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*Full Length Research Paper*

# Effect of explant type, source and genotype on *in vitro* shoot regeneration in Macadamia (*Macadamia* spp.)

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***Macadamia*, (*Macadamia* spp.) is conventionally propagated by grafting scions from selected superior cultivars onto rootstocks raised from seed, a process that takes about two years in addition to high inputs thus ending up with costly seedlings. A study was conducted to investigate the possibilities of applying tissue culture techniques in mass production of clean planting materials. Different types of explants sourced from either grafted seedlings or from intact or cut-back field grown trees were evaluated in view of their regenerative potential. The effects of age and genotype of the explant were also investigated. Results indicated the significant effect of explant type in mass production. Only nodal segments gave satisfactory results producing two to three shoots within four to six weeks. Nodal segments at the 1st, 2nd and 3rd bud position performed better than those obtained at the 4th, 5th and 6th bud position. Explants from mature field-grown trees displayed similar properties to those from grafted green-house seedlings and were better than those obtained from cut-back trees. Explants from cultivars of *Macadamia integrifolia* performed better than (*M. integroifolia* × *Macadamia tetraphylla)* hybrids. These results suggest that optimization of these factors can help in development of a commercially viable tissue culture system for *Macadamia*.**

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

## INTRODUCTION

*Macadamia* is a dark green spreading semi-hardwood evergreen crop (Duke, 2001) that belongs to the family Proteaceae and is grown for its edible kernel (Peace et al., 2003). Macadamia was introduced in Kenya in 1946 as an alternative cash crop to tea and coffee (Waithaka, 2001; Harries, 2004). Kenya is the fourth largest producer after Australia, Hawaii and South Africa with an annual production of 10,000 tons (Wilkie, 2008). The scope for increasing the production of *Macadamia* nuts exists, especially through expanded acreage, but availability of good quality planting materials is still a limiting factor. *Macadamia* is preferentially out-crossing (Sedgley et al., 1990) and plants resulting from open-pollinated seeds are variable in yield and nut characteristics and therefore of little value for commercial planting. Hence, conven-tional vegetative propagation method is by grafting scions from selected parents to rootstocks raised from seed (Nagao and Hirae, 1992; Gitonga et al., 2002). The pro-cess of grafting takes between 18 and 24 months besides requirement for skilled manpower, material inputs and space (Nyakundi and Gitonga, 1993; Gitonga et al., 2001) and therefore ending up with costly seedlings.

Tissue culture techniques are now widely used for mass multiplication of nut crops such as cashew nut (Thimmappaiah et al., 2007), chestnut (Vieitez et al.,

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2007) and walnuts (Leal et al., 2007). Mulwa and Bhalla (2000) reported successful tissue culture of *Macadamia tetraphylla* using nodal segments. Mulwa and Bhalla (2006) later reported on regeneration of shoots from somatic embryos of *M. tetraphylla*. Gitonga et al. (2008) succeedded in initiation of shoots from *Macadamia integrifolia*. However, to date, no reports are available on an efficient tissue culture system for *M. integrifolia* or (*M. integrifolia* x *M. tetraphylla*) hybrids forming the main commercial cultivars in Kenya. Some studies have shown that explant characteristics such as type, source, genotype and history affect the success and commercial viability of tissue culture systems (Bhau and Waklu, 2001; Chin and Chang, 2002; Hoy et al., 2003). The objectives of the current study therefore were to investigate the effect of type, source and genotype of explant on *in vitro* regenerative potential of *Macadamia*.

## MATERIALS AND METHODS

### Surface sterilization of explants and tissue culture media

Explants were surface sterilized by washing them in tap water with a few drops of Tween 80 and rinsing 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 (quick dip) and rinsed with sterile distilled water five times of 3 - 5 min each with agitation. Explants were then sterilized in 10% Jik® (Reckitt and Benkiser, Nairobi, Kenya) (3.5% NaOCl equivalent to 0.35% pure Chlorine) for 10 min. This was followed by five rinses with sterile distilled water and air drying in the laminar flow hood before inoculating the explants on tissue culture medium. Tissue culture medium consisted of MS (Murashige and Skoog, 1962) macronutrients, micronutrients, iron-EDTA solution and MS vitamins. The medium was prepared from stock solutions. All media supplements were added before adjusting the pH to 5.7 and autoclaving the media for 20 min at 121˚C and 100 kPa.

### Testing of explant types

Explant materials were obtained from variety KRG-15 (*M. integrifolia*) as the test variety. Different explant types namely leaf sections, stem nodal segments and cotyledonary sections were tested on their ability to maintain and initiate shoots on MS medium without any hormone supplements. Leaves were obtained from young shoots of current season's growth. Leaf sections, one centimeter long and one centimeter wide were excised from fully expanded leaves avoiding the mid ribs and placed abaxially onto ½ MS (macronutrients at half strength) culture medium. Stem segments about one centimeter long and containing single nodes were excised from young sprouting shoots of mature field-grown trees and placed onto culture medium after surface sterilization. Freshly fallen nuts were collected from the orchard and their husks removed. While in the laboratory, nuts were surface sterilized and shells cracked in the air flow chamber using a nut cracker. The kernels were aseptically removed and the embryo detached from the cotyledons and discarded. The cotyledons were subdivided to provide sections of about 0.5 $cm^3$. After surface sterilization, explants were placed onto culture medium making sure the freshly cut surfaces were in contact with medium. All explants were maintained under growth room conditions for four weeks.

Shoots regenerated from mother explants and surviving cotyledonary explants were sub-cultured on ½ MS medium supplemented with 6-benzylaminopurine (BAP) at seven levels (0, 0.5, 1.0, 1.5, 2.0, 4.0 and 5.0) mg/L BAP and 1 mg/L indolebutylic acid (IBA) shoot regeneration and multiplication. Shoots were further sub-cultured on 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 to initiate rooting and maintained under growth room conditions for additional four weeks.

### Testing the effect of explant source

Nodal segments were obtained from young sprouting shoots from three sources. These included the current season's flush from mature field-grown trees of more than 15 years of age, coppice shoots from trees that had been cut back at a height of one and half meters just before the rainy season, and sprouting shoots from two-year-old seedlings grafted with variety KRG-15 and maintained under green house conditions for one year. Explants were initiated on hormone-free ½ MS medium after surface sterilization and maintained under green house conditions for eight weeks.

### Testing the effect of explant age

The effect of age of explant was tested by comparing apical (tip) shoots with nodal segments obtained from either the 1st, 2nd or 3rd bud position from the tip coinciding with current season's growth flush and the 4th, 5th or 6th bud position from the tip coinciding with previous season's growth of twigs obtained from mature field-grown trees. Explants were initiated on ½ MS medium after surface sterilization and maintained under green house conditions for eight weeks.

### Testing the effect of genotype as a source of explants

The regenerative potential of five cultivars; KRG-15, MRG-25, EMB-1, EMB-2 and KMB-3, was evaluated by inoculating surface-sterilized nodal segments obtained from young shoots of the respective mature field-grown trees onto hormone-free ½ MS medium. Explants were maintained under green house conditions for eight weeks.

### Growth room 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 × 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 and all experiments were arranged in a completely randomized design. Culture period ranged between four to eight weeks depending on individual experiment. Data were recorded on bud breaking frequency, shoot number per explant and shoot length. Analysis of variance was performed using SAS (2001) and means separated by Student Newman Keuls (SNK) test at $p < 0.05$.

## RESULTS AND DISCUSSION

### Effect of explant type

Only nodal segments and cotyledonary explants

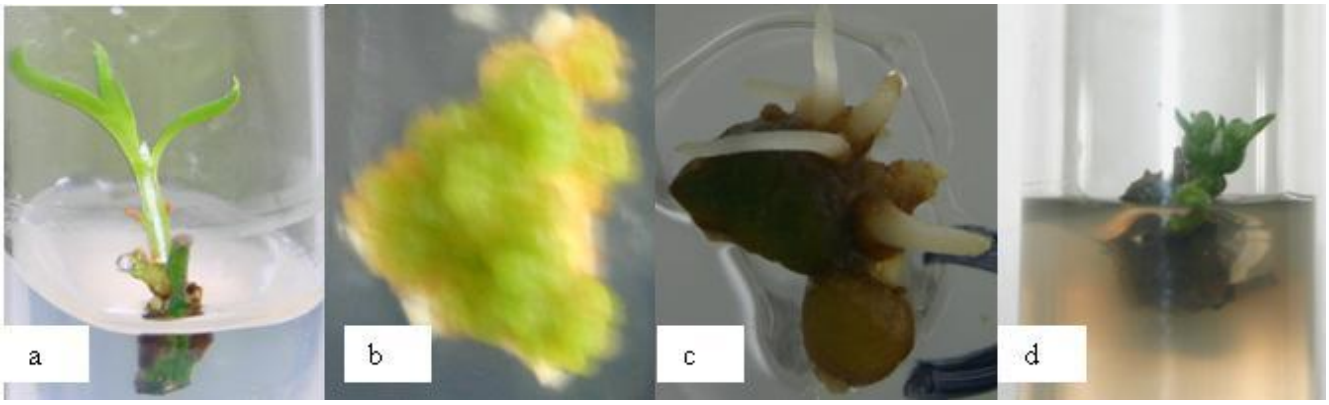


**Figure 1.** Performance of different Macadamia explants types in culture (a) Shoot regeneration from nodal segments after four weeks on hormone-free ½ MS medium, (b) Greening of surviving cotyledonary explants after two weeks on ½ MS medium with 0.5 mg/L 2,4-D, (c) Root regeneration from cotyledonary explants after four weeks on ½ MS medium with 2 mg/L 2,4-D and (d) formation of green shoot primordia from cotyledory explants after four weeks on ½ MS medium with 0.5 mg/L BAP.

**Table 1.** Performance of *Macadamia* explants sourced from three sources after eight weeks on ½ MS medium.

| Explant source | Bud break (%) | Shoots per explant |
|---|---|---|
| Two year old grafted seedlings | 72.0a | 1.88a |
| Mature field-grown trees | 75.0a | 1.59a |
| Coppice shoots from mature trees | 28.0b | 0.28b |

Means followed by the same letter in a column are not significantly different at $p < 0.05$.

indicated possibility of shoot regeneration while leaf sections did not regenerate any shoots and they dried within the first week. Nodal segments gave satisfactory results as they maintained well in culture with 63.0% bud break within two to three weeks and shoots fully formed between four to six weeks (Figure 1a). Shoots multiplied to two or three shoots per explant in nodal segments after transfer to ½ MS medium supplemented with 2 mg/L BAP and 1 mg/L IBA. However, when explants were transferred to ½ MS medium supplemented with NAA, IBA, IAA and 2,4-D no root regeneration was achieved within the test period. Eighty five percent (85%) of the cotyledonary explants browned within the first week and died. However, surviving cotyledonary explants started greening and produced green embryogenic calli from the second week after transfer to medium supplemented with 0.5 mg/L 2,4-D (Figure 1b), while explants transferred to medium supplemented with 2 mg/L 2,4-D produced roots other than shoots (Figure 1c). When calli were transferred to medium supplemented with 0.5 mg/L BAP, some cotyledons produced green shoot primordia (Figure 1d) but no shoot regeneration was achieved within the test period.

The effect of explant type on successful tissue culture of various crops has been reported (Gubis et al., 2003; Blinstrubiene et al., 2004; Tsay et al., 2006). Nodal segments have been widely used for *in-vitro* shoot proliferation of woody plants such as *Citrus limon* (Rathore et al., 2004), rough lemon (Ali and Mizra, 2006) and miracle berry (Ogunsola and Ilori, 2008). Mulwa and Bhalla (2000) also reported on successful tissue culture of *M. tetraphylla* using nodal segments. This is probably due to the readily available axillary buds in nodal segments that only require a trigger for bud break in contrast to leaf and cotyledonary tissue that would otherwise require initiation of adventitious buds (Sivanesan et al., 2007) and somatic embryos (Naz et al., 2008) before any shoot regeneration is achieved.

## Effect of explant source

There were no significant differences in bud break frequency and shoot number per explant in explants sourced from either grafted greenhouse-grown seedlings or mature field-grown trees (Table 1). Explants from these two sources achieved over 70% bud break and between one and two shoots per explant. However, when explants were sourced from coppice shoots of cut back trees, bud break was significantly reduced to 28%. Most (50%) of the coppice shoot-derived explants browned from phenolic exudation and died within the first week.

These results contrast those of Mulwa and Bhalla (2000) who reported significantly better performance of

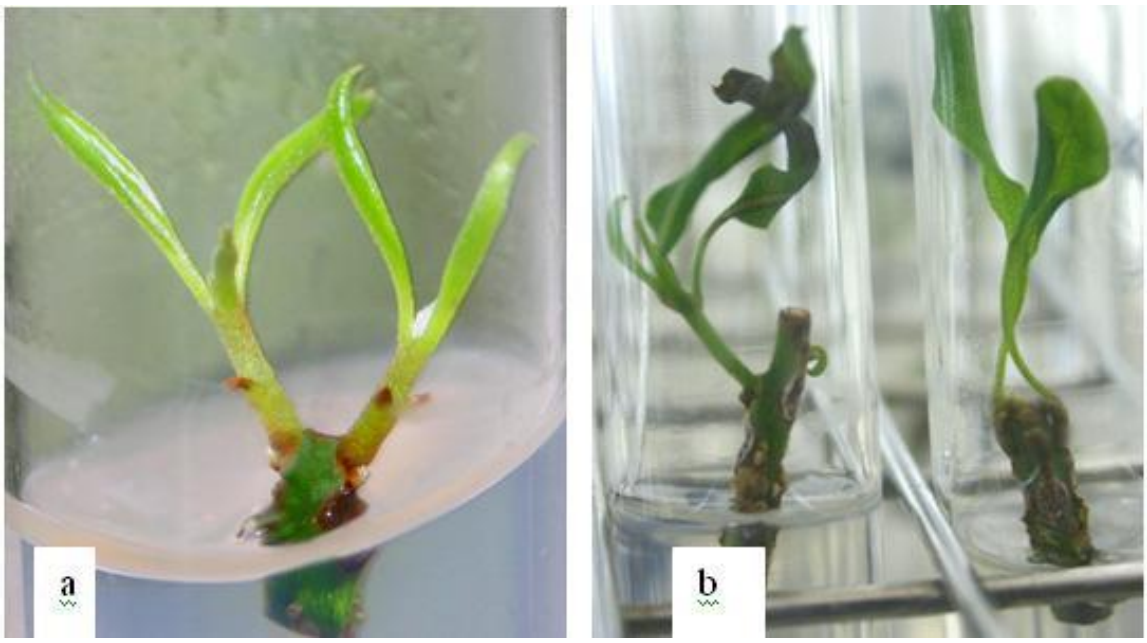


**Figure 2.** Shoot regeneration from nodal segments obtained from a- the 3rd bud position of a twig and b- the 5th bud position of a twig of a mature field-grown KRG-15 tree after eight weeks on ½ MS tissue culture medium.

explants derived from grafted seedlings. However, they compared performance of shoots from grafted seedlings to those obtained from green-house grown seedlings and not mature field-grown trees as in the present study. Their explants were obtained from *M. tetraphylla* and not *M. integrifolia*, suggesting genotypic differences in *in vitro* response of Macadamia. Oxidation of polyphenols from explants is a drawback reported in *in vitro* regeneration systems of woody plants (Mackay et al., 1995; McCown, 2000). Chevre and Salesses (1983) reported that peak flush of growth in chestnut coincided with maximum quantities of phenolic compounds while Dubravina et al. (2005) indicated that annual shoots of European yew had the highest capacity for synthesizing these compounds. Explants from cut-back *Macadamia* trees exhibited high levels of phenolic exudation and it is possible that cutting-back the trees triggered the production of polyphenols.

## Effect of explant age

The age of explant had an influence on its performance in culture. Nodal explants obtained from the 1st, 2nd and 3rd nodal position of the twig were more suitable than those obtained from the 4th, 5th and 6th nodal position. They maintained well in culture and 65.9% of them produced between one and three shoots within four to six weeks (Figure 2a). Those obtained from the 4th, 5th and 6th bud position were too hard to cut and they produced weaker shoots (Figure 2b). Most of them browned from phenolic exudation, had fungal infection and 50% died within the first week. Apical shoots did not regenerate any shoots and all of them withered and suffered apical die back within two weeks.

Phenolic exudation from young explants derived from mature trees into the culture medium is a common problem reported in tissue culture of woody species (McCown, 2000). Mathur et al. (1999) investigated the qualitative and quantitative estimates of phenolic compounds in different explant types of jamun (*Syzygium cumini* (L.) Skeels responsible for tissue browning in tissue culture. They demonstrated that shoot tips and nodal segments have almost similar amounts of the three types of phenolic compounds while Ozyigit (2008) indicated a positive direct relationship between age of explant and phenolic exudation in tissue culture of cotton. However, in the present study, shoot tip explants suffered die back and explants close to the apex; 1st, 2nd and 3rd nodal position were not affected by phenolic exudation. This suggests that these compounds accumulate with age in *Macadamia* shoots. Shoot tip necrosis of *Macadamia* has also been reported by Mulwa and Bhalla (2000) who suggested that it may be related to the relative humidity levels in the culture vessels.

## Effect of genotype

Among the five cultivars tested, only KRG-15, MRG-25 and EMB-2 survived in culture while EMB-1 and KMB-3 died within the first two weeks in culture. There were no significant differences in shoot number per explant in

**Table 2.** Effect of genotype of *Macadamia* on bud break frequency, mean shoot number per explant and mean shoot length after eight weeks on hormone-free ½ MS tissue culture medium.

| Treatment (Variety) | Macadamia species | Bud break (%) | Shoots per explant | Shoot length (cm) |
|---|---|---|---|---|
| KRG-15 | *M. integrifolia* | 62.2a | 2.6a | 3.60a |
| MRG-25 | *M. integrifolia* | 58.9ab | 2.3a | 0.21b |
| EMB-2 | *M. integrifolia* | 8.0c | 0.1b | 0.20b |
| EMB-1 | *M. integrifolia* | 2.0cd | 0.0b | 0.0b |
| KMB-3 | *M. hybrid* | 1.6cd | 0.0b | 0.0b |

Means followed by the same letter in a column are not significantly different at $p < 0.05$ (SNK test).

KRG-15 and MRG-25 (Table 2) but shoots from KRG-15 were more vigorous than those from MRG-25. The effect of genotype on successful tissue culture has been previously reported (Gubis et al., 2003; Brinstrubiene et al., 2004). Since explants of all the genotypes were cultured onto hormone-free medium, it can be assumed that the differences in their response in tissue culture were determined by the balance of their endogenous hormones (Razdan, 2003).

## Sub-culture and rooting

When individual shoots less than one centimeter were sub-cultured onto hormone-free medium similar to inoculation medium, more than 50% of the cultures browned and died within two weeks of sub-culture. Shoot masses were therefore sub-cultured twice to obtain substantial elongation before separating individual shoots. Efforts to root the *in vitro* shoots were fruitless. 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). Koyuncu and Balta (2004) stated that formation of adventitious root primordia in cuttings of *Camellia sinensis* L. was preceded by callus formation. However, cuttings with excellent callus failed to root while those with poor callus formed root initials that later elongated suggesting that excessive callogenesis may not be favourable to rooting. Vantekeswarlu (2000); Ongunsola and Iroli (2007) reported difficult or low frequency *in vitro* rooting of neem and miracle berry, respectively. The ability to vegetatively propagate woody plants depends on the physiological juvenility of the starting material (Pareek and Mathur, 1999; Ozkaya et al., 2003). Hence, the difficulty in rooting of *in vitro* shoots derived from adult material of woody plants is often asso-ciated with lack of juvenility (Preece and Read, 2003). In previous experiments on *in-vivo* rooting of Macadamia cuttings, rooting only occurred after six to nine months on moist sand and there was no advantage of using rooting hormones (Gitonga et al., 1997). It is possible that *Macadamia* requires a longer time *in-vitro* to root and the tender *in-vitro* shoots would probably need further procedures for maintenance for long periods to initiate rooting. The present study shows that nodal segments are the most suitable explants for shoot regeneration of *Macadamia* and that both grafted seedlings and mature field grown trees can be used as source of explant material. Cotyledonary explants also showed possibility of shoot regeneration as they produced green embryo-genic calli, roots and shoot primordia after sub-culture on medium supplemented with different auxins and cyto-kinins. The ratio of auxin to cytokinin is important in morphogenesis in tissue culture systems (Razdan, 2003) and further studies are needed in this area. The study further showed that *M. integrifolia* is amenable to *in-vitro* shoot multiplication using nodal segments but different cultivars respond differently and therefore, tissue culture protocols should be fine-tuned to be species and cultivar specific. However, shoot regeneration frequency was low and there was difficulty in rooting of *in vitro* shoots. There is need therefore to investigate other methods to improve shoot multiplication and rooting. Regeneration of shoots from somatic embryos of *M. tetraphylla* has been reported by Mulwa and Bhalla (2006) and the application of this method to *M. integrifolia* and the *(M. tetraphylla* x *M. integrifolia*) hybrids should be explored to improve shoot regeneration. Further, methods such as serial grafting (Mneney et al., 2001) and use of epicormic shoots sprouting from the tree truck that are expected to have reverted to juvenile phase (Pereira, 2009) have been suggested for tissue rejuvenation and therefore overcoming rooting of *in vitro* shoots of woody plants.

The advantage of micropropagation over other propagation methods lies in its ability to multiply thousands of elite clonal material within a relatively shorter time. Once an adequate protocol for micropropagation of *Macadamia* has been developed, propagules may be provided to farmers at a more affordable price. Elite *Macadamia* varieties may also be conserved for future breeding through slow growth *in vitro* storage (Hassan et al., 2007) or cryopreservation (Panis and Lambardi, 2006) at cheaper cost compared to field and on-farm collections (Bekheet, 2000). Development of meristem tip culture techniques may enhance regeneration of virus-free plants

(Grout, 1990) while the tissue culture plantlets may also be enriched *in vitro* using endophytes to induce resistance of the resultant Macadamia trees to other disease phytotoxins (Saikkonen et al., 2004). With an efficient tissue culture protocol, *Macadamia* improvement can also be achieved by introduction of superior genes in to existing varieties through genetic transformation techniques (Krichevsky, 2008). Pena and Séguin (2001) reported on the successful incorporation of transgenes for shortening the juvenile phase in to forest trees and this can be of great benefit to *Macadamia*. Tissue culture techniques may also aid in development of varieties resistant to pests and diseases through somaclonal variation (Pontaroli and Camadro, 2005).

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