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Full Length Research Paper

Factors influencing *in vitro* shoot regeneration of *Macadamia integrifolia*

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A study was carried out to investigate the effect of culture medium factors that influence the shoot regenerative potential of Macadamia nodal segments *in vitro*. Explants were obtained from shoots of current growth flush of *Macadamia integrifolia* and inoculated onto different test media. Woody plant medium (WPM) gave results comparable to MS medium whose macronutrients had been reduced to half rate. Explants cultured on media gelled with Biotec agar No. 1 and Purified agar had significantly higher bud breaking frequency and shoot number per explant than Phytagel and Gelrite. Optimum culture performance was obtained on MS medium enriched with 30 g/L sucrose. Highest bud breaking frequency (98%), shoot number per explant (8.1) and shoot length (3.3 cm) were obtained when WPM was supplemented with 2 mg/L BAP, 1 mg/L IBA and 1 mg/L GA_3. When elongated shoots were cultured on to medium supplemented with cytokinins for rooting, only excessive callusing was obtained but no roots were formed within the culture period. The results of this study indicate that *M. integrifolia* is amenable to tissue culture but further studies are required to obtain rooting of *in vitro* shoots to come up with an optimized commercially feasible protocol for Macadamia tissue culture.

Key words: *Macadamia integrifolia*, *in vitro*, explant, nodal segments.

INTRODUCTION

Macadamia (*Macadamia* spp.) is the most important nut crop in Kenya, with an annual production of about 10,000 metric tons produced by over 100,000 small-scale farmers who depend on them for income and livelihood as it is a low-input crop (Waithaka, 2001). There are also about 500 large-scale growers with at least 1000 trees each (Muhara, 2004). It is a growing agro-processing industry that targets niche markets in Europe and the Orient (Rotich, 2004). Other growing countries include Hawaii, Australia, South Africa, Malawi, Zimbambwe, Guatemala, Brazil, Costa Rica and Fiji (Kiuru et al., 2004).

Macadamia is conventionally propagated vegetatively by grafting scions from selected parents onto rootstocks raised from seeds. This process is constrained by requirement for long nursery period of between 18 - 24 months, skilled manpower, material inputs and space (Nyakundi and Gitonga, 1993; Gitonga et al., 2001) compared to seedlings of other fruits which take 12 - 15 months in the nursery. The resultant increase in input costs together with other inherent inefficiencies of this system such as graft incompatibilities makes macadamia seedlings very costly. One grafted seedling is currently sold at USD 1.75 at subsidized rate in a government's operated nursery and over USD 2.00 in other commercial

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Abbreviations: WPM, woody plant medium; **MS,** Murashige and Skoog (1962) medium; **BAP**, 6-benzylaminopurine; **IBA**, indolebutylic acid; **GA_3**, gibberellic acid A3.

nurseries. This is far beyond the reach of many small scale resource challenged farmers who live on less than a dollar a day and would require at least 30 trees to make economic returns. This calls for development of a more efficient propagation procedure that would make planting materials more affordable. Tissue culture techniques are now widely used for the *in vitro* multiplication of nut crops such as chestnut (Chevre et al., 1983; Piagnani and Eccher, 1988; Chevre and Salesses, 1987; Radojevic et al., 1987; Mullins, 1987) and walnuts (Grussel and Boxus, 1990; Turemis and Comlekcioglu, 1994). Mulwa and Bhalla, (2000) reported successful tissue culture of *M. tetraphylla*. However, there are no reports to date on a viable tissue culture system for *M. integrifolia* or (*M. integrifolia* * *M. tetraphylla*) hybrids which form the main commercial cultivars in Kenya. In plant tissue culture, it is well known that no two genotypes give similar responses under a given set of culture conditions (Nehra et al., 1990). Successful *in vitro* culture of any species require basic empirical experimentation to optimize on the nutritional, growth regulator and culture growth conditions of the plant at each stage of the culture process (Williams and Taji, 1989; Williams, 1996). The objectives of the current study were therefore to determine the effect pH of growth medium, gelling agents, sucrose concentration, basal medium composition and level of phytohormones on the *in vitro* shoot regenerative potential of *M. integrifolia.*

MATERIALS AND METHODS

Explant preparation and sterilization

Shoots were obtained from twigs of current growth flush of mature field grown trees of cultivar KRG-15 (*M. integrifolia*) in early morning and put in moist plastic bags for transfer to laboratory. In the laboratory, leaves were removed taking precautions not to damage the axillary buds. Explants were washed in tap water with a few drops of TWEEN® 80 and rinsed thoroughly under running water for at least one hour. Other sterile manipulations were carried out in the laminar flow hood. The explants were sterilized in 70% ethanol for 15 s (just dip) and rinsed with sterile distilled water five times of three to five minutes each with agitation. Explants were then sterilized in 10% JIK® (Reckitt and Benkiser, Nairobi, Kenya) (providing 3.5% NaOCl equivalent to 0.35% pure NaOCl) for 10 min. This was followed by five rinses with sterile distilled water and air drying in the laminar flow hood before excising single node segments and inoculating them on tissue culture medium.

Culture medium

Culture medium consisted of either MS (Murashige and Skoog, 1962) or woody plant medium (WPM) (Lloyd and McCown, 1980) depending on experiment. Media were prepared from stock solutions of macronutrients, micronutrients, vitamins and iron-EDTA. The pH was adjusted before autoclaving the media for 20 min at 121ºC and 100 kPa.

Culture growth conditions and experimental design

Growth room conditions were maintained at 25 ± 3°C, 16 h photoperiod provided by cool white fluorescent bulbs (≈ 50 µmols s^{-1} m^{-2}). All inoculations were carried out in 16 x 150 mm test tubes half-filled with culture medium and sealed with aluminum foil and two layers of Parafilm (American National Can™). Each treatment contained three to five replicates with five to ten explants per replicate and all experiments were arranged in a completely randomized design. Culture period ranged between two to eight weeks depending on individual experiment.

Determination of optimum pH levels

Nodal explants of cultivar KRG-15 were inoculated on to MS (Murashige and Skoog, 1962) medium with the pH levels adjusted to either 5.2, 5.5, 5.8, or 6.0 before autoclaving the medium. Cultures were maintained under growth room conditions for 4 weeks and data taken on bud breaking frequency and general shoot growth.

Determination of suitable gelling agents

Preliminary experiments were initially carried out to determine to optimum gelling concentration of four gelling agents namely; PHYTAGEL® (Sigma-Aldrich, Inc 3050 Spruce street, St Louis MO63103 USA 314-771-5765), GELRITE™ (Duchefa Biochemie B.V, Haarlem, The Netherlands), purified agar (Sigma-Aldrich, Inc 3050 Spruce street, St Louis MO63103 USA 314-771-5765) and BIOTEC® agar No.1 (Biotech laboratories, U.K) using MS-based medium. Hence, the treatments applied consisted of MS medium gelled with either PHYTAGEL® at 4 g/l, GELRITE™ at 2 g/l, purified agar at 14 g/l or Biotech agar No.1 at 9 g/l. The media were then used for inoculation of nodal explants obtained from mature field-grown trees. Cultures were maintained under growth room conditions for seven and a half weeks and data taken on bud break frequency, shoot number per explant, length of longest and shortest shoot, and percentage of strong and weak shoots.

Determination of optimum sucrose concentration

Four levels of sucrose; 15, 20, 25 and 30 g/l were used to supplement MS medium with macronutrients at half strength. pH was adjusted to 5.7 and media were gelled with 9 g/l Biotech agar No 1. Nodal explants obtained from mature field-grown trees were cultured on this media and maintained under growth conditions for seven weeks and data taken on bud breaking, shoot number per explant, shoot length including length of longest and shortest shoot, and percentage of strong shoots.

Effect of tissue culture basal medium composition on culture performance

The initiation medium consisted of either Murashige and Skoog (1962) medium based salts with the macronutrients either at half or full strength depending on treatment and all other components at full strength, or woody plant medium (WPM) (Lloyd and McCown, 1980) without any phtohormones. This was enriched with 30 g/L sucrose and 9 g/L Biotech agar No.1.

Effect of phytohormones on culture performance

The effect of phytohormones was evaluated by supplementing WPM with 6-benzylaminopurine (BAP) and indolebutylic acid (IBA) with or without gibberellic acid A3 (GA_3). BAP was used at seven levels; 0, 0.5, 1.0, 1.5, 2.0, 4.0 and 5.0 mg/L while IBA and GA_3 were used at 1.0 mg/L.

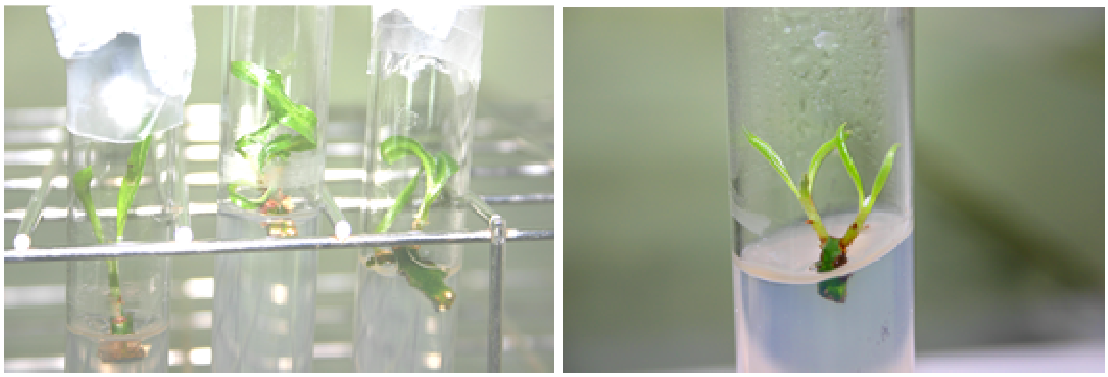

Figure 1. Nodal explant of *Macadamia integrifolia* cultivar KRG-15 initiated on hormone-free ½ Murashige and Skoog (1962) medium (only macronutrients at half rate) with (A) pH adjusted to 5.2 and (B) adjusted to 5.7 after 8 weeks in culture

Table 1. Performance of *Macadamia integrifolia* cultivar KRG-15 explants cultured on Murashige and Skoog (1962) medium gelled with four different agents after seven and a half weeks in culture.

Gelling agent	Bud break frequency (%)	shoot number per explant	Length of longest shoot (cm)	Length of shortest shoot (cm)	Strong shoots (%)	Weak shoots (%)
PHYTAGEL®	24b	0.68b	0.44a	0.07b	16ba	8b
GELRITE™	24b	0.64b	0.29a	0.07b	0b	24ba
Purified agar	56ba	1.68a	0.69a	0.52a	29a	20ba
BIOTEC® agar No. 1	71a	2.08a	0.73a	0.15b	36a	42a

Values are means of 5 replicates with 5 explants each. Means followed by the same letter in a column are not significantly different at α = 0.05 (SNK test).

Data analysis

Analysis of variance was done using SAS (SAS, 2001) and means separated by SNK test at α = 0.05.

RESULTS AND DISCUSSION

Effect of pH

The best pH range was determined as 5.5 - 6.0 with pH below 5.5 producing abnormal shoots which were either weak of watery (Figure 1A). Explants cultured on medium at pH level of 5.7 produced strong and normal shoots (Figure 1B) and was adopted for all subsequent experiments.

Effect of basal media

Woody plant medium yielded 85% successful cultures. There was 70% survival of cultures initiated on ½ MS-based medium (macronutrients reduced to half rate) compared to less than 30% of those initiated on full MS. Compared with MS, WPM is low in ammonium, nitrate, potassium, chloride and high in sulphate indicating that *M. integrifolia* nodal segments preferred low salt concentration (Pareek and Mathur, 1999). McCown and Sellmer (1987) reported that high ionic strength has an inhibitory effect on growth of many woody species. Skirvin (1980) and Griffis et al. (1983) found it beneficial to reduce the strength of MS by half in tissue culture of fruit trees. Piagnani and Eccher (1988) obtained highest proliferation rates of chestnut *in vitro* on media containing low total nitrogen levels and low NH_4^+/NO_3 ratios. In the present study reduction of MS macronutrients alone by half gave results comparable to WPM.

Effect of gelling agents

Results of culture performance on the four gelling agents are shown in Table 1. Explants cultured on BIOTEC® Agar No. 1 performed consistently better than those cultured on Purified agar, PHYTAGEL® and GELRITE™ in terms of bud break frequency and shoot number per explant. BIOTEC® Agar No. 1 and Purified Agar are both cloudy agars and they both performed better than PHYTAGEL® and GELRITE™ both of which are clear agars. There was no significant difference between PHYTAGEL® and GELRITE™ in all the parameters tested. Though shoot regeneration was low, explants cultured on PHYTAGEL® were mostly strong while those

Table 2. Effect of sucrose concentration on performance of *Macadamia integrifolia* cultivar KRG-15 explants on MS medium after six weeks in culture.

Sucrose level	Bud break (%)	Shoot number per explant	Shoot length (cm)	Length of longest shoot	Length of shortest shoot	Strong shoots (%)
15	16b	0.28b	1.10b	0.10b	0.02b	0.0d
20	40ba	0.88ba	0.95c	0.22b	0.04b	30c
25	52a	0.64ba	2.14a	0.56a	0.05b	69ab
30	60a	1.28a	2.14a	0.60a	0.22a	73a

Values are means of 5 replicates with 5 explants each. Means followed by the same letter in a column are not significantly different at α = 0.05 (SNK test).

Table 3. Effect of BAP on *in vitro* performance of nodal segments of *Macadamia integrifolia* cultivar KRG-15 after 8 weeks in culture in WPM medium supplemented with IBA and GA_3.

BAP level (mg/L)	Bud break (%)	Mean number of shoots per explant	Mean shoot length (cm)
0.0	75c	5.2cd	0.5e
0.1	61de	5.8c	0.6e
1.0	85b	6.2bc	2.3c
1.5	69d	6.5b	2.1d
2.0	98a	8.1a	3.3a
4.0	63d	5.4c	2.8b
5.0	55e	4.9d	2.3c

Values are means of 5 replicates with 5 explants each. Means followed by the same letter in a column are not significantly different at α = 0.05 (SNK test).

cultured on GELRITE™ produced only weak shoots.

These results indicate that *M. integrifolia* preferred cloudy forming gels other than clear forming gels. The choice of appropriate gelling agent in the appropriate concentration was found to be an important factor in tissue culture of confers (Von Arnold and Eriksson, 1984) and broad leafed trees (Pasqualetto et al., 1986). In comparing agar (Sigma type A) and GELRITE™ in tissue culture of Mexican redbud, Markay et al. (1995) reported different effects with cultures on GELRITE™ having higher dry mass than those on agar only when media were supplemented with activated charcoal. Without activated charcoal, explants on GELRITE™ were occasionally vitrified and often had small, white or pink leaves that became necrotic.

Effect of sugar concentration

High sucrose concentration favored bud break frequency, shoot number per explant, length and strength of shoots with the highest being attained at 30 g/L as summarized in Table 2. Increasing concentration from 25 to 30 g/L did not significantly improve shoot length but slightly increased the percentage of strong shoots and it also improved general shoot growth. In tissue culture of *M. tetraphylla*, Mulwa and Bhalla (2000) used 20 g/L sucrose while in the present study, 30 g/L was found to be significantly better than 20 g/L in tissue culture of *M. integrifolia*. The difference can be attributed to the difference in the species; *M. tetraphylla* and *M. integrifolia* probably require different levels of exogenous carbon source. Moreover, Molnar (1988) indicated that the optimum level of sucrose may depend on other media additives. Seingre et al. (1991) also reported an interaction between sugar and gelling agents and the difference in sugar requirement could be attributed to the different gelling agents used.

Effect of phytohormones

The level of BAP had a significant effect on bud breaking frequency, shoot number per explant and shoot length. Results are shown in Table 3. The results showed that, although bud breaking frequency was not consistent with the level of BAP used, the highest bud break frequency was obtained when MS medium was supplemented with 2 mg/L. The mean shoot number per explant and the mean shoot length generally increased with the increase of BAP level and optimized at 2 mg/L (Figure 2) beyond which further increase in the level of BAP was not beneficial. Explants cultured on medium supplemented with BAP alone produced weak shoots indicating this hormone encouraged shoot multiplication but not shoot growth. Inclusion of GA_3 in the culture medium did not

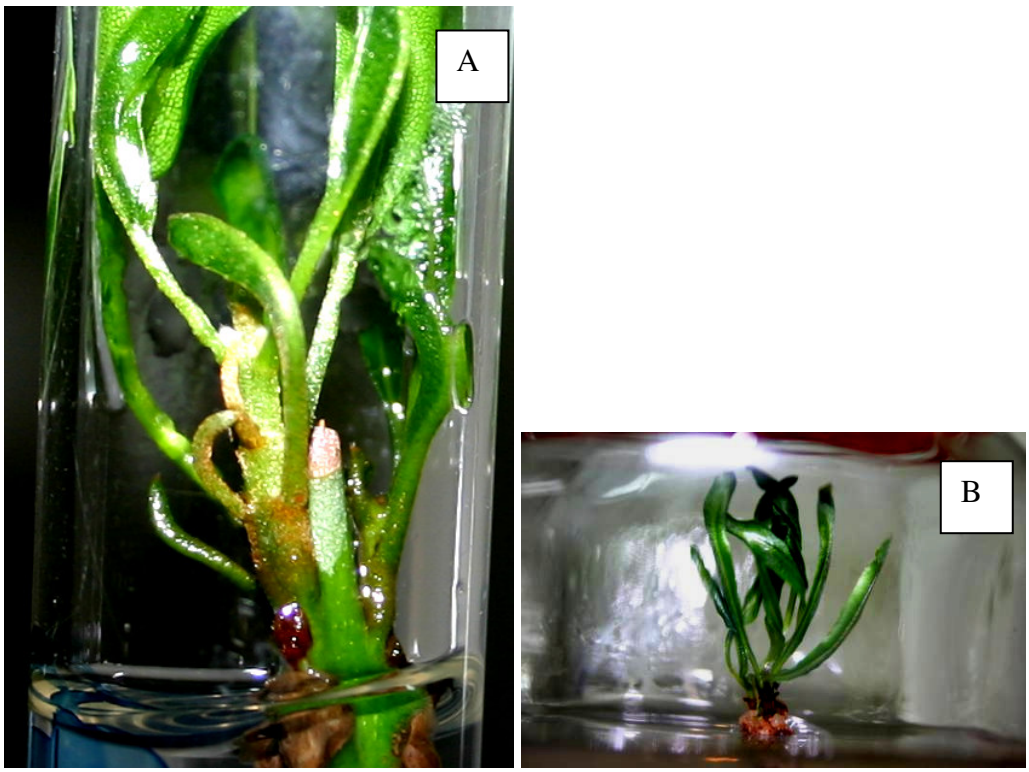

Figure 2. Nodal explant of *Macadamia integrifolia* cultivar KRG-15 cultured on WPM supplemented with 2 mg/L BAP, 1 mg/L IBA and 1 mg/L GA_3. (A) initial shoot initiation from mother explant and (B) further multiplication after sub-culture on to similar medium.

significantly increase shoot multiplication but improved general shoot growth. The synergetic effect of GA_3 to BAP has also been reported by Mulwa and Balla (2000) in tissue culture of *M. tetraphylla*.

Sub-culture and rooting

When individual shoots less than 1 cm were sub-cultured onto medium similar to inoculation medium, more than 50% of the cultures browned and died within 2 weeks of subculture. Hence shoot masses were subsequently sub-cultured 2 - 3 times for substantial elongation before separating individual shoots. New shoots developed 3 - 6 shoots when sub-cultured in the same medium for another 4 weeks.

In an attempt to initiate rooting, elongated shoots were sub-cultured onto ½ MS medium supplemented with Naphthalene acetic acid (NAA) at (0.5, 1.0 or 4.0) mg/L or Indole butyric acid (IBA) (0.5, 1.0, 4.0 mg/L) or Indole acetic acid (IAA) at 0.5 mg/L or 2,4 Dichlorophenoxyacetic acid (2,4-D) at 0.5 or 2.0 mg/L and maintained under growth room conditions for 4 weeks. There was 90 - 100% friable callusing on all media tested but no root formation was achieved within the test period.

Excessive callogenesis at the bases of *in vitro* shoots without rooting has been reported for chestnut (Chevre et al., 1983) and *Populus* x *Euamerica* trees (Agrawal and Gupta, 1999). Difficult or low frequency *in vitro* rooting has also been reported in a number of woody crops (Mullins, 1987; Mackay et al., 1995; Ventakeswarlu, 2002; Ongunsola and Iroli, 2007). Damiano and Monticelli (1998) stated that several woody plants often show poor rooting efficiency both in conventional and *in vitro* propagation. In tissue culture of chestnut, Piagnani and Eccher (1988) also reported better rooting of shoots derived from embryos than those derived from axillary buds. Ogunsola and Ilori (2007) achieved some rooting on embryo-derived shoots but none from nodal-derived shoot in the tissue culture of miracle berry (*Synsepalum dulcificum* Daniel). The ability to micropropagate fruit trees depends on the physiological juvenility of the explants (Pareek and Mathur, 1999) and according to Deburgh (1988); there is loss of rootability in woody species where non juvenile explant material is used for tissue culture.

Conclusion

The results of the current study show that *M. integrifolia* is amenable to tissue culture and can be proliferated on either WPM or ½ MS (macronutrients at half rate). However, in the present study difficulties were experienc-

ed in gelling WPM-based medium with Phytagel® and the reasons for that are not yet clear and should be investigated. Hence for now ½ MS medium can be adopted for further tissue culture work. Medium supplemented with 2 mg/L BAP, 1 mg/L IBA and 1 mg/L GA_3 and enriched with 30 g/L sucrose (pH 5.7) gives satisfactory shoot multiplication of 8.1 shoot per explant in eight weeks and a further 3 - 6 shoot in another four weeks. However, further improvement in shoot multiplication is required to achieve the economic benefits of a commercial tissue culture system. Rooting of *in vitro* shoots is still a major hurdle and is still under investigation. This is believed to be the first report on successful multiple shoots regeneration from *M. integrifolia*.

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