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Physio-biochemical Changes during Adventitious Root Formation in *Gmelina arborea* Roxb. Bud Sprout Cuttings

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ABSTRACT

Changes in the endogenous moisture content and biochemical viz., soluble sugar), phenols, o-phenol and peroxidase activity at different stages of adventitious rooting in auxin-treated (5 mM IBA) and non-treated miniature bud sprout cuttings of Gmelina arborea Roxb. were investigated at 0 h, 24 h, 3 days, 7 days, 2 weeks and 3 weeks of planting to elucidate the process of adventitious root formation at physio-biochemical level. Simultaneously adventitious rhizogenesis parameters viz., proportion of live green sprouts (%), callusing (%) and rooting (%) were recorded. Two weeks after planting, the marked difference for adventitious rhizogenesis was noticeable between treated and non-treated sprout cuttings. More than 20% rooting and 75% callusing were recorded in treated sprout cuttings. In contrast, only 7.4% non-treated sprout cuttings exhibited callusing. At the end of Week 3, more than 90% rooting was observed in treated sprout cuttings while it was absent in non-treated ones. All biochemicals exhibited changes in the first 24 h, indicating early incidence of consequential process(s), which influenced the differential response of cuttings to adventitious rhizogenesis in auxin-treated and non-treated state. Overall, the results indicate two possible phases of adventitious rhizogenesis - Phase I of Induction -the initial phase exhibiting low endogenous moisture and o-phenol but elevated phenol and peroxidase activity and Phase II of Initiation - the later phase coinciding with higher endogenous moisture and low phenol content and peroxidase activity. Both phases appear to be separated by an intervening unstable duration (Gap Phase of Stabilization) which may vary among different types of cuttings/species.

Keywords: *Bud sprouts, Moisture, Phenols, Peroxidase activity, Rooting*

INTRODUCTION

Adventitious rhizogenesis in shoot cuttings carries a great potential for rapid multiplication of superior stock for increasing demand for afforestation, commercial plantation activities and tree improvement endeavors. It is the most reliable, convenient, and cost-effective method for cloning specific genotypes but the apparent simplicity of the procedure conceals the inherent intriguing phenomenon deserving a comprehensive study of the factors regulating the process. The process of adventitious root formation needs to be studied as a dynamic series of distinct biochemical, physiological and histological events [1] because the poorly understood mechanism of adventitious rhizogenesis in shoot cuttings constitutes an impasse in cloning of woody perennials due to inadequate and unreliable root induction.

Different stages of *de novo* adventitious root formation have been suggested by researchers. The process has been reported to consist of four-stages: 1) Dedifferentiation or remeristemation, 2) initiation or inception as cells begin division to form slightly organized groups *i. e.* root initials, 3) differentiation of root primordial, and 4) primordial elongation [1-2]. Five histologically distinct stages of rooting were observed in *Vigna* hypocotyl cuttings [3]. Smith and Thorpe [4] reported three phases in the differentiation of *Pinus radiata* seedling root primordia as prinitiative phase with no histological change; the initiative phase forming a meristematic locus and post initiative phase with meristemoids differentiating into root primordia. Based on the rooting in cuttings of herbaceous species sequential interdependent physiological phases with varied auxin requirement and changed endogenous biochemical levels have also been suggested [5-13].

However, limited information is available on such phases of adventitious root formation in economically important woody perennials. Thus, the present investigation was carried out to elucidate the process of adventitious root formation at the physio-biochemical level taking *Gmelina arborea* Roxb., as a test tree which is one of the best and most reliable Indian timbers. It is a fast growing species of multiple utilities including tea chest plywood, paper and match making and medicinal properties [14-15]. The constraints of limited seed availability, poor germinabilty, [16] and viability of only about 12 months [17] necessitates development of a clonal procedure for its large-scale plantlet production.

MATERIALS AND METHODS

The experiment was carried out to investigate changes in the endogenous moisture content and biochemical *viz*., soluble sugar [18], phenols [19], o-phenol [20] and peroxidase activity [21] at different stages of adventitious rooting in auxin-treated (5 mM IBA) and non-treated miniature bud sprout cuttings (1-1.5 cm long with 1-3 unexpanded leaves) produced from semi-hardwood shoot cuttings from approximately 6-year-old trees [22].

Three hundred sprout cuttings were planted in the first week of May in randomized block design with each treatment replicated thrice of 50 cuttings. Three cuttings were randomly removed for biochemical estimation of each parameter at 0 h, 24 h, 3 days, 7 days, 2 weeks, and 3 weeks of planting for biochemical estimations. Simultaneously adventitious rhizogenesis was also recorded, separately planting two groups each of 90 (3 x30) cuttings administered with water or 5 mM IBA treatment in randomized block design.

Sprout cuttings were maintained in inert rooting medium, Soilrite - TC (Keltech Energies Ltd., Bangalore), in root trainers covered with aluminium frame polyhuts (4'x2.5'x2.5'), which were mounted on sunken beds of soil and sand saturated with water by flooding and provided with 75% shade with agro-shade net. Conditions of temperature and humidity were recorded manually that ranged 30-35^0 C and 60-80%, respectively. Cuttings were sprayed with fine mist using a hand sprayer at every 2 h during day time. Observations were recorded on the proportion of live green sprouts (%), callusing (%) and rooting (%) at 0 h, 24 h, 3 days, 7 days, 2 weeks and 3 weeks of planting.

Statistical analyses were done employing Analysis of variance (ANOVA), 'F' test for significance, and least significant difference at p=0.05 ($LSD_{0.05}$). Arc Sine √percentage transformations were carried out for percentage data [23].

RESULTS

Significant change in moisture content was recorded during different stages of histogenesis in sprout cuttings (Fig. 1). Moisture content of treated as well as non-treated sprout cuttings rose sharply within 24h of planting, by 15.9 % in the treated cuttings and 14.2 % in non- treated cuttings. The increment continued until Week 2 (coinciding with rooting in treated cuttings) of planting when the moisture content of both types of sprouts reached a maximum level. The moisture content of treated sprout cuttings was 25% higher than the initial moisture level and also 2.5% higher than the moisture content of non-treated sprout cuttings during the same period. In the next week (Week 3), a decline of 6.3% occurred in non-treated sprout cuttings and 2.3 % in treated sprout cuttings from the previous week. However, the decline in moisture content was significant only in non-treated sprout cuttings.

Endogenous levels of soluble sugar in both treated and non-treated sprouts also exhibited significant change during different stages of adventitious rhizogenesis. An increase in endogenous soluble sugar was recorded in first 24 h which significantly declined in non-treated sprout cuttings at Week 2 and early in-treated sprout cuttings at Week 1 by 74.9 % and 47.8 %, respectively over their initial values. However, the lowest values for endogenous soluble sugar in treated cuttings were recorded only at Week 2, which did not significantly differ from the previous week but was declined by 72.7% over the initial value. Week 3 registered a non-significant gain of endogenous soluble sugar in both non-treated and treated sprout cuttings (Fig. 2).

Significant change in the endogenous content of phenol was registered in the treated sprout cuttings only (not in non-treated sprouts) during different stages of the progression of experiment. The significantly lowest value for phenol in treated sprout cuttings was recorded at Week 3, denoting only 34.8% of the Initial value (Fig. 3). Though the changes in the levels of endogenous o-phenol in non-treated as well as treated sprout cuttings during different stages were not significant, a comparative analysis presented a contrasting scenario where o-phenol content gradually decreased till Week 2, reaching the lowest level which was 73% less than the initial o-phenol content in treated sprout cuttings. Further, the level was also 81.5% lower than that of non-treated sprouts in the corresponding week. Non-treated sprouts registered a very steep increase from Week 1 to Week 2 before showing a 33% decline the next week (Fig. 4).

Peroxidase activity exhibited an increase of 155.7% in treated sprout cuttings and 67.8% in non-treated cuttings within 24h of planting. After that it remained stable in both treated and non-treated cuttings till day 3. In non-treated sprout cuttings, peroxidase activity declined at successive sampling stages; very rapidly from Day 3 to Week 1 and slightly from Week 1 to Week 2. Peroxidase activity of the treated sprouts also declined rapidly from Day 3 to Week 1 followed by an elevation of 1.97 times in Week 2 and 11.7 times in Week 3. At this stage, peroxidase activity in treated sprouts was about 13.4 times higher than initial activity of the sprout at the start of experiment. In comparison to the peroxidase activity of non-treated sprouts in the corresponding week, the activity in treated sprouts was gained by 7.3 times. Overall, higher peroxidase activity was observed in treated sprout cuttings than the non-treated ones throughout the experiment (Fig. 5).

No appreciable change in the condition was observed in treated and non-treated sprout cuttings in the first 3 days. However, treated sprout cuttings recorded 7.4% callusing on the day 3. Some sprout cuttings belonging to both treatments exhibited wilting at Week 1. By this time, about 25% treated sprout cuttings developed callusing. In contrast, callusing was negligible in non-treated sprout cuttings. Two weeks after planting, marked difference for adventitious rhizogenesis was noticeable between treated and non-treated sprout cuttings. More than 20% rooting and 75% callusing was recorded in treated sprout cuttings. In contrast, only 7.4% of non-treated sprout cuttings exhibited callusing. At the end of Week 3, more than 90% rooting was observed in treated sprout cuttings while it was absent in non-treated ones (Fig. 6; Table. 1).

DISCUSSION

Adventitious rhizogenesis at the base of detached shoot cuttings is a complex multifactorial response leading to the establishment of a complete and autonomous plant. This vital developmental process in the growth and subsistence of cuttings involves the initiation of several new meristematic capacities in different tissues of stem cuttings [24]. Shoot cuttings severed from the mother trees represent an unstable metabolic state which undergo considerable morphological, physiological and biochemical changes subsequently towards adventitious root initiation or otherwise, guided by complex interactions of numerous internal and external factors. Significant fluctuations in the endogenous levels of moisture and biochemicals were recorded during the different stages of sampling indicating a complex process of adventitious root formation marked with various successive stages in the present study. All biochemicals exhibited changes in the first 24 h, indicating early incidence of consequential process(s), which influenced differential response of cuttings to adventitious rhizogenesis in auxin-treated and non-treated state.

A marked decline in endogenous level of moisture was recorded in auxin-treated sprout cuttings (but not in non- treated sprout cuttings) between Day 1 to Day 3 (Fig.1) indicating that auxin stimulate rooting *via* water stress related signal (change) leading to initiation of rhizogenesis. As per some earlier reports adventitious rooting is promoted by water stressing of cuttings/stockplants [25-27]. At the advent of adventitious rooting in auxin-treated cuttings, the endogenous moisture content was elevated to much higher level than initial content of the same cuttings as well as of non-treated cuttings at the corresponding stage without rooting. Seemingly, in later stages adequate endogenous moisture status facilitates induction of

adventitious roots. It corroborates with the observation that one of the most important factors favoring root development is the availability of water, as maintaining a positive water balance in cuttings during root development is essential [28].

Phenolic compounds protect plants from oxidative stress [29] to allow containment of excessive wound response that may impede subsequent processes of regeneration [30]. Endogenous o-phenol registered decline in the first 24 h in treated sprout cuttings while total phenol content exhibited an increase in treated and non-treated cutting. Such Increase in total phenol content within the region of root regeneration has been demonstrated in cuttings of *Phaseolus aurens* at the first 24 h [31] and *Phaseolus mungo* at 16 h of rooting [32]. The levels of endogenous phenolics has been reported to increase during *in vitro* root formation in apple shoots [33]. *In vitro* rooting has been reported to be associated with enhanced accumulation of phenolic acids and some flavonoids [9]. A pronounced basipetal movement of soluble phenols has been reported before rooting in Jack pine seedling cuttings, suggesting *de novo* synthesis of phenolics [34]. Reportedly, the phenolic content of cuttings increases during phase I followed by a decrease in phase II of rooting [32]. Decline in endogenous levels of both o- phenol and total phenol to the lowest levels in the later stages of rhizogenesis in the present investigation conform to this pattern. Earlier investigations have implicated low endogenous levels of simple phenolics for promotion of adventitious rooting in shoot cuttings [33, 35]. A contrasting pattern in the initial stage for o-phenol and total phenol observed in the present study suggests that changes in individual phenolics *vis-à-vis* total phenols may have role in adventitious root formation.

The activity of heme-containing enzymes, peroxidases having catalytic action on diverse organic compounds, including indole-3-acetic acid (IAA), has been used as a biochemical marker of the phases in adventitious rooting [36]. In cuttings of *Hydrangea sps*. peroxidase was the first enzyme exhibiting increased activity during root initiation [37]. The rise in total peroxidase activity of the whole plant during the initiation phase of rooting has been recorded [38]. A similar initial increase in peroxidase activity has also been reported during *in vitro* rooting of cuttings of *Sequiadendron giganteum* [39] and *Vitis* [40]. An elevation in peroxidase activity in all types of cuttings was noticed within 24 h of planting. However, auxin-treated sprout cuttings exhibited higher peroxidase activity in comparison to non-treated cuttings. Minimum level of peroxidase activity coincided with advent of adventitious rooting in auxin-treated cuttings, a pattern observed previously in *Theobroma cacao* [41] and *Populus deltoids* Marsh. [42]. Peroxidase activity was high at root initiation and exhibited a sharp decline thereafter, during the rooting of stem cuttings of *Casurina equisetifolia* [43]. Some authors, however, observed a continuous increase of peroxidase activity [44-45]. Several conditions have been created by which the rooting performance decreases. Sometimes the expected reduction of the peroxidase peak occurs [46], but in other cases not [45,47].

Carbohydrates provide energy and carbon chains for biosynthetic processes in new meristems and roots. Low carbohydrate allocation to the root formation site may limit adventitious rooting. However, more than the carbohydrate content, its allocation and distribution within the cutting seem significant [48-50]. Adventitious rooting is promoted through efficient partitioning of carbohydrates between the new sink of developing roots at the cutting base and the shoot meristem sink [49].

Essential parts of the sugar sensing and signalling network are the interactions between phytohormones and carbohydrates [51]. Root development and growth in *Arabidopsis thaliana* seedlings were observed to be guided by a glucose and auxin signalling crosstalk [52]. Early establishment of a carbohydrate sink at the rooting site was a key metabolic event in *Petunia hybrida* adventitious root formation [53]. In the present investigation soluble sugar levels recorded an increase in first 24 h which was followed by a gradual decline with the progress of experiment till the induction of adventitious roots. After induction of rooting in Week 2 soluble sugar content again registered increase in next week. Depletion of sugar content has been reported during adventitious rhizogenesis in cuttings of various poplars [54] and Laryx [55]. The observation that auxin-treated cuttings exhibiting efficient utilization of soluble sugars than non-treated cuttings was in agreement with the report that exogenous auxin increases the supply of carbohydrates at the base of cuttings for root induction [56].

Successive interdependent physiological phases of adventitious rhizogenesis commensurate with specific physio-biochemical flux have been identified in different species [38, 57-65]. Assumedly each of these phases has specific requirements which can even be antagonistic, but operate in a complementary manner. During all phases and almost every developmental step, auxins have been revealed to regulate adventitious rhizogenesis [12,66].

There is considerable agreement on three developmental phases of adventitious root formation, i.e., induction, initiation and extension [8,11-12]. Marked by the immediate consequences of the wounding response caused by severance, the induction phase in cuttings (or detached organs, such as leaves), comprises the first few hours after cutting removal. There is a local increase in jasmonate, phenolic compounds and auxin at the cutting base, frequently linked with a transiently lower peroxidase (EC 1.11.1.7) activity, and the establishment of a sink for carbohydrates [53,67]. During the induction phase, the depending upon the species and the age of the shoot cutting, primordium initial cells are established *via* de-differentiation of pericycle cells or cambium cells followed by cell division [68-69].

Regeneration consists of three successive phases: i) dedifferentiation during which cells attain competency to respond to the organogenic or embryogenic stimulus, ii) induction during which the stimulus acts and the cells become committed to a developmental pathway and iii) differentiation during which the stimulus is no longer required and the previously determined cells form the organ/embryo [70]. The timing of the three phases was determined in shoots and stem slices of the apple rootstock 'Jork 9' [71-73]. Other authors have reported similar results [6-7,74].

The results in *G. arborea* sprout cuttings indicate two possible phases of adventitious rhizogenesis - Phase I of *Induction* -the initial phase exhibiting low endogenous moisture and o-phenol but elevated phenol and peroxidase activity and Phase II of *Initiation* - the later phase coinciding with higher endogenous moisture and low phenol content and peroxidase activity. Both phases appear to be separated by an intervening unstable duration (Gap Phase of *Stabilization*) which may possibly vary among different types of cuttings/species (Fig 7). Two main phases of adventitious rhizogenesis: root induction and root formation (with varied nomenclature) differing in auxin requirements, the former requiring a higher auxin concentration while in the latter phase in which anatomical

changes take place high auxin inhibiting the process, have been previously suggested. Two distinct phases of adventitious root formation have been discerned in *Azukia* cuttings: the auxin-insensitive root initiation phase and the auxin-sensitive root induction phase [58-59]. The former is presumably associated with the development of sensitivity of target cells towards auxins for initiation of adventitious roots.

Off the physiological phases of adventitious root formation, in the induction phase, occur all the necessary biochemical events that precede the initiation of cell divisions start formation of root meristems and primordia organization. The duration of the root inductive phase may vary [62,75], being achieved in less than a day or up to several days in some species. A prerequisite for these phases is that the target cell(s) must be competent or acquire competence to respond to the external rooting signal. It is agreed that adventitious root formation begins with the induction phase that lacks any discernible cell divisions but involves the reprograming of target cells for establishment of groups of fresh meristematic cells. Hormonal changes may be triggered through wounding and water stress responses in severed organs reprograming target cells towards competency of responding to rooting stimuli. Further separation of early and late phase in induction phase has been postulated [76], where early induction phase may have dedifferentiation (0– 24 h, taking place before the induction phase) of founder cells [9, 77].

In the present study also the duration of 0– 24 h, where sharp changes in endogenous moisture and biochemicals were recorded indicate towards an early induction phase of dedifferentiation in *G. arborea.* A lower auxin and phenolic concentration and higher peroxidase activity has often been recorded during initiation apart from cell division and development of root primordia. Low phenolic concentration and higher peroxidase activity in also happened in the final phase of rooting in our study.

During the first hours after excision of cuttings, there occurs the stimulus that triggers the initiation of adventitious rooting owing to the breakdown of the vascular continuum, inducing accumulation of auxin near wounding [78]. This stimulus was independent of exogenously applied auxin. However, the auxin treatment speeded cambial cell division and amplified the sucrolytic activities at the base of the stem, contributing to establishment of the new root primordia. Profound changes in endogenous biochemicals were registered in *G. arborea* sprout cuttings during the adventitious root formation especially in the first 24 h, indicating incidence of consequential process(s), which influence subsequent rhizogenesis in auxin-treated cuttings but not in non-treated ones due to inadequate endogenous auxin. Marked decline in endogenous moisture was recorded in auxin –treated sprout cuttings between Day 1 to Day 3 indicating that auxin stimulates rooting *via* water stress related signal leading to initiation of rhizogenesis.

CONCLUSION

Profound and varied changes occur during the course of adventitious rhizogensis in *G. arborea* sprout cuttings resulting in root formation in auxin-treated cuttings and no rooting in non-treated ones. The findings implicate two possible phases of adventitious rhizogenesis separated by an intervening unstable duration. Phase I of *Induction* -the initial phase exhibits low endogenous moisture and o-phenol but elevated phenol and peroxidase activity while Phase II of *Initiation* - the later phase coincides- with higher endogenous moisture and low phenol content and peroxidase activity. Further studies are however, warranted in woody perennials to pinpoint mechanism of adventitious rhizogenesis to evolve efficient clonal procedures for large-scale application.

Table 1: Changes in G. arborea sprout cuttings during different stages of adventitious rhizogenesis

Period after planting	Non-treated cuttings	Treated cuttings
0 h	Green sprouts (100%)	Green sprouts (100%)
24 h	Green sprouts (100%)	Green sprouts (100%)
3 days	Green sprouts (100%)	Green sprouts (100%) Callus (7.40 %)
Week 1	Green sprouts (90%) Callus (3.70 %)	Green sprouts (90%) Callus (25.93 %)
Week 2	Green sprouts (90%) Callus (7.40 %)	Green sprouts (90%) Callus (78.78 %) Rooting (22.22%)
Week 3	Green sprouts (75 %) Callus (18.52 %)	Green sprouts (85%) Callus (8.40 %) Rooting (92.59%)

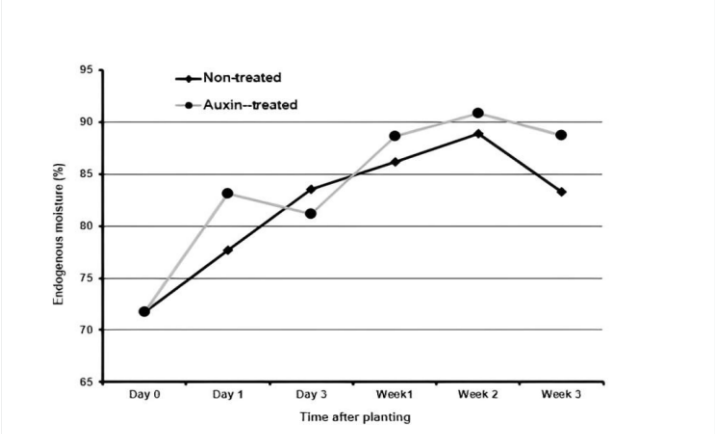

Figure 1: Endogenous levels of moisture (%) in sprout cuttings. Data are mean of five replicates at each point

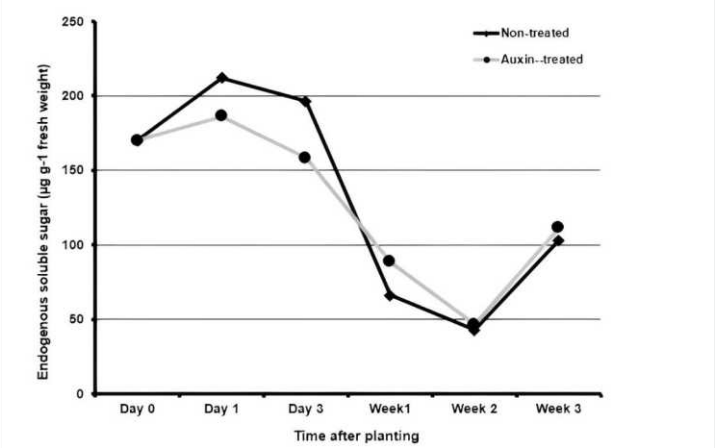

Figure 2: Endogenous levels of soluble sugar (µg g^{-1} fresh weight) in sprout cuttings. Data are mean of five replicates at each point

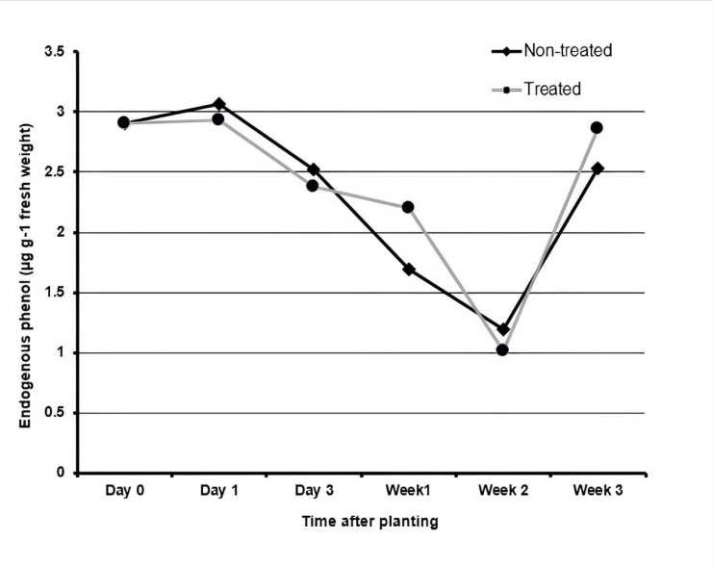

Figure 3: Endogenous levels of total phenol (µg g^{-1} fresh weight) in sprout cuttings. Data are mean of five replicates at each point

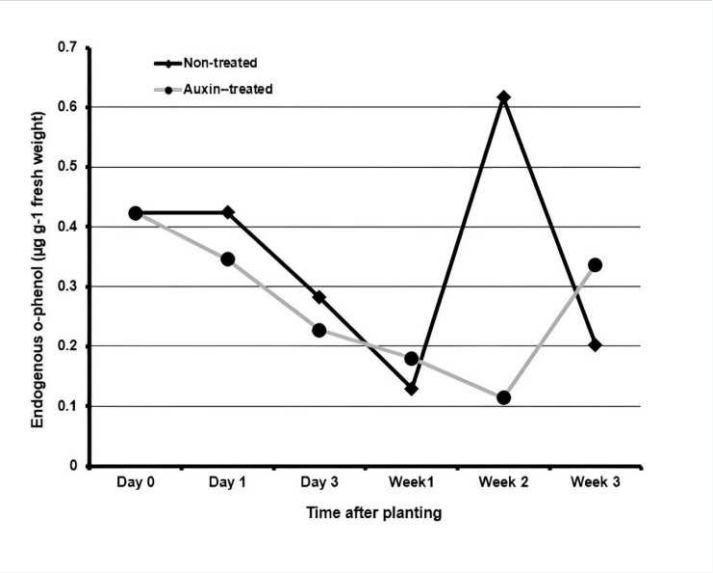

Figure 4: Endogenous levels of o-phenol (µg g^{-1} fresh weight) in sprout cuttings. Data are mean of five replicates at each point.

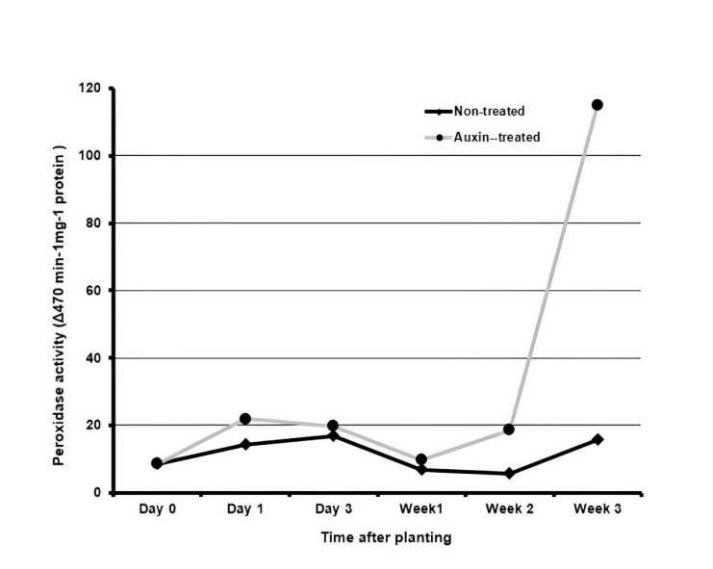

Figure 5: Endogenous levels of peroxidase activity (Δ_{470} min^{-1} mg^{-1} protein) in sprout cuttings. Data are mean of five replicates at each point.

Figure 6: Rooted auxin-treated Gmelina arborea Roxb. sprout cuttings after 3 weeks of planting

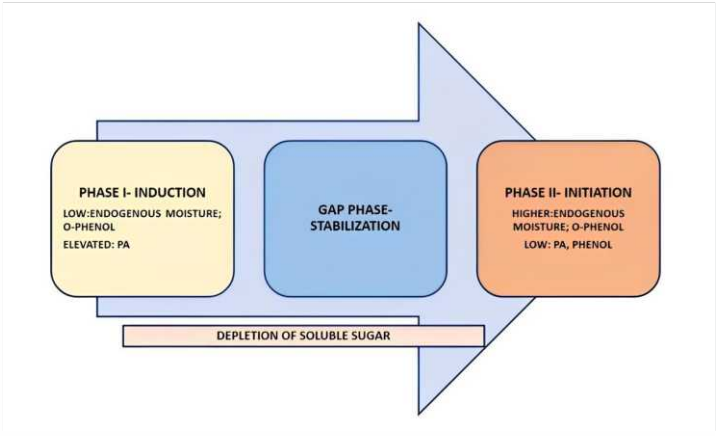

Figure 7: Possible phases of adventitious rhizogenesis in Gmelina arborea Roxb. sprout cuttings

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