

DEVELOPING AN OPTIMAL METHOD FOR THE ROOTING AND SEEDLING GROWTH OF MEDICINAL SPECIES *BUDDLEJA POLYSTACHYA* FRESEN: A STEP TOWARDS MASS PRODUCTION

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Abstract. For the improving regeneration and conservation of medicinal *B. polystachya*, stem cuttings were obtained from trees in Aseer area, Saudi Arabia, and two experiments were conducted to investigate factors affecting rooting (season and auxin type and concentration) and assessing growth performance of rooted cuttings. To test rooting factors, cuttings were collected during autumn, winter, and spring and treated with 500, 1000, and 2000 ppm indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and naphthyl acetic acid (NAA) during each season. Following the evaluation of rooting, the second experiment was conducted using the best rooted cuttings, to test growth performance of seedlings under the effect of different auxins and four types of growth media. Cuttings collected during spring and treated with IBA had the highest rooting percentage (91%–98%), number of roots (23–29 roots cutting⁻¹), and root length (26–39 cm). Regarding seedling growth, planting cuttings in growth substrate containing clay, sand, and peat moss (1:1:1 v/v) resulted in highest quality seedlings in terms of leaf number, stem height and relative growth rate. We concluded that spring cuttings collection treatment with 1000 ppm IBA, and transplantation to fertile growth media can be a useful technique for mass production of *B. polystachya*
Keywords: propagation, auxins, cuttings, growth media, season

Introduction

The development of vegetative propagation methods is a useful tool for the conservation of genetic resources, domestication of tree species, and national and international forest conservation plans. Hence, vegetative techniques are used as an essential tool for the mass multiplication of superior phenotypes/genotypes and production of true to type uniform plants (Thakur et al., 2008). Rooting of stem cuttings is a simple and economically feasible method of vegetative propagation usually utilized for many tree species (Mewar and Naithani, 2016). Several factors affect rooting such as the mother plant age, season, and application of root exogenous promoting hormones. Therefore, it is essential to understand the critical factors influencing rooting (Shekhawat and Manokari, 2016; Siddiqui and Hussain, 2007).

Roots development of cuttings differs from species to species. A variety of pre-treatments are performed to facilitate the successful rooting of cuttings such as, application of auxins which is the most common pretreatment in the vegetative propagation of plants. Many studies suggested that auxins play detrimental role in rooting capacity by facilitating the earlier production of rooted cuttings that will necessary for vegetative propagation (Fogaca and Fett-Neto, 2005; Topacoglu et al., 2016). Adekola and Akpan (2012) and Sardoei et al. (2013) concluded that these auxins

included indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and naphthyl acetic acid (NAA). The rooting period of plants following auxin application varies from species to species. In addition to other factors, auxins play a vital role in controlling plant growth and development influence the production of primary, secondary, and adventitious roots; they are the most frequently used plant growth hormones for rooting of cuttings in nursery practice. The commonly used auxin is IBA is synthetic auxin commonly used as an effective hormone to promote adventitious roots initiation compared to natural IAA (Pop et al., 2011). Successful propagation with the use of cuttings needs to select the right time cuttings collection, and this is intimately related to the natural phytohormones contents (Cristofori et al., 2010). In general, the growth medium is the most important factor determining seedling quality in the nursery (Baiyerin and Mbah, 2006).

Buddleja is a genus of flowering plants comprising approximately 100 species native to the tropics in America, Asia, and Africa (Corte's et al., 2006; El-Sayed et al., 2008). *B. polystachya* Fresen. is a multi-branched shrub that grow up to five meters; however, it may irregularly reached twelve meters height under favorable environment. This tree is widespread in the semi-arid highlands near the Red Sea coastal and distributed naturally in some countries like Eritrea, Ethiopia, Saudi Arabia, and Yemen where grown around forest, usually beside watercourse, at elevations 2200-3600 m above sea level (Chaudhary, 2001; Mohamedkassm et al., 2013). Several *Buddleja* species have been used in traditional medicine in many parts of the world, and the roots, leaves, and flowers of various *Buddleja* species have been used in folk medicine (Corte's et al., 2006; El-Sayed et al., 2008; Mohammed et al., 2016) reported that crude extracts of *B. polystachya* leaves contain certain chemical constituents that could possibly lead to antimalarial drug development and further more Al Ati et al. (2015) isolated and evaluated fifteen compounds from *B. polystachya* growing wild in Asir, one of these compound ethyl acetate fraction showed the most significant anti-inflammatory activity. Unfortunately, this valuable species is now present in a list of seriously endangered species according to International Union for Conservation of Nature and Natural Resources (IUCN, 2016).

This research work represented step to develop efficient and easy methods for the rehabilitation and conservation of *B. polystachya*, an ecologically and medicinally valuable species, by identifying the appropriate season for collection of cuttings and determining the types and concentrations of auxins for vegetative propagation and factors improving seedling growth such as growth media and auxin types.

Material and methods

First experiment

Site and seasons of cuttings collection: Healthy and uniform woody stem cuttings (average length 15 ± 3.5 cm) were collected during specific months of each season, autumn (September), winter (December), and spring (March), from stock trees of *B. polystachya* growing wild as scattered trees among others tree species in the Aseer region ($18^{\circ}12'63''$ N, $42^{\circ}31'01''$ E; 2093.4 m above sea level), in the southwest of Saudi Arabia. This region is characterized by an average annual rainfall of 373.4 mm; monthly mean, minimum, and maximum temperature of 19.2 °C, 7.56 °C, and 31.0 °C, respectively; and relative humidity between 14.5% to 91.6%. All stem cuttings were collected in the early dawn (07:00 am) and kept in a plastic sealed ice container to

minimize drying. This experiment was designed to test the interaction effects of auxin type, their concentrations, and season of collecting cuttings on vegetative propagation of *B. polystachya* using stem cuttings. This experiment was repeated thrice during autumn, winter, and spring.

Experimental site

The study was conducted in a greenhouse at the College of Food and Agricultural Science, King Saud University, Saudi Arabia under controlled temperature (25 ± 1 °C) conditions with 12/12 h of light/darkness, and irrigated whenever needed during the period of the experiment. The vegetative propagation unit inside the greenhouse was covered using transparent plastic and equipped with a mist device.

Description and application of treatments

Three phases of the first experiment were conducted using three auxin types and concentrations, during autumn (September, October and November 2017), winter (December 2017, January and February 2018), and spring (March, April and May 2018) in order to test major factors affecting rooting ability of this species. Thirty treatments were applied, comprising three growth auxins, three concentrations of each auxin, and three seasons of cuttings collection (3 auxins $\times$ 3 concentrations $\times$ 3 seasons + 3 controls) (Table 1). The plant growth auxins (IAA, IBA, and NAA) were imported from Sigma. Stock solutions of the three auxins were prepared at concentrations of 500, 1000, and 2000 ppm by weighing the respective fresh weight (g) of each auxin and dissolving in distilled water. Cuttings were immersed in each auxin concentration for 5 min before planting in nursery beds (3 $\times$ 0.5 $\times$ 2 m) with sand rooting media and cuttings were inserted 5–6 cm deep using a wooden stick.

Measurements

After 12 weeks, the assessment of rooting success was performed by harvesting five replicates from each treatment to evaluate the root formation of stem cuttings collected for the period of the first collection season (autumn) by manually counting the number of rooted cuttings (for rooting percentage computation) and number of roots per cutting, whereas root length was measured from the point of emergence to the tip using a linear meter. The evaluation of rooting was performed in the same way for the remaining two seasons (winter and spring).

Second experiment

Following screening for the factors improving rooting from the results of the first experiment, the second experiment was performed to test the effect of auxin type and seedling growth media on the growth of seedlings. Results of the first experiment showed approximately higher rooting percentage, number of roots and root length occurring in cuttings treated with 1000 ppm of the three auxins and collected during spring than in those of the other treatments and seasons. Therefore, three replicates of cuttings from each treatment of 1000 ppm of IAA, IBA, and NAA were transplanted into four growth media (clay, sand, combination of clay + sand (1:1 v/v), and clay + sand + peat moss (1:1:1 v/v)). Table 2 showed the growth media properties, such as soil organic carbon percentage (OC %) was obtained as described by Nelson and

Sommers (1982), soil texture was determined using the micropipette method (Miller and Miller, 1987), and water-holding capacity of soil was determined by the pressure plate method as described by Topp et al. (1993). After 12 weeks, for the evaluation of seedling growth, number of leaves per cutting were counted manually and relative growth rate of seedling height RGR(H) was estimated according to *Equation1* (Ostos et al., 2008).

$$\text{RGR(H)} = (\text{LnH2} - \text{LnH1}) / t_2 - t_1 \quad (\text{Eq.1})$$

where: RGR(H) = seedling height relative growth rate; H1 and H2 = initial and final seedling height, respectively; t2 and t1 = initial time and final time in month respectively.

Table 1. Treatment descriptions

Treatments	Treatment description		
	Auxin	Auxin concentration (ppm)	Season
IAA*500*Autumn	IAA	500	Autumn
IAA*500*Winter	IAA	500	Winter
IAA*500*Spring	IAA	500	Summer
IAA*1000* Autumn	IAA	1000	Autumn
IAA*1000*Winter	IAA	1000	Winter
IAA*1000*Spring	IAA	1000	Summer
IAA*2000* Autumn	IAA	2000	Autumn
IAA*2000*Winter	IAA	2000	Winter
IAA*2000*Spring	IAA	2000	Summer
IBA*500*Autumn	IBA	500	Autumn
IBA*500*Winter	IBA	500	Winter
IBA*500*Spring	IBA	500	Summer
IBA*1000*Autumn	IBA	1000	Autumn
IBA*1000*Winter	IBA	1000	Winter
IBA*1000*Spring	IBA	1000	Summer
IBA*2000*Autumn	IBA	2000	Autumn
IBA*2000*Winter	IBA	2000	Winter
IBA*2000*Spring	IBA	2000	Summer
NAA*500*Autumn	NAA	500	Autumn
NAA*500*Winter	NAA	500	Winter
NAA*500*Spring	NAA	500	Summer
NAA*1000*Autumn	NAA	1000	Autumn
NAA*1000*Winter	NAA	1000	Winter
NAA*1000*Spring	NAA	1000	Summer
NAA*2000*Autumn	NAA	2000	Autumn
NAA*2000*Winter	NAA	2000	Winter
NAA*2000*Spring	NAA	2000	Summer
DW*0*Autumn	Distilled water	0	Autumn
DW*0*Winter	Distilled water	0	Winter
DW*0*Spring	Distilled water	0	Summer

Table 2. Growth media properties

Growth media	Property		
	Grain size (mm)	WHC (mm/m ²)	OC (%)
Sand	0.1-2.0	60-113	0.10
Clay	< 0.01	122-195	1.20
Sand + Clay (1:1)	0.075-0.10	106-148	1.32
Sand + clay + peat moss (1:1:1)	0.90-0.14	125-181	20.50

Experimental design

For the first experiment a factorial experiment ($3 \times 3 \times 3$) was set up using three different factors (three plant growth auxins, three concentrations of each auxin, and three seasons of cutting collection). The experiment was laid out in a completely randomized manner with five replicates per treatment. Then, the second experiment was designed as a factorial (3×4) of three auxins and four growth media.

Statistical analysis

The following statistical analyses were used: ANOVA was used to test the effect of treatments, the least significant difference multiple range-tests were used to identify differences between means, and the general linear model was used to test the interaction between the three tested factors (auxins, concentrations, and seasons of collecting cuttings). All statistical analyses were achieved by using SAS version 9.3.

Results

Rooting percentage

The interaction of auxin types, auxin concentrations, and time of collection of cuttings had highly significant ($p < 0.05$) effects on rooting percentage. All cuttings treated with the different concentrations of auxins showed a high mean rooting percentage (72%) regardless of collection season. However, although there is no significant difference between means of 1000, 2000 and 500 ppm IBA treatments, but the highest rooting percentages (98, 95% and 91%) were obtained in cuttings collected in spring when treated with the three levels of IBA (1000, 2000 and 500 ppm receptively), followed by cuttings treated with 2000 ppm of IAA and NAA (89.5%, and 86.4%) The minimum rooting percentages (59%, 63%, and 65%) were obtained in the control cuttings collected during the three season's autumn, winter and spring, respectively (*Fig. 1.*)

Number of roots per cutting

The interaction among auxin types, auxin concentrations, and collection season of cuttings on number of roots per cutting was highly significant. However, the combination of IBA, its concentration, and collection season resulted in differences in the mean number of roots per cutting, and cuttings collected during spring and treated with 500 or 1000 ppm of IBA, had the maximum roots per cutting (28.6 and 26.4 roots, respectively), followed by treatment with 2000 ppm IAA (24.6 roots) in comparison to the other treatments. In contrast, the minimum number of roots per cutting (1.0–2.8 roots) was recorded in the control cuttings during all seasons (*Fig. 2.*)

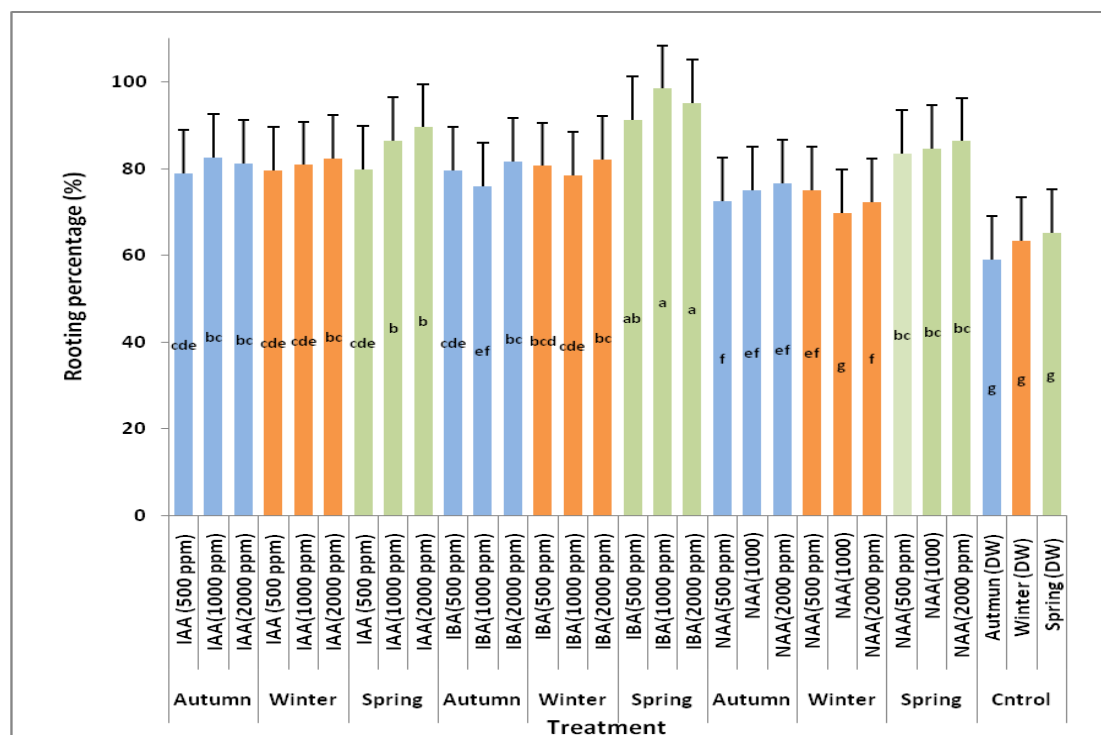


Figure 1. Effect of treatments (three concentrations of IAA, IBA, and NAA and three seasons of cuttings collection) on rooting percentage of *B. polystachya* after 120 days. Columns with different letters indicate least significant differences ($P < 0.05$). DW = Distilled water

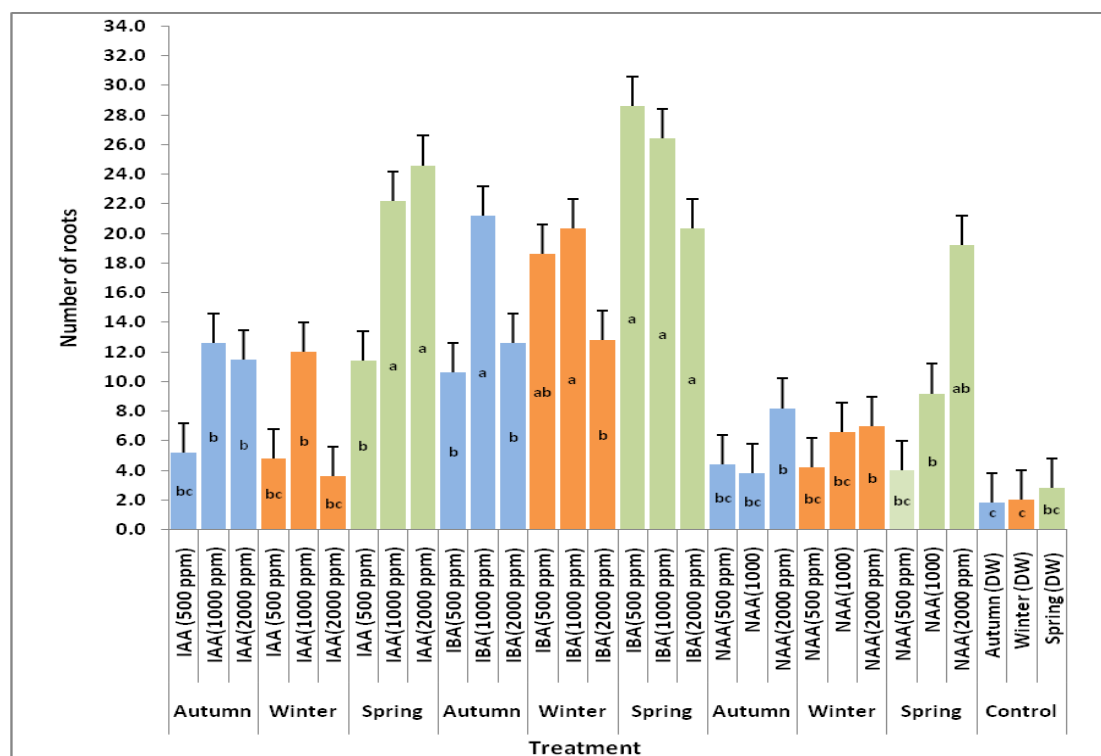


Figure 2. Effect of treatments (three concentrations of IAA, IBA, and NAA and three seasons of cuttings collection) on number of roots per cutting of *B. polystachya* after 120 days. Columns with different letters indicate least significant differences ($P < 0.05$). DW = Distilled water

Root length (cm)

The interaction effects of factors significantly improved root length in cuttings. The longest roots were 39.8, 27.5, 26.3, and 24.9 cm, recorded in cuttings collected during spring and treated with 500, 1000, and 2000 ppm of IBA and 2000 ppm of IAA, respectively. In addition, some cuttings collected during autumn had longer roots, especially under treatments with 1000 and 2000 ppm IBA. All control cuttings had the least root lengths (Fig. 3).

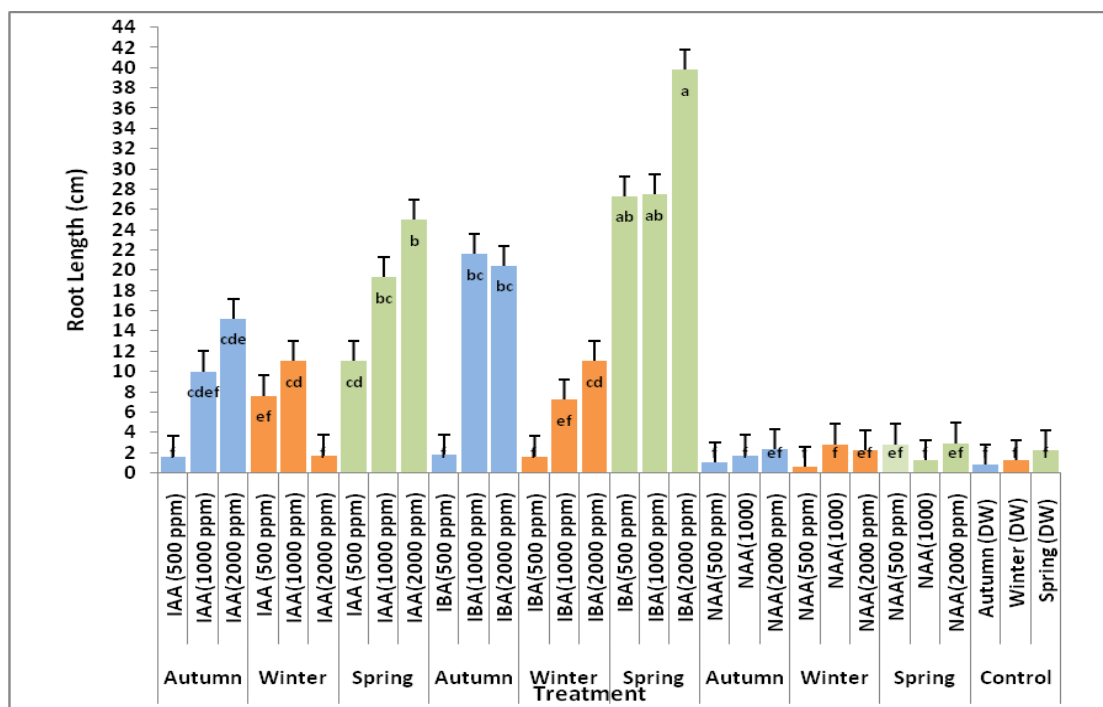


Figure 3. Effect of treatment (three concentrations of IAA, IBA, and NAA and three seasons of cuttings collection) on root length of *B. polystachya* after 120 days. Columns with different letters indicate least significant differences ($P < 0.05$). DW = Distilled water

Factors enhancing seedlings growth

Type of auxins and growth media significantly increased leaves per seedling. However, cuttings treated with IAA, IBA, and NAA grown in a mixture comprising peat moss with clay and sand (1:1:1 v/v) had the highest number of leaves (number of leaves increased by 144, 135, and 130%, respectively) compared to that in the control cuttings grown in the same media. In addition, cuttings treated with IBA grown in clay + sand (1:1v/v) had a higher percentage (115%) of number of leaves per cutting than the control seedlings grown in the same media. RGR(H) had the same trend as number of leaves per seedling, i.e., seedlings grown in media comprising a mixture of clay, sand, and peat moss had the highest rate of growth, followed by those grown in clay + sand media (Table 3).

Discussion

This study found that vegetative propagation of *B. polystachya* is considerably easy with the use of stem cuttings. However, for mass vegetative propagation aimed at

obtaining a higher rooting percentage and vigorous seedlings with the highest number of long roots within the shortest time, the best time for collection of cuttings is spring and 1000 ppm IBA and 2000 ppm IAA and NAA are the best concentrations for treatment with auxins. The vegetative propagation of *B. polystachya* from stem cuttings is to some extent easy with or without the application of auxins, but cuttings treated with auxins had significantly higher number of roots and root length, especially the treatment with IBA. All three auxins promoted adventitious roots formation in the stem cuttings (Fig. 4), which is necessary for the vegetative propagation of many plant species (Druege et al., 2019). Auxins or plant growth hormones were used effectively in many plant species to promote the rooting of cuttings (Soundy et al., 2008; Singh et al., 2011; Sağlam et al., 2014).

Table 3. Interaction effect of Auxin and growth media on leaves per cutting and RGR(H)

Treatment	Leaves per seedling	RGR(H) (cm cm ⁻² month ⁻¹)
IAA*clay	14.85 ± 1.30 ^{bc}	0.395 ± 0.035 ^{bc}
IAA*sand	18.67 ± 2.01 ^b	0.624 ± 0.015 ^{ab}
IAA*clay-sand (1:1v/v)	11.94 ± 0.96 ^{dc}	0.177 ± 0.011 ^{cd}
IAA*clay + sand + peat moss (1:1:1v/v)	28.41 ± 3.41 ^a	1.044 ± 0.118 ^a
IBA*clay	15.66 ± 2.07 ^{bc}	0.449 ± 0.084 ^{bc}
IBA*sand	15.66 ± 1.93 ^{bc}	0.468 ± 0.074 ^{bc}
IBA*clay-sand (1:1v/v)	25.10 ± 3.05 ^{ab}	0.920 ± 0.070 ^{ab}
IBA*clay + sand + peat moss (1:1:1v/v)	27.36 ± 2.65 ^{ab}	0.982 ± 0.03 ^{ab}
NAA*clay	14.47 ± 1.98 ^{cd}	0.369 ± 0.002 ^{bc}
NAA*sand	17.25 ± 2.11 ^{bc}	0.545 ± 0.012 ^{bc}
NAA*clay-sand (1:1)	13.00 ± 1.74 ^{bc}	0.262 ± 0.004 ^{bc}
NAA*clay + sand + peat moss (1:1:1v/v)	26.70 ± 3.07 ^{ab}	1.006 ± 0.027 ^a
DW*clay	10.50 ± 2.50 ^{cd}	0.049 ± 0.002 ^d
DW*sand	10.96 ± 3.22 ^{cd}	0.092 ± 0.041 ^d
DW*clay-sand (1:1v/v)	11.63 ± 1.87 ^{cd}	0.151 ± 0.023 ^{cd}
DW*clay + sand + peat moss (1:1:1v/v)	11.63 ± 1.74 ^{cd}	0.163 ± 0.024 ^{cd}
Level of significant (p-value)	< 0.0141	< 0.0001



Figure 4. Root density of stem cuttings collected during spring and treated with 1000 ppm of (a) IAA, (b) IBA, (c) NAA and (d) control

Various studies investigated the effects of different auxins on vegetative propagation via cuttings rooting and plant growth. Sevik and Guney (2013) study the effects of IAA, IBA, NAA, and gibberallic acid (GA3) in *Melissa officinalis*; Stefancic et al. (2005) investigated the effects of IAA and IBA in *Prunus spp.*; and Chhun et al. (2003) study the effects of IAA, IBA, and NAA in *Oryza sativa*. These studies generally confirmed that the auxins or hormones have a significant effect on rooting, and consistent with these findings, the present study confirms that auxins can be a useful substance for increasing rooting in cuttings (Salmi and Hesami, 2016). The results revealed that increasing the level of the IAA, IBA, and NAA enhanced rooting percentage, number of roots per cutting, and root length. The role of increasing levels of auxin is well documented. An increased auxin concentration in the stem cutting produced by active auxin transferred from the leaves activated adventitious root creation from the cambium (Justamante et al., 2019). We found that the appropriate time for *B. polystachya* cuttings collection is spring, and our results further confirmed that the season of cuttings collection is a critical factor for successful rooting. Our results confirmed the findings of other studies, the time of cuttings collection to be a major factor influence propagation of plant (Klein et al., 2000; Swamy et al., 2002; Haile et al., 2011). Ling and Zhong (2012) reported enhancing in rooting percentage of the Tetraploid Locust during May. Seasonal differences in successful rooting are common in woody plants, and the optimal season for rooting must be established independently for each species (Hussain et al., 2014). The rooting medium considered as proper atmosphere for rooting of cuttings (Tchinda et al., 2013), we used sand as the rooting medium in the first experiment, following Adugna et al. (2015) who used fine sand as rooting medium for *V. planifolia* stem cuttings and obtained 99.3% percentage of rooting. Although all seedlings were treated with the same concentrations of auxins and collected in the same season (spring), the interaction between IBA and combination of clay, sand, and peat moss was found to be more effective compared to that of the other two auxins and media (Table 3 and Fig. 5).

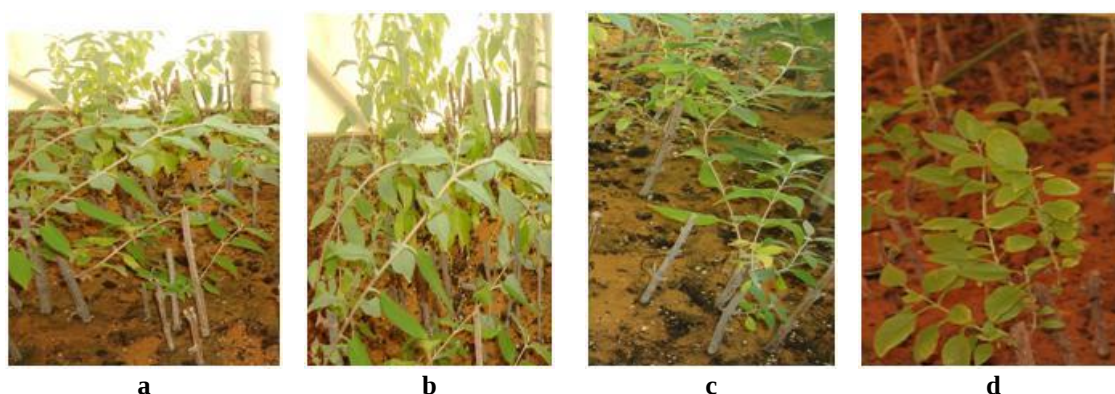


Figure 5. Growth performance of seedlings under the effect of media (clay, sand, and peat moss (1:1:1 v/v) treated with 1000 ppm of (a) IAA, (b) IBA, (c) NAA, and (d) control

The combination of clay, sand, and peat moss was the best growth medium in comparison to the other media. This interaction enhanced seedling growth in terms of increasing number of leaves and RGR(H). This can be due to the higher content of organic matter and water holding capacity of the medium. These results are previously conformed by findings of by Neelam et al. (2001) who reported that media with improved soil physical

and chemical traits and aeration led to superior plant growth and consistency with previous findings of Ahmed et al. (2017) who reported that media consisting of clay, sand and peat moss in equal ratios enhanced seedlings growth of *B. aegyptiaca*. Furthermore, there are several studies indicating the effect of organic matter on soil fertility (Martinez et al., 2003), survival and growth (Larchevêque et al., 2006), biomass (Moreno-Peñaranda et al., 2004), and seedling quality (Mañas et al., 2009).

Conclusions

This study developed an optimal method for the vegetative propagation of *B. polystachya* via woody stem cuttings. The results confirm that woody stem cuttings collected during the spring season and treated with 1000 ppm of IBA or 2000 ppm IAA or NAA had a high rooting percentage with high quality rooted cuttings, i.e., dense and longer roots. Rooted cuttings planted in a combination of clay, sand, and peat moss in equal proportion had dense, long roots. However, in general, seedlings resulting from stem cuttings treated with IBA were superior to seedlings from the other treatments in terms of shoot development (the highest number of leaves and stem height relative to the growth rate) after 6 months. Rooting of stem cuttings of *B. polystachya* is a feasible propagation method for producing high quality seedlings, which can be used as an adequate stock for rehabilitation, conservation, and future studies on the medicinal properties of this valuable tree species. Further research work should be needed to investigate mass propagation of this species using other methods of propagation as micro-propagation or crafting. More research will be needed to evaluate growth performance and or success of seedlings resulting from vegetative propagation under field or nursery conditions.

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