Changes In Peroxidase Activity During Adventitious Root Formation At The Base Of Mung Bean Cuttings

S. Nag, A. Paul, M. A. Choudhuri

Abstract: - An attempt was made to identify the role of peroxidase activity as well as the changes of peroxidase (POX) isozymes that control adventitious root formation at the base of hypocotyl cuttings of mung bean (*Vigna radiata* L. cv. 105). Three phases of the adventitious root formation process were identified; induction (0-24 h), initiation (24-72 h) and expression (after 72 h). The lower peroxidase activity during the induction period (0-24 h) corresponded with the first peak of IAA at 24 h which indicated termination of the induction phase. A peak of peroxidase activity with low IAA levels at 72 h signaled the termination of the initiation phase. After 72 h, peroxidase activity declined and IAA levels increased slowly and this was characterized as the expression phase. Exogenous application of auxin IBA (10^{-5}M) or polyamine putrescine (PUT, 10^{-4}M) had an effect on increasing the number of adventitious root than control. Compared with controls IBA (10^{-5}M) or PUT (10^{-4}M) treated cuttings exhibited increased levels of peroxidase activities also. The change in isozymes pattern were observed after 24 h of cuttings. The Relative Mobility Fraction (Rmf) of isozymes also increased and extra peaks of band intensity (OD) were also appeared during initiation and expression phases of rooting in *Vigna* hypocotyl regions treated with IBA (10^{-5}M) or PUT (10^{-4}M) than control.

Index Terms:- Adventitious root, Auxin, Expression Phase, Induction Phase, Initiation Phase, Mung bean, Peroxidase (POX) isoenzymes, Polyamines, Rmf

1 INTRODUCTION

Rooting seems to be mediated through a chain of biochemical reactions localized in the nucleus and cytoplasm. It also appears that the genetic programming which controls rooting depends on the effect of interacting external and internal factors. Thus variation in rooting of cuttings has been associated with changes in levels of endogenous growth regulators and metabolites in cuttings. Such regulatory processes are controlled through qualitative and quantitative changes in enzymes. Many basic studies on adventitious root formation have been carried out under *in vitro* [1], [2], [3], [4], [5], [6] and also *in vivo* [7], [8], [9], [10] conditions to distinguish and delineate the successive phases of adventitious root formation and regulation. Rooting appears to be a developmental process consisting of distinct stages, each with its own requirements and characteristics [11].

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Although some research has been conducted on enzymes during rooting but only limited information is available about macromolecules associated with different pathways prior to and during rooting. Several peroxidases have been shown to catalyze oxidation of various phenols and amines by peroxide, and also aerobic oxidation of considerably different substances without H_2O_2. Classical plant peroxidases (POX) (E.C.1.11.1.7) are heme containing enzymes that catalyze the oxidation of a diverse group of organic compounds [12]. It has been suggested that peroxidases play significant roles in plant growth, differentiation and development [1], [13], in hormone catabolism [7] and in lignin polymerization [14]. Many studies of adventitious root formation have shown that peroxidase isozymes play a fundamental role in the rooting of cuttings [1], [15], [16], [17], [18], [19]. Therefore, changes in the activity of this enzyme could be used as analytical measures of the root initiation process [1], [18], [20], [21]. The goals of this study were (i) to investigate the role of both auxin and polyamine (Putrescine) in adventitious root formation at the base of mung bean cuttings, (ii) to study the changes of peroxidase isozymes as marker under auxin and polyamine treatments in order to improve rooting procedure for mung bean cuttings *in vivo* condition.

2 MATERIALS AND METHODS

2.1 Preparation of plant samples

Mung bean (*Vigna radiata* L. cv. 105) seedlings were grown in sand in a controlled growth room with 16 h photoperiod at 222 μ mole m^{-2} s^{-1} intensity (400-700nm) for 7 days. The hypocotyls of 7-day old seedlings were excised 3 cm below from the cotyledonary node, the cotyledons were removed, and the resulting cuttings 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 put into 50 ml glass beakers containing 30 ml of test solution that covered the entire hypocotyl. The test solutions of IBA (10^{-5}M) or PUT (10^{-4}M) were separately renewed from time to time. Control

solutions were distilled water (p^H 7.0). Cuttings were maintained in a controlled growth chamber (26 ± 1^0C temperature, 16 h photoperiod and 80% RH) for 12 days, after which the adventitious roots were counted. The endogenous levels of free indole-3-acetic acid (IAA), peroxidase activity and peroxidase isoenzymes were analysed from the hypocotyl region of cuttings from 0 to 5 day after excision at intervals of 24 h.

2.2 Fluorimetric Determination of free IAA content in Mung bean Cuttings

A more specific fluorimetric assay method based on the reaction of IAA with acetic anhydride in the presence of perchloric acid as a catalyst, to form the fluorescent tricyclic derivative 2-methyl indole-α-pyrone [22], was standardized. The assay conditions were standardized using the hypocotyl of *Vigna radiata* L. cv. 105. For IAA extraction 1 gm of hypocotyl tissue was collected randomly from 12 hypocotyls from 0-5 day after excision of seedlings and was extracted following the method of Knegt and Bruinsma [23] slightly modified by Nag *et al.* [7]. After appropriate reaction time (optimum 10 min) the reaction was stopped by adding 4 ml of distilled water. The aqueous solutions were mixed and read immediately in an LS 30- Luminescence spectroflurimeter (Perkin-Elmer, UK) with the excitation wave length fixed at 440 nm and emission wave length fixed at 520 nm. In the blank, methanol was used before acetic anhydride and perchloric acid. The amount of IAA was analyzed by a standard calibration curve prepared for the pyrone flurimetric assay of IAA and expressed in terms of $ng.g^{-1}f$.

2.3 Extraction and Assay of Peroxidase (POX)

Peroxidase activity was measured according to the method of Kar and Mishra [24] using pyrogallol as the substrate. The activity of the enzyme (Enzyme Unit i.e. E.U.) was calculated as ΔAXTV/t X v, where ΔA is the absorbance of sample after incubation minus absorption at zero time control. TV is the total volume of the filtrate, t is the time (min) of incubation with substrate, and v is the volume of filtrate actually taken for incubation [25]. The enzyme activity was expressed in terms of E.U. mg^{-1} protein min^{-1}.

2.4 Polyacrylamide gel electrophoresis

Non-denaturing gel electrophoresis (PAGE) was performed according to the modified method of Davis [26] using vertical slab gels (1mm thick) and was set up to form a discontinuous system of two layers: (i) a resolving gel: an 8 cm layer of 10% polyacrylamide and (ii) a stacking gel: A 3cm layer of 3.75% polyacrylamide. Then 200 µg of protein per well was loaded and electrophoresis was carried out using two constant voltages. Samples were run first at 120 mV until the color reached the dividing line between the two gel types, then at 140 mV. POX bands were detected by immersing the gels in a solution of 0.2M acetate buffer, p^H 4.8 containing 0.04M benzidine and 0.3% H_2O_2 [27]. Data were collected after gel staining and only clear and consistent isoenzyme bands were scored. All the results are the mean of at least three samples from three independent experiments. The data were statistically analyzed for standard error.

3 RESULTS

Data from Table 1 shows that IBA (10^{-5}M) treatment had greater effects on the parameters of adventitious root formation than PUT. PUT (10^{-4}M) only increased the total root length (14.6 ± 1.9 cm) than IBA (10^{-5}M) (13.6 ± 2.0 cm). However, in other parameters the effect of IBA was significantly better than PUT. Similar observations were also reported by Nag *et al*. [28]. The present study shows that the endogenous content of free IAA in control cuttings of mung bean hypocotyls increased during the first 24 h and then decreased up to 48 h during adventitious root formation (Figure 1). After that, free IAA levels increased slowly up to 96 h and decreased thereafter. On the other hand, peroxidase showed lower activity during 0-48 h but a peak of peroxidase activity occurred at 72 h which corresponded to a decreased level of free IAA. After 72 h, peroxidase activity declined in control cuttings. Figure 2 shows that there were no marked differences in POX activities during 0-24 h between control and treated (IBA, 10^{-5}M; PUT, 10^{-4}M) cuttings. POX activity increased sharply from 48-72 h, reaching a peak at 72 h and declining thereafter. The POX activity in treated cuttings (IBA or PUT) was greater than controls but increased after 96 h rather than decreasing as observed in controls.

Table 1: Effect of IBA (10^{-5}M) and PUT (10^{-4}M) on adventitious Root formation of Stem Cuttings of *Vigna radiata* L. cv. 105

Treatment and Concentration	No. of root primordium	No. of primary roots	No. of secondary roots	Total root length (cm)
Control (H_2O)	12.6±1.1	10.4±1.4	10.1±0.9	10.5±1.3
IBA (10^{-5}M)	38.3±2.1	14.4±2.2	16.3±1.6	13.6±2.0
PUT (10^{-4}M)	12.8±1.0	10.8±1.4	13.9±1.2	14.6±1.9

Data are expressed as average value for 20 cuttings ± standard error

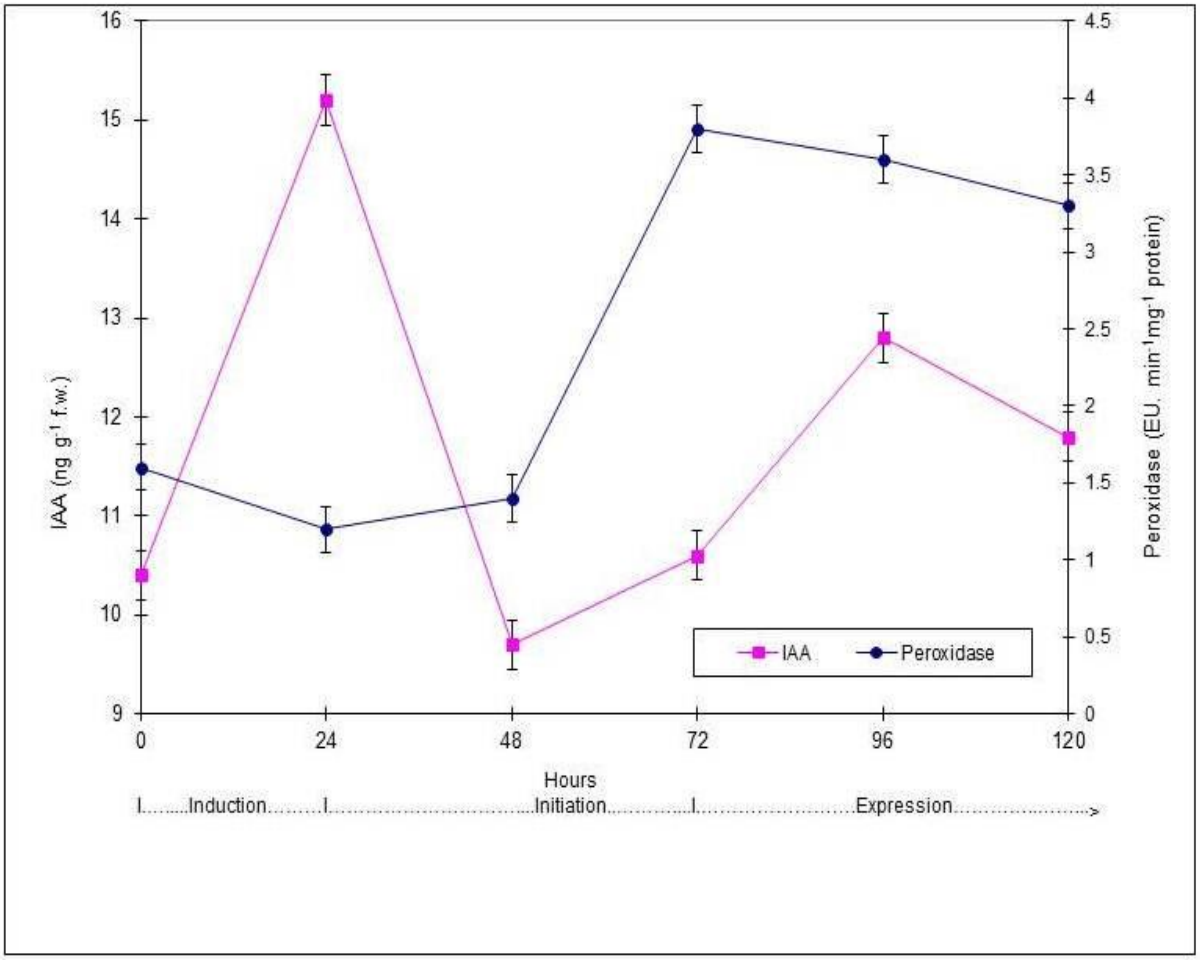

Figure 1: Phases and durations of adventitious root formation based on endogenous IAA levels and peroxidase activity in mung bean cuttings

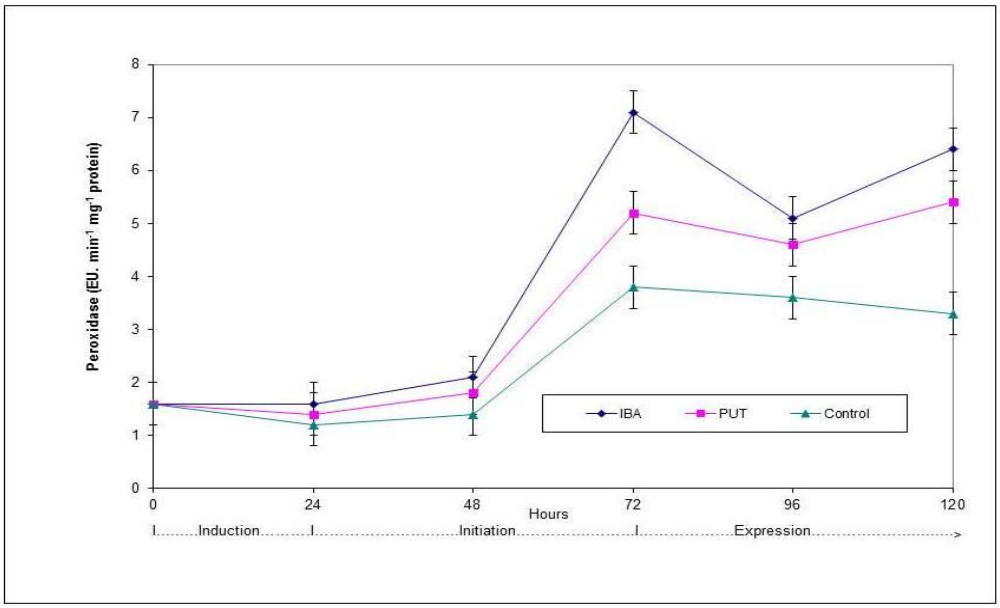

Figure 2: Changes in endogenous peroxidase (POX) activity with time in mung bean hypocotyls treated with IBA (10^{-5} M) or PUT (10^{-4} M) at intervals of 24 hours

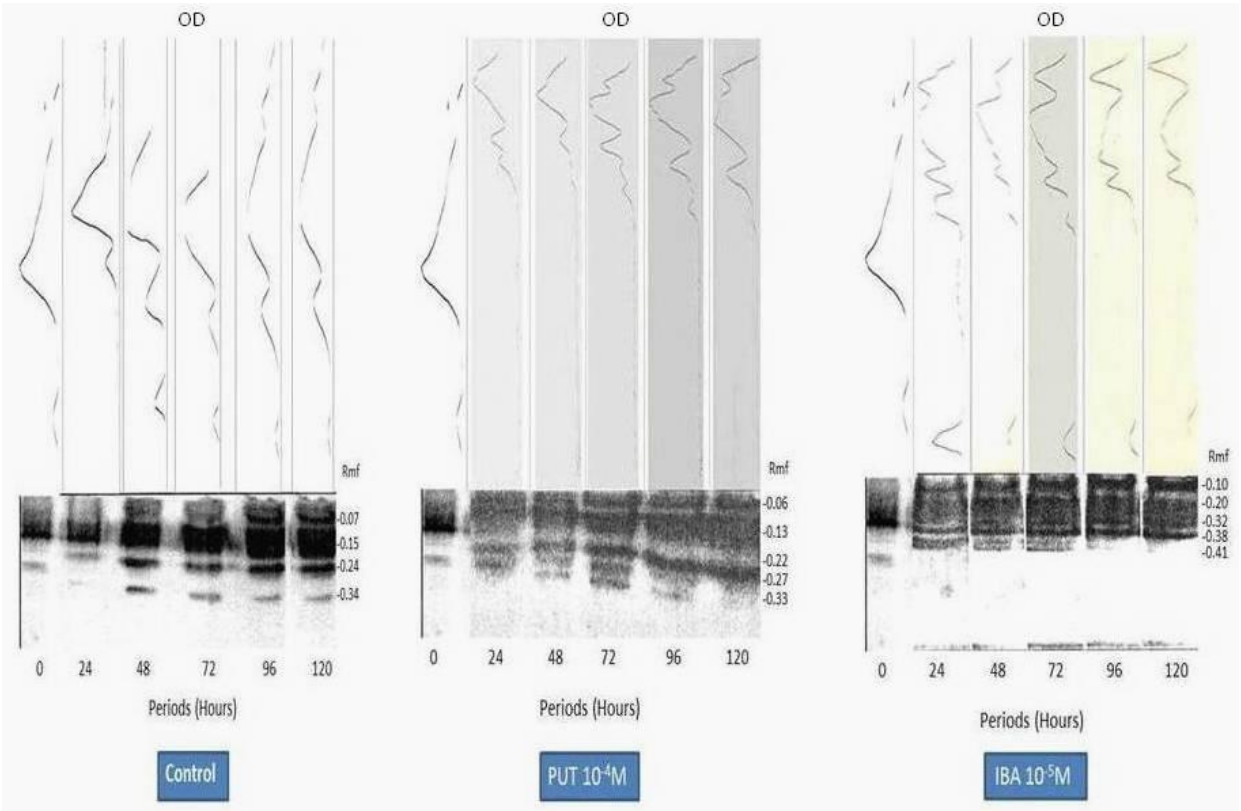

Figure 3: Peroxidase isoenzymatic bands, Band Intensity (OD) and Relative Mobility Fraction (Rmf) of isozymes from hypocotyl cuttings of mung bean (*Vigna radiata* L. cv. 105) at different time periods (from 0 – 120 hours)

Electrophoresis showed (Figure 3) four clear bands in control cuttings and five clear bands in IBA (10^{-5}M) or PUT (10^{-4}M) treated cuttings during and after 48 h of treatment periods and onwards. The results revealed that in all the cases number of isoforms, band intensity (OD) and Relative Mobility Fraction (Rmf) value were higher in treated cuttings (IBA or PUT) than control. The peroxidase isoenzyme profile of the hypocotyl of *Vigna* plants were greater in IBA (10^{-5}M) treatment than in PUT (10^{-4}M) treatment in respect of band intensity and Rmf value. Isoperoxidase bands resolved in control as well as in treated cuttings exactly in the order of enhancement of total peroxidase activity at the same hours. Band analysis showed a unique peak only in treated cuttings with extra and higher Rmf value than control cuttings.

4 DISCUSSIONS

The data in Table 1 clearly reveal that IBA treatment produced significant effects on the parameters (number of root primordium, number of primary roots, number of secondary roots and total root length) of rooting in hypocotyl cuttings of *Vigna radiata* L. cv. 105 compared to controls. Among the rooting parameters studied, IBA had a pronounced effect on the number of root primordium. PUT, on the other hand, produced marginal improvement over controls. Interestingly, the effect of PUT on total root length was more pronounced than IBA. Similar observations were also reported by Nag *et al*. [7], [28]. Gaspar and Hoffinger [31] demonstrated in a number of different plant species that increasing endogenous free IAA levels always occur in the inductive phase of rooting (0-24 h) with first peak of free IAA terminating the inductive phase and signaling the beginning of the initiation phase. A gradual increase (from 72 h) in free IAA content, reaching a peak at 96 h and declining thereafter, designates the expression phase. They also showed that POX activity generally peaked in a reverse trend compared to endogenous IAA levels. In the present study with mung bean hypocotyl cuttings, nearly identical trends in endogenous- free IAA levels and POX activities were observed as those demonstrated by Gaspar *et al*. [3], [20] and Nag *et al*. [7].Thus the present study further corroborates the different rooting phases described by Gaspar and his coworkers [3], [20] and Nag and his coworkers [7] based on endogenous IAA levels and POX activities. A sharp increase in POX activity was observed at the late initiation phase (72 h) in IBA-treated cuttings. This might be taken as an index of better rooting performance by mung bean cuttings and might serve as a good marker for rooting ability in cuttings [1], [6], [7], [15]. In PUT-treated cuttings, a similar rise in POX activity was also observed although POX activity was lower than that of IBA-treated cuttings. Plant peroxidases are known to be involved in auxin metabolism as well as cell wall synthesis in the presence of H_2O_2 and phenol, and POX activity is more involved in cell wall genesis at the later phase and obligatory step in root formation [29], [30]. After 48 h of initiation, four isozymes were detected and this pattern remained unchanged thereafter (Figure 3).These results suggest that increased total peroxidase (Figure 2) is due to the formation of new isozymes (Figure 3). During the first 24-48 h of root initiation, a qualitative change in isozyme pattern was established in the hypocolyl by Chandra et al. [32]. They also found that at least four new isozymes of peroxidase were associated with rooting in *Phaseolus mungo* cuttings. Synchronous changes in isozyme patterns of peroxidase have been positively correlated with induction and initiation of rooting in several plant species [1], [6]. An increase in number of isozymes, OD and Rmf value in the later stages in IBA or PUT treated cuttings might be taken as an index of better rooting performance by mung bean cuttings. In both the treatments studied, more intense peroxidase electrophoresis bands during the different phases matched a higher ability to root. So the increase in soluble peroxidase activity could be due to a general increase in all isozymes or to an increase in particular isozyme already present or to the appearance of new isoenzymes [1], [33]. Based on our results, the increase in soluble peroxidase activity at 48 h and onwards was probably due to an increase in particular previously existing (only two isozymes at 0 h) isozymes.

5 CONCLUSIONS

The changes in the activities of total peroxidase and peroxidase isozymes in mung bean hypocotyl cuttings is proportional. It is tempting to speculate that the more rise in peroxidase activity during the late initiation phase in IBA or PUT treated cuttings might be associated with the appearance of extra isozyme bands which correlate with better rooting performance and peroxidase might serve as a good marker for rooting ability in cuttings [6], [7], [15].

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