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Callus formation impedes adventitious rhizogenesis in air layers of broadleaved tree species

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Abstract. Callusing and root induction in air layering was evaluated aiming at evolution of procedure for mass clonal propagation of mature ortets of five tropical broadleaf species differing in their potential for adventitious root formation in shoot cuttings as: *Anogiessus latifolia* < *Boswellia serrata* < *Dalbergia latifolia* < *Gmelina arborea* < *Dalbergia sissoo*. Two experiments were conducted in rainy season during consecutive years; without application of growth regulators in the first year and with growth regulators (T_1 - water, T_2- 100 ppm indole-3-acetic acid, T_3-100 ppm thiamine- HCl and T4 -combination of T_2 + T_3) in the next year. Air layered branches were detached from the trees to record percentage of alive air-layers, callusing and rooting (%) as well as root number and root length. Response to air layering was found to be highly variable in five tree species but appeared to be feasible procedure for clonal propagation of mature ortets of *B. serrata* and *D. sissoo* with 100% (in auxin + thiamine treatment) and 83.3% (in auxin treatment) success, respectively. Maximum callusing (%) was found in *D. latifolia* while no callusing was observed in *D. sissoo*, which is most easy-to-root among all five species. Callus formation impedes adventitious rhizogenesis in air layers as significant negative correlation of callusing (%) and adventitious root formation was recorded in air layers of five tropical broadleaved tree species. Application of exogenous auxin alone or in combination with thiamine circumvents callusing to ensure direct development of roots for successful air layering.
Keywords auxin, callusing, girdling, rooting, thiamine.

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Introduction

Clonal propagation of selected superior genotypes of tropical tree species is difficult owing to maturity related loss in adventitious root potential which constitutes a serious constraint, in their *ex situ* conservation and mass multiplication for sustainable use. Air layering involving production of *in situ* adventitious roots on girdled tree-shoots, which are subsequently detached and out planted, may serve as an effective mean of clonal propagation of such rooting recalcitrant woody perennials. The girdled shoots with continuous supply of water and mineral nutrients also obtain complex of endogenous biochemicals from apices and expanding leaves for root induction and differentiation leading to plantlet production. The procedure is simple, economical and space saving as no separate cloning area, controlled environmental conditions and intermittent misting are required. Air layering also offers the benefit of higher survival rates and faster rooting process.

Air layering technique has been successfully used to propagate some of the most recalcitrant forestry species such as *Albizia procera* (Ansari et al. 1998), *Anogeissus pendula* (Gupta et al. 1997), *Elaeocarpus ganitrus* (Bhojvaid & Negi 2003) *Osyris lanceolata* (Mwang'ingo et al. 2006) and *Prosopis cineraria* (Solanki et al. 1986). However, on several instances in place of adventitious roots profuse callus formation occurs in the air layers even after ensuring optimal propagation conditions (moisture conservation and darkness) in air layers and treatment with growth regulators usually implicated in root induction and lapse of substantial time. In shoot cuttings of many species callus formation has been indicated as the precursor of root induction (Hartmann et al. 2010) but air layers behave in different manner showing callusing and adventitious rooting as independent physiological events (Sanjay Singh et al. 2004). Thus in the present study, callus and root induction in air-layering was evaluated aiming at evolution of procedure for mass clonal propagation of mature ortets of five tropical broadleaf species.

Materials and methods

Mature trees of five important tropical broadleaved species differing in their potential of adventitious root formation in shoot cuttings as: *Anogiessus latifolia* < *Boswellia serrata* < *Dalbergia latifolia* < *Gmelina arborea* < *Dalbergia sissoo* revealed in our earlier investigations (Ansari & Sanjay Singh 2005) were used to conduct two experiments in consecutive years. In the first experiment on the onset of monsoon (rainy) season in the month of July three trees of each species in approximately 15 year old plantations were selected and air layered on 10 shoots (i.e. 3 replications of 10 each) by removing bark, cambium and phloem with the help of a sharp knife. Moist Soilrite® (inert rooting media) was placed at the girdled area of the branch, which was subsequently covered with non-transparent black polythene sheet. No growth regulator treatment was provided to air layers.

In the next year, the influence of growth regulators was investigated. Four treatment viz., water (T_1), 100 ppm indole-3-acetic acid (T_2), 100 ppm thiamine-HCl (T_3) and combination of $T_2 + T_3$ (T_4) were administered on the girdled area with the help of cotton before covering with soilrite and polythene sheet as described in the first experiment. Each treatment for a single species contained 40 layers in four trees (10 per tree) in randomized block design.

After 45 days the air layered branches were detached from the trees to record percentage of live air-layers, callusing and rooting (%) as well as root number and maximum root length i.e. the length of longest adventitious root. The data were statistically analyzed employing analysis of variance (ANOVA), '*F*'-test to ascertain significance at $P \leq 0.05$ and least significant difference ($LSD_{0.05}$) values to separate means in different statistical groups (Gomez and Gomez 1984). The percentage data were transformed employing arc sine square root transformation prior to statistical analysis.

Results

A good number of air layered shoots remained alive after 45 days in first experiment where no exogenous treatment was employed (Table 1). Only *D. sissoo* and *B. serrata* responded positively to air layering with 68 % and 52 % adventitious rooting, respectively. Better root number and root length was recorded for*D. sissoo* than *B. serrata.* Formation of callus at the girdled areas was also observed especially in *D. latifolia.*

Significant interaction of species and treatments was recorded for live shoot (%), callusing (%) and rooting (%) but not for root number and length. Combined dose resulted in significantly superior (100%) rooting in *B. serrata* but in *D. sissoo,* IAA proved to be the best with 83% rooting (Table 2). Maximum

Table 1 Response of air layering in tree species without application of growth regulators

Species	Live shoot (%)	Callusing (%)	Rooting (%)	Root Number	Root length (cm)
A. latifolia	68.0	36.00	0.00	-	-
B. serrata	52.0	16.00	52.00	6.62	4.90
D. latifolia	68.0	58.00	0.00	-	-
G. arborea	44.0	24.00	0.00	-	-
D. sissoo	72.0	20.00	68.00	30.34	10.64
$LSD_{0.05}$	NS	8.92	11.37	17.75	3.46

Table 2 Interaction of tree species and growth regulator treatments on characteristics of air layering in Experiment 2 for the species; AL (*A. latifolia),* BS (*B. serrata),* DL (*D. latifolia),* GA (*G. arborea)* and DS (*D. sissoo*)

Interaction	Characteristics				
	Live Shoot (%)	Callusing (%)	Rooting (%)	Root Number	Root Length (cm)
AL x T_1	99.7	0.0	3.1	0.7	1.7
BS x T_1	50.0	0.0	91.7	24.8	10.1
DL x T_1	99.7	91.5	3.1	0.3	0.3
GA x T_1	50.0	75.0	0.0	0.0	0.0
DS x T_1	75.0	0.0	75.0	30.1	13.9
AL x T_2	99.7	32.9	11.7	2.0	3.0
BS x T_2	75.0	3.1	91.7	23.6	8.8
DL x T_2	100.0	0.0	0.0	0.0	0.0
GA x T_2	0.0	11.7	25.0	1.3	1.3
DS x T_2	91.8	0.0	83.3	17.1	14.5
AL x T_3	0.0	0.0	0.0	0.0	0.0
BS x T_3	75.0	11.7	91.7	8.2	7.2
DL x T_3	100.0	32.9	3.1	0.3	0.3
GA x T_3	58.7	99.7	0.0	0.0	0.0
DS x T_3	25.0	0.0	75.0	16.4	8.2
AL x T_4	91.8	3.1	11.7	2.0	4.5
BS x T_4	100.0	0.0	100.0	27.9	10.0
DL x T_4	100.0	100.0	0.0	0.0	0.0
GA x T_4	41.4	67.1	25.0	1.0	1.0
DS x T_4	91.8	0.0	75.0	16.0	12.8
$LSD_{0.05}$	21.3	18.4	27.2	NS	NS

callusing (%) was found in *D. latifolia* while no callusing was observed in *D. sissoo* (Table 2).

In five species exogenous application of IAA alone or in combination with thiamine ensured better success in rooting of air layers. However, the influence of individual thiamine treatment or combined with IAA was more prominent for live shoot (%) as observed in *D. latifolia* and *G. arborea* (Figure 1).

Discussion

Air layering technique is successful in propagating plants because the layered branch is not separated from the mother plant and, therefore, receives continuous supply of water and mineral nutrients through the xylem and remains alive (Hartmann et al. 2010) and intact shoots (with leaves) possibly synthesize some unknown auxillary substances which help in induction of adventitious roots (Sanjay Singh et al. 2004). Branch girdling also causes blockage in the acropetal and basipetal flow of photoassimilates in the phloem, causing an accumulation of several metabolites (organic compounds, carbohydrates, auxins, etc.) above this region (Alves-de Oliveira et al. 1999) that favors the formation of callus and development/activation of adventitious root primordia to develop into roots (Sanjay Singh et al. 2004). In addition the absence of light in the area of root formation, use of substrates to provide continuous moisture, and moderate temperatures are factors that favor rooting in the girdling zone (Ramirez-Villalobos & Urdaneta-Fernández 2004). Still different tree species differ in their potential of plantlet regeneration through air layering. In the present study also the response to air layering was found to be highly variable in five tree species. Two species *D. sissoo* and *B. serrata* appeared amenable for air layering even without growth regulator treatments but better rhizogeneis was in the later reflected in better root number and length.

Exogenous application of auxin-type growth regulators can speed up the rooting process in air layers (Hackett 1988, Bacarín et al. 1994, Rahman et al. 2000, Naz & Aslam 2003). Auxins promote meristematic differentiation, and thus, their accumulation contributes to the formation of root primordia/callus in the girdling zone of the branch (Tchoundjeu et al. 2002). Improvement in the success of air layering occurred in all species in treated condition in our second experiment. The overall success of plantlet production was observed as *B. serrata*

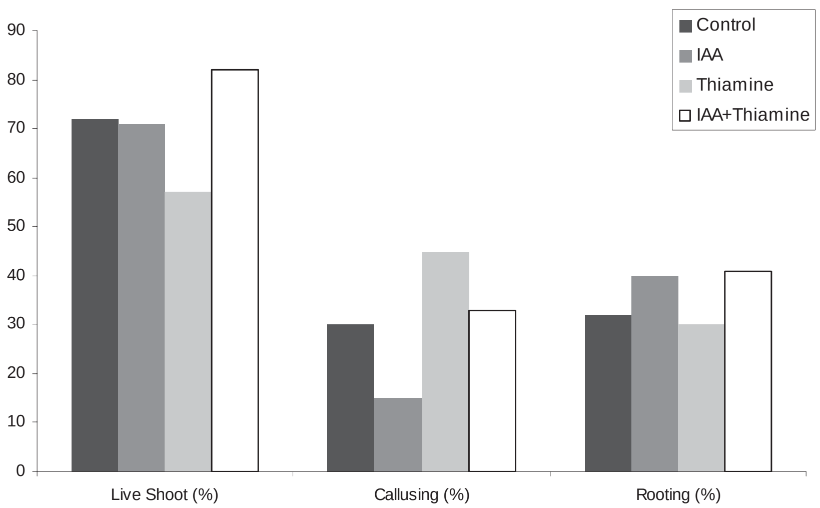

Figure 1 Percentage of live shoot, callusing and rooting in different treatment employed for air layers in five tropical trees

> *D. sissoo* > *G. arborea* = *A. latifolia* > *D. latifolia.* Combined dose of IAA and thiamine resulted in optimized rooting (100%) in *B. serrata.* However, IAA alone was effective with 83% rooting in *D. sissoo* (Table 2). *D. sissoo* is most easy-to-root among all five species and no callusing was observed here while maximum callusing (%) was found in *D. latifolia* a difficult to root species.

Callus development is common with wound healing where it appears as a thin layer around the wounded end of cuttings or girdled parts of air layers. This newly formed callus consisted of irregular unspecialized parenchymatous cells. The callus cells could further differentiate and form root primordia or serve as a protective layer for the formation of adventitious roots from other tissues of the layered branch (Colodi et al. 2008, Hartmann et al. 2010). Adventitious roots frequently emerged through the callus, leading to the belief that callus formation is essential for rooting because differentiation within callus led to formation of root initials (Hartmann et al. 2010). Adventitious roots are able to originate from callus tissue formed at the base of cutting, although connection with the main vascular system of these initials was extremely difficult (Davies et al. 1982). Skolidis et al. (1990) reported that, callus developed as a first step in process of root regeneration, followed by induction of root initials or primordias, indirect regeneration and root emergence in plum rootstock and cultivars. Kyonku & Balta (2004) reported in tea leaf bud cuttings adventitious roots can initiate in callus, thus callus formation must precede root initiation. However, cuttings with excellent callus often failed to root, but many cuttings with poor callus developed root primordia and elongated. Evidently callus formation is an event that generally precedes adventitious rhizogenesis but callus and adventitious root formation appear to be independent phenomena in most plants. Simultaneous appearance of these tissues may happen because both are influenced by the same environmental factors (Salisbury and Ross 1986, Burch & McGraw 1996, Acosta et al. 2000, Colodi et al. 2008). Not much work has been conducted on the relationship of callusing and adventitious rooting in air layers but in case of shoot cuttings callus formation has been reported to hinder root formation process (Spethmann & Hamza 1988) and it can be the consequence of unusual hormonal proportion due to higher exogenous treatments (Stefancic et al. 2005). Our findings reveal that callus formation impedes adventitious rhizogenesis in air layers as significant negative correlation of callusing (%) and adventitious root formation in air layers of five tropical broadleaved tree species was recorded (r = 0.556, p <0.05; n = 20) (Figure 2).

In air layers profuse callusing usually occurs on the upper part of the girdled shoot indicating towards accumulation of photosynthates, auxins and auxiliary substances at abnormally high levels. Although IBA increases the elasticity of cell walls, accelerating division, excessive concentrations of the hormone may inhibit this process (Rahman et al. 2000). Exogenous auxin application in air layers during periods of active growth (high endogenous auxin levels) could raise hormone levels above the optimal concentrations, leading to a decrease in rooting (Moreira et al. 2009). A similar trend was obtained in air layers of *M. rupestris*, where concentrations higher than 1,500 mg L-1 IBA resulted in a reduction of dry and fresh weights of the roots (Durán-Casas et al. 2013).

Both callusing and adventitious root formation occur in same time and space on shoot cuttings and/or girdled branches of air layers and apparently have similar growth requirements. Thus, it is possible that the processes compete for the available resources in plant. Appearance of callusing prior to adventitious rooting seems to be immediate response of the plant to heal the wounded part and not a perquisite for adventitious root induction. According to Sánchez et al. (2009), in air layers performed on guava (*Psidium guajava*) plants, with increasing concentrations of IBA (2,000, 4,000, and 6,000 mg L-1), callus formation was reduced, and its formation was completely inhibited at the concentration of 6,000 mg L-1 IBA. Mishra & Jaiswal (1993) also observed that only the air layers those treated with IBA exhibited rooting while other showed callusing. Thus, administration of exogenous auxin usually circumvents callusing to direct development of adventitious roots in air layers.

Adventitious root induction primarily depends on interaction between external environment and internal factors and interaction of the two. However, it is usual practice in forestry and horticulture to treat shoot cuttings with auxins which generally improve rooting. In addition a number of other biochemicals act either as auxin synergists to promote rhizogenesis or deterrent to rooting process (Fege and Brown 1984, Haissig 1984, Pythoud et al. 1986, Gasper & Coumans 1987, Kling et al. 1988, Del Rio et al. 1991, Tchoundjeu & Leakey 2000). Some reports have established augmenting role of auxins in rooting success in air layers too. For example exogenous application of IBA enhanced the root initiation and induced rooting in a shorter duration in *Elaeocarpus ganitrus, Osyris lanceolata* and some mangrove trees (Eganathan et al. 2000, Bhojvaid & Negi 2003, Mwang'ingo et al. 2006). However, no effect of exogenous auxin was recorded in other species e. g. *Cecropia obtusifolia, Inga feuillei* (LaPierre 2001, Brennan & Mudge 2004). Thiamine, with its established indispensability for *in vitro* growth and development of excised roots in many plants (Aberg 1961), may have a direct or indirect (as a synergist) role in rhizogenesis (Ansari & Kumar 1994, Palanisamy et al. 1998, Ansari et al. 2002, Sanjay Singh et al. 2005). We observed in the five species under study that exogenous application of IAA alone or in combination with thiamine enhanced rooting of air layers. However, the influence of individual thiamine treatment or combined with IAA was more prominent for live shoot (%) as observed in *D. latifolia* and *G. arborea* suggesting a synergistic role of thiamine rather than root initiating one, possibly by ensuring vigour of the air layers following wounding and removal of vital parts.

In conclusion, callusing may or may not precede adventitious rhizogenesis in air layers of tropical trees. Callusing and adventitious rooting appear to be independent phenomena

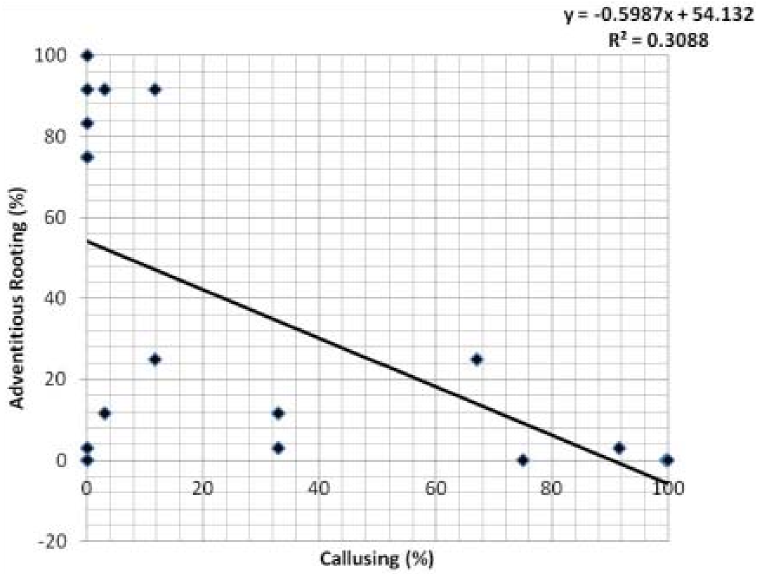

Figure 2 Linear regression analysis and specific correlation coefficient (R^2) between callusing and adventitious rooting in five tropical broadleaved tree species

occupying same time and space, competing for similar resources. Thus, callusing impedes adventitious root formation in air layers. Application of exogenous auxin alone or in combination with thiamine circumvents callusing to ensure direct development of roots for successful air layering.

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