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ADVENTITIOUS ROOT FORMATION AND ASSOCIATED BIOCHEMICAL CHANGES IN MICRO SHOOTS OF *TECTONA GRANDIS* CLONES

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Globally, *Tectona grandis* (teak) is mass-produced through tissue culture. Being a perennial species, an understanding of adventitious rooting would significantly increase its production. This study investigated biochemical and histological changes and media compaction associated with rooting in eight tissue-cultured *T. grandis* clones. Root emergence was observed from the 3rd to 8th day in 4–5 cm long shoots treated with 5 mM indole-3-butyric acid (IBA). Root primordia became visible after 3 days in good rooters while root initiation, elongation and root emergence occurred between the 3rd and 7th day depending on the clone. Grade 5 vermiculite facilitated root formation and improved primary and secondary root thickness and length. Sugars and proteins increased during root initiation from day 1 to 3, reflecting active cell division using available energy sources. An initial low, followed by increased indole-3-acetic acid (IAA) levels revealed inhibition of endogenous auxin due to external supply, replaced later by the shoot's auxin. The polyphenol oxidase (PPO) and indole-3-acetic acid oxidase (IAAO) levels increased during rhizogenesis. Therefore, it is clear that sugars, enzymes and proteins play a significant role during various rooting phases. The findings indicated the influence of biochemical pool composition on root system architecture. Genotype variation demands clone-specific protocols for successful commercial propagation of the species.

Keywords: Teak, genotypes, tissue culture, root system, clone-specific protocols, vermiculite

INTRODUCTION

The commercial success of micropropagation technology depends on achieving high transfer rates of plants from the laboratory, on a large scale and lower cost, and achieving a high survival rate in the field. The application of tissue culture (TC) technology for mass propagation of hardwoods is limited. The primary difficulties encountered are variation in rooting in different genotypes and hardening. Though appearing to be physiologically fully functional, the plantlets are unlikely to actively photosynthesise. When transferred to the nursery for rooting, such shoots undergo tremendous stress. Understanding micro shoot physiology is essential to overcome difficulties during the transition process where predetermined cells redifferentiate to root primordia-initiating cells. Many reports describe changes occurring during adventitious root formation (Husen & Pal 2001, Metaxas et al. 2004).

Root architecture is crucial in establishing plantlets to ensure their survival in the field. Vermiculite, a natural mineral, is suitable for rooting teak (*Tectona grandis*) in commercial TC (Mahalakshmi et al. 2018, Tamin 2019). However, its different grades on root formation have not been addressed.

Metabolic changes in cut zones inhibit or promote adventitious root regeneration. It varies between genotypes within a species, leading to asynchrony in commercial TC. The changes further vary as new root tissues appear and start elongating (Husen & Pal 2006). Auxins play a crucial role in mobilising carbohydrates in the shoots (Veierskov et al. 1982). They also increase transport to the root zone, solubilise proteins and enhance cellular division enzymes and differentiation during root primordium initiation or development in the cut zones (Husen & Pal 2006). Hence, metabolic changes in the rooting zone may serve as easy, fast and reliable markers to assess cellular differentiation.

Rooting in teak depends on the age of the donor trees. The auxin requirement increases with increasing age (Husen & Pal 2006). The influence of genotypes in eliciting rooting responses is also poorly understood. Variation in metabolic changes has not been studied previously in teak, especially in micro shoots.

Further, information on the influence of media compaction on poor rooters is lacking. Therefore, the present study investigated the adventitious rooting of teak micro shoots as influenced by genotypes and endogenous levels of biochemicals. It analysed the metabolic changes over a ten-day interval in the rooting zone of micro shoots during adventitious root development in different genotypes.

MATERIALS AND METHODS

Eight clones of teak (TG 1 to TG 8) from the TC laboratory, IFGTB, Coimbatore, India, were used for the study. As described by Mahalakshmi et al. (2018), single nodal cultures were maintained. All culture experiments were conducted in 300 mL culture bottles containing 30 mL medium, incubated at 23 ± 2 °C under 16 h photoperiod with a light intensity of 112 µmol m^{-2} s^{-1} provided by cool fluorescent tube lights.

Medium compaction and root growth

Shoots from the slowest rooter were excised from 45-day-old cultures of teak. The shoots were pulse-treated for 10 mins with 5 mM indole-3-butyric acid (IBA) and transferred to varying grades of vermiculite, large (> 12 mm), medium (6–12 mm), fine (3–6 mm), superfine (1–3 mm) and micron (< 1 mm and fine powder). The cuttings in vermiculite were moistened daily with 50 mL Hoagland solution. After 60 days, the plants were dried, weighed and their roots preserved in 70% ethanol. Each treatment consisted of five replicates of 10 explants each and the experiment was repeated twice. For analysis, each root system was spread out on a tray to avoid overlap, and images of the roots were captured. The images were later analysed using ImageJ and RootTrace to determine the following root morphology parameters: the number of main and secondary roots, average main root and secondary root length (cm), average main root and secondary root diameter (mm), and total root surface area (cm^2). Manual counting determined the number of adventitious roots (Roman-Aviles et al. 2004).

Histological analysis

Samples (5 mm from base) grown on Murashige and Skoog (MS) medium and treated with 5 mM IBA were fixed in FAA (HCHO: CH_3COOH: 50% C_2H_5OH, 5:5:90, v/v) following different periods of incubation for 10 days. They were then dehydrated using 70% alcohol, ethanol, isopropanol and xylene, and embedded in the paraffin wax. Tissue sectioning (10 µm) was carried out using a rotary microtome, and the slides were then stained in toluidine blue adopting the procedure of Sharma and Millam (2004). Sections were mounted in Canada balsam and observed in a compound microscope 10 × and 40 × for root initiation.

Biochemical changes

Samples (basal portions of nodal explants 0.8 to 1.0 cm) were taken daily (i.e. 24 h interval) till the emergence of roots (up to 10 days after culture) and stored at 4 °C until further analysis. All biochemical investigations were repeated thrice in triplicates. Thirty explants per clone were used for each treatment. Total soluble protein contents were estimated, adopting the method of Lowry et al. (1951). Indole-3-acetic acid oxidase (IAAO) and polyphenol oxidase (PPO) activities were estimated using protocols described by Sadasivam and Manickam (2004). Soluble sugars and total reducing sugars were estimated based on Dubois et al. (1956).

Statistical analysis

A two-way ANOVA was used to analyse data. Graphs were plotted using best fit regression analysis and equations, and R^2 values were incorporated in figures. All statistical tests were performed using SPSS version 20.0. The data presented in percentages were subjected to arcsine transformation before they were analysed, and were converted back to percentages for convenience of presentation in tables and graphs. The experiments were arranged in a completely random design (CRD).

RESULTS

This study investigated the effect of clonal variation on *ex-vitro* rooting of the micro cuttings of *T. grandis*. Various stages of rooting were observed in the microshoots through histological studies. Roots emerged from the third day extending to the sixth for some clones. A fully developed root system was observed in all the

clones within 10 days. The influence of medium compaction on slow rooters and variation in adventitious rooting between clones correlated with biochemical changes evaluated during the rooting period.

Effect of clones on rooting

Pulsing with 5mM IBA for 10 mins induced root formation in all the clones. More than 95% of the explants rooted within seven days. A significant difference was observed in the rooting % of the different clones over the rooting period. Roots emerged fastest in TG 3 (3 days, Figures 1c & 2a) while clone TG 1 was the slowest to emerge (7 days) (Figures 1a & 2a). A significantly higher number of roots per explant (8–9) was observed in clones TG 6 and 7 (Figures 1f & g), whereas clone TG 1 had a lower number of roots. The ANOVA showed the significant effects of clonal variation on rooting percentage and the number of roots formed per explants (Table 1).

Root length was observed to be low in clones with more roots per explant (TG 7, 7.5 cm) and the responses varied significantly (Table 1, Figure 2b) with time and clones. The length of roots also varied with clones and the maximum root length was observed in TG 1 (11.25 cm). The survival of the plantlets following hardening was 100% in all the clones.

Histology in TG 7, a profuse rooter, revealed cell division initiation in the endodermis after 3 days (Figures 3a–d). This period (1–3 days) corresponded to the induction and initiation phases of rooting. After 4 days, well-developed root primordia at endodermis (Figures 3d & e) was observed, which started growing as a root (Figure 3f). This corresponds to root organisation and elongation phases. Subsequently, roots started appearing from the lower end of micro shoots after 5 days of exposure to rooting medium.

Media compaction and root formation

The adventitious roots developed from the slowest rooter, TG 1, revealed significant differences in root morphology when grown in different vermiculite grades. The number of primary roots produced was highest in Grade 5, and both primary and secondary roots were longer and thicker (Table 2). The average root length (cm) was almost twice the other grades. However, the total root surface area was maximum for Grade 3, revealing denser root mass (Figure 4).

Table 1 Analysis of variance (ANOVA) among biometric traits in different teak clones subjected to rooting

Source	SS	df	MS	F	P
Explant rooting (%)					
Between clones	0.002	7	0.020	3.658	0.00
Between days	0.005	9	0.001	8.762	0.00
Interaction between clones and days	0.004	47	0.030	1.596	0.02
Error	0.008	130	0.040		
Number of roots per explant					
Between clones	0.245	7	0.035	5.723	0.00
Between days	0.33	9	0.037	5.983	0.00
Interaction between clones and days	0.61	47	0.013	2.118	0.00
Error	0.796	130	0.006		
Root length (cm)					
Between clones	74.661	7	10.666	5.985	0.00
Between days	130.166	9	14.463	8.115	0.00
Interaction between clone and days	160.384	47	3.412	1.915	0.00
Error	231.688	130	1.782		

SS = sum of squares, df = degrees of freedom, MS = mean sum of squares, F = F test value, P = probability (5%)

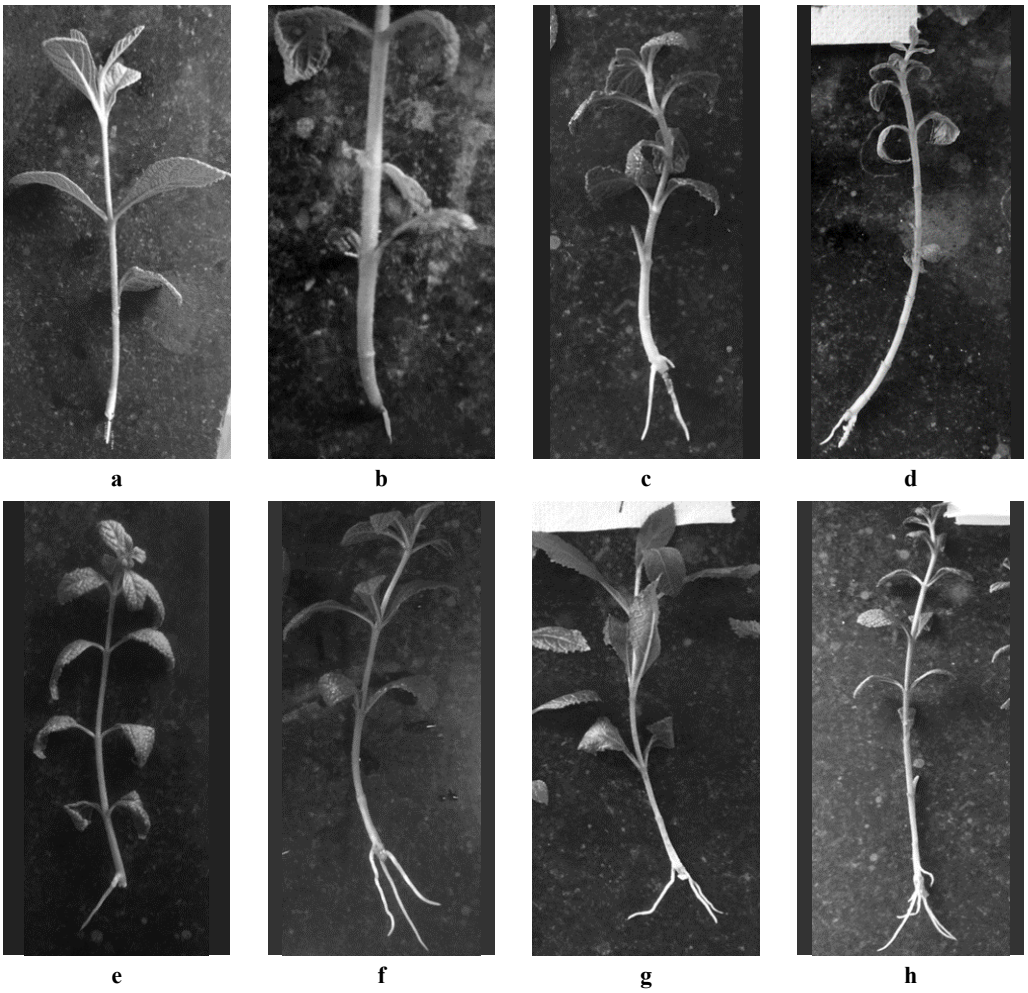

Figure 1 Rooting of different teak clones (a) TG 1 (b) TG 2 (c) TG 3 (d) TG 4 (e) TG 5 (f) TG 6 (g) TG 7 (h) TG 8

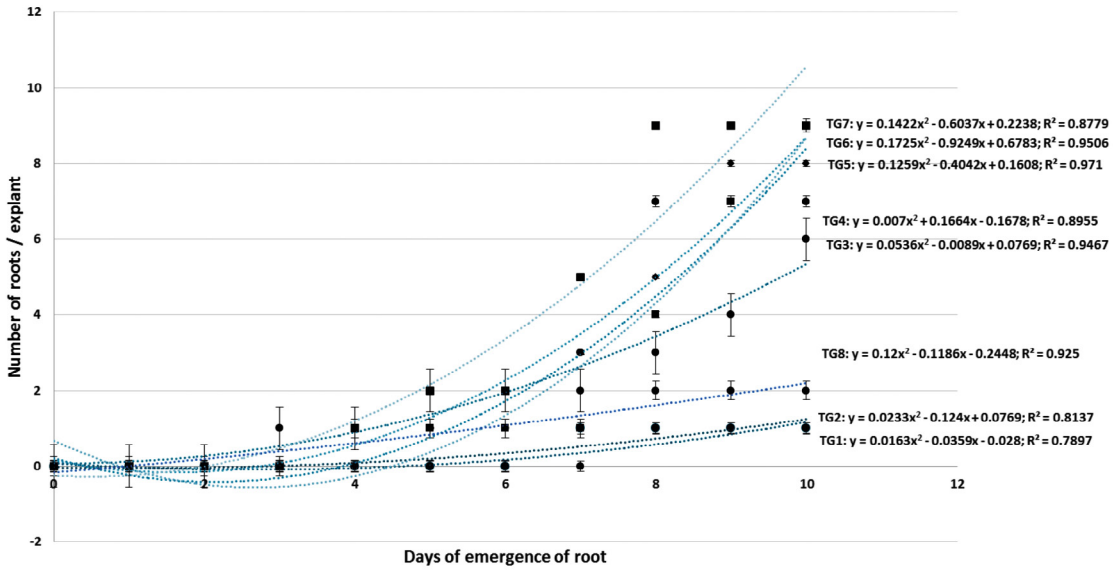

Figure 2a The rooting response of different genotypes of teak, showing the number of roots developed per explant at the end of 10 days

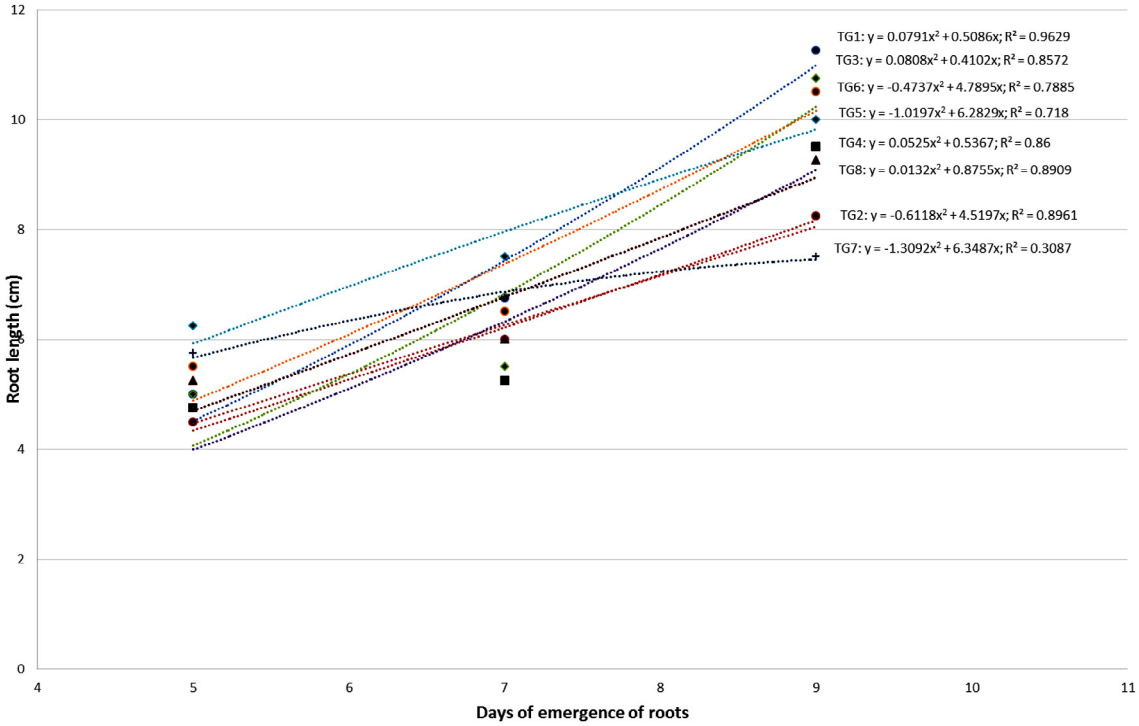

Figure 2b The rooting response curve of different genotypes of teak based on the root length developed per explant at the end of 10 days

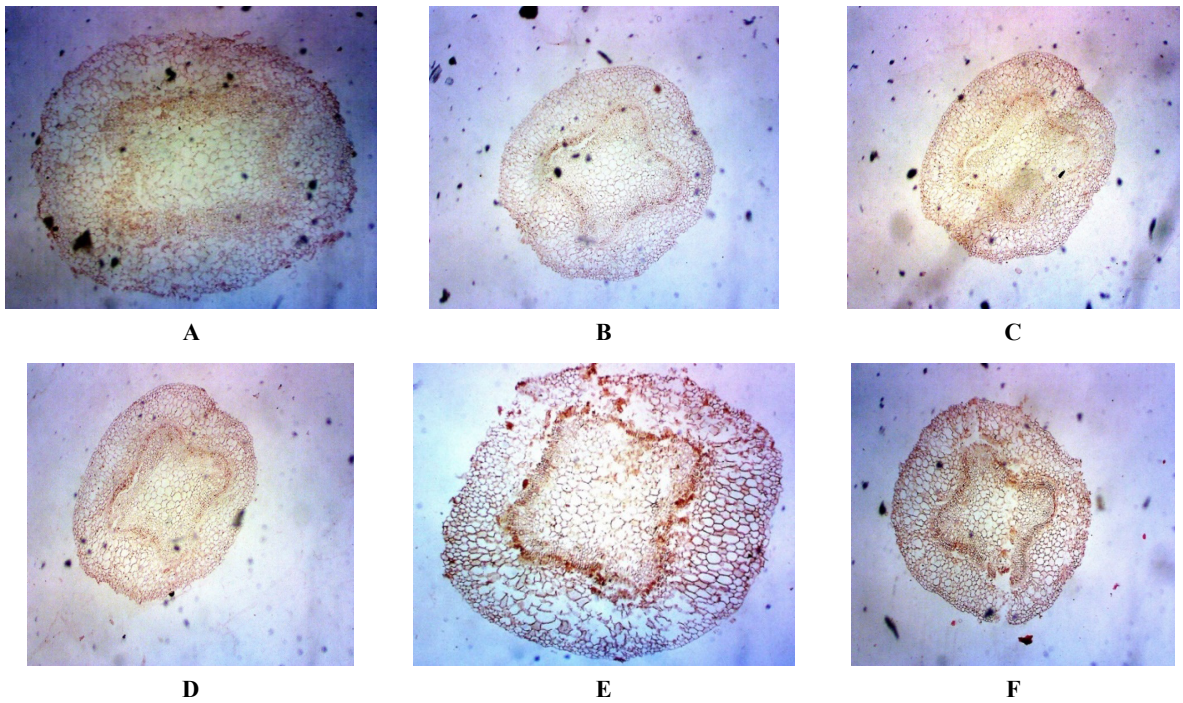

Figure 3 Histological observations during rooting of micro shoots at various stages of root initiation in *in vitro* teak clone (TG 7); sections of stem (a) at the start of the experiment, (b) after 24 h (induction), (c) after 48h (initiation); cells with dense cytoplasm indicated by arrows suggest induction phase of rooting (d) after 72h, initiation of root primordia (arrow) formation of meristematic tissues (e) after 96h, root primordia extending towards the epidermis (f) root emergence

Table 2 Analysis of variance (ANOVA) among root morphology parameters in teak micro shoots grown in different grades of vermiculite

Source	SS	df	MS	F	P
Primary roots number	622.96	4	155.74	38.76	0.00
Error	80.36	20	4.02		
Secondary roots number	39.65	4	9.91	16.27	0.00
Error	12.18	20	0.61		
Primary root length (cm)	207283	4	51820.70	166.96	0.00
Error	6208	20	310.4		
Primary root thickness (mm)	130.68	4	32.67	18.55	0.00
Error	35.22	20	1.76		
Secondary root length (cm)	2365.7	4	591.42	28.22	0.00
Error	419.2	20	20.96		
Secondary root thickness (mm)	133.36	4	33.34	25.65	0.00
Roots	26	20	1.3		
Total root surface area(cm^2)	49797796	4	12449449	30.2	0.00
Error	8244210	20	412210		

SS = sum of squares, df = degrees of freedom, MS = mean sum of squares, F = F test value, P = probability (5%)

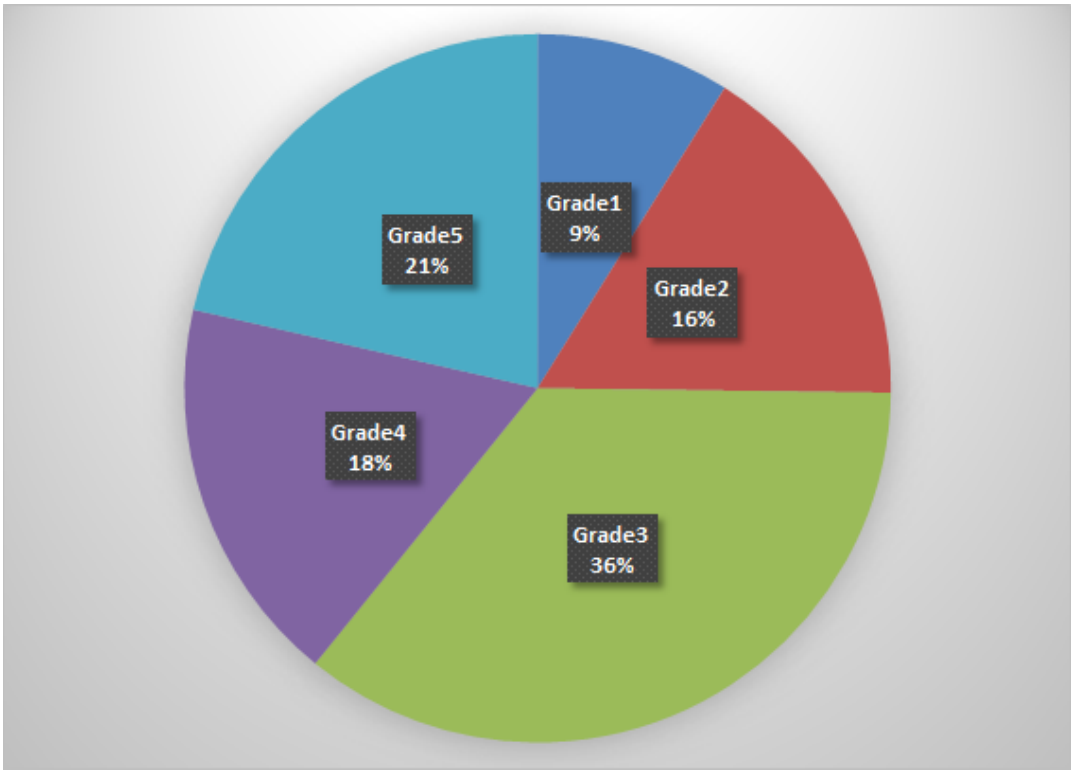

Figure 4 Percentage of total root surface area (cm^2) of root mass when grown in different grades (1–5) of vermiculite, measured using Image J and RootTrace

Biochemical analysis

The biochemical changes were monitored at every 24 h interval for 10 days. The changes in biochemical parameters were compared during the rooting process.

Protein content

Significant differences in total protein content were recorded in microcuttings in all the clones (Figure 5). The total soluble protein content was maximum during the initiation of rooting (3–6 days) and it gradually decreased. It remained steady in all the clones during this period. The TG 2,3,4 and 8 recorded a balanced level of proteins during the period of observation; TG 1 recorded a slow and steady decrease with time while TG 5,6 and 7 recorded low values initially but gradually increased by day 6 or 7 (Figure 5a). Clones TG 1 and TG 5 showed high levels, TG 4 and 6 were optimal, while clones TG 2,3,7 and 8 recorded values lower than the average throughout the experiment (Figure 5b). The levels were low at the start of the experiment, i.e. day 0, which increased sharply until day 5 (Figure 5c). Thereafter, the protein content showed a slight decline followed by a steady increase in the microcuttings until the end of the experiment.

Total soluble sugar (TSS) and reducing sugar (RS)

At the beginning of the experiment (day 0), the total sugar contents were minimal, steadily increasing by day 3, and this remained constant up to day 8, following which it increased sharply (Figure 6c). All the clones had a constant level of total sugars except clones TG 2 and 3. The TG 2 showed a sharp and steady increase in its sugar levels during rooting, while TG 3 initially decreased until day 6 and later increased (Figure 6a). Both clones TG 1 and 2 showed low levels of sugars; it was moderate in TG 3, whereas all other clones (TG 4–8) recorded high values (Figure 6b). The levels of TSS were significantly high in the microshoots of different clones subjected to

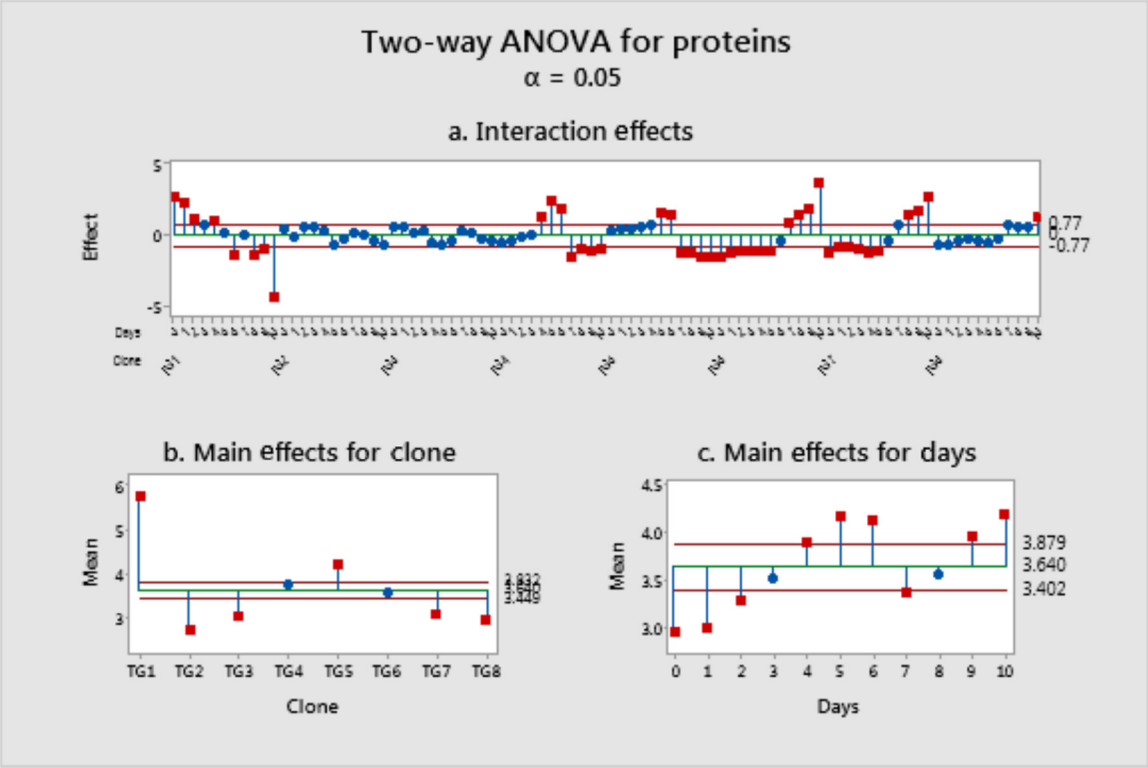

Figure 5 Two way ANOVA for total proteins, (a) interactive effects for clones and days, (b) main effects for clones and (c) main effects for days

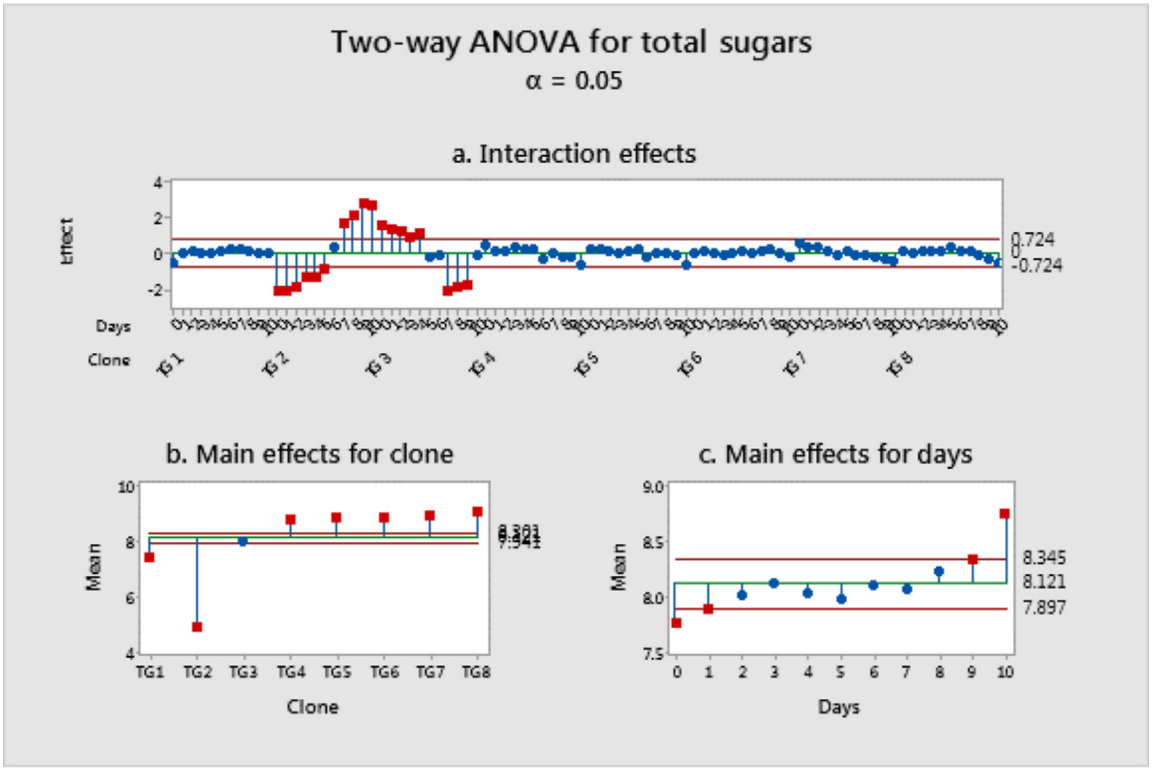

Figure 6 Two way ANOVA for total sugars, (a) interactive effects for clones and days, (b) main effects for clones and (c) main effects for days

rooting (Figure 6); the sugar levels were also maximum (about double the lowest levels) after day 10 (Figure 6c).

The RS content decreased during the observation period in clones TG 1,3,4 and 8 while those of TG 5 recorded a slight increase up to day 5, followed by a steady decrease. Clones TG 2, 6 and 7 recorded a steady increase in the reducing sugar levels (Figure 7a). Further, the decrease in RS levels on this medium was observed during subsequent stages. The clones recorded varying levels of reducing sugars, i.e., it was low in clones TG 1, 4, 7 and 8 while it was moderate in TG 6, while high RS levels were recorded in TG 2, 3 and 5 (Figure 7b). The levels of RS contents differed significantly during rooting from day 0 to day 10 (Figure 7c). A linear and steadily increasing trend was observed during the period of rooting from day 0 to 10.

Indole-3-acetic acid (IAA) content

Variation in IAA content in the plant tissues was observed during the entire study period from the first to the 10th day. On day 2, a sharp increase in IAA content was observed but it decreased slightly on day 6 followed by a sharp increase on day 8, registering about fourfold increase at this stage (Figure 8c). Significant differences in IAA contents of the microshoots were observed both clone-wise and over days (Figure 8). Clones TG 1, 2 and 8 recorded high IAA contents while the rest recorded low levels (Figures 8a & b).

Changes in enzymatic activities

The PPO and IAAO are considered as markers of adventitious rooting. High activity of PPO was recorded in clones TG 2 and 3 at the start of the experiment (Figure 9) while it was within the average limits in all other clones. Clones TG 1, 7 and 8 recorded low levels (Figure 9b). The mean effect of days (Figure 9c) pointed to high PPO activity at the beginning of the experiment, which decreased during the subsequent period until day 6. A sharp increase (almost two-fold) was observed in day 7 but declined sharply in day 8 during the root elongation phase. The PPO activity significantly differed in the different clones (Figure 9a).

Though the initial IAAO levels were high, they dropped drastically by days 1 and 2, later recording a slight increase on days 3 and 4. It remained almost constant between days 3–8

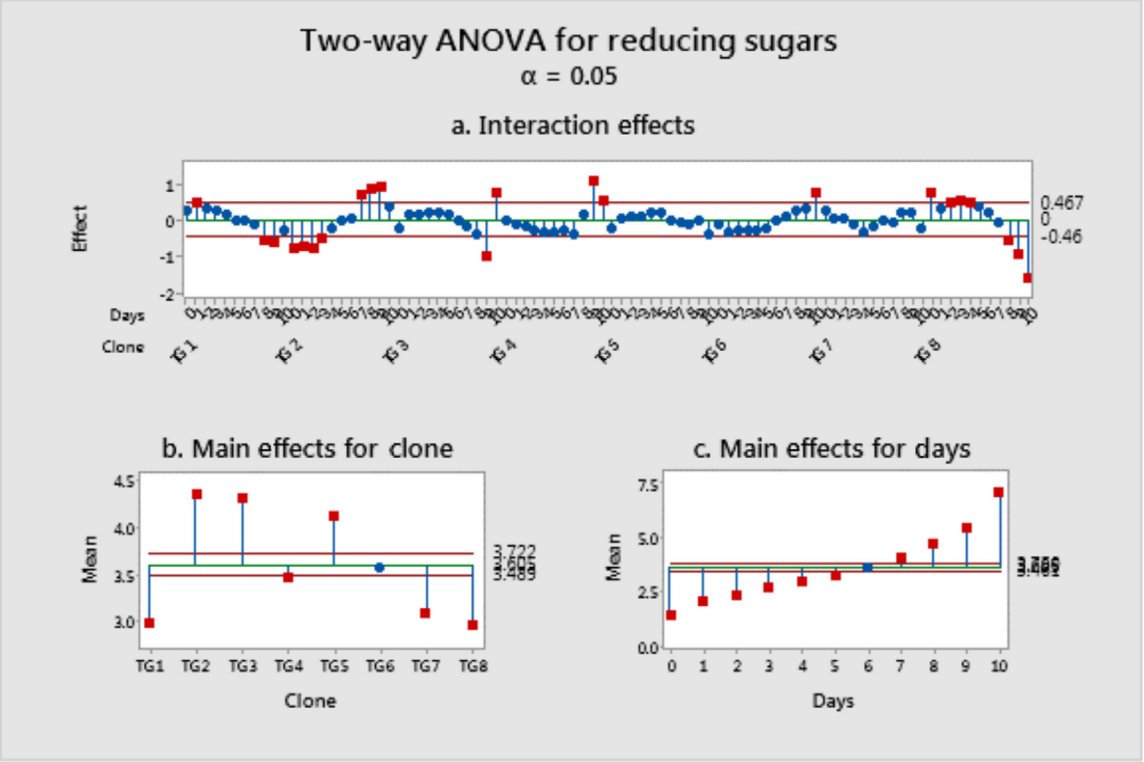

Figure 7 Two way ANOVA for reducing sugars, (a) interactive effects for clones and days, (b) main effects for clones and (c) main effects for days

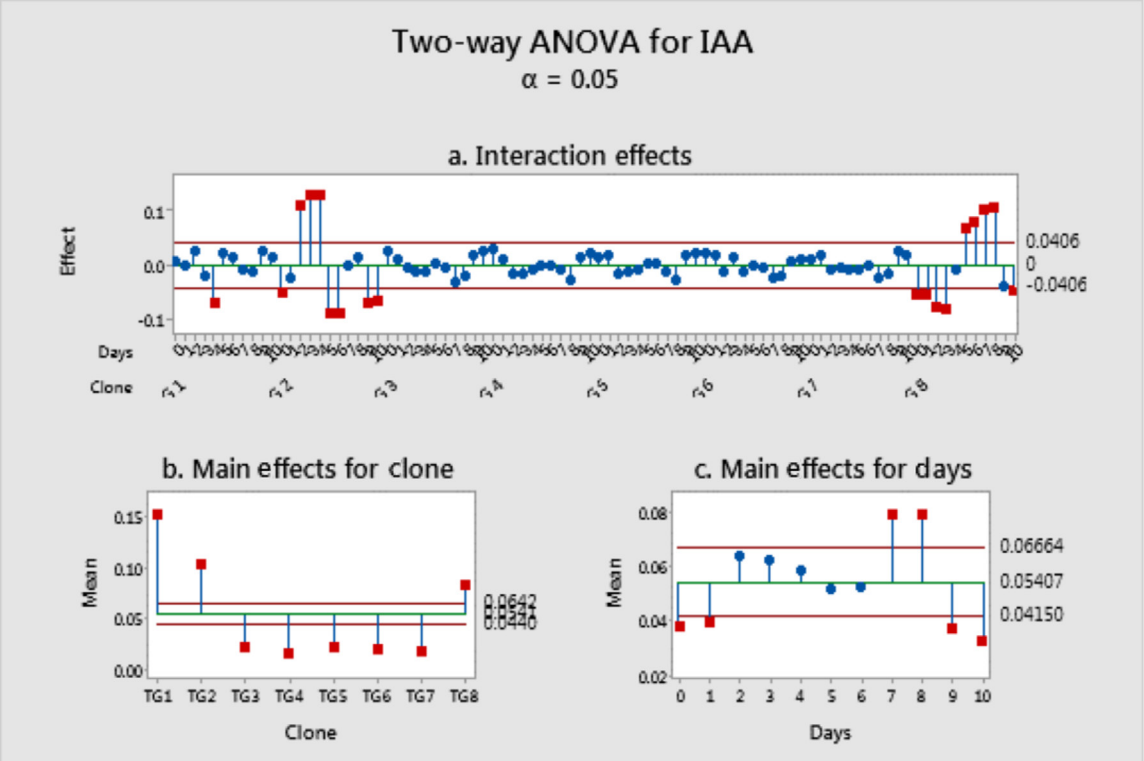

Figure 8 Two way ANOVA for IAA content, (a) interactive effects for clones and days, (b) main effects for clones and (c) main effects for days

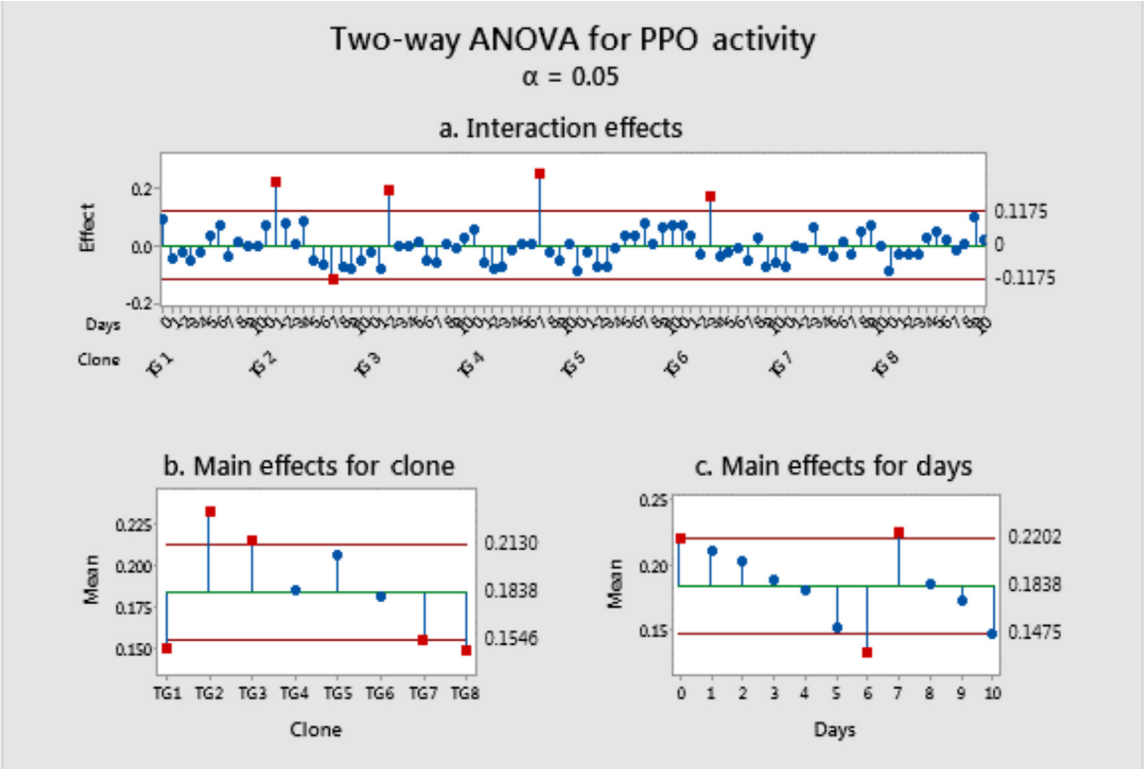

Figure 9 Two way ANOVA for polyphenol oxidase (PPO) activity, (a) interactive effects for clones and days, (b) main effects for clones and (c) main effects for days

(the root induction, initiation and emergence period), following which the levels increased (Figure 10c). Except for TG 2 and 3, all clones recorded low IAAO activity levels (Figure 10a & b).

DISCUSSION

Adventitious rooting plays a crucial role in micropropagation. The success of commercial TC depends on the efficiency of rooting of micro cuttings and the quality of roots. Various *in-vitro* propagation protocols have been developed for teak (Monteuuis et al. 1998, Husen & Pal 2001, Chia 2003, Mahalakshmi et al. 2018). However, reports on the influence of genotypes on the rooting process is limited (San Jose et al. 1992). Although root formation was observed in all the clones by the seventh day of the study, their root emergence varied. Clone TG 3 produced roots in three days, while clone TG 1 required seven days for emergence (Figure 1a). This information is crucial while planning large scale multiplication as variations in time leads to asynchrony, causing time lags in commercial production. Sawitri et al. (2020) reported that 11 clones identified for superior performance in Indonesia showed variations in their ability to root vegetatively. Clones TG 6 and 7 showed profuse rooting. Therefore, clone TG 7 was analysed histologically to understand the cellular differentiation process in teak roots.

Application of exogenous auxins influence the biochemical pool, triggering root formation. This leads to anatomical changes resulting in adventitious rooting (Heloir et al. 1996). Here, roots started emerging on the fifth day. Ahkami et al. (2009) reported different phases during adventitious rooting. In this study, the histological investigations revealed cell division induction (24 h), initiation (48 h), initiation of root primordia (72 h) and elongation (after 96 h); all four phases of rooting (Figure 3).

Medium compaction, a common problem encountered in nurseries, occurs when an external force applied to the growing medium's surface or even the medium's weight causes the mix volume to decrease. In vermiculite, as mentioned earlier, chances of compaction tend to occur when varied particle sizes are used. This affects root formation and architecture (Rogers & Benfey 2015). The different grades of vermiculite affected the root growth of the slowest rooter selected (TG 1). Root branching (lateral and short

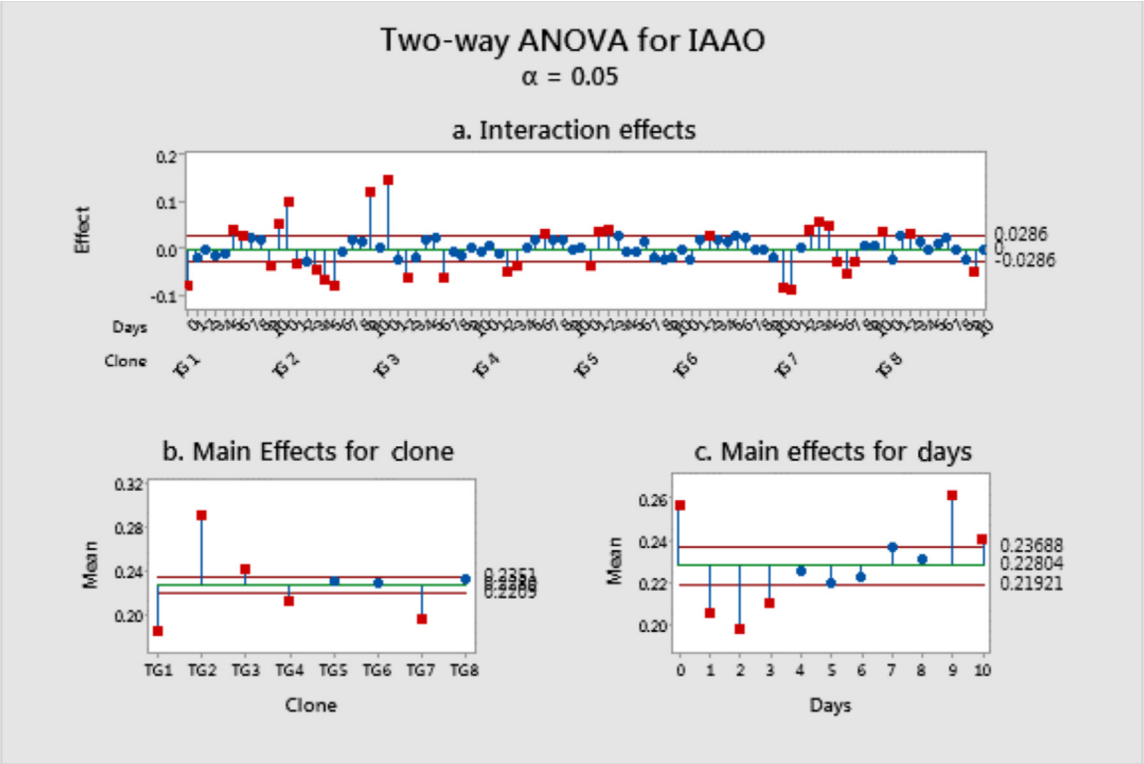

Figure 10 Two way ANOVA for indole acetic acid oxidase (IAAO) activity, (a) interactive effects for clones and days, (b) main effects for clones and (c) main effects for days

roots), permeability and water-holding capacity are interrelated (El Amrani & Amraoui 2020). Low root formation and elongation in Grades 1 and 2 in the current study could be attributed to compaction due to their small particle size. Fine roots were higher in Grade 3, suggesting better permeability and less compaction. An increase in particle size impedes lateral root extension (Garg & Ng 2015). It reduces the formation of smaller roots (Kormanek et al. 2015). In the case of perennials where root architecture plays a key role in the long-term establishment, maintaining a good root architecture is essential (Hartmann et al. 1997). The quality of the adventitious root system formed from the teak micro shoots requires focused attention. This study found that Grade 3 and above is optimal for better root formation from micro shoots. El Amrani and Amraoui (2020) observed that a medium rich in organic matter and vermiculite is optimal for better root architecture.

Biochemical variations observed during the entire rooting period indicated that root induction was related to variations of TSS, protein, endogenous auxin and enzyme activities. A sharp increase in the total soluble proteins over time from the day of planting up to day 6, followed by a slight decline on day 7 was noted. From day 8, it increased further (Figure 6c). The protein levels are reported to peak during the root induction phase, which might be attributed to different enzyme reactions triggering root formation (Rout et al. 1996, Haissig 1986). However, the PPO levels showed a sharp decline during the rooting period. Dalet and Cornu (1998) reported that PPO directly regulates primordial root development. However, in the current study, the findings could not correlate PPO activity with rooting. The PPO levels decreased steadily during the root initiation and induction stages, confirming that PPO activities do not define root primordia.

Interestingly, total soluble and reducing sugar contents increased, indicating that carbohydrates were produced and utilised in the micro shoots during adventitious root formation. Being an energy-demanding process, rapid solubilising of carbohydrates to total soluble sugars could have occurred (Sivaci & Yalcim 2007). Husen and Pal (2006) observed similar results in stem cuttings of teak. The RS, which steadily increased, may have triggered root primordia formation. Haissig (1986) reported auxin-treated cuttings to accumulate RS at the basal region, consistent with the current

observations (Figure 8c). Further increase up to day 10 suggests mobilising these energy sources for further development of the shoots.

The beneficial role of auxins, specifically IBA, in adventitious root induction in teak has been reported in many studies (Husen & Pal 2001, 2006). The IAA activity has also been used as a biochemical marker of the rooting phases (Corrêa et al. 2012). In the present study, endogenous levels of auxin were low during the first two days, namely day 0 and 1 (Figure 8c). This might be due to the inhibitory effect of the external auxin application (IBA, 500ppm). Accordingly, the IAA oxidase levels were also low (Figure 10c). From the third day onwards, IAA levels within the base cutting increased and remained high during root induction, initiation and elongation phases. A similar trend was observed in IAAO (Figures 8c & 10c). Once the roots emerged (day 8), the IAA levels decreased, triggering a similar enzyme IAAO production trend. This brings to light the stimulating effects of low concentrations of external auxin application, enhancing endogenous auxin production. Exogenous auxin application triggers lateral root primordium initiation (Sreevidya et al. 2010, Suraj & Varghese 2019). The IBA is commonly used due to its lower toxicity and ability to increase endogenous IAA levels (Han et al. 2009).

The beneficial role of IBA on rooting of micro cuttings or micro shoots has been well documented (San Jose et al. 1992). Between-clone differences also referred to as inter-clonal differences, are observed during the teak clonal propagation process (Monteuuis 1995). This requires adapting propagation methods to reluctant genotypes by understanding the physiological aspects of the cuttings. Though day-wise effects revealed a trend in biochemical changes, the biochemical levels varied substantially at the start of the experiment in the different clones. This may have contributed to the difference in the time duration of the emergence of the eight clones. Total soluble and reducing sugar levels were high in clones TG 3, which could have led to an early trigger of IAA, leading to a rapid emergence of roots. Though TG 1 had high levels of proteins, hydrolysis could have been slow.

Although the endogenous levels of IAA were high, the IAAO levels were still low. Further, the sugar levels were low. Resetting the biochemical pool to favourable metabolites could have contributed to the delay in root emergence. Another feature was the long roots in TG 1 compared with numerous and dense roots in TG 7 and 8. This indicates that sugar/protein hydrolysis influences root quality. Therefore, the metabolic pool has a direct bearing on the root architecture of the shoots.

CONCLUSION

This study revealed that in teak, an external stimulus of IBA triggers *de novo* synthesis of endogenous auxins. It triggers a sequence of events leading to changes in metabolic pool, culminating in root primordia initiation and root formation. The root architecture is influenced by the media used for propagation and biochemical. Genotype variation exists, hence basic information on the biochemical pool at the start of the experiment for different clones would help tailor the root architecture of teak. Being a perennial species, these initial efforts in planning would enable an efficient and cost-effective method for large-scale propagation. This would ensure synchrony in the TC process, reducing time lags in commercial production.

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