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ROLE OF ENDOGENOUS AUXIN AND POLYAMINES IN ADVENTITIOUS ROOT FORMATION IN MUNGBEAN CUTTINGS

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SUMMARY

Exogenous application of putrescine (10^{-4} M) markedly improved the number of second order root and second order root length per cutting, whereas, spermidine (10^{-9} M) and spermine (10^{-5} M) had no significant effect on adventitious root formation in mungbean cuttings. IBA (10^{-5} M) was more effective than putrescine in rooting performance. Three phases of adventitious root formation process could be identified namely, induction (0-24h), initiation (24-72h) and expression phase (after 72h). High levels of free IAA, Putrescine (PUT) and low peroxidase (POX) activity were observed during the induction phase. IBA (10^{-5} M) treated cuttings showed higher levels of IAA and PUT as well as POX activity than control cuttings. In short-term estimation (0-24h), the free IAA peak (6h) which preceded the PUT peak (12h), might be the reason of initiation of the induction phase of rooting. Experimenting with the inhibitors of polyamine biosynthesis, DFMO, (10^{-4}M, α- difluoromethyl ornithine) and DFMA (10^{-4} M, α- difluoromethyl arginine) it was observed that DFMO was more inhibitory than DFMA in adventitious root formation and hence ODC (ornithine decarboxylase) pathway might be the preferred pathway for puterscine biosynthesis during adventitious root formation in mungbean. AG (amino guanidine), which inhibits the conversion of putrescine to Δ- pyrroline and then to GABA (γ- aminobutyric acid), inhibited rooting. CHA (cyclohexylamine), which inhibits the conversion of puterscine to spermidine, on the contrary, favoured rooting. Further, exogenous application of GABA also promoted rooting. The results thus point to the involvement of putrescine and its degradation product GABA in adventitious root formation in mungbean cuttings.

Key words: **Adventitious root, auxin, mungbean, peroxidase, polyamines**

INTRODUCTION

The process of adventitious root formation consists of successive interdependent physiological phases (Gaspar *et al.*1992, Klerk and Brugge 1992, Couee *et al.* 2004). The measurement of endogenous auxin concentrations during its time-course study has greatly contributed to the understanding of this developmental process (Moncousin *et al.* 1998, Nag and Choudhuri 2001). An early and temporary increase in the endogenous level of indole-3- acetic acid (IAA) is the major event of the inductive phase of adventitious rooting, since it appears necessary for the reactivation of cell divisions (Gaspar *et al.* 1994, Heloir *et al.* 1996). In addition, several studies have emphasized that polyamines play a role in rooting (Hausman *et al.* 1994, Nag *et al.* 1999). Correlations between polyamine accumulation and the initial stages of adventitious root formation have been observed in *Phaseolus* (Jarvis *et al.* 1983), apple (Wang and Faust 1986) tobacco callus

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culture (Tiburcio *et al.* 1987), *Prunus avium* (Biondi *et al.* 1990), poplar (Hausman *et al.* 1994) and in walnut shoots (Heloir *et al*. 1996). It is also known that peroxidase activity regulates IAA catabolism and acts as a marker for successive phases with a typical minimum at rooting induction and a typical maximum at initiation phase (Gaspar *et al*. 1997).

The aim of this work, therefore, is to study the possible interactions between polyamine metabolism and the auxin level in mungbean cuttings during different rooting phases in control and IBA treated plants. Producing different rooting conditions by treating of cuttings with auxin and polyamines and also using inhibitors of polyamine biosynthesis, such as CHA (inhibitor of spermidine synthase) or AG (inhibitor of diamine oxidase) and GABA (one of the catabolic products of puterscine) to reveal the specific role of auxin and polyamines in different rooting phases. Furthermore, the effects of all these compounds, either alone or in combination, on the rooting parameters were studied in mungbean hypocotyl cuttings.

MATERIALS AND METHODS

Mung bean (*Vigna radiata* (L.) Wilczek cv. B105) seedlings were grown in sand in a controlled growth room with 16h photoperiod at 222 μmol m^{-2} s^{-1} intensity (400-700 nm) for 7 days. The hypocotyls of 7-d-old seedlings were excised 3 cm below the cotyledonary node, the cotyledons were removed, and the resulting cutting consisting of the hypocotyl and the intact epicotyl, with a pair of primary leaves were used in rooting experiments reported here. Freshly prepared hypocotyl cuttings were dipped into 50ml glass beakers containing 30ml distilled water (control) or test solutions comprising of polyamines, viz. puterscine (10^{-4}M), spermidine (10^{-9}M) and spermine (10^{-5}M); auxin, viz. Indole-3-butyric acid (IBA 10^{-5}M); inhibitor of polyamine biosynthesis, viz. α-difluoromethyl ornithine (DFMO, 10^{-4}M) or α-difluoromethyl arginine (DFMA, 10^{-4}M), cyclohexylamine (CHA, 10^{-4}M) and aminoguanidine (AG, 10^{-4}M) and the catabolic product of puterscine, viz. - γ aminobutyric acid (GABA, 10^{-4}M) either alone or in combination with each other. Cuttings were maintained in a controlled growth chamber (26 ± 1^0 C temperature, 16h photoperiod and 80% RH) for 12d, after which the adventitious roots were counted. The endogenous levels of free indole-3-acetic acid (IAA), and puterscine (PUT) were analysed from 0 to 5d after excision at intervals of 24h.

For IAA extraction, one g hypocotyl tissue was collected randomly from 12 hypocotyls from 0 to 5d after excision of seedlings and were extracted following the method of Knegt and Bruinsma (1973), slightly modified by Sinha and Basu (1981). After extraction of IAA, it was assayed following the method of Mousdale *et al.* (1978) by the indole-a- pyrone fluorescence method with some modifications (Nag *et al.* 2001). The extracted IAA was dissolved in 2ml redistilled methanol and then evaporated to 0.2ml. After cooling, the reaction was initiated by adding 0.1ml acetic anhydride followed by 0.1ml 60% (w/v) perchloric acid as a catalyst. After 10 min, the reaction was stopped by addition of 4ml of distilled water. The aqueous solutions were mixed and read immediately in a LS 30- Luminescence Spectrofluorimeter (Perkin-Elmer, U.K.) with the excitation wave length fixed at 440nm and the emission wave length fixed at 520nm. In blank, methanol was used before acetic anhydride and perchloric acid. Amount of IAA was analysed by a standard calibration curve prepared for the pyrone fluorimetric assay of IAA expressed in terms of ng g^{-1} fw.

Polyamines were extracted, separated and detected after dansylation as described by Reggiani *et al.* (1990) using silica plates ($60F_{245}$, Merck, Germany) with cyclohexane-ethylacetate (3:2 v/v) as the solvent. Spots, demarcated under UV light, were scraped from the plates and extracted with ethylacetate. Fluorescence was measured in the LS 30- Luminescence Spectrofluorimeter at an excitation wave length 360nm and emission wave length 506nm and the results were compared with dansylated standards. The amount of polyamine content was expressed in terms of nmol mg^{-1} protein. The amount of protein was determined by the method of Bradford (1976) with bovine serum albumin as the standard.

All the results presented here are the mean of at least three samples from three independent experiments. The data were statistically analysed for standard error.

RESULTS AND DISCUSSION

The data indicated that IBA (10^{-5}M) treatment exhibited maximum effect on rooting of explants of *Vigna radiata* except the second order root length (Table 1). IBA produced significantly better effects than polyamines (PUT, SPD and SPM) and inhibitors of polyamine biosynthesis (DFMA and DFMO) inhibited all the rooting parameters. Wiesmann *et al.* (1989) also suggested that IBA is more effective in overall improvement of rooting performance. Polyamines had no effect on root primordium formation. Only PUT (10^{-4} M) increased significantly the number of second order root (13.9) and first order (11.35cm) and second order root length (3.25 cm) per cutting. Treatment with SPD (10^{-9} M) and SPM (10^{-5}M) did not produce any promising effect on any rooting parameters studied. This is quite in agreement with the observation of Jarvis *et al.* (1983) in mungbean. On the other hand DFMO (10^{-4} M) significantly reduced the rooting parameters in mungbean hypocotyl explants than control, while DFMA had only slight inhibitory effect on first order (8.53cm) and second order (1.33 cm) root length and number of second order root (7.64) also. These results indicate that PUT is intimately involved in rooting of mung bean explants and that the ODC (orthine decarboxylase) pathway of PUT biosynthesis appears to be playing a more important role than the ADC (arginine decarboxylase) pathway (Tiburcio *et al.* 1987).

The perusal of the data showed that PUT (10^{-4} M), GABA (10^{-4} M) and CHA (10^{-4} M) had no significant effect on number of rooting characteristics of mung bean hypocotyl explants over control (Table 2). AG (10^{-4} M) gave significantly higher inhibitory effect on all the parameters studied during root growth. But it was observed that CHA +AG and GABA +AG produced at par effect and slightly lowered the inhibitory effect of AG. However, application of AG in combination with IBA induced significantly the number of root primordium (18.57) and the number of first order root (12.28) but decreased the first order root length (6.85 cm), number of second order root (5.99) and second order root length (0.85 cm). Further, significant increase in number of root primordia and number of first order root was noted over control when CHA was added with IBA and AG, i.e. IBA + CHA + AG. But in case of first order root length (8.09 cm), number of second order root (10.4) and second order root length (1.6 cm), the effect of IBA + CHA + AG had marginal effect over the control (8.99, 10.1 and 1.5 cm respectively). Treatment of cuttings with CHA in presence of IBA (IBA + CHA) gave significantly the best result pertaining to rooting performance among the treatments made in mungbean cuttings, closely followed by the promotive effect of IBA + PUT and IBA treatment alone. It is interesting to note the AG always inhibited the rooting performance even in presence of IBA or PUT, whereas, CHA always promoted rooting in presence of IBA or PUT. Hence,

Table 1. Effects of IBA (10^{-5}M) and putrescine (10^{-4}M), spermidine (10^{-9}M), spermine (10^{-5}M) and their biosynthetic inhibitors on rooting of cuttings of mungbean cv. B105. Data are expressed as mean values for 20 cuttings ± standard error.

Treatment & concentration	No. of root primordium	No. of first order roots	First order root length (cm)	No. of second order roots	Second order root length (cm)
Control	12.7	10.3	8.99	10.1	1.5
PUT (10^{-4} M)	12.8	10.8	11.35	13.9	3.25
SPD (10^{-9} M)	12.6	10.4	8.4	9.7	1.9
SPM (10^{-5} M)	12.9	10.2	8.8	10.1	2.0
IBA (10^{-5} M)	38.3	14.4	12.0	16.3	1.6
DFMO (10^{-4} M)	7.28	6.02	5.85	4.12	0.64
DFMA (10^{-4} M)	8.72	6.02	8.53	7.64	1.33
CD at 5% level	0.37	0.06	0.06	0.17	0.28

Table 2. Effects of puterscine (10^{-4}M), IBA (10^{-5}M), GABA (10^{-4}M) and the inhibitors of polyamine biosynthesis alone or in combination with each other on rooting of cuttings of mungbean cv. B105. Data are expressed as mean values for 20 cuttings ± standard error.

Treatment & concentration	No. of root primordium	No. of first order roots	First order root length (cm)	No. of second order roots	Second order root length (cm)
Control	12.7	10.3	8.99	10.1	1.5
IBA (10^{-5}M)	38.3	14.4	12.0	16.3	1.6
PUT (10^{-4} M)	12.8	10.8	11.35	13.9	3.25
IBA + PUT	39.5	20.8	12.9	17.01	3.35
CHA (10^{-4} M)	12.82	10.98	11.58	14.2	3.31
IBA + CHA	41.0	31.0	16.1	17.8	3.8
AG (10^{-4} M)	6.85	5.23	5.21	5.75	0.80
CHA + AG	7.97	6.35	7.0	7.48	1.0
GABA (10^{-4} M)	12.84	11.5	12.10	14.92	3.32
GABA + AG	8.97	7.32	7.12	7.52	1.1
IBA + AG	18.57	12.28	6.85	5.99	0.85
IBA + CHA + AG	29.4	13.2	8.09	10.4	1.6
CD at 5% level	0.16	0.11	0.14	0.05	0.12

it can be noted that auxin, a primary trigerring agent of root initiation (Haissig 1974), acts in conjunction with PUT or rather with its degradation products which would be evident from previous several experiments (Medhy 1994, Penel 1997).

From the Fig. 1 it may be noticed that the elevation of endogenous free IAA was observed in the inductive phase (0-24h) of rooting and the first peak of free IAA terminated the inductive phase and also marked the commencement of initiation phase. The peak of peroxidase (POX) activity at 72h indicated the terminaton of initiation phase which was followed by expression phase (72h onwards). The similar trends of rise and fall of free IAA level and POX activity in different phases of root development were also observed by Gaspar *et al.* (1997) under *in vitro* conditions. It is also evident from the results of IBA treatment which promoted rooting that both IAA (Fig. 2A) and PUT (Fig. 2B) levels were augmented, suggesting that these two endogenous plant growth regulators play an important role in adventitious root formation and that IAA probably controls the endogenous level of PUT at the initial phase. Further, in mungbean hypocotyl cuttings, there must be an interrelationship between endogenous IAA and PUT levels. Thus the results point out (Fig.3) that the early elevation of IAA peak (18h) in the induction phase (0-24h) followed by PUT peak (24h), might be responsible for induction and initiation of adventitious roots in mungbean hypocotyls.

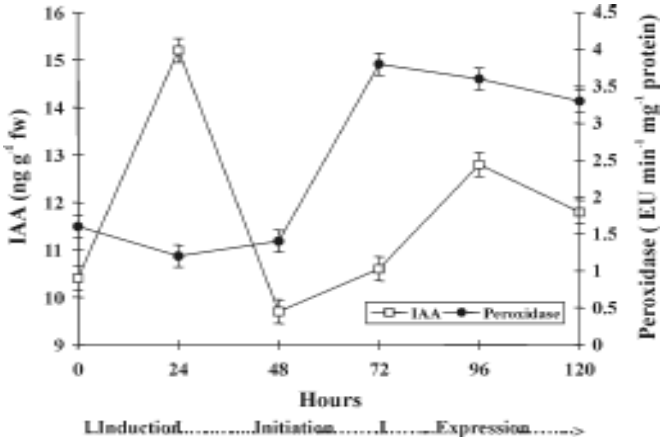


Fig. 1. Changes in IAA levels and peroxidase activities with time in mungbean hypocotyl explants at intervals of 24 h

Hence, it can be concluded that auxin acts as the prime trigger for root initiation (Haissig 1974) and the

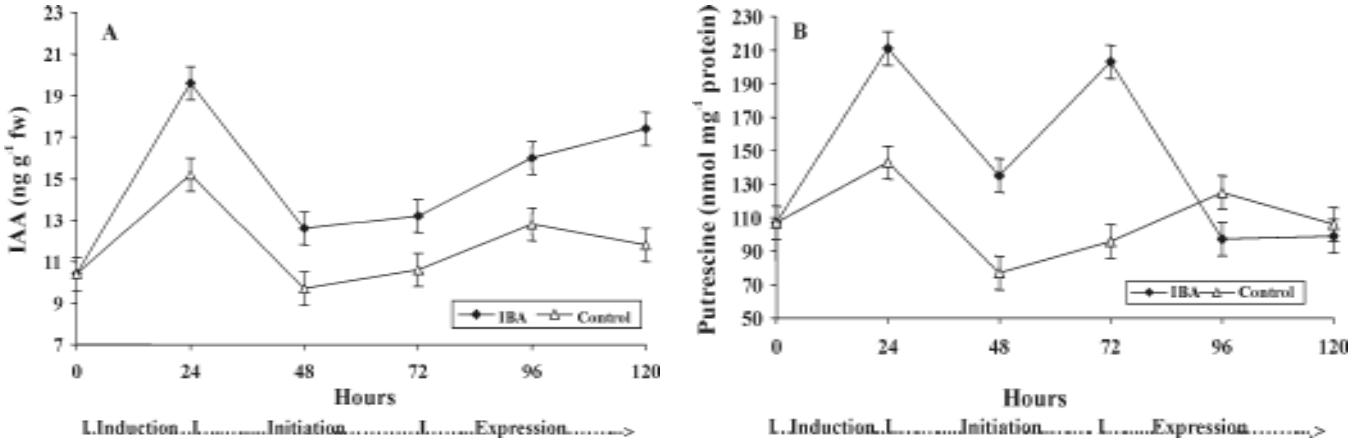
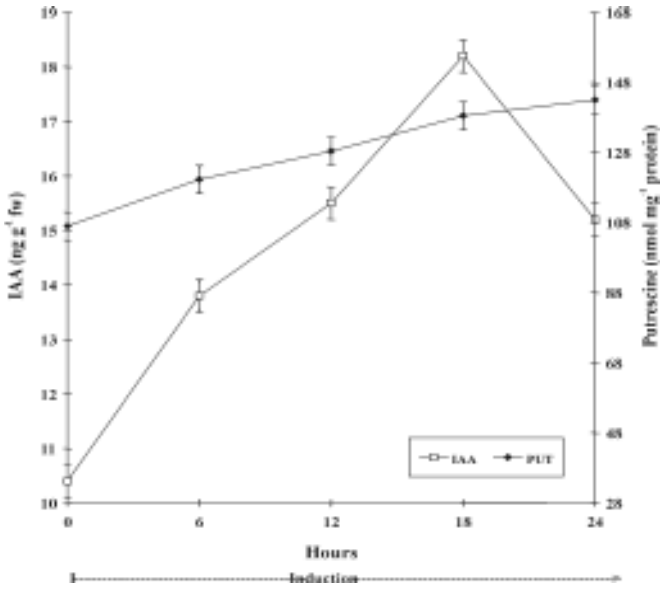


Fig. 2. Changes in IAA levels (A) and putrescine levels (B) with time in mungbean hypocotyl explants treated with IBA (10-5M) at intervals of 24 h



Fig, 3. Changes in IAA and putrescine (PUT) levels with time in mungbean hypocotyl explants at intervals of 6 h

diamine PUT (which prefers ODC pathway for its synthesis rather than ADC pathway in mungbean) acts as a second messenger of auxin (Tiburcio *et al.* 1989). It is also evident from the present study that GABA, the degradation product of PUT, are likely to act as second messengers of PUT (Penel 1997), in this process.

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