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Effect of Explants Density on the *in vitro* Proliferation and Growth of Separated and Cluster Shoots of Smooth Cayenne Pineapple (*Ananas comosus* L. Merr.)

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Abstract: Multiplication rate was always used as the sole parameter for assessment of the optimal treatment for *in vitro* shoot formation. However, beside its effect on the shoot formation, different treatments had different impact on the production cost. Separated shoots and shoot cluster of different sizes (1, 2, 3 and 4 shoots per cluster) of Smooth cayenne pineapple were cultured at density of 1, 2, 3 and 4 separated shoots and 1 cluster on agar solidified full strength MS medium enriched with sucrose at 30 g L^{-1} and BAP at 2.25 mg L^{-1} for 2 months to investigate the effect of explants density and explant types on shoot formation per explant, total per liter and cost per shoot. Density of one separated shoot per culture resulted in the highest shoot formations per shoot (11 shoots) but lowest total shoot per liter (550 shoots) and highest cost per shoot (13 cents), while density of 4 shoots per culture resulted in lowest rate (4 shoots) but higher total per liter (1400 shoots) and lowest cost per shoot (10 cents). Cluster of two shoots had higher rate (7 shoots) and lower cost (16 cents) than cluster of 4 shoots (4 shoots and 20 cents) but both resulted in equal total shoot per liter (750 shoots). Separated shoot had higher shoot formation capacity, higher total per liter and lower cost per shoots than cluster of shoots and using of total per liter instead of rate per explant was the best parameter for selection of treatment of lower cost and higher shoots production.

Key words: Pineapple, *Ananas comosus*, micropropagation, explants density, total shoot production, MS medium, smooth cayenne, cost per shoot, shoot formation rate

INTRODUCTION

In vitro multiplication of pineapple is usually conducted either by transferring of the whole multiple shoots as one unit (Almeida *et al*., 2002), separation of the multiple shoots into individual shoots and subculturing at density of 1 (Hamad and Taha, 2008; Sripaoraya *et al*., 2003; Devi *et al*., 1997), 2 (Be and Debergh, 2006; Soneji *et al*., 2002), 3 (Hamad and Taha, 2003) and 5 (Firoozabady and Gutterseen, 2003; Daquinta *et al*., 1997) shoots per culture. The multiple shoots could also be cut into 2, 4 and 8 pieces (shoot clusters) each consisted of 2 to 3 shoots per piece and cultured at density of 1 shoot per culture in solid as well as agitated liquid medium cluster (Firoozabady and Gutterseen, 2003; Hirimburegama and Wijesinghe, 1992) and at density of 5 clusters 1 L^{-1} bioreactor (Escalona *et al*., 1999) and 50 clusters 10 L^{-1} bioreactors (Fairoozabady and Gutterseen, 2003; Escalona *et al*., 1999). However, neither the effect of explants density nor the explants type (cluster and separated shoots) and cluster size (1, 2, 3 and 4 shoots per cluster) on the shoot formation on the conventional micropropagation system were reported. Using bioreactors system, Escalona *et al*. (1999) reported that the density of clusters and incubation period effect the shoot formation and the frequency of shoot sizes.

In vitro production of ten of thousands of pineapple propagation materials per year using solid (Sripaoraya *et al*., 2003), stationary (Be and Debergh, 2006; Soneji *et al*., 2002; Almeida *et al*., 2002) and agitated (Kofi and Adachi, 1993) liquid cultures and bioreactor systems (Firoozabady and Gutterson, 2003; Escalona *et al*., 1999) have been reported. In all of these reports, shoot formation per explant was the sole parameter of assessment. Although, shoot formation per explant does indicate a different proliferation potential of different plants and explants, effectiveness of different treatments and large scale production, commercial feasibility of a protocol depend on the cost per shoot and cost per liter of medium rather than the shoot formation per explant and expected total production per year.

Cost of 1 L of medium was used as basis for comparison of the cost effectiveness of different cytokinins in multiplication of banana (Arinaitwe *et al*.,

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2000) and the effect of different medium states on the cost per shoot of *Rauwolfia serpentina* (Goel *et al*., 2007). The objective of this study was to compare the effect of shoots cluster size (number of shoots per cluster) and density of separated shoots per culture on the average shoot formation, shoot length, expected total shoots 1 L^{-1} of medium and the cost per single shoot.

MATERIALS AND METHODS

Medium preparation: MS medium (1.3 L) was prepared from stock solutions (Murashige and Skoog, 1962) and supplemented with sucrose at 30 g L^{-1} and BAP at 2.25 mg L^{-1} and adjusted to pH 5.7 before adding agar at 7.0 g L^{-1}. The beaker containing the medium placed over magnetic stirrer hot plate and heated to boiling to dissolve the agar and then dispensed equally (20 mL jar^{-1}) into 63 glass jars (5×15 cm) with screw rim and autoclaveable plastic lid. The medium was then autoclaved at 121°C and 1.5 kg cm^{-2} for 25 min.

Plant materials: Stock pineapple cultures were originally established from crown tip following the protocol described by Hamad and Taha (2003). Briefly, the crown tips were sterilized by soaking in 25% Clorox for 30 min, 80% alcohol for 3 min and then rinsed in sterilized distilled water for 5 min, trimmed to 1 cm^3 and cultured on agar solidified full strength MS enriched with sucrose at 30 g L^{-1} and BAP at 2.25 mg L^{-1}. The stock cultures maintained by subculturing every 60 days on fresh medium. To investigate the effect of explant types and density on the *in vitro* shoot formation, multiple shoots from the stock cultures were either separated into individual shoots or into clusters of 2, 3 and 4 shoots and cultured at density of 1, 2, 3 and 4 separated shoots and one cluster consists of 2, 3 and 4 shoots (Cluster of different size) per glass jar tube containing 20 mL of agar solidified full strength MS medium enriched with sucrose at 30 g L^{-1} and BAP at 2.25 mg L^{-1}. Each treatment of different density of separated shoots and cluster of different size consisted of 9 cultures. The cultures were layout on the incubation room shelves as RCBD and kept under constant temperature of 25°C and photoperiod of 16 h of light provided by fluorescence lamps. All of the experiments were conducted to the Institute of Biological Sciences, University of Malaya at Kuala Lumpur, Malaysia.

Data collection and analysis: After 60 days of incubation, the multiple shoots removed from the cultures, weighed, separated into individual shoot for counting the number and measuring the length of shoots. In cases when there were more than one separated shoot and when a cluster of shoots were used per culture, the total shoot number were divided by the number of the separated shoot or number of shoots per cluster to compute the average per single shoot. The length of all shoots were summed and divided by the total number of shoots to compute the average length of shoot. The total and average shoots of each three of the nine cultures of each treatment were computed and considered as one replicate. Total shoot production 1 L^{-1} of medium were estimated by multiplication of average of each replicate by 50. Finally, three tables of three replicates; two tables for average of shoot number and length and one for total shoots 1 L^{-1} of medium were established and used for statistical analysis of the data. The experiment was layout as RCBD and the data were subjected to analysis of variance and testing of the significant difference between treatments mean at $p \leq 0.05$ by Duncan Multiple Range test using SPSS statistical package No. 11. The cost analysis were calculated based on the cost of 1 L of MS medium, quantity of culture tubes, time of autoclaving and laminar use, labor working hour and the electricity cost of 60 days of incubation and the total shoots produced 1 L^{-1} of medium.

RESULTS

The average shoot formation and shoot length and the estimated total per liter of medium per one shoot of the separated and cluster shoots (Table 1) showed that increasing the number of shoots per cluster (cluster size) from 2 to 4 shoots reduced the capability of shoot formation per single shoot from 7 to 4 shoots and increased the shoot length from 13 to 16 mm. But using cluster of different sizes did not induce any significant different in the estimated total of shoot production 1 L^{-1} of medium. The total shoots per liter using cluster of 2, 3 and 4 shoots were 700, 750 and 800 shoots, respectively. However, the cost of shoot production increased from 13 to 20 Malaysian cents per shoot (about $ 0.04 to 0.07). Similarly, the shoot formation capability of the separated shoot decreased from 11 to 7 shoots as the density increased from 1 to 4 shoots per culture. The shoot length increased from 12 to 15 mm as the density increased from 1 to 2 shoots per culture. However, the density lost its effect on shoot length when 3 and 4 shoots were used per culture. Equal shoot lengths of about 14 mm were obtained at either density. Contrary to shoots cluster in which the estimated total shoots per liter were not affected, increasing the density of separated shoot per culture to 3 and 4 shoots resulted in significantly higher total per liter (1200 and 1400 shoots) than using 1 and 2 (550 and 800 shoots) shoots per

Table 1: Effect of explants density (shoots per culture) and explants manipulation (separated and cluster of shoots) on the *in vitro* shoot formation and shoot length (mm) per single shoot, total shoots 1 L^{-1} of medium and cost per shoot of Smooth cayenne pineapple

	Explants manipulation							
	Shoots/explant		Shoot length (mm)		Total shoots L^{-1}		Cost/shoot (RM)	
Explants/culture (No.)	Clustered	Separated	Clustered	Separated	Clustered	Separated	Clustered	Separated
1	11.00a	11.00a	12.0b	12.0b	550b	550.0b	0.13	0.13
2	7.00bc	8.00ab	13.0ab	15.0ab	700b	800.0b	0.16	0.12
3	5.00bc	8.00ab	13.0ab	13.0ab	750b	1200.0a	0.18	0.10
4	4.00c	7.00bc	16.0a	14.7ab	800b	1400.0a	0.20	0.10
Average	6.75B	8.5A	13.5NS	13.7NS	700B	987.5A		

Means of the same column followed by the same small letter(s) and the average followed by same capital letters were not significantly different at $p \leq 0.05$ as tested by Duncan multiple range test. RM Malaysian Ringgit (1 USA $ = 3.25 RM). The calculated cost included only the variable items of cost (MS, agar, sucrose, hormones, electricity cost of autoclave, laminar operation and incubation room and the wages of labor)

culture and reduced the cost from 13 and 12 cents at density of 1 and 2 shoots to 10 cents at density of 3 and 4 shoots. The total of shoots produced using cluster of 3 and 4 shoots (700 and 750 shoots) was not significantly different from that at density of 1 (550 shoots) and 2 (800 shoots) separated shoots but was two times less and two times more expensive than that at density of 3 (1200 shoots at 10 cents) and 4 (1400 shoots at 10 cents) separated shoots per culture (Table 1).

DISCUSSION

The cost per single shoot as well as the total shoots production and the rate of shoot formation per explant of Smooth cayenne pineapple was significantly affected by both of the explants density and size of the shoot clusters. The result (Table 1) indicated that the cost per single shoots could be reduced to as low as 10 cents by using 3 and 4 separated per culture. Although, easy to test and simple to apply, the effect of explants density and explants manipulation on the *in vitro* multiplication of pineapple was totally ignored in the previous study of pineapple. In most of the studies, the attention was focused on optimization of hormones and medium states. Treatment with highest shoot formation per explant was considered optimal and suggested for large scale production of propagules. However, higher shoot formation per single explant is not the best indicator for possibly commercial large scale production. The total shoot production and cost per single shoot rather than the rate per single explant are the most important and essential parameters.

At any density, separated shoots resulted in higher shoot formation than cluster of shoots (Table 1). In both types of explants, assessment based on shoot formation per explant is not enough for selection of best treatment. According to shoot formation per single shoot, density of one separated shoot and cluster of two shoots per culture would be recommended over higher shoot density and larger cluster size. However, compared to density of 1 shoot per culture, separated shoots at density of 3 and 4 shoots per culture increased the total by two to three times and reduced the cost per shoot by two times. Similarly, increasing the cluster size (shoots per cluster) reduced the average of shoot formation per each shoot of the cluster, but had no effect when estimated total 1 L^{-1} of medium was compared. It is interesting that whether the explants were separated shoots or cluster of shoots, higher explants density decreased the shoots formation per single shoot. However, while increasing the density of separated shoots increased the total shoots per liter of medium, the estimated total of shoots of the different cluster sizes (2, 3 and 4 shoots/cluster) were not significantly different.

Cost of production was always stressed as the main obstacle of micropropagation. Nevertheless, the cost items such as medium volume and medium use efficiency (total shoot per liter), autoclaving and laminar operation time, labor working hours and shelving space, was not taken in to consideration during assessment of different *in vitro* multiplication treatments. All of these cost items are related and could be manage through selection of the explant types and density of explant per culture. Yet the effect of these two factors on the rate of shoot formation and its relation to the different cost items were not reported. Arinaitwe *et al.* (2000) used the cost of 1 L of medium as basis to compare the cost effectiveness of different cytokinin on banana shoot formation. Using the same approach, Goel *et al.* (2007) reported that cost per shoot of *Rauwolfia serpentina* in semi solid was Rs. 0.126 and could be reduced to Rs. 0.004 (Indian currency) using glass bead with Daurala sugar. Our estimation of cost per pineapple shoot included all cost items of multiplication (medium, culture tubes, electricity cost for operating autoclave, laminar and incubation room and the labor wages).

Longitudinal segmentation of multiple shoots into cluster of shoots is much simpler and easier than separation into individual shoots and hence it would take shorter laminar operating time (electricity cost) and

subculturing into fresh medium (labor cost). Since, random cutting of multiple shoots is not expected to cause significant difference in the estimated total shoot per liter, (cluster of different sizes resulted in statistically equal total per liter (Table 1), the use of shoot cluster for subculturing into fresh medium would be favored over separated shoots for saving electricity and labor cost. However, using of the shoot cluster, on the other hand, resulted in lower rate per single shoot, lower total shoots per liter of medium and higher cost per shoot. The lower rate per shoot of cluster may be due to domination effect of one shoot of the cluster over the others. Separation of shoots eliminated the domination effect of one shoot over the other and consequently improved the shoot formation rate per single shoot. Using bioreactor system, Escalona *et al*. (1999) reported that the density effect of clusters depended on the incubation period. Explant at density of 1 (Hamad and Taha, 2008; Sripaoraya *et al*., 2003; Devi *et al*., 1997), 2 (Be and Debergh, 2006; Soneji *et al*., 2002) and 3 (Hamad and Taha, 2003) separated shoots and 1 and 3 clusters (Firoozabady and Gutterson, 2003; Escalona *et al*., 1999) per culture on conventional and 50 clusters (Escalona *et al*., 1999) on bioreactor system and a rate range of 3 to 25 shoots on conventional and 60 shoots per cluster on bioreactor system were reported. However, neither the effect of different explants density nor the effect of different size of clusters on the shoot formation rate of pineapple and the expected total of shoots was compared in the previous studies. The shoot formation rate obtained in this study at density of 1 and 2 separated shoots per culture were within the rate range previously reported for Smooth cayenne on solid full strength MS medium. The little different on the rate could be attributed to different hormone treatments and incubation periods.

Which explant types and explant density would be the best depended on the availability of culture stocks, amount and time of propagules delivery and the available budget. If the amount of the available shoots were limited and few stock cultures were successfully established as it is usual during the establishment stage, subculturing of shoots at density of one shoot per culture is essential to take the advantage of high shoot formation per single shoot. However, by the second cycle of multiplication stage, more shoots would be available and subculturing at higher density, in addition to higher shoots total per liter of medium, would reduce the cost per shoot (less jars, medium, shelving space and working time). Because of its simplicity and expected saving of laminar operation (electricity cost) and culturing working hours (labor cost), random cutting into shoot clusters deserve further investigations to improve the shoot formation capacity per liter of medium. Investigation on the effect of clusters of different size as well as of separated shoots at density higher than 4 clusters of shoots and 4 separated shoots per culture on the shoot formation and cost on conventional micropropagation system could reduce the cost to lower than the estimated 10 cents per shoot obtained in this study. In this study both separated and cluster shoots were treated by the same hormone and equal concentration (BAP at 2.25 mg L^{-1}). If higher cytokinin were used for cluster, it may overcome the domination effect of one shoot over the other and improve the proliferation capacity of the cluster. Other hormone types, concentrations and combinations may promote the shoot cluster proliferation.

In conclusion, compared to explant density of one shoot per culture, the cost of the *in vitro* shoot production of Smooth cayenne pineapple could be reduced to half by culturing at density of 3 separated shoots per culture. Although, optimization of hormones and medium states are very important for proliferation studies, adoption of a shoot multiplication system for commercial application require using of proper explants density and explants manipulation. In addition to the rate per explant, estimated total per unit of medium volume should be taken in to consideration. Total shoots per liter and total at each cycle of multiplication is very essential and more practical for estimation of cost and for better management and planning of commercial micropropagation than the rate per explant.

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