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Full Length Research Paper

Effect of plant growth regulators and activated charcoal on *in vitro* growth and development of oil palm (*Elaeis guineensis* Jacq. var. Dura) zygotic embryo

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The effect of plant growth regulators and activated charcoal (AC) on *in vitro* regeneration and plantlet development of oil palm (*Elaeis guineensis* Jacq. var. Dura) zygotic embryos were assessed. Zygotic embryos were cultured on Murashige and Skoog (MS) medium supplemented with a blend of 0.05 or 0.1 mg/L of each plant growth regulators (PGR) (gibberellic acid, 6-benzlaminopurine and α-naphthaleneacetic acid) without or with 2 g/L AC. The growth and development of the embryos were affected by the types of media formulations. Zygotic embryos cultured on MS medium supplemented with both PGR and AC enhanced shoot initiation and subsequent plantlet development, while PGR supplemented MS media without AC led to abnormal growth, suggesting that AC is indispensable for oil palm plantlet regeneration *in vitro*. The best medium for growth and development of plantlets was MS medium supplemented with 0.1 mg/L PGR and 2 g/L AC which significantly increased plantlet height (9.4 cm) as well as root length (4.4 cm) than the remaining media formulations.

Key words: Activated charcoal, oil palm, plant growth regulators, zygotic embryo.

INTRODUCTION

Malaysia is known to be one of the largest producer and exporter of palm oil in the world. The crop contributes significantly to the country's gross domestic product. Palm oil holds huge economic importance in the food, non-food derivatives, oleochemical and biofuel industries (Mya et al., 2010). Thus, breeding for high yielding elite oil palm (***Elaeis guineensis*** Jacq.) clones is necessary to remain competitive in the global market. Success of breeding programs however is mainly dependent on the availability of a wide gene pool in the crop's germplasm. Seeds of oil palm are intermediate in nature hence it is not possible to store the seeds for long time (Ellis et al., 1990; Hor et al., 2005). Following a substitute approach, oil palm germplasm is being maintained in field genebanks (Rajanaidu, 1994) but these require a huge investment in land, labour and time. In this context ***in vitro*** conservation of embryo could be a promising alternative to ensure the availability of germplasms as and when required (Grout et al., 1983; Engelmann et al., 1995). It is noteworthy to mention that revival of zygotic embryo is influenced by ***in vitro*** culture media composition. Therefore, it is necessary to establish a suitable media for the regeneration of the embryo in culture.

Successful culture of palm oil embryos requires a growth medium that can supply all the essential mineral ions, carbon source, vitamins and other organic supplements

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Abbreviations: MS, Murashige and Skoog (1962); **AC,** activated charcoal; **PGR,** plant growth regulators; **GA_3,** gibberellic acid; **BAP,** 6-benzlaminopurine; **NAA,** α-naphthaleneacetic acid.

Figure 1. (a) Depericarped seed, (b) kernel, (c) position of embryo and (d) excised embryo.

including growth regulators that can influence growth and morphogenesis. ***In planta***, many plants produce their own hormones to regulate their growth. However, under ***in vitro*** conditions, synthetic growth regulators (hormones) are supplied to ensure optimal growth of the plants. Murashige and Skoog (1962) basal medium (MS) has been widely used as a culture medium for growth and development of oil palm zygotic embryos (Thawaro and Te-chato, 2010). However, variation can occur due to the type and concentration of growth regulator used. Patcharapisutsin and Kanchanapoom (1996) suggested that half strength MS medium enriched with either NAA or 2,4-D, sucrose and activated charcoal (AC) can be used to initiate callus cultures from matured embryos of oil palm; while, same culture suggested that an increased concentration of NAA and 2,4-D in the same culture medium resulted in the production of embryoids from embryogenic calli.

Another factor which seems to influence growth and development of explants in culture is the presence or the absence of AC. In general, AC has been used in plant tissue culture media to improve and/or promote morphogenesis in a wide variety of species (Malhotra et al., 1998; Madhusudhanan and Rahiman, 2000; Gantait et al., 2008, 2009a, b). Growth promoting effects of AC have been attributed to its capacity to adsorb unwanted phenolic exudates which accumulates in culture media, especially in the early stages of culture initiation (Fridborg et al., 1978), In addition, AC also absorb growth-inhibiting substances produced by media break down during autoclaving (Gantait et al., 2009a). Therefore, in this study, we investigated the role of different concentrations of gibberellic acid (GA_3),6-benzlaminopurine (BAP) and α-naphthaleneacetic acid (NAA) as well as AC on the growth and development of zygotic embryos of oil palm var. Dura.

MATERIALS AND METHODS

This study was conducted in the Cryopreservation Laboratory in the Department of Crop Science, Universiti Putra Malaysia (UPM). Mature ***E. guineensis*** Jacq. var. Dura seeds were obtained from Sime Darby Seeds and Agricultural Services. Depericarped oil palm seed were cracked with a hammer to remove the endocarp. The kernels were surface sterilized with 20% commercial Clorox for 20 min then rinsed with several changes of sterile distilled water. The embryos were excised aseptically using sterile secateurs, forceps and scalpel. Figure 1 shows the depericarped seed kernel; partially cut open kernel showing the position of the embryo and the actual embryo measuring 3 to 5 mm in length.

Culture medium

MS basal culture medium supplemented with 30 g/L (w/v) sucrose, 100 mg/L (w/v) myoinositol, with PGR (GA_3, BAP and NAA) at 0.1, 0.05 mg/L or without PGR and with or without 2.0 g/L AC was dissolved individually in sterile distilled water. The pH of the medium was adjusted to 5.7 followed by addition of 8.0 g/L agar prior to sterilization at 121°C for 20 min. Six types of culture medium (T1 to T6) were prepared in accordance with the required treatments. The treatments in this study are as shown in Table 1.

Culture and growth conditions

Excised embryos were cultured on 15 ml of MS culture medium supplemented with different concentrations of PGR and AC (Table 1). All culture tubes with explants were incubated in the growth room at a temperature of 26 ± 2°C, 16 h photoperiod and light

Table 1. Treatments used in this study which consist of MS basal medium supplemented with the required amount of PGR (NAA, BAP and GA_3) and/or AC.

Treatment	MS Culture medium composition (mg/L)
T1	0.1 NAA + 0.1 BAP + 0.1 GA_3 + 2000 AC
T2	0.05 NAA + 0.05 BAP + 0.05 GA_3 + 2000 AC
T3	No PGR + 2000 AC
T4	0.1 NAA + 0.1 BAP + 0.1 GA_3 + No AC
T5	0.05 NAA + 0.05 BAP + 0.05 GA_3 + No AC
T6	No PGR + No AC

The acronym PGR used in this study refers to a mixture containing GA_3, BAP and NAA at the same concentration and the acronym AC stand for activated charcoal.

intensity of 60 $\mu molm^{-2}s^{-2}$. The numbers of viable embryos were recorded. Embryos were considered viable when they had expanded and showed signs of haustorium formation two weeks after culture. Further records on embryo development were taken four weeks after culture when they had developed shoots or roots. Only embryos that developed into plantlets were considered as having survived. Furthermore, morphological characteristics including plant height, root length and stem diameter were also recorded.

Statistical analysis

The data were subjected to analysis of variance (ANOVA) and means were separated using Duncan's multiple range tests (DMRT) at a probability of 0.05. The randomized complete block design (RCBD) was used with five replications per treatment and with each treatment having 10 embryos.

RESULTS AND DISCUSSION

The pattern of oil palm zygotic embryo development and morphogenesis during the first two weeks of culture is shown in Figure 2. Swelling and expansion of embryos cultured on T1 medium was observed within the first 5 days (Figure 2b) and it was followed by the formation of haustorium, approximately 7 to 10 days of culture (Figure 2c). The haustorium enlarged and the embryonic axis began to turn green (Figure 2d, e and f) and finally shoot emergence was observed (Figure 2g) leading to the emergence of plumule from the shoot apex within 14 days of culture in T1 medium. Similar pattern of growth and development was also observed by Thawaro and Te-chato (2010) who reported that swelling of the zygotic embryo occurred at 10 days of culture followed by initiation of shoots at 14 days after culture and complete plantlet formation approximately one month after culture in ***E. guineensis*** Jacq. var. Tenera. The well developed embryos which are shown in Figure 2c to g were considered viable. The viability of the cultured oil palm zygotic embryos differed based on the culture medium used. The best culture medium was T1 which enhanced complete plantlet development after two weeks. The remaining treatments resulted in embryos with restricted growth (Figure 2e).

Figure 3 shows the percentage viability at four weeks after culture. All culture media supplemented with AC (T1, T2 and T3) irrespective of the presence or absence of PGR had almost 100% viability. Contrarily, embryos cultured on media devoid of AC (T4, T5 and T6) showed poor viability, ranging from 23 to 63%. In the culture medium without AC, the addition of PGR inhibited the ability of the embryos to develop into plantlets, although at this period embryo development is confined up to expansion stage only (Figure 2b), without extensive shoot or root development.

As the embryos further developed, percentage survival (that is number of embryos that developed into plantlets) was recorded at four weeks after culture. The results on survival percent were presented together with the viability in Figure 3. The percentage survival of the embryos was lower than the viability in all the treatments. Between T2 and T3, there were significant differences between viability and survival. The growth promoting effect of PGR in media amended with AC was demonstrated significantly. The culture medium supplemented with 0.1 mg/L of each of the PGR and 2 g/L AC (T1) had the highest percentage (97%) of survival, while in the remaining culture media, percentage survival was less than 53%.

The morphology of the developing plantlets varied greatly according to the medium used after one month in culture. Plantlets which developed from embryos cultured on T1 grew vigorously than the remaining treatments after four weeks of culture (Figure 4). Also, embryos cultured on T2 and T3 which contained AC devoid of PGR developed into plantlets without roots. But embryos cultured on the medium without AC showed minimal development (Figure 4); whereas, embryos cultured on T4 and T5 eventually died with those on medium T6 having abnormal growth. Based on the results obtained in this study on growth and development of zygotic embryo of oil palm var. Dura, it can be mentioned that activated charcoal is necessary to ensure the initial ability to germinate, while the presence of PGR in the medium is vital for further development of the embryo. The inclusion of AC appeared to play a decisive role, offering an added advantage. In an earlier study by Gantait et al. (2008)

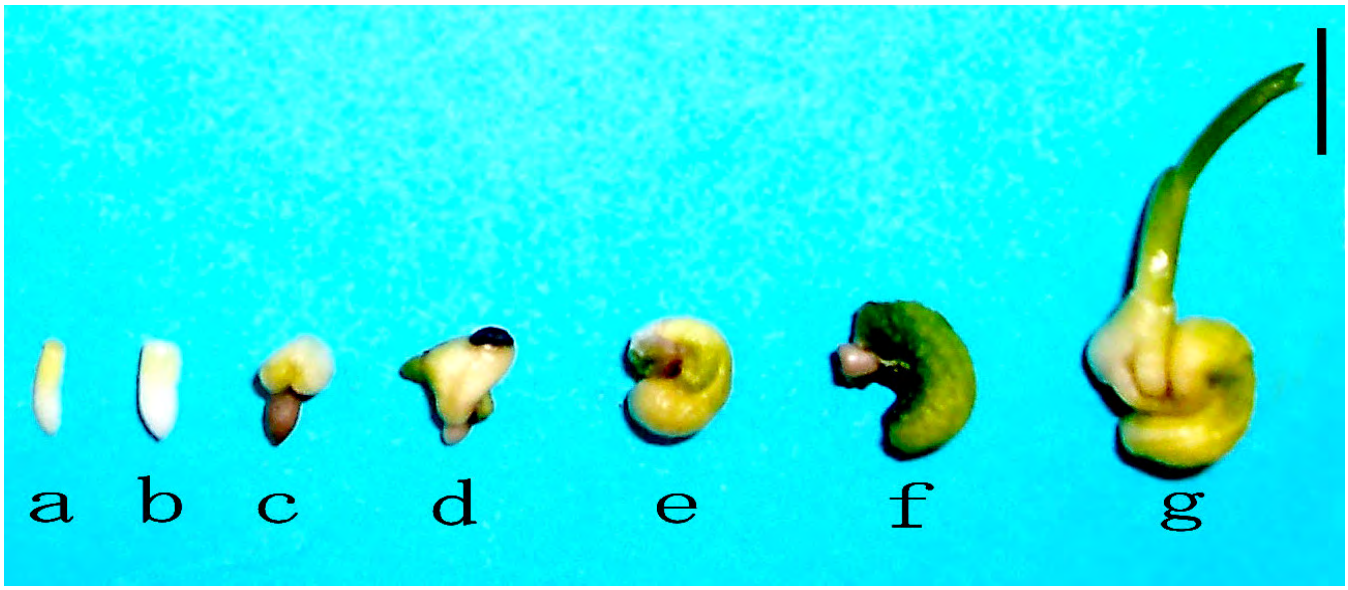

Figure 2. Growth and developmental pattern of oil palm zygotic embryos within two weeks of culture on T1 medium (Bar = 5 mm).

with anthurium, they reported that apart from the physiological regulation of the plant growth and absorbance of growth inhibiting phenolic compounds, AC provides a reasonable physical environment for root growth by eliminating light. This statement was further supported by Gantait et al. (2009a, b) in Dendrobium and Vanilla, respectively, where they observed the enhanced effect of AC in rhizogenesis ***in vitro***. The positive effect of AC on root growth had been shown by Sarma and Rogers (2000) on their studies on ***Juncus effusus*** L. Similar effect of AC on shoot elongation and root induction has also been reported in shoot cultures of ***Ulmus campestris*** (Biondi et al., 1984) and ***Picea galuca*** (Rumaray and Thorpe, 1984).

As a consequence of failure of T5 and T6 in the survival of the embryos, data on plant height, root length and stem diameter for T4, T5 and T6 could not be shown. Hence, the significant impact of PGR on plantlet height, root length and stem diameter is presented in Table 2. The maximum plant height (9.43 cm), root length (1.63 cm) and stem diameter (0.20 cm) was achieved in zygotic embryos cultured on T1 medium after four weeks of culture (Table 2) whilst embryos cultured on T2 and T3 medium had no root formation and the plant height (3.23 and 3 cm, respectively) and stem diameter (0.12 and 0.08 cm, respectively) was significantly less in comparison with embryos cultured on T1 medium. The radicle emerged four weeks after culture (Figure 4) and developed into secondary root from six weeks onwards (Figure 5). The vigorous and extensive growth of roots for the zygotic embryos cultured on T1 medium is necessary to ensure high rate of survival after post flask transfer to the field. The extensive growth of root in T1 medium might be due to synergistic effect of AC and PGR, especially NAA which stimulates the production of roots (Figure 5). It seems that low concentrations of auxin and cytokinin combination are established to be effectual for the establishment of oil palm zygotic embyo. The inducing effect of NAA with BAP was also reported earlier by Gantait et al. (2010), where MS medium fortified with a low level of NAA and BAP promoted earliest bud initiation in gerbera. The synergistic effect of BAP, NAA and GA_3 in the establishment of zygotic embryo and its development during this study was evident. Samarina et al. (2010) also reported the similar incident during their significant study in lemon micropropagation. They found that the combining effect of low concentrations of NAA, BAP and GA_3 plays decisive role in the enhanced morphogenesis in all four lemon cultivars under their study. Nevertheless, it can be assumed that NAA and BAP basically enhanced the shoot initiation and GA_3 helped in elongation of shoots regenerated from oil palm embryo. This study also corroborates the earlier report of Bhattacharya et al. (2003) on papaya ***in vitro*** regeneration where they established that NAA and BAP had a conjugated effect on shoot initiation from mature embryo and later showed shoot elongation effect when it was transferred to the GA_3 supplemented MS medium separately. Interestingly, another pioneer work of Chen et al. (2008) on ***Eucommia ulmoides*** demonstrated the impact of BAP and GA_3 in shoot initiation and proliferation, whereas NAA proved to be effective for root development, significantly. So, it was worthy to use the combination of these three PGR (BAP, NAA and GA_3) accordingly to achieve the complete regeneration of oil palm zygotic embryo in this study.

The reduction in plant growth and development (plant height, root length and stem diameter) is directly related to composition of the medium, whereby the growth reduction was in relation to the concentration of PGR and

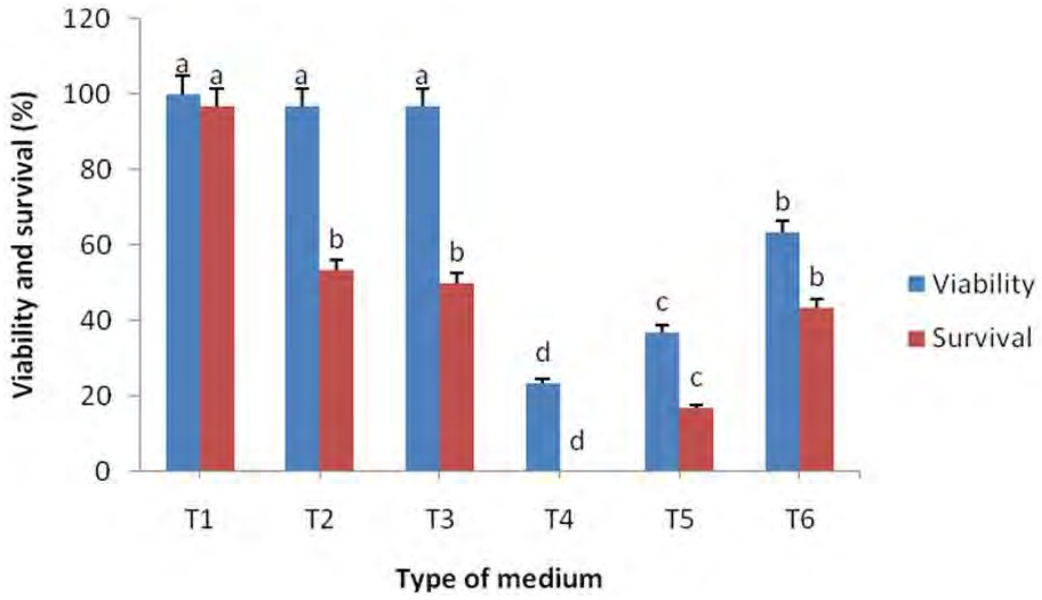

Figure 3. Viability and survival of embryos cultured on different media two and four weeks after culture. Bars represent the standard error of viability and survival percentage. Values are means of five independent experiments, each containing 10 embryos.

Figure 4. Effect of culture media on morphology of oil palm four weeks culture (Bar = 5 mm).

Table 2. Effect of media formulations on plant height, root length and stem diameter in oil palm zygotic embryos after four weeks of culture.

Treatment	Plant characteristic					
	Plant height (cm)		Root length (cm)		Stem diameter (cm)	
	W2	W4	W4	W6	W2	W4
T1	3.80[a]	9.43[a]	1.63[a]	4.40[a]	0.05[a]	0.20[a]
T2	1.60[b]	3.23[b]	0.00[b]	0.00[b]	0.00[b]	0.12[b]
T3	1.55[b]	3.00[b]	0.00[b]	0.00[b]	0.00[b]	0.08[b]

W2, W4 and W6 indicate data collected at the end of two, four and six weeks, respectively, after culture. Different letters within the same column indicate significant differences between morphological characteristics at $p < 0.05$. Values are means of five independent experiments, each containing 10 embryos.

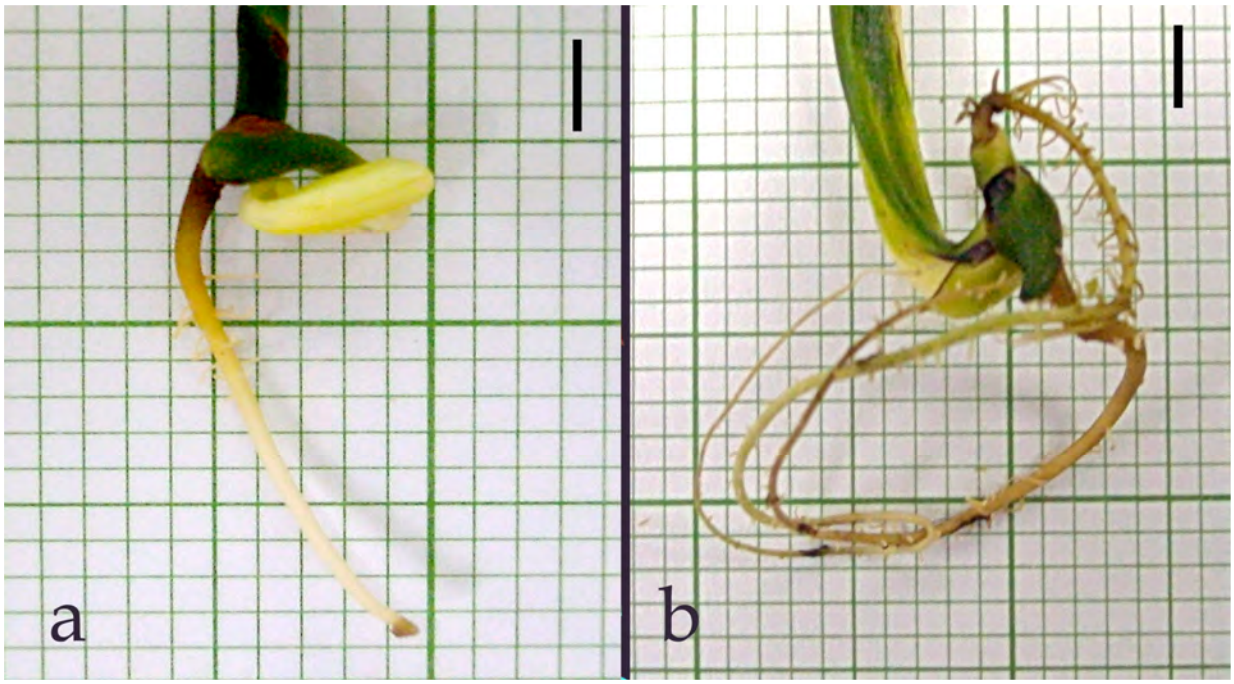

Figure 5. Well developed plantlets with roots obtained from palm oil embryos cultured on T1 medium six (a) and 12 (b) weeks after culture (Bar = 5 mm).

activated charcoal. Interestingly, in the report of Thawaro and Te-chato (2010), they observed good growth and development when embryos were cultured on a medium without AC and PGR, contrary to our observation in Dura where there was abnormal growth on such medium. In this study, we observed an improved vigorous growth and development of zygotic embryos of oil palm on the medium supplemented with PGR and AC.

Conclusion

In this study, we showed that AC with PGR (gibberellic acid, 6-benzlaminopurine and α-naphthaleneacetic acid) played essential role for successful regeneration of plantlets from oil palm zygotic embryos. A very low concentration (0.1 mg/L) of PGR and comparatively higher concentration of AC (2.0 mg/L) is required for healthy plantlet development from the embryos. The successful regeneration of plantlets achieved in this study can be used to complement conventional seedling development for the oil palm industry.

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