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Full Length Research Paper

In vitro shoot induction of *Acacia auriculiformis* from juvenile and mature sources

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Nodal segments of *Acacia auriculiformis* from juvenile (5 month-old and 14 month-old) and mature (72 month-old) genotypes were micropropagated through regular regime for more than 36 months *in vitro*. Shoot induction utilized six concentrations of BAP for 5 month-old and 72 month-old sources while 14 month-old and 72 month-old sources were treated with a combination of similar concentrations of BAP and 0.1 mg/l and 0.5 mg/l Kn respectively in Murashige and Skoog medium supplemented with 3 % sucrose and 3.75 g/L Gelrite. Nodal segments from both the juvenile and mature sources can be sustainably micropropagated in tissue culture. Overall, the juvenile materials displayed higher potential for axillary shoot induction and growth than the explants from mature sources for both treatments. Age of stock plant (juvenility of the explant) plays an important role on the success of tissue culture initiation. The highest shoot regeneration (100%) was obtained from the explants of 5 month-old seedlings whereas 85.71% of shoot regeneration was from explants of 72 month-old tree. For the combination of BAP with Kinetin, the highest percentage of shoot regeneration of 14 month-old seedling was 66.67 % with mean length of shoots 6.80 mm were obtained from explants cultured in 0.5 mg/L BAP plus 0.1 mg/L Kn whereas for nodal segments of 72 month-old tree, the highest percentage of shoot regeneration was 50% and mean length of shoots was 2.00 mm were in 0.1 mg/L BAP plus 0.1 mg/L Kn. It was observed that higher percentages of shoot regeneration were achieved from explants taken from younger stock plants than mature ones. The results also showed that low concentration of BAP (0.1-0.5 mg/L) were sufficient for shoot induction from explants of both sources. Similar pattern was observed when using combination of BAP and Kinetin. However, explants from mature sources (72 month–old tree) required a longer gestation period up to 15 months to start shoot multiplication whereas for explants from juvenile sources required no gestation period.

Key words: Acacia auriculiformis, micropropagation, juvenile, mature, BAP, Kn.

INTRODUCTION

Acacia auriculiformis A.Cunn. ex Benth. is native to savannas of Papua New Guinea, Islands of Torres Strait and northern Australia. In natural stands it is a vigorous tree, reaching a height of 30 m with a trunk up to 60 cm in diameter. Because of its ability to grow on very poor soil and in areas with an extended dry season, it has been introduced into countries such as India, Indonesia, Malaysia, Tanzania and Nigeria (Shukla *et al.*, 2007). The results from international trials of *Acacia* species and provenances have shown that *Acacia auriculiformis* is a useful multi-purpose tree species, being fast-growing and suitable for timber and pulp production (Phi, 2009). It was also reported that the oil from the seeds produced some medicinal properties such as spermicidal and anti-HIV properties along with the safe use on vaginal epithelium (Girljashankar, 2011).

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Abbreviations: BAP, 6-benzylaminopurine; **Kn**, Kinetin; **MS**, Murashige and Skoog.

In the past, the majority of forest trees have been propagated through the traditional family forestry method, where trees are grown from seeds and propagated sexually. Clonal propagation through tissue culture offers an alternative to vegetative practices and has the potential to provide high multiplication rates of uniform genotypes, resulting in short-term gains (Beck and Dunlop, 2001). The ease of *in vitro* cloning in many tree species depends on the degree of juvenility of the explanted tissues. Maturation is one of the key obstacles to successful vegetative propagation (Biondi and Thorpe, 1981). Vegetative propagation through micropropagation techniques is important for *A. auriculiformis* tree improvement and breeding programmes. Genetic gain could be achieved in a shorter rotation cycle by utilising improved materials selected from matured mother trees or sources which have proven to exhibit and acquire favourable quantitative and qualitative characteristics. Regeneration from seeds usually exhibit continuous variation to many characters. In this regard, *in vitro* shoot multiplication is worth and practical to consider as it will result in production of uniform stocks with high genetic stability. In addition, as indicated by a recent review by Pijut *et al*. (2012), most micropropagation of tropical hardwood has been mainly limited to short-term studies using juvenile plant sources.

The objective of this study was to evaluate the effects of different ages of sources on shoot induction of *A auriculiformis* utilizing different concentrations of BAP and its combination with Kinetin.

MATERIALS AND METHODS

Plant material and surface sterilization

Nodal segments (axillary bud) were collected from 5 and 72 month- old selected plus tree from a provenance trial at University Putra Malaysia while nodal segments from 14 months old tree were collected at Field 29, Forest Research Institute Malaysia. The nodal segment explants were surface sterilized in 70% ethanol for 30 seconds which were followed with three times rinsing in sterile distilled water. The explants were later soaked in 0.1 % Mercuric chloride followed by three thorough rinsing in sterile distilled water. Standard with strict safety procedures were adopted when handling mercuric chloride during surface sterilization and disposal of its waste.

Medium and culture conditions

The explants were then cultured onto standard Murashige and Skoog medium (1962) supplemented with cytokinin such as BAP (6 – Benzylaminopurine) alone at concentrations of 0, 0.1, 0.5, 1.0, 2.0 and 5.0 mg/L (0, 0.44, 2.22, 4.44, 8.88 and 22.22 µM) and combination of these concentrations of BAP with 0.1 or 0.5 mg/L (0.47 and 2.33 µM) Kinetin. The medium was also incorporated with 3 % Sucrose, solidified with 3. 75 g/L Gelrite and the pH was adjusted between 5.6 – 5.8 prior to autoclaving. The cultures were grown at 23 ± 2° C under 16-h photoperiod with light intensity of 22.22 µmol $m^{-2}sec^{-1}$ supplied by white Philips flourescent lights. The former experiment on the effects of BAP on shoot induction was done on nodal segments from 5 months old seedlings and 72 month-old sources; while the latter experiment on the effects of BAP and Kinetin was conducted on nodal segments from 14 months old tree and 72 month-old sources. Shoot induction capacities on the multiplication medium were assessed by recording the percentage of successful shoot regeneration, the multiplication rate corresponding to the number of new shoots produced and the length of shoot growth at each transfer. Results are expressed as means. Shoot production data were analyzed using SAS Statistical Analysis System package (ANOVA procedures, SAS Institute, Inc 2000), after appropriate transformation for homogeneity of variance when necessary. Further mean separation tests were evaluated using the Least Significant Difference (LSD) Test and means differing at a probability of $\leq$ 0.05 were considered to be significantly different.

RESULTS AND DISCUSSION

Effects of BAP on the shoot induction

Generally, nodal segments from both 5 month-old and 72 month-old sources are able to multiply shootlets in all concentrations of BAP tested including the control (Table 1). In general, those from 5 month-old sources produced significantly better shoot proliferation and growth when compared to those from 72 month-old sources (Figure 1). The time taken to proliferate was also shorter for those from juvenile sources. The best shoot proliferation for both sources is when being treated with 0.1 mg/L BAP where nodal segments from juvenile sources recorded 100 % shoot regeneration with shoot number of 1.00 and mean shoot length of 5.07 mm (Table 1). Qualitative observation on the shoots produced when treated in 0.1 mg/L BAP after one month clearly indicated that shootlets from 5 month-old sources were greener and prolific when compared to those from 72-month old sources (Figure 1). The shoots in MS (Murashige and Skoog) medium without any growth substance were less elongated and did not produce any multiple shoot. The growth of shoots produced from nodal segment explants of 72 month-old tree were rather inhibited. However, when the shoots were transferred to fresh medium monthly, these shoots then became elongated and produced multiple shoots after 15 months of transfers. On the other hand, the shootlet produced from nodal segment explants of 5 month-old sources were always healthy and produced multiple shoots right after first transfer.

Longer gestation period (long phase of surviving without further shoot multiplication) was observed when using explants from mature stock plants. Eventhough shoot induction and multiplication were successful when treated with cytokinin but subsequent growth was inhibited. Longer period was needed for the rejuvenation process to take place to enable the cells to divide and differentiate to produce new shoots. Thus, the problem of

Table 1: Effects of different concentrations of BAP on the shoot induction from nodal segment explants of 5 month-old seedling (left) and 72 month-old tree (right column) after a month in culture.

	Percentage of shoot regeneration		Mean number of axillary shoot		Mean length of shoot (mm)	
Age (month)						
BAP (mg/L)	**5**	**72**	**5**	**7 2**	**5**	**72**
0	80.00 ^{cd}	52.38 ^{c}	0.73 ^{bc}	0.90 ^{bc}	1.33 ^{b}	3.53 ^{b}
0.1	100.00 ^{a}	85.71 ^{a}	1.00 ^{a}	1.33 ^{a}	5.07 ^{a}	4.90 ^{a}
0.5	93.33 ^{ab}	61.90 ^{b}	0.93 ^{ab}	0.86 ^{c}	3.60 ^{a}	2.95 ^{bc}
1.0	86.67 ^{bc}	61.90 ^{b}	0.87 ^{abc}	0.95 ^{bc}	3.47 ^{ab}	2.43 ^{cd}
2.0	66.67 ^{e}	66.67 ^{b}	0.67 ^{c}	1.10 ^{b}	3.07 ^{ab}	2.43 ^{cd}
5.0	73.33 ^{de}	38.10 ^{d}	0.93 ^{ab}	0.62 ^{d}	3.50 ^{a}	1.62 ^{d}
Average	88.33	61.11	0.86	0.96	4.50	2.98

Note: Values having the same superscripts within the column are not significantly different at $P \leq 0.05$

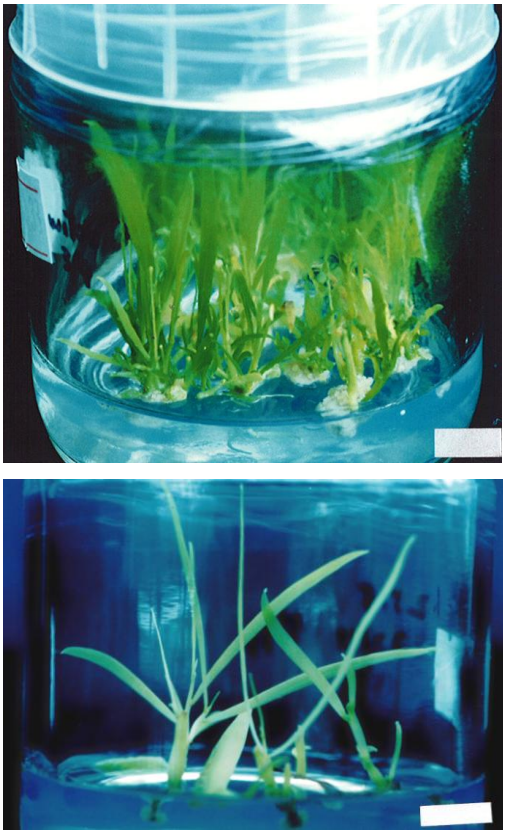

Figure 1: Shoot multiplication of *A. auriculiformis* in MS medium supplemented with 0.1 mg/L BAP after a month. Nodal segment explants was taken from 5 month-old seedling (top) and 72 month-old selected plus tree (bottom).

maturity versus juvenility of the explants is obvious in this study.

Comparing different levels of BAP (0 - 5.0 mg/L) on the shoot induction from two different ages of stock plants revealed that low concentrations of BAP (0.1 and 0.5 mg/L) were optimum and sufficient to produce the highest growth in terms of number of multiple shoots and shoots elongation for both ages of stock plants. According to

Table 2: Effects of BAP and Kinetin and their combinations on the shoot induction from nodal segment explants of 14 month-old sources after a month in culture

Hormone				
BAP (mg/L)	**Kn (mg/l)**	**Percentage of shoot regeneration**	**Mean number of shoot**	**Mean length of shoot (mm)**
0	0.1	33.33 [c]	0.47 [b]	3.13 [bc]
0.1	0.1	40.00 [c]	0.60 [b]	4.40 [b]
0.5	0.1	66.67 [a]	1.13 [a]	6.80 [a]
1.0	0.1	40.00 [c]	0.60 [b]	2.00 [c]
2.0	0.1	60.00 [ab]	0.80 [ab]	4.27 [b]
5.0	0.1	46.67 [bc]	0.53 [b]	2.67 [bc]
BAP (mg/L)	**Kn (mg/l)**	**Percentage of shoot regeneration**	**Mean number of shoot**	**Mean length of shoot (mm)**
0	**0.5**	46.67 [ab]	0.67 [ab]	3.73 [ab]
0.1	0.5	46.67 [ab]	0.60 [b]	3.40 [ab]
0.5	0.5	60.00 [a]	0.93 [a]	4.53 [a]
1.0	0.5	33.33 [bc]	0.47 [bc]	2.47 [bc]
2.0	0.5	46.67 [ab]	0.53 [bc]	3.40 [ab]
5.0	0.5	20.00 [c]	0.27 [c]	1.07 [c]

Note: Values having the same superscripts within the column are not significantly different at $P \leq 0.05$

Darus (1989) and Monteuuis (2004), juvenile and mature *Acacia mangium* genotypes can be sustainably mircropropagated. Darus (1991) also reported that low levels of BAP (0.5 mg/L) were sufficient to encourage for shoot multiplication and elongation. This is supported by Galiana *et al.* (1991) who reported that for juvenile explants of *A.mangium*, the optimal BAP concentration for good rooting rate and growth was 0.5 mg/L. Similarly in the case of *A.nilotica*, low levels of BAP (0.25 - 2.0 mg/L) either with or without Kn/ 2iP have induced axillary bud development (Singh *et al.,* 1993). In this study, for explants from juvenile and mature source of *A.auriculiformis*, low concentrations of BAP (0.1 – 0.5 mg/L) were sufficient for both shoot induction and multiplication.

Effects of BAP and Kn on the shoot induction

To encourage the growth of axillary buds and reduce apical dominance in shoot cultures of broad-leafed plants, one or more cytokinins are usually incorporated into the medium at initial establishment stage (George, 1993). Effects of combination of growth regulators (cytokinin) were also included in this study with combination of BAP and Kn. In fact, Rocha and Quoirin (2004) and Fotso *et al.* (2007) have also used kinetin in micropropagation of *Ricinodendron heudelotii* and *Swietenia macrophylla* respectively. Certain concentrations of BAP were best when combined with Kn, but some resulted in leaf defoliation and browning. For example culture supplemented with BAP 2.0 mg/L and 0.1 mg/L Kn induced callus growth instead of shoots. The obvious effect of combining BAP and Kn in this study was the tendency of shoots having leaf defoliation or which sometimes turned brown after several subcultures. Similarly, Singh *et al.* (1993) reported similar phenomenon in *A.nilotica* i.e. defoliation in shootlets upon subsequent second subculture when the shoot proliferation medium containing Kinetin (0.5 mg/L BAP, 0.25 mg/L Kn and 0.25 mg/L IAA).

Overall, nodal segments from younger sources i.e. 14 month-old produced better percentage of shootlet regeneration and better growth when compared to those raised from mature 72 month-old sources. For instant, nodal segments from 72 month- old sources recorded the highest percentage of shoot regeneration (50.00 %), the highest mean number of shoots (0.60) and mean length of shoots (2.00 mm) when they were cultured in 0.1 mg/L BAP plus 0.1 mg/L Kn. On the contrary, nodal segments of 14 month- old , recorded 66.67%, being the highest percentage of shoot regeneration, the highest mean number of shoots (1.13) and mean length of shoots (6.80 mm) when cultured in 0.5 mg/L BAP plus 0.1 mg/L Kn (Tables 2 and 3). In addition, it was also observed that culture treated with these combination required longer gestation period to initiate shoot multiplication for both sources.

Table 3: Effects of BAP, Kinetin and their combinations on the shoot induction from nodal segment explants of 72 month-old selected plus tree after three months in culture

Hormone				
BAP (mg/L)	**Kn (mg/l)**	**Percentage of shoot regeneration**	**Mean number of shoot**	**Mean length of shoot (mm)**
0	0.1	0.00 ^{c}	0.00 ^{c}	0.00 ^{c}
0.1	0.1	50.00 ^{a}	0.60 ^{a}	2.00 ^{a}
0.5	0.1	20.00 ^{b}	0.20 ^{b}	0.90 ^{b}
1.0	0.1	30.00 ^{b}	0.50 ^{a}	0.50 ^{b}
2.0	0.1	0.00 ^{c}	0.00 ^{c}	0.00 ^{c}
5.0	0.1	0.00 ^{c}	0.00 ^{c}	0.00 ^{c}

BAP (mg/L)	**Kn (mg/l)**	**Percentage of shoot regeneration**	**Mean number of shoot**	**Mean length of shoot (mm)**
0	**0.5**	10.00 ^{ab}	0.00 ^{c}	0.00 ^{c}
0.1	0.5	30.00 ^{a}	0.50 ^{a}	1.80 ^{a}
0.5	0.5	20.00 ^{a}	0.20 ^{b}	0.20 ^{bc}
1.0	0.5	0.00 ^{b}	0.00 ^{c}	0.00 ^{c}
2.0	0.5	0.00 ^{b}	0.00 ^{c}	0.00 ^{c}
5.0	0.5	20.00 ^{a}	0.20 ^{b}	0.70 ^{b}

Note: Values having the same superscripts within the column are not significantly different at $P \leq 0.05$

Conclusion

The study demonstrates that both juvenile and mature sources can be sustainably micropropagated. In this study, there was obvious effect of stock plant's age on the response of plant *in vitro*. Explants from 5 month-old seedlings responded better and quicker to micropropagation compared to 72 month-old tree. Longer period was needed to micropropagate plants from mature source. MS medium with BAP alone at 0.1 – 0.5 mg/L is sufficient to induce and multiply shoots of *A.auriculiformis in vitro*.

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