EFFECT OF GROWTH REGULATORS ON *IN VITRO* GERMINATION OF COCONUT MATAG 2 ZYGOTIC EMBRYOS IN LIQUID AND SOLID CULTURE MEDIA

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ABSTRACT

A protocol for the *in vitro* germination of coconut embryos was developed. Coconut MatagF2 embryos were screened for *in vitro* germination on MS and Y3 media containing different concentrations (0.5, 1.0, 3.0, 5.0 10.0 mg/L) of BAPor NAA. Less tissue browning was observed when the culture media contained less than 1.0 mg/L BAP or NAA. The best embryo expansion (250%) was observed using Y3 medium containing 0.5 mg/L BAP, which caused only 20% tissue browning. Compared with solid culture media, a liquid medium (with agar excluded) was more conducive to embryo germination, resulting in 90% bud shoot initiation and 480% embryo expansion being achieved when supplemented with 0.5mg/L each of BAP and NAA..This protocol could facilitate the production of large numbers of germinated embryos useful in an *in vitro* micropropagati on system for Matag coconut.

Key words: BAP, *in vitro*, Matag coconut, NAA, plant growth regulator, Y3 medium, zygotic embryo

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INTRODUCTION

The coconut palm (*Cocosnucifera*) is a major agricultural crop in the humid tropics. It is estimated that over 11 million farmers, mostly smallholders with low income, grow the palm in

90 countries. More than 80% of the total world production of coconuts comes from countries in the Asia–Pacific region. Whereas coconut plantations have been established in the past, the palm is now grown on a smaller scale,largely used for ornamental purposes (Core, 2005).The downturn in the coconut industry has been documented for decades (Wright and Persley 1988), but little effort has been made to prevent further decline.Productivity in the planting of coconut has not been increased, and more than half of the existing plantations are too old for continued profitable copra production (Samosir et al., 2005). The decline in coconut production is also made worse by new devastating pests and diseases of the palm. Hence, replanting with new, high-yielding, disease-resistant cultivars would be an important part of re-establishing the traditional coconut-based farming system. Because coconut is not amenable to common vegetative propagation methods, it is usually propagated through seed germination. However, this is both cumbersome and costly. Propagation by germinating coconuts does not provide a sufficient or consistent supply of planting materials for cultivation. To overcome these setbacks, the tissue culture approach can be utilized to produce large quantities of genetically identical plants, to increase efficiency of propagation, to maintain virus-free stock, or to produce a marketable size planting material within a short period.

In this study, the *in vitro* germination of Matag coconut embryos on different media containing various plant growth regulatorswasexamined.

MATERIAL AND METHODS

Plant Material and Aseptic Culture

Zygotic embryos of Matag (Figure 1a) coconut were and sterilized by immersion in 50% ethanol for 2 min, thenin 1% Vircon for 30 min. This was followed by further sterilizationin Clorox (sodium hypochlorite, 5.2%), first with the embryos immersed in 20 to 100% Clorox for 40 min, followed by immersionin 5, 10 or 20% Clorox for 20 min (*Table 1*). Two drops of Tween 20 (a polysorbate surfactant that serves as a detergent and emulsifier)were added to the sterilizing agent in each of the abovementioned steps.After rinsing three times in steriledistilled water, the embryos were culturedon the specified mediaand incubated for 6 weeks at26 ± 2 °C in light.The cultured embryos were thenobserved after 6 weeks and the percentagesurvivaland contamination recorded.

Effect of BAP and NAA

Surface sterilizedembryos were cultured individually under aseptic conditions on basal MS or Y3 media containing 0.1 g arginine, 0.1 asparagine, 0.1g glutamine, 3.0% sucrose, 0.3% gelrite agar for gelling, and supplemented with five different concentrations of benzylamino purine (BAP) (0.5, 1.0, 3.0, 5.0 10.0 mg/L) or naphthalene acetic acid (NAA)(0.5, 1.0, 3.0, 5.0 10.0 mg/L). Medium sterilization was performed by autoclaving at 121^{o}C for 20 min. The medium pH was

adjusted to5.7-5.8 before adding agar. The embryos were inoculated in a 150 mL flask containing 40mL of the desired medium. The cultures were incubated in a plant growth room maintained at a temperature of 25 ^{o}C±1 and with lighting provided by cool-white fluorescent lamps (1000–2000lux) on a 16-hour photoperiod. Observations were recorded at weekly intervals. Results were expressed as the percentage of tissue browning that appeared and the expansion of embryos after 45 days of culture.

Effect of coconut water, agar, and plants growth regulators

The effects of different concentrations of coconut water and agar on embryo expansion and bud shoot initiation were studied. The embryos were cultured on Y3medium with three different concentrations of coconut water (0, 30 and 50%) in combination with various concentrations of phytogel agar, *viz*. 0 g/L (liquid medium), 0.75 g/L, 1.5 g/L and 3.0 g/L. Each medium was supplemented with 30 g/L sucrose and combination of 0.5 mg/L BAP and 0.5 mg/L NAA. All the embryos were subcultured onto the same fresh medium at 1 month intervals. Results were expressed as percentage of embryo expansion and bud shoot initiation. The experiment was carried out over 4-6 months of culture. In a separate experiment, different types of plant growth regulators, *viz*. BAP, NAA, kinetin (Kin), indole-butyric acid (IBA), indole-acetic acid(IAA), and gibberellic acid (GA3),at 1 mg/L concentration were tested for their effects on bud shoot initiation.

Statistical analyses

All statistical analyses were performed using SPSS software. The experiment followed a completely randomized design, using 15 flasks for each treatment. Each flask contained two samples and all experiments were repeated thrice. The means and standard errors (indicated as ±values) were calculated for the treatment responses.

RESULTS AND DISCUSSION

Embryo sterilization

Embryos immersedin 50-100% Clorox generally showed higher survival rates (90-100%) than those sterilized with20% Clorox (Table 1). The latter had survival rates of less than 50%, with contamination of up to 70%. The optimum sterilization technique for embryo development was with the use of 50% Clorox for 40min, followed by 10% Clorox for 20 min to achieve a100% survival rate without contamination. The results in the sterilization technique were similar to that of Zuraida et al.(2011), which showed that the rice seeds treated with 100% Clorox achieved good survival rates (73 - 85%), whereas those treated with50% Clorox did not survive.

Table 1: Effect of different sterilization techniques on survival of Matag F2 coconut embryos after 6 weeks of culture

First sterilization (40 min)	Second sterilization (20 min)	% contamination	% survival
20	5	70	30
	10	60	40
	20	60	40
35	5	40	60
	10	40	60
	20	20	80
50	5	10	90
	10	0	100
	20	0	100
75	5	0	100
	10	0	100
	20	0	100
100	5	0	100
	10	0	100
	20	0	100

Results represent means± standard errors(SEM) of 15 replicates

Effect of BAP and NAA

Coconut Matag F2 embroys were cultured as explants on MS media supplemented with different concentrations of BAP (0.5-10 mg/L) and NAA(0.5-10 mg/L) to evaluate their effect on embryo expansion (Figure 1b-c). Two media, MS and Y3, were tested over 50 days to examine their effects on embryo expansion and incidence of browning. In general, MS medium supplemented with either BAP or NAA was effective in inducing embryos expansion. Less tissue browning was observed with concentrations of BAP or NAA at 1 mg/L or below in both the media tested (Table 2). In MS medium, supplementation with 0.5-1.0 mg/L NAA resulted in embryo expansion of up to 150-160%. In addition, this media tended to produce relatively less tissue browning. In terms of the expansion of embryos, Y3 medium performed better as compared with MS media. Embryo expansion (250%) was highest in Y3 medium supplemented with 0.5 mg/L BAP while tissue browning remained low at 20%. Y3 media was hence selected as the optimum media for further experiment studies. Higher concentrations of BAP or NAA (5-10 mg/L) resulted in more serious browning and poor response in embryo expansion. From the results obtained, Y3 medium was more suitable than MS medium for the development of Matag embryos. According to Muniran et al. (2008), modified Y3 medium was superior to N6 and MS media in the regeneration of *Elaeisguineensis* (oil palm). They also found this medium suitable for callus induction and rooting. Sukendah and Cedo (2005) using Y3 medium in their study on germinated coconut embryos which may germinated or exhibited. Maria Buena et al. (2003), in their study on the use of zygotic embryos of coconut for mass propagation,found Y3 medium

supplemented with various additives useful. The results in the present study showing that BAP at lower concentrations (0.5 mg/L) promoted the expansion of Matag embryos are in agreement with those of Ruzic and Lazic (2006), who reported that 0.5–2 mg/LBAP promoted shoot induction in *Ribesnigrum*. A similar finding was made in the study on *Taxodium*spp. where medium supplemented with 0.4 mg BAP/L facilitated better shoot multiplication (Abou Dahab et al., 2010). The effect of BAP in promoting shootlet number was also observed by Boulay (1989) in *Sequoia sempervirens* where shoot proliferation was best in media with 0.5-1.0 mg BAP /L and 0.02 mg NAA. Similar to the results in our studywas the finding by Koriesh *et al.* (2003) that the highest rate of *Eucalyptus citriodora* shoot multiplication was obtained using a medium supplemented with 0.5 mg BAP /L.

Table 2:Effect of BAP and NAA on percentage of tissue browning and embryo expansion

Medium	BAP (mg/L)	NAA (mg/L)	% Browning	% Embryo expansion
MS	0	-	35±5	80±9
	0.5	-	25±8	90±12
	1.0	-	30±9	60±6
	3.0	-	65±21	50±12
	5.0	-	100±11	0
	10.0	-	100±12	0
	-	0.5	20±3	160±12
	-	1.0	20±4	150±14
	-	3.0	55±6	80±8
	-	5.0	90±10	0
	-	10.0	95±11	0
Y3	0	-	20±2	180±21
	0.5	-	20±4	250±20
	1.0	-	30±6	190±21
	3.0	-	50±7	170±7
	5.0	-	75±11	120±15
	10.0	-	100±11	0
	-	0.5	20±2	230±21
	-	1.0	30±6	230±12
	-	3.0	40±8	70±11
	-	5.0	60±11	0
	-	10.0	80±12	0

Effect of coconut water and agar concentration

In view of the excellent results obtained with low concentrations of either NAA or BAP (Table 2), further experiments were carried out using a combination of both growth regulators, each at

0.5 mg/L. Embryos cultured on medium containing 0.5 mg/L NAA+0.5 mg/L BAPwere observed for bud shoot initiationand embryo expansion when the concentrations of coconut water and agar in Y3 culture medium were varied. In general, bud shoot initiation (90%) (Figure 1d-e) and embryo expansion (480%) were best in liquid medium (without addition of agar). Among the agar treatments, the lowest concentration tested, 0.75 g/L, gave the second best response with up to 50% bud shoot initiation. Higher agar concentrations of 1.5-3.0 g/L did not support bud shoot initiation.

Coconut water did not affect the initiation of shoot buds in Matag coconut. Our finding is in agreement with that of Suthar et al. (2011) who reported that cultures in liquid medium produced two times as many shoots as compared to culture media containing agar. They also mentioned that maximum shoot length was recorded in the liquid medium. The promotion of shoot proliferation in liquid medium may be due to the faster uptake of BAP and better absorptionof water by plants (Gurel and Gulsen,1998; Klimaszewska et al., 2000). Suthar et al. (2011) also found that increasing agar concentration (1.0%) in the culture medium caused a reduction in the overall growth of shoots of *Boswellia serrata.* Abdoli et al. (2007) reported that the percentage of explants forming shoots increased at a low concentration of agar (0.4%), although an increase in the occurrence of hyperhydric shoots was also observed. Increasing agar concentration to 0.8%)reduced the incidence of hyperhydric shoots from 53.3 to 7.8%. Gaspar et al. (1987) state that excessive uptake of water from the growth medium is the most important cause of hyperhydricity.However, Gurel(1998) demonstrated that agar content of 0.5-0.8%was effective in promoting earlyshoot proliferation, shoot development and growth of almond at all stages. In a separate experiment, BAP, NAA, kinetin, indolebutyric acid, indoleacetic acid, and gibberellic acid added separately at 1 mg/L did not improve the rate of success of bud shoot initiation as compared with 0.5 mg/L each of BAP and NAA applied in combination (results not presented).

Table 3: Effect of different concentrations of coconut water and agarin Y3 medium supplemented with 0.5 mg/L BAP+0.5 mg/L NAA on embryo expansion and bud initiation

Coconut water (%)	Agar concentration (g/L)	Embryo expansion(%)	Bud shoot initiation (%)
0	0 (Liquid)	480±60	90±11
	0.75	230±30	50±8
	1.5	190±14	0
	3.0	150±11	0
30	0 (Liquid)	430±35	90±9
	0.75	350±45	50±5
	1.5	180±20	0
	3.0	80±21	0
50	0 (Liquid)	340±18	80±11
	0.75	280±13	40±8
	1.5	220±18	30±5
	3.0	160±9	0

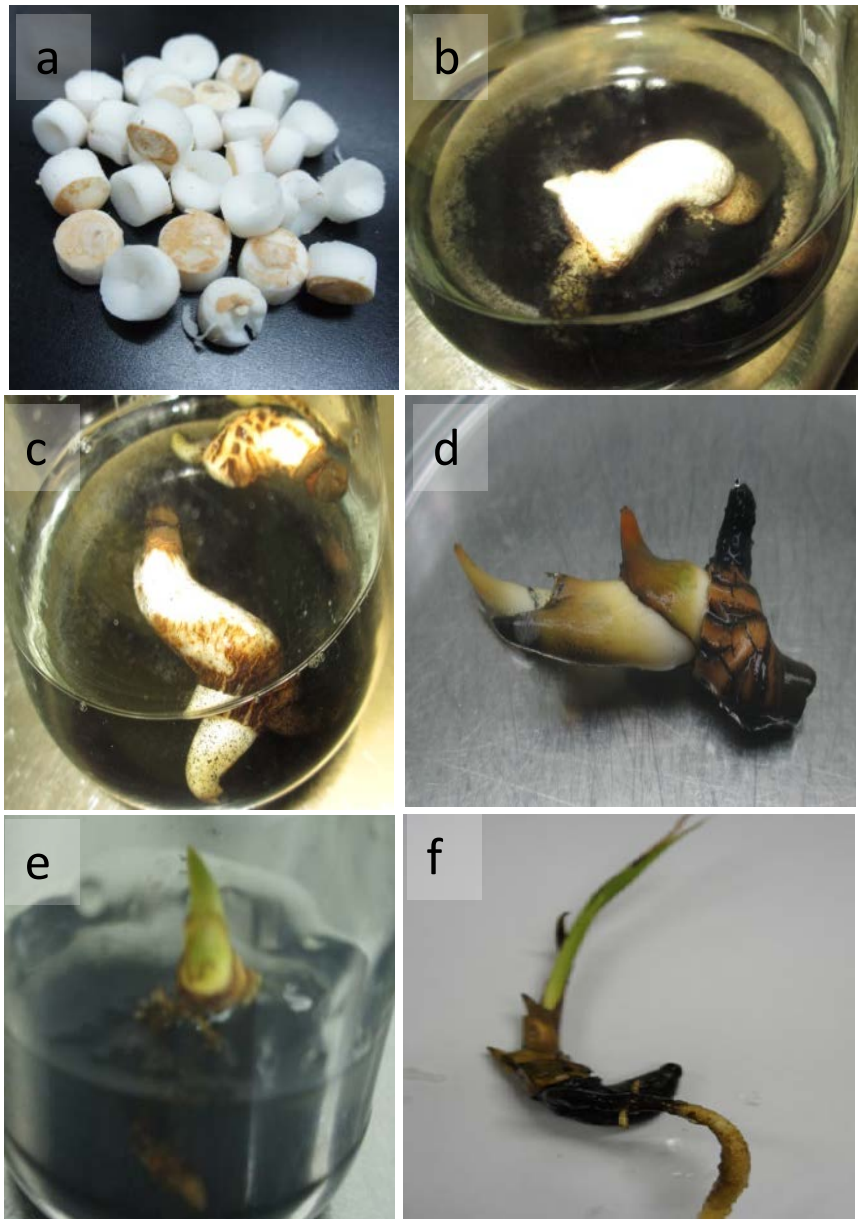

Figure 1: Germination of coconut Matag F2 zygotic embryos.

(a) embryos in kernel, (b-c) development of embryos, (d-e) shoot bud initiation and (f) complete regenerated seedling.

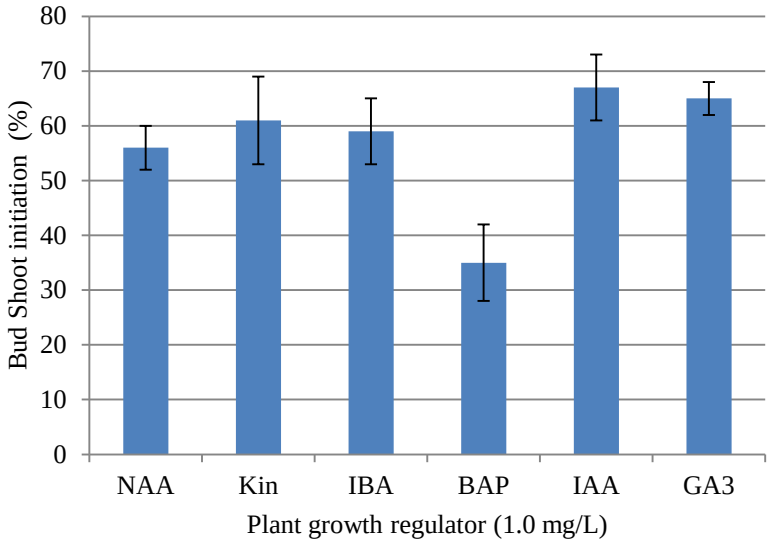

Figure 2: Effect of various plant growth regulators on bud shoot.

Conclusion

Y3 culture medium, especially in the absence of added agar, was found to be a better medium for embryo expansionthan MS medium.Addition of a low concentration of BAP (0.5 mg/L) or NAA (0.5 mg/L) to Y3 medium supported embryo expansion of coconut Matag by up to 250%..In the presence of both BAP and NAA, embryo expansion of 480% and bud shoot initiation of 90% were achieved. This protocol is suited for use in the *in vitro* production of fully developed coconut palm (Matag) seedlings.

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