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DIRECT AND INDIRECT PLANT REGENERATIONS OF PINEAPPLE *VAR.* MD2 *(Ananas comosus* L.)

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ABSTRACT

The variety MD2 of pineapple *(Ananas comosus* L.) was used in this study. This variety is highly demanded in the international market and known for possessing harvest quality with aroma, high sugar content (14% Brix), vitamins and longer shelf life. However, shortage of planting material has limited the production in Malaysia. *In vitro* mass propagation using the direct and indirect shoot proliferation techniques was tested on variety MD2. The plantlets were successfully initiated from sucker on solid MS basal medium containing 30 g/L sucrose, 0.1 g/L Myo-inositol and 3 mg/L BAP after one month of culture. The highest direct shoot tips regeneration was obtained on solid MS medium when added with 30 g/L sucrose, 0.1 g/L Myo-inositol, 3 mg/L BAP and 1 mg/L NAA. Indirect shoot regeneration was obtained on medium containing 3 mg/L Zeatin after one month of culture. In average, 10 shoots were regenerated from approximately 1 gram of calli. The techniques can produce 100-200 number of plantlet within 4 to 6 months of culture, and ready for planting after 7 months of culture.

Key words: Direct micropropagation, indirect micropropagation, pineapple, MD2, plant regeneration

INTRODUCTION

Pineapple is listed as a major tropical fruit in world production (FAOSTAT, 2010). One of the highly successfully commercialized pineapple varieties is MD2 variety because it is traded in about 75% of the European Union market (Anonymous, 2006). MD2 is a hybrid pineapple produced by Pineapple Research Institute (PRI), Hawaii and currently being the most demanded variety compared to Smooth Cayenne variety (Danso *et al.,* 2008). In fact, MD2 is a very demanding tropical fruit in both domestic and export for international markets (Davey *et al*., 2007). In Malaysia, MD2 have been listed as one of the seven tropical fruits in focusing the output production via the National Key Economic Area (NKEA) initiative under the Malaysian Permanent Food Production Areas (LPNM, 2012). The variety is in high demand due to its sweets aroma, blemish-free flesh and deep golden fruit with high sugar content (14% Brix) with vitamin A, B, and C, ripen evenly and longer shelf life (Danso *et al.*, 2008 & Akbar, 2003). There is an increase in the planting areas and the demand for planting materials. Among the problems faced by pineapple industry in Malaysia is shortage of MD2 planting material because MD2 needs a long period of time to produce slips/suckers. As a result, the slips/suckers are expensive and this will burden the farmers with the increase of capital. This problem could be overcome by producing large number of plantlets through tissue culture techniques. The study of MD2 pineapple had been done by Danso *et al.,* 2008 through direct *in vitro* micropropagation. Indirect regeneration was done since plantlet production can be scaled up manyfold through the intervening callus phase, compared to direct regeneration (ShaValli Khan *et al.,* 2002).

Indirect regeneration through callus culture has been proven to be more efficient in plantlet production as compared to organogenesis (Akbar *et al*., 2003 & Khan *et al*., 2002). The culture will undergo the formation of morphogenic callus and somatic embryo prior preceded to organogenesis.

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However, organogenesis has remainedas one of the important processes in callus regeneration pathway since it is a process of forming and developing shoot from somatic cells (Pardal, 2002). Other propagation technology such as bioreactor system can be used to upscale the production rate of pineapple culture in order to increase further demand of high quality and clonal planting materials of pineapple (Escalona *et al*., 1999).

This paper focuses on both direct and indirect regeneration of MD2 pineapple in various combination of hormone that will enhance the mass propagation of pineapple MD2 in order to fulfill the market demand. In order to accommodate with mass production of pineapple planting material through direct and indirect culture process, Danso *et al.* (2008) have proven high number of shoot multiplication rate by culturing pineapple variety MD2 on a solid MS media supplemented with BAP. Hence, in this study, the explants were cultured on solid MS media supplemented with BAP and zeatin to facilitate the initiation of shoot propagation for the purpose of organogenesis and callus culture. The effect of MS media supplemented with BAP and NAA on the multiplication rate of pineapple var. MD2 was also reported.

MATERIALS AND METHOD

Plant Materials

Twelve sucker of pineapple var. MD2 were bought from JTP Trading Sdn. Bhd (distributor of fresh fruit and vegetable company) in Johor and used as explants with approximately 20 leaves and 30 cm in length. The outer leaves were removed and washed under running tap water for 1 minute before sterilized in 10% (v/v) Benlate® (systemic benzimidazole fungicide that is selectively toxic to microorganisms) solution for 3 minutes. After that the explants were rinsed with sterile distilled water and followed by soaking them in 70% (v/v) Clorox^{TM} (5.25% sodium hypochlorite) solution for 15 minutes and then rinsed with sterilized distilled water. Then, the explants were transferred into the laminar-air flow and soaked in 100% (v/v) ethanol for 10 minutes, before rinsed three times with sterile distilled water. Sterilized explants were then excised until 1-2 cm in sizes.

Initiation Media for Direct Shoot Regeneration

Twelve explants were cultured on MS (Murashige & Skoog, 1962) media containing 30 g/L (w/v) sucrose, 0.1 g/L Myo-inositol, solidified with 2 g/L phytagel and supplemented with three different concentrations of 6-benzylaminopurine (BAP) at 1, 3 and 5 mg/L. Negative control was carried out for each experiment with three replicates. All explants (12 suckers) (Figure 2a) of pineapple var. MD2 were introduced into MS basal media supplemented with various BAP concentrations (1, 3 and 5 mg/L) and after 1 month of culture, numbers of shoots were recorded. The cultures were grown under cool-white fluorescent lamps providing 30 µmol s-1, m-2 light intensity at 25±1ºC with a photoperiod of 16 hours daylight.

Shoot Multiplication Media

After 4-6 weeks, adventitious shoots (Figure 2b) were initiated from the initiation media cultures and were transferred into MS medium containing 30 g/L (w/v) sucrose, 0.1 g/L Myo-inositol, 2 g/L phytagel and supplemented with various combination of plant growth regulators (PGRs) have been used to determine the highest multiplication rate as in Table 2. All cultures were incubated in condition as mentioned above.

Initiation Media for Indirect Shoot Regeneration

Approximately 1.5 cm in length of *in vitro* leaf base from direct initiated cultures were used to induce callus on MS media containing 30 g/L (w/v) sucrose, 0.1 g/L Myo-inositol, 2 g/L phytagel with various PGRs, (Table 3) on the Petri dishes with 10 leaves per dish. The materials were incubated under 16 hour photoperiod of cool-white fluorescent lamps with a light intensity of 30 µmol^{m-2s-1} at 25±1ºC. The pH of all media was adjusted to 5.8 with 1.0 M NaOH or 1.0 M HCl prior to autoclaving.

Statistical Analysis

Analysis of variance (ANOVA) was used for statistical analysis such as mean and standard errors (SE).

RESULTS AND DISCUSSION

Explants cultured in MSB3 showed the highest number of shoots initiation (5 ± 0.2) as compared to other initiation media (Table 1 and Fig. 1). The average of five shoots per explants produced in MSB3 culture media were higher than previous findings, 3.1 ± 0.1 for Queen variety (Sripaoraya *et al.,* 2003) and 2.95 ± 0.22 for Smooth Cayeen variety (Khan *et al*., 2004).

Table 1. The number of shoots produced from explants cultured in direct shoot initiation medium

Media	BAP concentrations (mg/L)	No. of shoots
MSB0	Negative control	1 ± 0.1
MSB1	1	2 ± 0.1
MSB3	3	5 ± 0.2
MSB5	5	4 ± 0.1

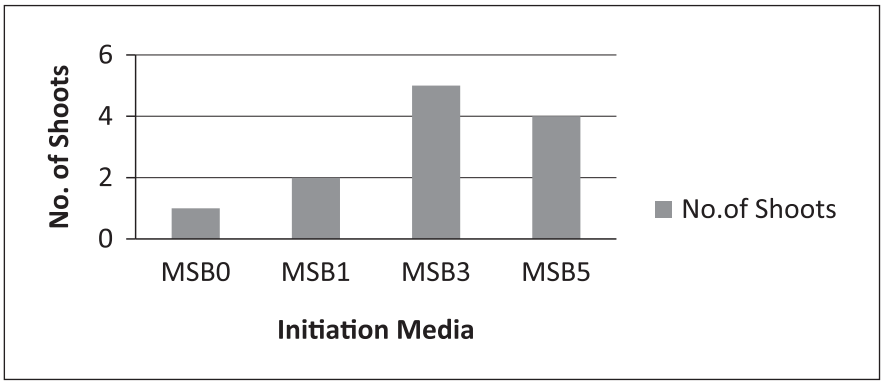

Fig. 1. Effecton the number of shoots produced indifferent initiation media

The highest number of shoot multiplication Figure 2e was in MSB3N1 (15 ± 0.1). This indicated that the combination of exogenous hormone (cytokinine, BAP and auxin, NAA) have facilitated in inducing and multiplying the MD2 pineapple plantlets (Table 2 and Fig. 3).

Table 3 showed the shoot produced on MS basal media containing various PGR from *in vitro* leaf base explants. *In vitro* leaf base cultured onto MST1, MST3, MST5 and MSB3T1 media produces unorganized white, watery callus formation (Figure 2d. Unorganized white, watery callus is classified as low quality callus for cell line with low regeneration potential through somatic embryogenesis (Chin, 1991) as compared to friable callus. In addition, no shoot buds were developed on *in vitro* leaf base explants culture in Thidiazuron (TDZ). Hence, the TDZ is not suitable in callus initiation for pineapple var. MD2 even though previously it has been reported that TDZ can facilitate the active cell division and regeneration either in direct or indirect tissue culture (Guo *et al*., 2011). *In vitro* leaf base explant cultured in Zeatin produced high number of shoots formations i.e. in MSZ3 media it produced 10 ± 0.1 numbers of shoot-like structure after 2 months of culture (Figure 2c). The cultures developed shoots (10 shoots per explants) within a further 18 days of incubation in 3mg/L Zeatin. There are two types of cytokinins which are adenine-type cytokinins (Zeatin, BAP and Kinetin) and phenylurea-type cytokinins (TDZ) involved. On that note, the type of cytokinin is playing a major role of promoting cell division and controlling plant development. This explained the high emerging of shoot buds of pineapple var. MD2 in solid MS media supplemented with zeatin while no shoot buds emerged from solid MS media supplemented with TDZ (Table 3 and Fig. 4).

CONCLUSION

The PGR combination described are promising approach for shoot multiplication of pineapple variety MD2. Both direct and indirect shoot regeneration techniques are successful in producing large number of high quality and uniform MD2 plantlets. However, the indirect technique shows superiority over direct regeneration technique because it can regenerate approximately 600 plantlets of pineapple variety MD2 within 4 to 6 months of culture.

Table 2. Effect of plant growth hormones on shoots multiplication from sucker of pineapple var. MD2

Culture media	Concentration				No. of shoot per explant
	BAP (mg/L)	NAA (mg/L)	Kinetin (mg/L)	IBA (mg/L)	
MSB0N0	–	–	–	–	3 ± 0.1
MSB1N3	1	3	–	–	5 ± 0.2
MSB2N3	2	3	–	–	7 ± 0.1
MSB3N1	3	1	–	–	15 ± 0.1
MSB3N2	3	2	–	–	10 ± 0.1
MSB3N3	3	3	–	–	7 ± 0.2
MSB2N1K1	2	1	1	–	6 ± 0.1
MSB2N2I1	2	2	–	1	5 ± 0.1

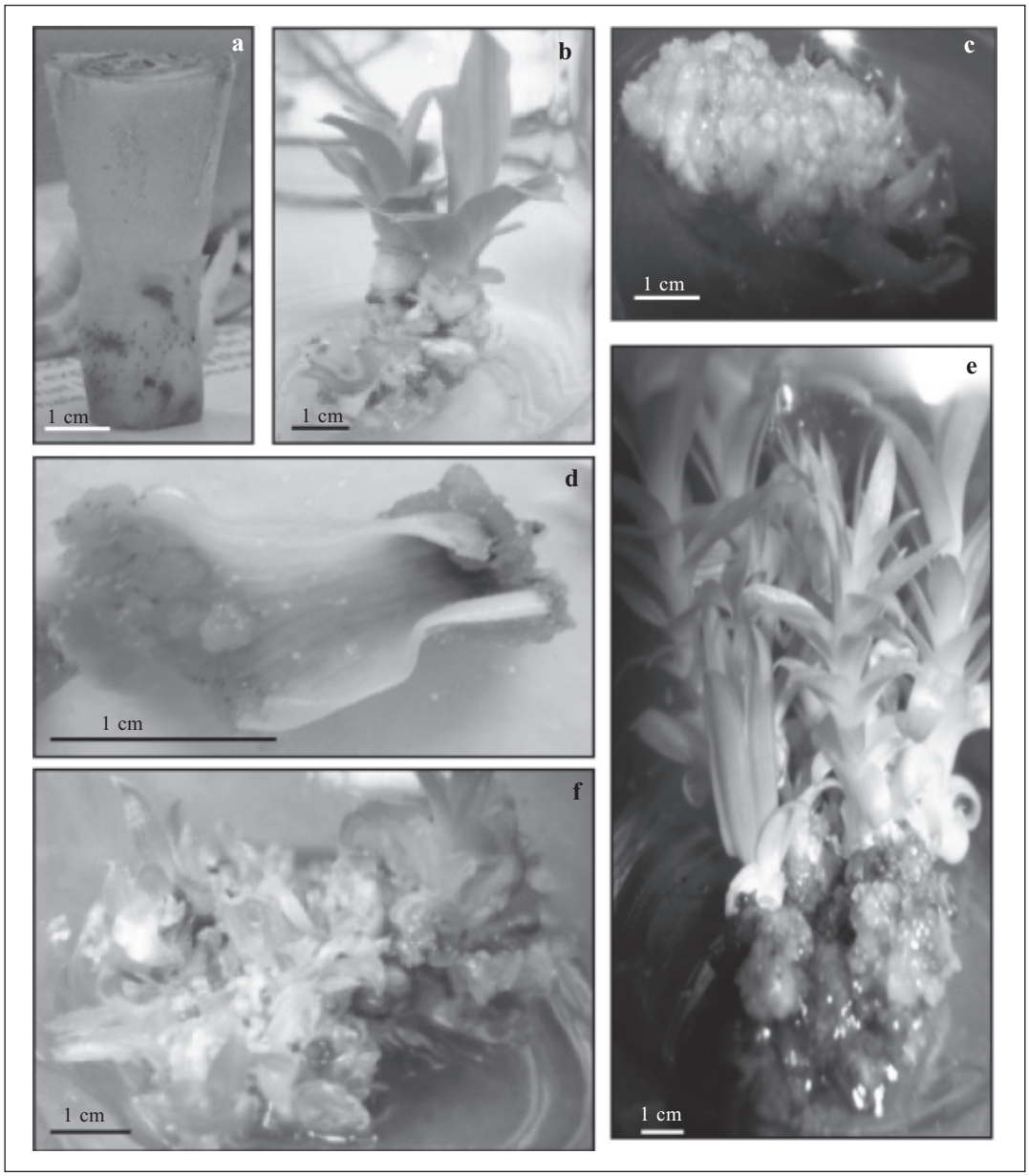

Fig. 2. Plant regeneration from sucker of pineapple var. MD2. a) Explant of pineapple var. MD2; b) Development of adventitious shoots in MS + 3 mg/L BAP after 1 month old culture; c) Shoot-like structure obtained in MS + 3 mg/L zeatin after 2 months of culture; d) Callus induced from the *in vitro* leaf based in 3 mg/L TDZ after 1 month of culture; e) Shoots regenerated in MS + 3 mg/L BAP + 1 mg/L NAA after 1 month of culture from direct regeneration pathway; f) Shoots regenerated in MS + 3 mg/L BAP + 1 mg/L NAA after 1 month of culture from callus (40x magnification).

Table 3. Effect of plant growth hormones on callus shoot regeneration media

Culture media	Concentration			No. of shoots produced from a clump of callus
	BAP (mg/L)	TDZ (mg/L)	Zeatin (mg/L)	
MSZ1	–	–	1	3 ± 0.2
MSZ3	–	–	3	10 ± 0.1
MSZ5	–	–	5	5 ± 0.1
MST1	–	1	–	0
MST3	–	3	–	0
MST5	–	5	–	0
MSB1T3	1	3	–	3 ± 0.1
MSB3T1	3	1	–	0

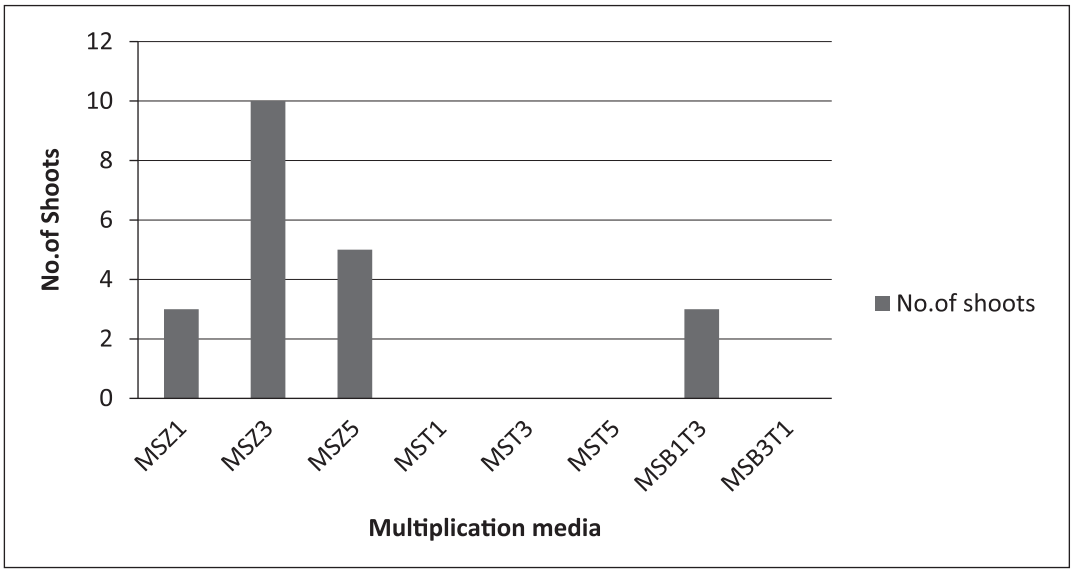

Fig. 3. Effect on the number of shoots produced in different multiplication media.

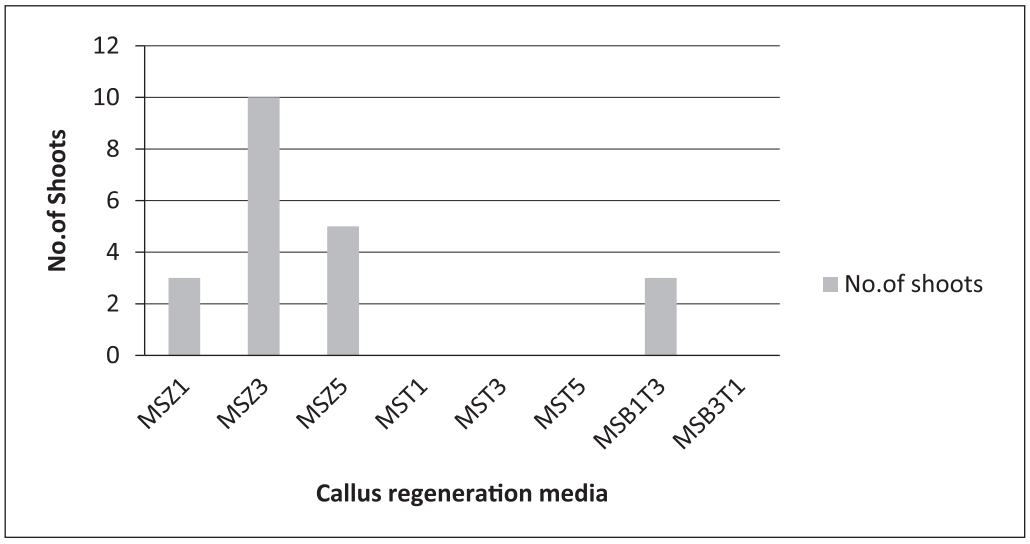

Fig. 4. Effect on the number of shoots produced in different callus shoot regeneration media.

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