

Recalcitrant effect associated with the development of microshoots on callus culture of *Adansonia digitata* L.

Mujeeb Ur Rehman¹, Manisha Chaudhary¹, Arvind Arya² and Sandeep Kumar^{1*}

¹Department of Biotechnology, Shobhit Institute of Engineering and Technology (Deemed-to-be-University), NH-58, Modipuram, Meerut, Delhi-NCR, Meerut-250110, (UP), India. ²Department of Biotechnology, Noida Institute of Engineering Technology, 19, Knowledge Park- II, Institutional Area, Greater Noida (UP), India.

*E-mail: dr.sandeepkumar@shobhituniversity.ac.in

Abstract

Adansonia digitata L. (Kalpvriksha, Baobab) of the family *Malvaceae* is an endangered flora in India. The micropropagation of woody plants, adventitious root and shoot development remain some of the major problems due to their recalcitrance to *in vitro* handling. In the present study, an efficient protocol for *in vitro* shoot regeneration and propagation of recalcitrant baobab trees was developed. Shoot induction on nodal explants was evaluated using various concentrations of 6-Benzylaminopurine (BAP), Kinetin (Kn) and Thidiazuron (TDZ) with NAA. The highest number of adventitious shoots per explants (3.2 ± 2.33) and longest shoots (3.3 ± 2.20) were recorded on 0.5mg/L BAP and 0.2mg/L NAA treatments. Three auxins, indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and Naphthalene acetic acid (NAA) were used in the induction of roots. The highest rooting was achieved with IBA-treated shoots at 1.0 mg/L concentration. Although *A. digitata* has previously revealed complexity in *in vitro* proliferation, the results proved efficient and reproducible. The 74% survival rates of plantlets was observed after three months.

Key words: *Adansonia digitata*; shoot culture; callus culture; recalcitrance; endangered species.

Introduction

The tropical African dry and hot climates are home to a plant known as baobab (*Adansonia digitata* Linn.). The generic name *Adansonia* commemorates French naturalist philosopher and explorer Michal Adanson (1727-1806). The specific name *digitata* refers with its finger like arrangement of the leaflets on the leaves. The large deciduous tree is one of the most beneficial in the savanna and may reach heights of over 25m from the ground. It has a variety of medicinal and non-medicinal characteristics. This tree is said to have uses for every part of it and is a highly economically valuable tree species (Rabiu and Murtala, 2013). Despite its economic potential, it is unlikely that the *A. digitata* species will continue to supply goods and services because of its mass value, and it cannot regenerate wildly due to constantly human pressure, seed dormancy, drought, and other environmental factors (Rahul *et al.*, 2015). The baobab has been said to be the most drought resistant of trees, during rains the trees swell greatly absorbing water can store thousands of liters absorbing water in their trunk for later use. Due to huge size, *Adansonia* is regarded as the “Queen of all carbon storage trees” (Sundarambal *et al.*, 2015).

The published surveys of baobabs in the Indian subcontinent attribute the introduction of baobabs to Arab traders (Patrut *et al.*, 2020) or medieval Muslim rulers in the subcontinent that maintained African slave armies (Wickens and Lowe, 2008), but without any supporting evidence from genetic, archaeobotanical or ethno-historical analyses (Davey *et al.*, 2003). These hypotheses imply a recent history of the baobab in the Indian subcontinent, which stand in contrast to significant evidence of a deep prehistory of biotic exchange between Africa and the

Indian subcontinent (Blench, 2003; Garcia *et al.*, 2011). These exchanges include the introduction of cereals such as sorghum (*Sorghum bicolor*), pearl millet (*Pennisetum glaucum*) and finger millet (*Eleusine coracana*), and legumes, such as cowpea (*Vigna unguiculata*), and hyacinth bean (*Lablab purpureus*) from Africa into the Indian subcontinent during the second and first millennia BCE (Fuller and Boivin, 2009; Lucek *et al.*, 2010), and the movement of zebu cattle (*Bos indicus*) in the reverse direction (Lesur *et al.*, 2014).

In vitro multiplication is becoming a powerful tool applied in research, for commercial production, and plant conservation. In terms of plant regeneration, recalcitrance may be a major limiting factor for the exploitation of medicinal plant species (Buendía-González *et al.*, 2007, Khezri *et al.*, 2024). Recalcitrance is the inability of plant cells, tissues, and organs to respond to the regeneration program (Benson, 2000b; Irshad *et al.*; 2017; 2018) Some recalcitrant species can be established and propagated in *in vitro* aseptic condition. Due to the physiology of donor plants and the physiological stress of *in vitro* plants (, 2000; Venkatachalam *et al.*, 2017), different cellular skills to respond by somatic embryogenesis and/or oxidative stress by organogenesis may contribute to the recalcitrance of *P. quadrangularis* and *P. cineraria* culture.

The micropropagation of woody plant species, adventitious rooting and high shoot multiplication remain some of the major bottlenecks due to their recalcitrance to *in vitro* manipulation (Feito *et al.*, 1996; Baltierra *et al.*, 2004; Tereso *et al.*, 2008). Recalcitrance occurs when plant cells, tissues or organs do not respond to *in vitro* manipulations. This is a major hindrance in plant biotechnological applications (Benson 2000a). A myriad of

factors influences or trigger recalcitrant responses in woody plant species, namely, whole plant physiology of the donor, *in vitro* manipulations and *in vitro* plant stress physiology. Because of the physiology of the donor plant, some species are amenable to *in vitro* regeneration, whereas others are more difficult. Another physiological stress factors responsible for *in vitro* recalcitrance is the oxidation of compounds in explants tissues at the time of excision, especially in woody species with high levels of phenolic compounds. Browning of excised explants and the resultant discoloration of culture media is a major challenge in plant tissue culture systems (Huang *et al.*, 2002). Correct manipulation of the *in vitro* environment can reduce or overcome the problem of recalcitrance. This may be achieved by having an optimum balance of applied exogenous cytokinins and auxins. The aromatic cytokinin, 6-benzyladenine, remains the most widely used in commercial micropropagation worldwide. Recently, hydroxylated 6-benzyladenine derivatives, namely, meta-topolin, meta-topolinriboside and meta-methoxytopolinriboside, have been detected and identified in different plants (Nowakowska & Pacholczak, 2020; Monokari *et al.*, 2021), and may be beneficial in the micropropagation of woody plant species due to their different physiological effects on plant tissues. Several studies (Werbrouck *et al.*, 1995; Magyar-Tabori *et al.*, 2010) have reported on the beneficial effects of the toposin-family of aromatic cytokinins, and their potential as alternatives to 6-benzyladenine in micropropagation.

In vitro propagation techniques are potential alternatives to conventional vegetative propagation methods and many reports have shown the achievement of *in vitro* propagation in many leguminous trees such as *Bauhinia variegata* and *Parkinsonia aculeate* (Mathur and Mukuntha Kumar, 1992), *Albizia odoratissima* (Rajeswari and Paliwal, 2006; Borthakur *et al.*, 2011), *Pterocarpus marsupium* (Husain *et al.*, 2007), *Acacia chundra* (Rout *et al.*, 2008), *Albizia chinensis* (Borthakur *et al.*, 2011) *Adansonia digitata* (N'Doye *et al.*, 2012) and *Dalbergia sissoo* (Vibha *et al.*, 2014). Several woody plants have been successfully regenerated through micropropagation techniques including *Allanblackia stuhlmannii* (Neondo *et al.*, 2011), *Pterocarpus santalinus* (Chaturani *et al.*, 2006) and *Hagenia abyssinica* (Feyissa *et al.*, 2005). Therefore, the objective of the present study is to develop *in vitro* propagation protocol for *A. digitata* through nodal culture from mature trees.

Materials and methods

Plant material: Nodal segments (2-4 nodes) were obtained from 05-06 years old *A. digitata* tree, located at Shobhit University, Meerut (U.P) India.

Sterilization of the explant: The explants were first cleaned by immersing them in a 5% Tween 20 solution, followed by rinsing with distilled water for one hour. After cleaning, the explants were cut into segments measuring 2-4 cm, each containing two or three nodes. Surface sterilization was then performed using 70% ethanol for 35 seconds, followed by treatment with a 0.1% (w/v) mercuric chloride solution for 3-4 minutes. The segments were then thoroughly rinsed with sterile distilled water four times. After trimming both ends, the sterilized explants were inoculated onto MS medium supplemented with varying concentrations of auxin and cytokinin to induce shoot formation.

Growth condition and media: MS (Murashige and Skoog, 1962)

basal medium were used in this study. The media were adjusted to pH 5.8, solidified with 8g/L agar and autoclaved at 121 °C for 15-17 min. The culture tubes contained 20 mL of MS medium for all experiments. Cultures were inoculated at a temperature of 25±2 °C under a 16/8 h photoperiod provided with cool white, fluorescent light.

Induction of shoot bud: Effect of different concentration of plant growth regulators on MS medium (semisolid/liquid) was compared. The conduct experiment was repeated three times; each treatment consisted of 10 explants. The concentrations were followed: BAP-0.2-6.0 mg/L and NAA-0.1-1.0 mg/L After four weeks of inoculation, the number of shoots, length of shoot and induction of callus were recorded.

Proliferation of shoots: Nodal segments were cut off and transferred into fresh MS medium supplemented with different concentration of BAP, TDZ, Kn (0.1-2.0 mg/L) in combination with NAA (0.1-0.5 mg/L) individually in order to standardize the highest rate of shoot proliferation; there were 18 treatments in total. The plant growth regulators were compared during the phase of subculture, and the optimum medium was selected, after four, six and ten weeks of inoculation, the multiplication rate percent shoot response, number of shoots and length of shoot were recorded.

***In vitro* rooting and acclimatization:** Healthy shoots were cut off and transferred into rooting MS medium supplemented with different concentration of IBA, IAA and NAA (0.1-1.0mg/l). Observations were recorded after every 5 days. To optimize the best root initiation medium was chosen as the rooting medium and auxins. The percentage of root induction, root number and root length were observed and recorded after 6 weeks and 10 weeks. After 10 weeks in culture, *in vitro* rooted plantlets were planted in small plastic pots in a 2:1:1 vermicompost: sand: garden soil mixture and placed in mist house for 2-3 months for acclimatize in *in vivo* environment. The survival rate was calculated after one month.

Data analysis: Each experiment was repeated three times with each treatment comprising 10 explants, resulting in a total of 30 explants per treatment. Data represent the mean of three experiments, and results on percentage culture response, number of shoots per explant, and rooting were observed at regular intervals. The results are presented as mean ± standard deviation of three replicates.

Induction rate (%) = The number of induced explants/The number of total initial explants × 100%

Average shoot or root number = The total number of shoots or roots/The number of shoots or rooted plantlets.

Rooting rate (%) = The number of the rooted plantlets/The number of total shoots × 100%

Results and discussion

Induction of shoot bud: The sterilized nodal explants were inoculated into the shoot induction media and then initiated growth after 10-20 days, while petioles began to fall off and small green buds appeared. The incubation period of the pathogens represents the time, when contamination was observed and it varies on average from 3-4 days. The pathogens have been shown to be both fungi and bacteria. The axillary bud induction phase was observed between 4-5 weeks. The lower concentration of BAP added, the earlier buds sprouted. Range analysis shows that

BAP had the most influence on the induction rate. The induction rate was only 34% in the case of higher BAP concentration and the lateral buds initiated late; furthermore, the new buds were thin and delicate in liquid semisolid media. The advantageous BAP concentration is 0.2 mg/L, NAA 0.3 mg/L with MS in semisolid and the induction rate reached 60%. Previous studies also showed that BAP induced the maximum response (Fig. 1, Fig. 4-A and B). Comprehensively considering growth and induction rate, semisolid and liquid MS medium supplemented with 0.5 mg/L BAP and 0.3 mg/L NAA, turned out to be a better medium for *in vitro* induction, as it supported maximum shoot bud induction. After six weeks old culture on TDZ with NAA were observed induction of brown callusing (Fig. 4- C and D).

In some studies, have reported difficulties in the aseptic of explants, which is the most important step in the micropropagation protocol. Generally, there are four possible sources of contaminants: internal or external contamination of the parent plant, insufficiently nutrient media sterilized, cleanness of laboratory, and erroneousness on the part of the researcher (Onwubiko *et al.*, 2013). Contamination with bacteria, fungi, yeasts, or viruses has been recognized as the most important

cause of *in vitro* culture failure (Cassells, 1991; Leifert *et al.*, 1994; Rehman *et al.*, 2023a; Chaudhary *et al.*, 2023)

This study was to establish a complete *in vitro* direct clonal propagation regeneration protocol in *Adansonia digitata* using nodal explants. Among the previous investigations of other authors, we did not find any reports containing results regarding a complete *in vitro* regeneration protocol in *A. digitata* from nodal explants. In *A. digitata* (baobab) shoots were obtained from nodal segments of *in vitro* germinated seeds on ½ QL medium containing 10 µM BAP (Ishii and Kambou, 2007). In another study in this same species the *in vitro* propagation was carried out from different types of explants taken from twenty-day aged sterile seedlings such as cotyledonary nodes, axillary nodes and terminal apex, and in the presence of 0.5 mg/L BAP, a multiplication rate of 2.31 was obtained for apex explants, 1.88 for axillary nodes and 2.0 for cotyledonary nodes (N'Doye *et al.*, 2012; Rehman *et al.*, 2023b). As observed in *A. digitata*, where an average of 2.0 shoots per explant was induced, only one shoot per explant was observed in *O. pyramidale*, which showed a very strong apical dominance, although the formation of nodes by seedling was higher (average number of nodes per shoot of 3.2).

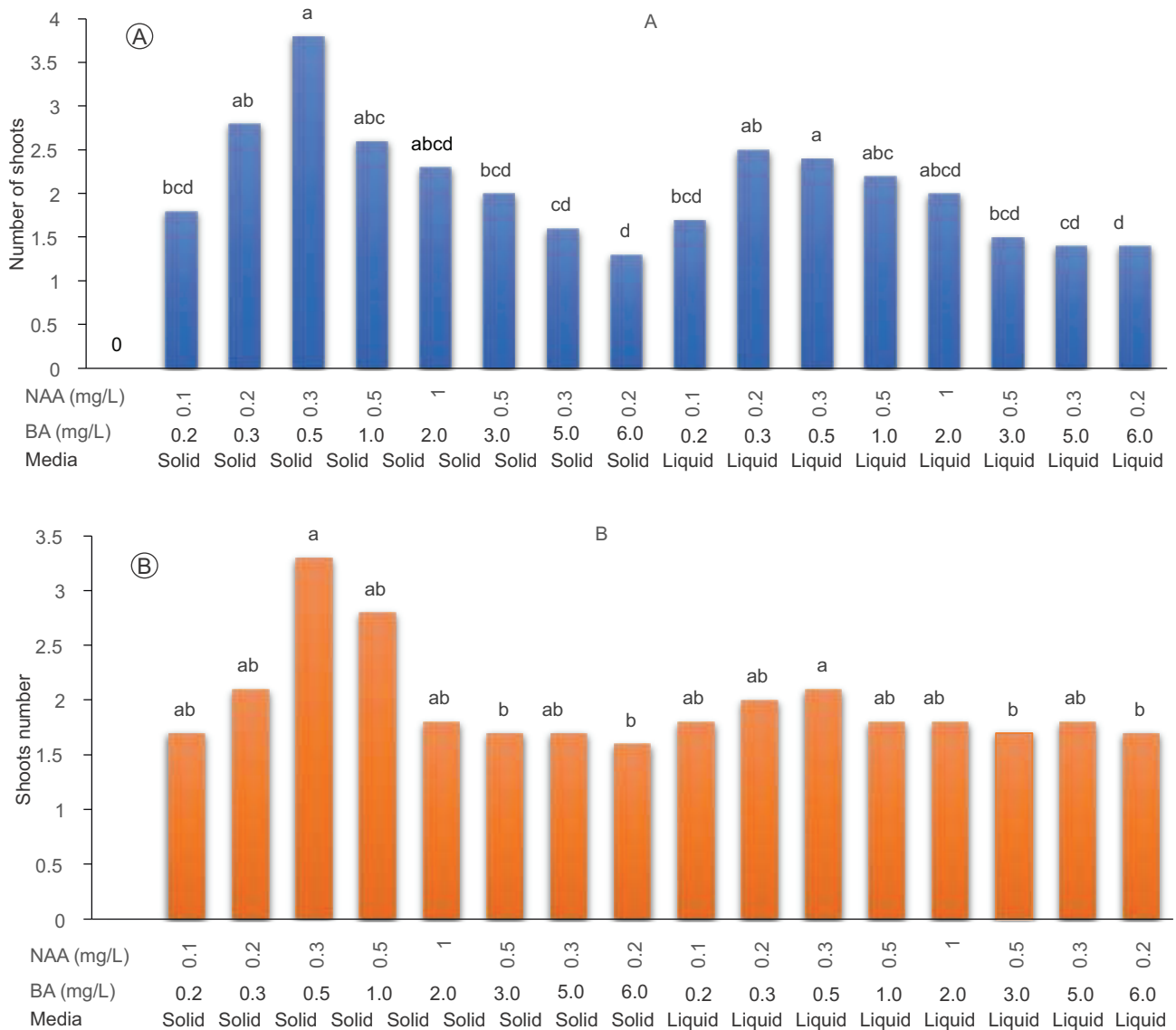


Fig. 1. Effect of plant growth regulators on the induction of shoots (A: Mean No. of shoot and B: Mean No. of shoot length in cm) of nodal explant of *Adansonia digitata* after 6 weeks (10 Explants in each concentration)

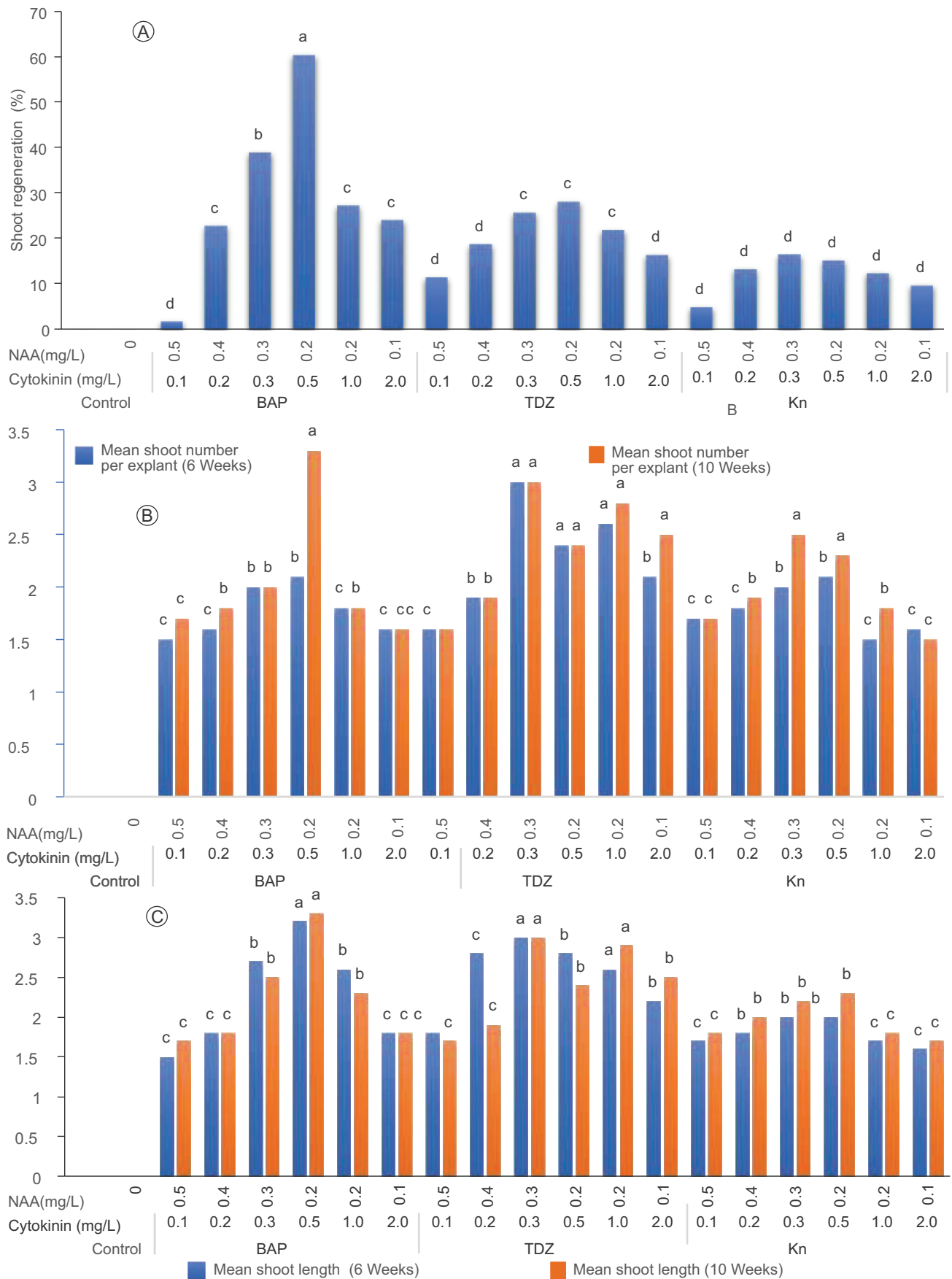


Fig. 2. Effect of cytokinin and auxin on MS media for shoot proliferation from node explants of *Adansonia digitata* [A: Regeneration %, B: Mean shoot number per explant 6 and 10 weeks, C: Mean shoot length (cm) in 6 and 10 weeks].

Proliferation of shoots: The MS medium is a significant substrate for plant tissue culture. Shoot buds from explants were subcultured on MS supplemented with BAP, TDZ, Kn in combination with NAA to screen the optimal medium. Among the 18 treatment media (Fig. 2), the best shoot bud proliferation and elongation were observed on MS medium, which proliferated to

2.8 times than the original after Six weeks (Fig. 2). While, there was no more significant difference observed in the multiplication rate and shoot numbers between MS with different combination of cytokinin and auxin, the growth state of the buds was totally different. The bud clusters on the MS medium with supplemented BAP-0.5 mg/L and NAA-0.2 mg/L were green and booming,

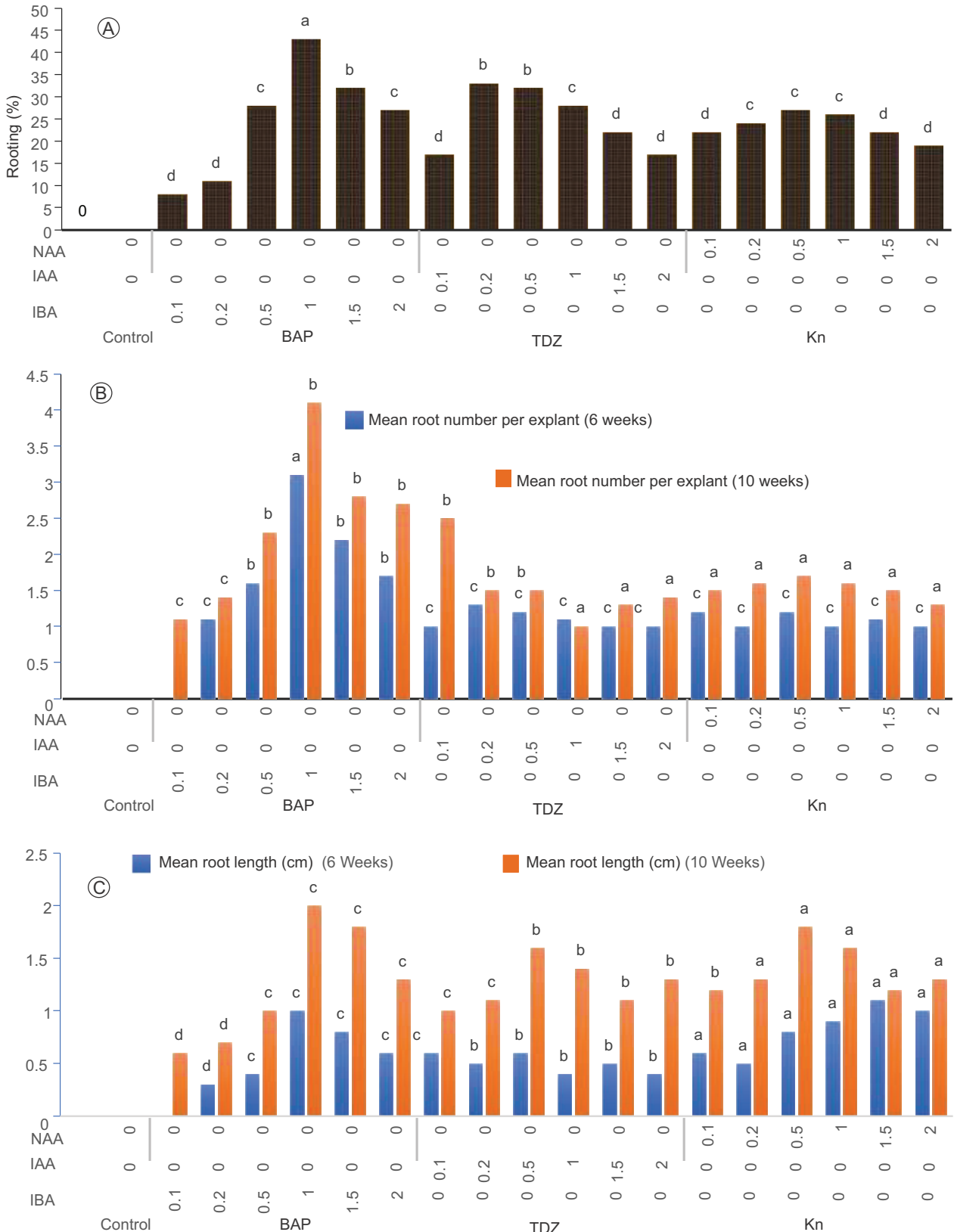


Fig. 3. Effect of different concentrations of auxins on root induction and multiplication of *A. digitata* [A: Rooting %, B: Mean root number per explant 6 and 10 weeks, C: Mean root length (cm) in 6 and 10 weeks].

showing no defoliation or callus. After six weeks, shoot buds were subcultured on MS medium supplemented with different combinations of BAP and NAA to screen for the optimal combination and concentration of BAP and NAA. It seems that, there was an increase in the multiplication rate with the decrease of BAP concentration under the same auxin level. Shoot proliferation, it decreased both in the multiplication rate and shoot numbers with increasing NAA-0.2 mg/L concentration under the BAP-0.5 mg/L level. Higher number of multiple shoots occurred on the media with low BAP concentrations (Fig. 4-E). Defoliation and vitrification occurred when NAA concentration reached 0.5 mg/L in the present study, plantlets do not grow well when the level of growth regulator is high. BAP promoted the optimal proliferation at low concentrations among the various combinations tested, the highest rate (64%) of multiplication was observed on the medium fortified with the combination of 0.5 mg/L BAP and 0.2 mg/L NAA. However, the optimal growth state of the buds as well as the shoot length (3.3cm) was found on the medium fortified with the combination of 0.5 mg/L BAP or 0.3 mg/L TDZ or and 0.2 or .03 mg/L NAA (Fig. 2). In conclusion, the latter two combinations were more suitable for shoot bud proliferation and elongation ((Fig. 4-E). Kn and TDZ all combination with MS media were observed no significant result for multiplication of shoot bud.

Werbrouck *et al.* (1995) found that BA-treated *Spathiphyllum floribundum* plants only produced high-quality 9-glucosides in the basal portions, as opposed to the free base-BA and its riboside derivatives, which were present throughout the plant. 9 weeks after transferring the plants *ex vitro*, the 9-glucosides were still detectable in the plant tissues (Werbrouck *et al.*, 1995). However, after being transferred to *ex vitro* conditions for one week, the free base-BA and its riboside derivatives (9R-BA), which were initially present in negligible amounts *in vitro* (Werbrouck *et al.*, 1995), could not be discovered. In the present study, larger adventitious shoot were produced with Kn and TDZ compared to BA at same concentration, suggesting that the levels of irreversibly sequestered cytokinins were lower in the topolin-treated plants. *In vitro* shoot proliferation is highly problematic for woody species exhibiting episodic growth patterns due to predetermined developmental cues that are inherent in plant tissues (McCown, 2000). Vieitez *et al.* (2009) observed very slow *in vitro* shoot growth due to the episodic character of the woody species, *Quercus alba* and *Quercus rubra*. The current observation suggests that the inherent episodic growth cues of *A. digitata* have a profound effect on shoot proliferation, and could partially be alleviated by using the topolins in culture.

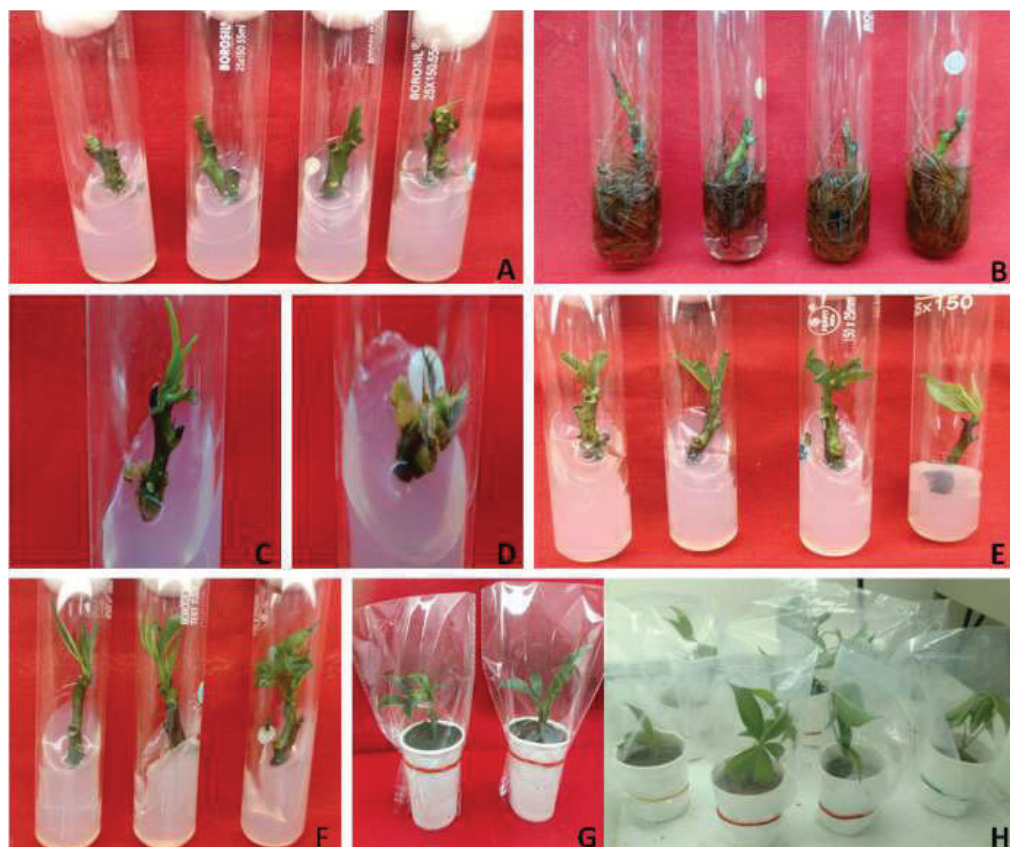


Fig. 4. *In vitro* propagation of *Adansonia digitata* using axillary node explants. (A, B) Shoot bud initiation. (C, D) Induction of callus after six weeks old culture (E) Multiple shoot bud regeneration. (F) Regenerated plantlets with well-developed roots. (G, H) Hardening and acclimatized plants.

In vitro rooting and acclimatization:

The incapability to initiate adventitious roots is often a limiting factor in conventional cuttings and tissue culture in *A. digitata*. The highest percentage of rooting (43%) with abundant lateral roots and no callus as well as the highest average root number of 3.1 was observed on MS medium supplemented with 1.0 mg/L or 1.5 mg/L IBA (Fig. 3). IBA, IAA and NAA, the percentage of rooting first better and then declined with the increasing concentration of auxin, which was reliable with the *in vitro* rooting in *A. digitata* (Fig. 4-F). Moreover, the quality of the subculture shoots evidently influenced *in vitro* rooting. As a result, it is important to obtain healthy normal shoots in the phase of multiplication culture. All the treatments were subcultured on same concentration of auxin as a previous medium. After ten weeks old culture, observed the root number and length of root (Fig. 4-G and H).

The hardening and acclimatization of tissue cultured plantlets was the most tricky and labor-consuming step because the newly transplanted plantlets were highly risk to fungal diseases. In the present study, the rooted plantlets were successfully transferred into plastic cups containing a vermicompost:sand:garden soil mixture at a ratio of 2:1:1 followed by a sequence of effective protection measures. After being transplanted, the plantlets must be watered and then covered with plastic film to maintain high humidity. Exposure to air and removal of fallen leaves and rotten seedling should be performed in a timely manner to prevent plant diseases and insect pests. The plastic film and shading net was removed after three weeks. The survival rates of plantlets reached 74% were observed after three months. Regenerated plants grew well in the pots and were morphologically similar to the mother plant.

In a similar study concluded on IBA is the most frequently utilized auxin in commercial micropropagation, and studies by Epstein and Ludwig-Muller (1993), De Klerk *et al.* (1999), and Fogaca and Fett-Neto

(2005) revealed that it was more stable and less prone to photo-oxidation than IAA. When compared to IAA and NAA in the current investigation, IBA consistently exhibited high rooting (50%) over a wider concentration range of 1.0-1.6 μM . That shows a minimal possibility of root induction at higher concentrations. De Klerk *et al.* (1995) observed that beyond a certain optimum level, higher concentrations of auxins could be inhibitory to root formation. Epstein and Ludwig-Muller (1993) attributed the superior rooting ability of IBA compared to IAA on the higher stability of IBA and possible differences in their metabolism and transport. New emerging evidence further suggests that part of the effects of IBA are caused solely by its direct action (Ludwig-Muller *et al.*, 2005), and this may partially account for the differences in rooting abilities observed in this study. *In vitro* rooted shoots were only able to persist in the *ex vitro* environment for two weeks before they succumbed. Success in *in vitro* rooting facilities acclimatization by improving water uptake (De Klerk 2002). However, the difficulties of *in vitro* rooting and acclimatization of woody species has been widely reported (Pullman and Timmis 1992). The *in vitro* rooting success in the present study was using IBA, but some of the roots were still brittle after 10 weeks, and could have matured after a longer culture period.

Practical implications: This study offers a novel, efficient protocol for the *in vitro* propagation of *Adansonia digitata*, which has significant implications for both conservation and commercial propagation. The ability to propagate *A. digitata in vitro* is particularly valuable for conservation programs aimed at preserving this culturally and ecologically important species. Furthermore, the developed protocol may support commercial endeavors by providing a reliable means of mass-producing *A. digitata* plantlets, which can be crucial in agroforestry systems and for the production of economically valuable baobab products.

The present study led to the establishment of a complete regeneration protocol by clonal propagation of *Adansonia digitata*. The nodal segments, the protocol proved to be efficient and reproducible. For the aseptic of nodal explants the most suitable formula proved to be the pretreatment with bavistin 1g/L, followed by the treatment with EtOH 70%, 30 min and HgCl_2 0.01%, 5 min. Regarding the induction and stabilization of the culture, the best phenotypic responses were obtained on the MS culture medium supplemented with 0.5 mg/L BAP and 0.2mg/L NAA. The highest shoot proliferation capacity of 64% and led to the maximization of biometric parameters like shoot length and the number of shoots per explant. Biometric parameters registered higher average values in the case of explants inoculated in a dark condition 4-5 days. BAP and NAA in subcultures induced the maximum multiplication of shoots. The healthy 10 weeks old multiple shoots for rooting, supplementation of MS medium with 1.0 mg/L NAA was shown to be the most efficient for induction and prolific. For hardening and acclimatization, the substrate proved to be important, thus, vermicompost: sand: garden soil 2:1:1 mixing substrate for the highest plant survival rate. Although *A. digitata* has been shown to be recalcitrant, *in vitro* propagation is suggested as a method of propagation for conservation of endangered species. On the basis of this study, it is recommended to apply the protocol both for the large-scale production of genetically uniform plant material, due to the ensured genetic stability, and also for the germplasm conservation.

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