 the crude enzyme extract was obtained by homogenising the weighed segments at 4°C in 0.067 M phosphate buffer (pH 7.0). After repeated centrifuging at 15,000 rpm the supernatant was collected and stored till used. The total activity was assayed with a Bausch and Lomb Spectronic 20 photo-colorimeter for peroxidase by the method of Mitra et al. (1970), for IAA-oxidase by that of Gordon and Weber (1951) and for NAD-dependent glutamate dehydrogenase (GDH) by that of Thurman et al. (1965).

Isoenzymes were separated by disc electrophoresis on 10% polyacrylamide gels at 4°C following the method of Ornstein (1964) and Davis (1964). The gels were stained by the methods cited above, except for IAA-oxidase where the technique of Endo (1968) was utilised.

Experimental details are given separately in each experiment.

EXPERIMENTATION AND RESULTS

Experiment 1

This experiment was designed to study the relative effectiveness of some soluble sugars and starch in the rooting process. Three hundred and sixty stem segments were divided into 18 groups of 20 segments each. Half the segments in each group were kept in continuous light (3200 lux) and the other half in continuous darkness.

The treatments and results are presented in Table 1. They show that no roots were initiated on segments in water or auxin solution alone, but roots developed on segments in sugar solutions. No segments rooted in starch in the light but a few rooted in darkness. Both the number of rooted segments and the number of roots per segment were similar in glucose and sucrose but were lower in ribose. Auxins added to sugar or starch increased both the number of rooted segments and the number of roots; IBA was more effective than IAA in both light and dark. The increase due to auxins was most pronounced with 1.0% starch and 0.5% sucrose, less so with glucose and least with ribose. With 0.5% starch the effect was markedly higher in light than in darkness.

Experiment 2

This experiment was conducted to study the effect on rooting of some inhibitors of protein and nucleic acid synthesis in relation to nutritional and regulatory factors. The inhibitors were 5-fluorodeoxyuridine (FUDR), 5-fluorouracil (FU), actinomycin-D and cycloheximide; the full range of treatments is shown in Table 2.

The results (also presented in Table 2) show that roots were not initiated on segments cultured in water or IAA alone but were produced in glucose and more profusely in IAA plus glucose. FUDR, actinomycin-D and cycloheximide inhibited rooting completely in glucose alone and decreased it appreciably in glucose plus IAA. The inhibitory effect increased with concentration until 5.0 mg/l where none of the segments rooted. FU at 1.0 mg/l did not affect the number of segments that rooted, but it did decrease the number of roots per segment slightly; at 5.0 mg/l it decreased both parameters.

TABLE 1—Effect of different sugars and starch alone, or together with IAA and IBA, on the number of etiolated stem segments of **Populus nigra** (2.5 cm) that rooted, the number of roots per rooted segment and per segment (figures within parentheses) in continuous light or darkness.

Treatment	No.of segments rooted		No. of roots per rooted segment (per segment)	
	Light	Dark	Light	Dark
Control	0	0	-	-
IAA 0.1 mg/1	0	0	-	-
IBA 0.1 mg/1	0	0	-	-
Ribose 0.5%	2	1	1.0 (0.2+0.1)	1.0(0.1+0.1)
Glucose 0.5%	6	4	1.2 (0.7+0.2)	1.7(0.7+0.3)
Sucrose 0.5%	5	5	1.6 (0.8+0.3)	1.6(0.8+0.3)
Starch 0.5%	0	3	-	1.0(0.3+0.1)
Starch 1.0%	0	2	-	1.0(0.2+0.1)
IAA 0.1 mg/1 + ribose 0.5%	2	4	1.5 (0.3+0.2)	1.5(0.6+0.2)
IAA 0.1 mg/1 + glucose 0.5%	8	10	2.4 (1.9+0.4)	2.6(2.6+0.3)
IAA 0.1 mg/1 + sucrose 0.5%	6	10	2.2 (1.3+0.4)	2.5(2.5+0.3)
IAA 0.1 mg/1 + starch 0.5%	0	2	-	1.0(0.2+0.1)
IAA 0.1 mg/1 + starch 1.0%	2	6	2.0 (0.4+0.2)	2.6(1.6+0.4)
IBA 0.1 mg/1 + ribose 0.5%	5	5	2.0 (1.0+0.2)	2.5(1.7+0.6)
IBA 0.1 mg/1 + glucose 0.5%	10	10	5.5 (5.5+0.5)	5.0(5.0+0.6)
IBA 0.1 mg/1 + sucrose 0.5%	10	10	7.2 (7.2+0.9)	8.6(8.6+0.1)
IBA 0.1 mg/1 + starch 0.5%	9	4	6.0 (5.4+0.4)	2.5(1.4+0.1)
IBA 0.1 mg/1 + starch 1.0%	10	10	10.4(10.4+0.6)	8.0(8.0+0.4)

TABLE 2—Effect of different concentrations of 5-fluorodeoxyuridine (FUDR), 5-fluorouracil (FU), actinomycin-D and cycloheximide singly and in combination with IAA, glucose or glucose + IAA on the number of etiolated stem segments of **Populus nigra** that rooted out of 10 and the number of roots per rooted segment (figures within parentheses) in darkness.

Treatment	Water	IAA 5.0 mg/l	Glucose 0.5%	Glucose 0.5% + IAA 5.0 mg/l
Control	0 (–)	0 (–)	8 (2.4 ± 0.3)	10 (6.1 ± 0.6)
FUDR 1.0 mg/l	0 (–)	0 (–)	0 (–)	1 (1.0 ± 0.0)
FUDR 5.0 mg/l	0 (–)	0 (–)	0 (–)	0 (–)
FU 1.0 mg/l	0 (–)	0 (–)	8 (1.0 ± 0.0)	8 (4.4 ± 0.6)
FU 5.0 mg/l	0 (–)	0 (–)	3 (1.0 ± 0.0)	6 (3.2 ± 0.2)
Act. 1.0 mg/l	0 (–)	0 (–)	0 (–)	3 (1.0 ± 0.0)
Act. 5.0 mg/l	0 (–)	0 (–)	0 (–)	0 (–)
Cyc. 1.0 mg/l	0 (–)	0 (–)	0 (–)	4 (1.6 ± 0.1)
Cyc. 5.0 mg/l	0 (–)	0 (–)	0 (–)	0 (–)

Experiment 3

This experiment was designed to study the effect of cycloheximide and actinomycin-D on nucleic acid and protein metabolism and their relationship with nutritional and hormonal factors in the rooting of stem segments. There were 4 treatments:

1. Control
2. 5.0 mg/l IAA plus 0.5% glucose
3. 5.0 mg/l IAA plus 0.5% glucose plus 5.0 mg/l actinomycin-D
4. 5.0 mg/l IAA plus 0.5% glucose plus 5.0 mg/l cycloheximide

The contents of DNA, RNA and proteins were determined at 24-hour intervals up to 96 hours. They are presented in Fig. 1.

The rooting results confirmed those shown in Experiment 2: all segments rooted in glucose plus IAA, with 5.8 roots on each but none rooted when either cycloheximide or actinomycin-D was added to the medium. Nor did any root in the control series.

Fig. 1 shows that DNA, RNA and protein levels of the segments cultured in glucose plus IAA were at all times higher than those in the control series, which in turn were higher than those in solutions containing actinomycin-D and cycloheximide. While over-all levels of DNA and protein increased with time, RNA levels decreased.

Experiment 4

This experiment describes the results of a time course study of changes in the activity and the isoenzyme patterns of peroxidases, IAA-oxidase and NAD-dependent GDH in stem segments cultured under 4 different treatment conditions. Treatments 1-3 were identical with those in Experiment 3. Treatment 4 differed only in containing 1.0 mg/l cylcoheximide (c.f. 5.0 mg/l in Expt. 3).

Rooting response:—The results confirmed the findings of the previous two experiments and showed that while all segments rooted in glucose plus IAA, rooting was completely inhibited when either cycloheximide or actinomycin-D was added.

Peroxidase activity:—The results presented at the base of each zymogram in Fig. 2 show that the activity of peroxidases increased in all cultures within 24 hours, the increase being marked in the treatment containing actinomycin-D but only slight in other cases. The activity increased in water and in actinomycin-D up to 72 hours.

Peroxidase isoenzyme pattern (see Fig. 2):—Thirteen isoenzymes were observed in the segments initially, and during the seven days of the experiment there were a number of changes in these. New peroxidases appeared in, and initial peroxidases disappeared from, all treatments. Two of the isoenzymes that appeared in the treatment with glucose plus IAA alone were suppressed by actinomycin-D and for a while by cycloheximide. Other isoenzymes appeared in the presence of the inhibitors but not the controls.

IAA-oxidase activity:—The activity was high initially but decreased in all cases at 24 hours (Fig. 3). Subsequently in the controls and the segments cultured in glucose + IAA the activity returned to the initial level. In segments cultured in cycloheximide and actinomycin-D, however, the activity reached levels above that obtaining initially.

IAA-oxidase isoenzyme pattern:—Fig. 3 shows that 7 isoenzymes were present initially. After 7 days there were fewer than this in all treatments except that containing cycloheximide, but there two of the seven present were not original. With time all treatments lost original isoenzymes and developed new ones. After 24 hours cyclo-

POPULUS NIGRA

o——o WATER
□——■ GLUCOSE + IAA
●——● GLUCOSE + IAA + ACTINOMYCIN-D
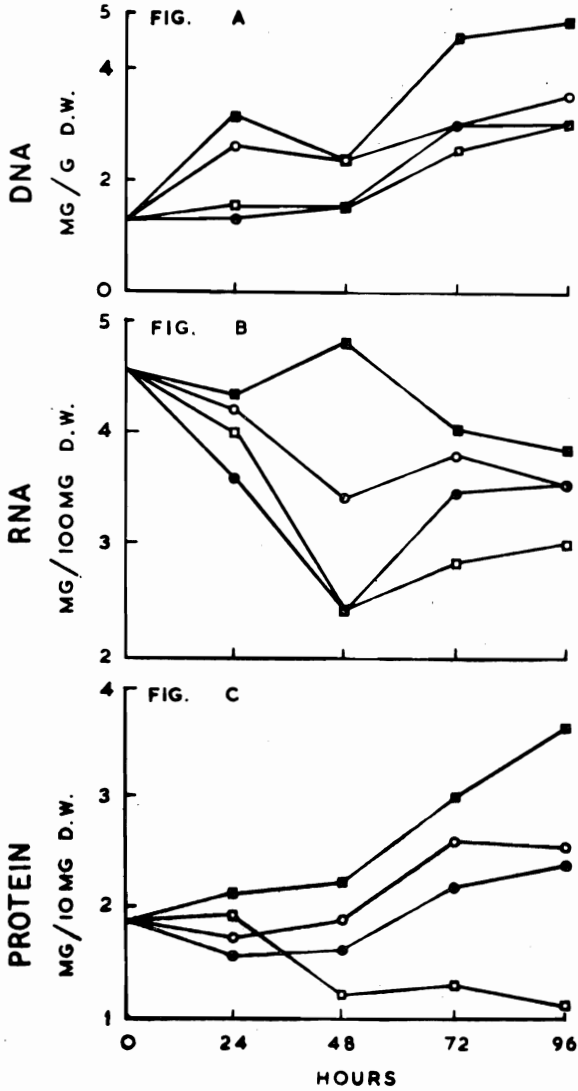□——□ GLUCOSE + IAA + CYCLOHEXIMIDE

FIG. 1—DNA (A), RNA (B) and protein (C) contents of etiolated stem segments of **Populus nigra** cultured under four different treatments. Rooting occurred only on segments cultured in glucose + IAA.
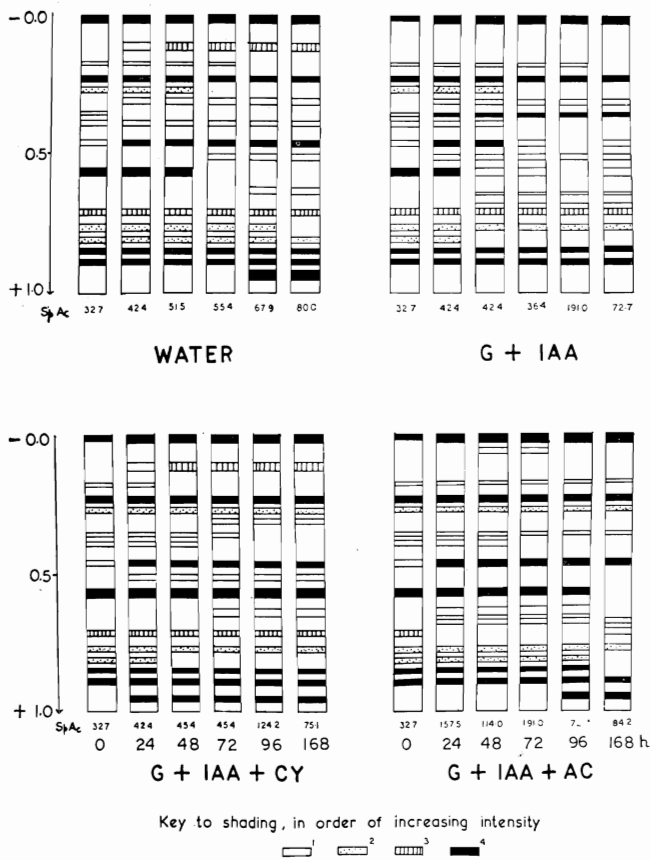
FIG. 2—Peroxidase isoenzyme pattern of **Populus nigra** etiolated
stem segments cultured in (i) water, (ii) glucose + IAA,
(III) glucose + IAA + cycloheximide and (iv) glucose
+ IAA + actinomycin-D.
G = glucose, AC = actinomycin-D,
CY = cycloheximide

heximide and actinomycin-D had suppressed some of the isoenzymes appearing in the
controls, and new ones (not present in the controls) subsequently developed.

GDH isoenzyme pattern:—Six isoenzymes could be observed initially in the segments
(Fig. 4). One new isoenzyme (Rf 0.10) developed in all treatments at 24 hours, but
disappeared at different rates from the different treatments. An existing isoenzyme
(Rf 0.28) also disappeared from the different treatments at different rates while another
isoenzyme appeared in the actinomycin-D treatment at 72 hours but in no other.

Experiment 5

This experiment was conducted to study the RNA pattern of etiolated stem segments
when cultured in water, glucose plus IAA, and glucose plus IAA plus actinomycin-D.
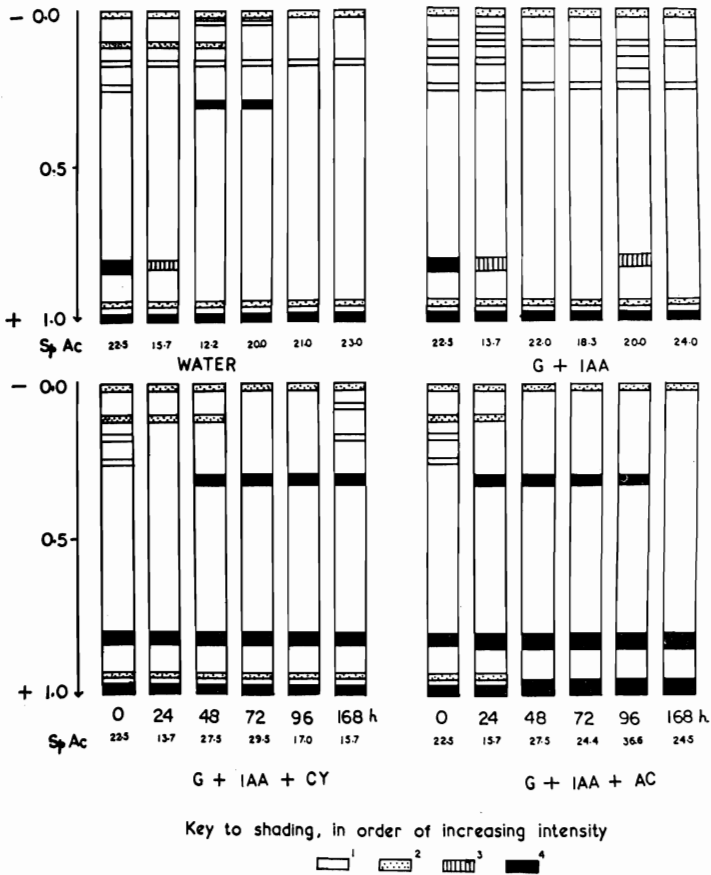
FIG. 3—IAA-oxidase isoenzyme pattern of **Populus nigra** etiolated
stem segments cultured in (i) water, (ii) glucose + IAA,
(iii) glucose + IAA + cycloheximide and (iv) glucose
+ IAA + actinomycin-D.
G = glucose, AC = actinomycin-D,
CY = cycloheximide

The results presented in Fig. 5 show that two new low-molecular-weight RNAs
were induced in segments cultured in glucose + IAA at 48 and 72 hours, respectively.
These were absent in controls as well as in the actinomycin-D culture.

DISCUSSION

The results presented in this paper confirm earlier findings (Nanda *et al.*, 1971;
Nanda and Jain, 1971) that rooting of etiolated stem segments of *Populus nigra* is
limited by nutritional factors. Stem segments did not root in water or in IAA but
rooted in glucose or sucrose and more profusely in glucose or sucrose + auxin. IBA
was a more effective auxin than IAA. It is interesting that starch can be used as source
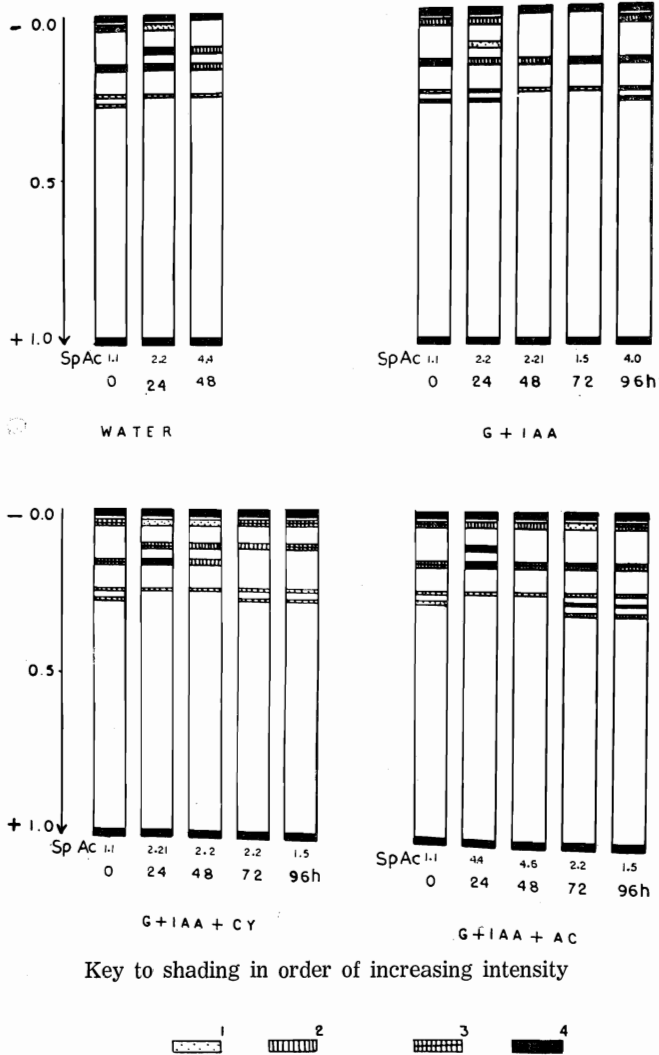
FIG. 4—NAD-dependent GDH isoenzyme pattern of **Populus nigra** etiolated stem segments cultured in (i) water, (ii) glucose + IAA, (iii) glucose + IAA + cycloheximide and (iv) glucose + IAA + actinomycin-D. G = glucose, AC = actinomycin-D, CY = cycloheximide

of carbon for the supply of energy required for root initiation: it is mobilized into sugars by the enzymes that leach out of the segments into the medium (Nanda and Jain, 1972).

The inhibition of rooting by FUDR, FU, actinomycin-D and cycloheximide at a time when rooting is possible nutritionally is suggestive that the magnitude of rooting is determined by the size of the protein pool available in the tissue at the time of root initiation. Alternatively the size of the pool of nucleotides or amino-acids con-
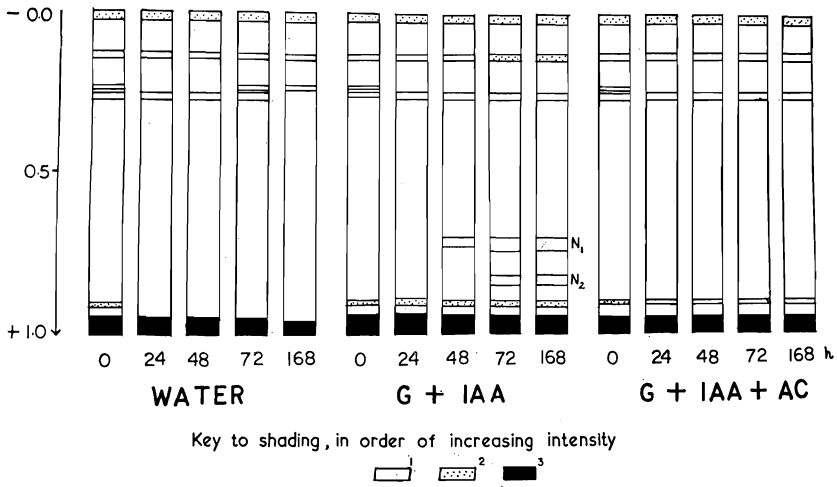
FIG. 5—Pattern of RNAs extracted from etiolated stem segments of **Populus** cultured in (i) water, (ii) glucose + IAA and (iii) glucose + IAA + actinomycin-D.
G = glucose, AC = actinomycin-D

tributing to this may be important. The decrease in inhibition with a delay in the application of cycloheximide shown in another paper is probably because the protein pool had already reached a high level prior to the cessation of fresh biosynthesis caused by the antimetabolite (Nanda *et al.*, 1973). The results of the protein content presented in Experiment 3 lend some support to this postulate. Thus, while the protein content of segments cultured in glucose plus IAA plus cycloheximide decreased that of segments cultured in glucose plus IAA increased appreciably (Experiment 3). The lack of decrease in actinomycin cultures may reflect the different modes of action of actinomycin (which inhibits RNA-polymerase action) and cycloheximide, which inhibits peptide elongation. It appears possible that the size of the pool of substances required for root initiation was reached only in glucose plus IAA solutions and remained lower in other cases. The differences in protein levels were associated with differences in RNA and DNA levels.

The induction of two new RNAs by IAA during the initiation and development of roots and their repression by actinomycin-D (which inhibits RNA-polymerase) reported in Experiment 5 is of considerable significance: it is possible that these RNAs may be concerned in some way with the production of adventitious roots. As these RNAs are of low molecular weight, they cannot be ribosomal in nature and therefore are either messenger or transfer RNAs. Attempts to characterize them are being made in order to understand any role they may have in differentiation of cambial derivatives into root primordia.

The induction of two new isoenzymes of peroxidase and IAA-oxidase by IAA and glucose and their repression in some cases by actinomycin-D is rather interesting. Chandra *et al.* (1971) also reported that some new peroxidases developed in cuttings that produced roots but not in those that were treated with streptomycin that suppressed rooting. The physiological significance of these isoenzymes is yet to be elucidated.

Possibly they control the level of endogenous auxin to balance with the available carbon supply.

The appearance also of some new isoenzymes of peroxidases and IAA-oxidases in segments cultured in water and even in media containing cycloheximide or actino-mycin-D is in itself interesting. These may increase IAA-oxidase activity causing oxidation of endogenous auxin to a level lower than that necessary for the differentiation of cambial derivatives into root primordia. Endo (1968) and Yoneda and Endo (1970) also reported that some peroxidases act as IAA-oxidases. Hoyle (1972) reported that IAA oxidase and guiacol peroxidase activities are dual catalytic functions of the single enzyme. Retig and Rudich (1972) found that the active sites of both peroxidases and IAA-oxidase on the gels were similar.

However, the most interesting point that emerges from this investigation is that cycloheximide is not an inhibitor of the syntheses of *all* proteins nor does actinomycin-D impair the syntheses of *all* RNAs. While they inhibit the production of some peroxidases, the development of some others is actually caused by them regardless of whether or not these are involved in rooting. Similar effects of cycloheximide have been reported by other workers (Macdonald and Ellis, 1969; Ellis and Macdonald, 1970; Lee, 1971). In fact, Nanda *et al.* (1973) have reported that cycloheximide stimulates gibberellic acid-induced differentiation of leaves and floral buds in *Impatiens balsamina.* Similarly Parish (1968) and Novacky and Wheeler (1971) also reported that low concentrations of actinomycin-D caused the development of isoperoxidases. Seitz and Lang (1968) suggest that stable messenger RNA may exist in amounts sufficient to maintain a certain level of protein synthesis even after the inhibition of fresh synthesis (Key *et al.,* 1967; Lin and Key, 1968).

The results, combined with the published literature, indicate that a proper balance between auxin and carbon nutrition is necessary for optimal production of roots. Auxin possibly acts as a triggering agent at the transcriptional level and carbon nutrition as a source of carbon regulates translation in the synthesis of specific enzyme proteins that are required for the initiation and development of roots. A quantitative relationship appears to exist between the contents of nucleic acids and proteins and the level of rooting.

REFERENCES

BURTON, K. 1956: A study of the conditions and mechanism of the Diphenylamine reaction for the colorimetric estimation of Deoxyribonucleic acid. **Biochem. J. 62:** 315-323.

CHANDRA, G. R., GREGORY, L. E. and WORLEY, J. M. 1971: Studies on the initiation of adventitious roots on mung bean hypocotyl. **Plant and Cell Physiol. 12:** 317-324.

DAVIS, B. J. 1964: Disc electrophoresis—II. Method and application to human serum proteins. **Ann. N.Y. Acad. Sci. 121:** 404-427.

ENDO, T. 1968: Indoleacetate oxidase activity of horseradish and other plant peroxidase isozymes. **Plant and Cell Physiol. 9:** 333-341.

ELLIS, R. J. and MACDONALD, I. R. 1970: Specificity of cycloheximide in higher plant systems. **Plant Physiol. 46:** 227-232.

GORDON, S. A. and WEBER, R. P. 1951: Colorimetric estimation of indoleacetic acid. **Plant Physiol. 26:** 192-5.

HOYLE, M. C. 1972: Indoleacetic acid oxidase: a dual catalytic enzyme?. **Plant Physiol. 50:** 15-8.

KEY, J. L., BARNETT, N. M. and LIN, C. Y. 1967: RNA and protein biosynthesis and the regulation of cell elongation by auxin. **Ann. N.Y. Acad. Sci. 144:** 49-62.

KUMAR, P. 1972: Effect of auxins on the seasonal rooting response of stem cuttings of some plant species. Ph.D. thesis, Panjab Univ. Chandigarh.

LEE, T. T. 1971: Promotion of indoleacetic acid oxidase isoenzymes in tobacco callus cultures by indoleacetic acid. **Plant Physiol. 48:** 56-59.

LIN, C. Y. and KEY, J. L. 1968: Cell elongation in the soybean root: the influence of inhibitors of RNA and protein biosynthesis. **Plant and Cell Physiol. 9:** 553-560.

LOWRY, O. H., RISEBROUGH, N. J., FARR, A. L. and RANDALL, R. J. 1951: Protein measurement with the folic phenol reagent. **J. Biol. Chem. 193:** 265.

MACDONALD, I. R. and ELLIS, R. J. 1969: Does cycloheximide inhibit protein synthesis specifically in plant tissues? **Nature, Lond. 222:** 791-792.

MEJBAUM, W. 1939: Über die Bestimmung kleiner Pentosemenger, insbesondere in Derivaten der Adenylsäure. **Z. Physiol. Chem. 258:** 117-20.

MITRA, R., JAGANNATH, D. R. and BHATIA, C. R. 1970: Disc electrophoresis of analogous enzymes in Hordeum. **Phytochemistry 9:** 1843-1850.

NANDA, K. K. 1971: Final rep. of PL 480 Res. Proj. A7-FS-11.

NANDA, K. K. and ANAND, V. K. 1970: Seasonal changes in auxin effects on rooting of stem cuttings of **Populus nigra** and its relationship with mobilization of starch. **Physiol. Plant. 23:** 99-107.

NANDA, K. K., ANAND, V. K. and KUMAR, P. 1970: Some investigations of auxin effects on rooting of stem cuttings of forest plants. **Indian For. 96:** 171-87.

NANDA, K. K. and JAIN, M. K. 1971: Interaction effects of glucose and auxins in rooting etiolated stem segments of **Salix tetrasperma. New Phytol. 70:** 945-8.

————— 1972: Utilisation of sugars and starch as carbon sources in the rooting of etiolated stem segments of **Populus nigra. New Phytol. 71:** 825-828.

NANDA, K. K., JAIN, M. K. and BHATTACHARYA, N. C. 1973: Rooting response of etiolated stem segments of **Populus nigra** to antimetabolites in relation to indole-3-acetic acid and glucose. **Biol. Plant 15 (6):** 412-8.

NANDA, K. K., JAIN, M. K. and MALHOTRA, S. 1971: Effects of glucose and auxins in rooting etiolated stem segments of **Populus nigra. Physiol. Plant. 24:** 387-91.

NANDA, K. K., KUMAR, P. and KOCHHAR, V. K. 1974: Role of auxins, antiauxins and phenol in the production and differentiation of callus on stem cuttings of **Populus. N.Z. J. For. Sci. 4 (2):** 338-46 (this issue).

NANDA, K. K., PUROHIT, A. N., BALA, A. and ANAND, V. K. 1968: Seasonal rooting response of stem cuttings of some forest tree species to auxins. **Indian For. 94:** 154-62.

NANDA, K. K., KUMAR, M., SAWHNEY, S. and SAWHNEY, N. 1973: Effect of cycloheximide on GA_3 response and on photoperiodic reactions of **Impatiens balsamina** L. **Ann. Bot. 37:** 107-111.

NOVACKY, A. and WHEELER, H. 1971: Stimulation of isoperoxidases by actinomycin D. **Amer. J. Bot. 58:** 858-860.

ORNSTEIN, L. 1964: Disc electrophoresis. 1. Background and theory. **Ann. N.Y. Acad. Sci. 121:** 321.

PARISH, R. W. 1968: Studies on senescing tobacco leaf disks with special reference to peroxidase. I. The effects of cutting, and of inhibition of nucleic-acid and protein synthesis. **Planta 82:** 1-13.

RETIG, N. and RUDICH, J. 1972: Peroxidase and IAA oxidase activity and isoenzyme patterns in cucumber plants, as affected by sex expression and ethephon. **Physiol. Plant. 27:** 156-160.

SEITZ, K. and LANG, A. 1968: Invertase activity and cell growth in lentil epicotyls. **Plant Physiol.** **43:** 1075-1082.

THURMAN, D. A., PALIN, C. and LAYCOCK, M. V. 1965: Isoenzymatic nature of L-glutamic dehydrogenase of higher plants. **Nature, Lond. 207:** 193-4.

YONEDA, Y. and ENDO, T. 1970: Peroxidase isoenzymes and their indoleacetate oxidase activity in the Japanese morning glory, Pharbitis nil. **Plant and Cell Physiol. 11:** 503-6.