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Effect of Explants Density, Medium Volumes and Subcultures on the *in vitro* Shoot Formation and Cost of Multiplication of Moris pineapple (*Ananas comosus L. Merr*)

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Abstract:

The effects of three medium volumes (4, 6 and 8 ml) and four explants density (one, two, three and four explants) on the in vitro shoot formation rate per explant and total shoots production of Moris pieapple were tested over four subcultures in full strength liquid MS medium enriched with sucrose at 20 g/l and BAP at 2.0 mg/l. Over four subcultures, the highest shoot formation per explant (6.75 shoots) and the highest total shoots (22680 shoots) obtained using one explant and 8 ml of medium per culture. However, at highest total cost (USA $ 1175.25) and cost per shoot (5.2 cents). Use of four explants in 6 ml of medium resulted in loss of 50 % of the total shoots (10080 shoots) but lowered the total cost seven times (USA $ 162.92) and the cost per shoot three times (1.6 cents). Use of three explants on 6 and 8 ml maintained the cost per shoot at (1.7 cents), reduced the total cost three times (USA $ 306.33 and 330.75) while the loss in total shoots was less than 20 % (17640 and 18144 shoots). At each subculture there was different optimum combination of medium volume and explants density per culture. At the first subculture, the highest shoot formation per explant was 7 shoots obtained at any explants density in 6 and 8 ml of medium while the highest shoot formation during the second subculture was 6 shoots obtained using three explants in 4 ml and three and four explants in 8 ml of medium. Use of one explants during the third and two explants during the fourth subculture on both of 6 and 8 ml of medium resulted in highest shoot formation per explants (9 and 7 shoots respectively). Density of three explants on 6 and 8 ml of medium was the best compromise between highest total shoots, lowest total cost and lowest cost per shoot.

Keywords: *Micropropagation; Ananas comosus; Total cost, Subcultures; Explant density; Medium volume.*

Introduction:

Many researchers have suggested micropropagation techniques as an alternative to overcome the slow propagation of pineapple. Starting with single explant, [1- 4] expected production of a total of 5000, 80000, 100000 and 121125 shoots per year and [5] expected production of one million shoots per 9 months. Starting with 80 [6], 42 [7], 40 [8], 21 [9], 10 [10] and 5 explants [11] expected production of 161080 shoots per 8 months, 24768 shoots per 6 months, 30000 shoots per 6 months, 15 000 shoots per 7 months, 1.2 million of shoots per 4 months and 24768 shoots per 6 months respectively. From 10 to 20 suckers, [12] expected 1.0 million shoots per year and from single crown, expected 10000 shoots per 6 months [13].

In these studies the focus was mainly on optimization of hormone treatment (concentrations, types, combinations) and medium states (solid, liquid) at fixed medium volume and fixed explant density per culture and the expected total of shoots was based on average of shoot formation rate overall subcultures and extrapolation to period not supported by actually conducted subcultures.

Results of these studies reflect the mass production potential. Unless different combination of explants density and medium volume per culture were used and at each and over consecutive subcultures the results assessed by comparison of total shoots, total cost and cost per shoot, commercial micro-propagation is questionable. Not only different rate of shoot formation and total shoots is expected at different combination of explants density and medium volume per culture but also at specific available amount of explants and medium, dispensing the medium at smaller volume and using of single explants per culture require more vessels, longer operation time of laminar, larger shelving space than dispensing the available medium at larger volume and using of higher explants density per culture. Cost is always emphasis as the main obstacle of commercial application of tissue culture and this obstacle could not be solved unless the cost of production is itemized and the percentage of each cost item of the total cost is determined.

The objective of this study was to investigate the effect combinations of four explants density (one, two, three and four explants) and three volumes of medium (4, 6 and 8 ml) per 25 ml- culture tube on the *in vitro* shoot formation per explant and total shoots and to estimate the total cost and cost per shoot at each and over four consecutive subcultures.

Materials and Methods

Full strength MS medium supplemented with sucrose at 20 g/l and BAP at 2.0 mg/l, adjusted to pH 5.0 and sterilized by autoclaving at 121 ^{0}C and 1.5 kg/ cm^2 for 20 minutes was used throughout the experiments [14]. The different volumes of sterilized medium [9, 8 and 15] sterilized medium was dispensed under laminar flow cabinet into 75 culture (each volume in 25 tubes) and marked as A, B and C. One, two, three and four explants obtained from Moris stock cultures were added to 12, 6, 4 and 3 of each of the 25 culture tubes marked A, B and C.

The cultures incubated under constant temperature 25 ^{0}C and 16 hours of light provided by cool white fluorescent lamps (30 mmol m^2 s^1). After 60 days of incubation, multiplied shoots were picked out from some of each of the 25 culture tubes marked A, B and C under laminar flow cabinet condition, placed on sterilized petri dish and separated into individual shoots for counting and measuring their length.

Twelve shoots from each combination of medium volume and explants density were sub cultured onto culture tubes containing the same medium volume and explant density the shoots were taken from. Counting and measuring the length of the shoots of the other culture tubes was done outside the laminar flow cabinet under room condition to complete recording the data of the first subculture. The same procedure was repeated to collect the data of the second, third and fourth subculture at 60 days interval. Table of total shoots per culture after first, second, third and fourth subculture was converted to table of expected total shoots after consecutive subcultures.

Data were subjected to three ways analysis of variance and the means significant tested by Duncan Multiple Range test at p 0.05 using SPSS statistical package No 11.0. Tables of expected total shoots production per subculture and over consecutive subcultures were used for estimation of total cost and cost per shoot at each and after consecutive subcultures.

Results

Average of shoot formation overall subcultures (Table, 1) showed that use of one explant in 8 ml of medium per culture produced the highest shoot formation (6.75 shoots) while four explants in 4 ml of medium produced lowest shoot formation per explant (5 shoots).

Generally, at each fixed combination of medium volume and explants density the shoot formation increased over the first three subcultures and decreased afterward. But, the pattern of shoot formation over subcultures depended on the combination of explants density and volume of the medium per culture (Table, 1). At density of one explant, in all medium volumes per culture (4, 6 and 8 ml), the shoot formation per explant increased over the first three subcultures from 5 shoots at the first subculture to 8 and 9 shoots at the second and third but declined at the fourth subculture to 6 shoots.

At density of two explants, the shoot formation in cultures containing 4 ml of medium increased over the first three subcultures from 5 to 6 shoots and remained stable at 6 shoots at the fourth subculture. In cultures containing 6 and 8 ml of medium, the second subculture resulted in the lowest shoot formation (4 shoots) and the explants regained back its shoot formation capacity during the third and fourth subculture.

At density of three explants per culture, the shoot formation in cultures containing 4 ml of medium increased over the first two subcultures from 5 to 6 shoots while in cultures containing 8 ml the rate decreased from 7 shoots at first to 6 shoots at the second subculture but remained stable at 6 shoots at the third and fourth subcultures.

At density of four explants, subcultures had no effect in shoot formation if the explants incubated in 4 ml of medium per culture. Rate of 4 shoots was obtained at each of the four subcultures. On the contrary, the shoot formation in cultures containing 8 ml of medium decreased over subcultures from 7 shoots at first to 6 shoots at second and third and 5 shoots at the fourth subculture.

At each subculture, there was different optimum combination of explants density and medium volume per culture for shoot formation (Table, 1). The highest shoot formation at the first subculture (7 shoots) obtained using any of the explants density in 6 and 8 ml of medium per culture while at the second subculture the highest shoot formation (6 shoots) obtained using three explants in 4 and 8 ml and four explants in 8 ml of medium per culture.

The highest shoot formation at the third subculture (9 shoots) obtained using one explants in 6 and 8 ml of medium and at the fourth subculture (7 shoots) obtained using two explants in 6 and 8 ml of medium per culture. Similar, the lowest shoot formation at the first, third and fourth subculture was 5 shoots obtained using respectively any of the explants density in 4 ml, four explants in 4 ml and four explants at any of the medium volume per culture. The lowest shoot formation at second subculture was 4 shoots obtained using one, two and four explants (any of the explants density) in 6 ml and two explants in 4 ml of medium per culture.

Table (1). Effect of medium volumes and explants density per culture on the *in vitro* shoot formation and total shoot of Moris pineapple over four subcultures.

MS (ml) / culture	**Explants** / culture	1	2	3	**Subcultures** 4	Average
					Shoots per explants	
4	1	5 cd	5 cd	8 ab	6 bcd	6 ABC
	2	5 cd	4 d	6 bcd	6 bcd	5.25 BC
	3	5 cd	6 bcd	6 bcd	5 cd	5.5 ABC
	4	5 cd	5 cd	5 cd	5 cd	5 C
	Average	5 DE	5 DE	6.25 ABC	5.5 CD	
6	1	7 abc	4 d	9 a	6 bcd	6.5 AB
	2	7 abc	4 d	7 abc	7 abc	6.25 ABC
	3	7 abc	5 cd	7 abc	6 bcd	6.25 ABC
	4	7 abc	4 d	6 bcd	5 cd	5.5 ABC
	Average	7 AB	4.25 E	7.25 A	6 BCD	
8	1	7 abc	5 cd	9 a	6 bcd	6.75 A
	2	7 abc	5 cd	6 bcd	7 abc	6.25 ABC
	3	7 abc	6 bcd	6 bcd	6 bcd	6.25 ABC
	4	7 abc	6 bcd	6 bcd	5 cd	6 ABC
	Average	7 AB	5.5 CD	6.75 AB	6 BCD	
					Total shoots	
4	1	60 m	300 kl	2400 fg	14400 b	
	2	60 m	240 l	1440 i	8640 cd	
	3	60 m	360 jk	2160 fg	10800 c	
	4	60 m	300 kl	1500 hi	7500 d	
6	1	84 m	336 kl	3024 ef	18144 ab	
	2	84 m	336 kl	2352 fg	16464 ab	
	3	84 m	420 jk	2940 ef	17640 ab	
	4	84 m	336 kl	2016 gh	10080 cd	
8	1	84 m	420 m	3780 e	22680 a	
	2	84 m	420 jk	2520 fg	17640 ab	
	3	84 m	504 j	3024 ef	18144 ab	
	4	84 m	504 j	3024 ef	15120 b	

Means followed by the same letter were not significantly different at $p \leq 0.05$ according to Duncan Multiple Range Test.

Starting with 12 shoots, estimation of expected total shoots over consecutive subcultures (Table, 2) showed that after two subcultures the highest total shoots (504 shoots) could be obtained using three and four explants on 8 ml of medium while the lowest total (240 shoots) could be obtained using two explants on 4 ml of medium. Statistically equal total shoots (322 shoots) obtained using one and four explants in 4 ml, one, two and four explants in 6 ml of medium and equal total shoots (405 shoots) obtained using three explants in 4 and 6 ml and one and two explants in 8 ml of medium per culture.

After three subcultures the highest total shoots (3780 shoots) obtained using one explant in 8 ml of medium and the lowest total (1440 shoots) using two explants on 4 ml of medium. Statistically equal total shoots (2358 shoots) obtained on four combinations (one and three explants on 4 ml, two explants on 6 and 8 ml) and equal total shoots (3003 shoots) obtained on other four combinations (one and three explants in 6 ml and three and four explants in 8 ml of medium per culture). The highest total shoots over four consecutive subcultures (22680 shoots) obtained using one explant and 8 ml of medium per culture and the lowest (7500 shoots) obtained using four explants on 4 ml of medium. Statistically equal total of about 17606 shoots obtained at five combinations of explants density and medium volumes (one, two and three explants in 6 ml and two and three explants in 8 ml of medium). Equal total shoots (14760 shoots) obtained in two combinations (one explant in 4 ml and four explants in 8 ml) and equal total shoots (9630 shoots) obtained on other two combinations (two explants in 4 ml and four explants in 6 ml of medium per culture). Generally, irrespective of the explants density, the highest total shoots at each subculture was obtained when the medium dispensed at 8 ml per culture and the lowest total shoots when the medium dispensed at 4 ml per culture.

Table (2). Effect of medium volumes and explants density per culture on the total cost and cost per shoot of Moris pineapple over four subcultures.

MS (ml) / culture	Explants / culture	Subcultures 1	2	3	4
Total cost (USA $)					
4	1	3.19	18.74	96.14	714.67
	2	1.64	9.47	40.49	226.11
	3	1.13	6.38	37.39	223.02
	4	0.87	4.83	24.25	120.98
6	1	3.28	25.69	115.02	918.22
	2	1.69	12.94	57.65	370.07
	3	1.16	8.69	45.97	306.33
	4	0.89	6.57	28.97	162.92
8	1	3.38	26.45	141.4	1175.25
	2	1.74	13.32	70.84	415.52
	3	1.19	8.94	54.98	330.75
	4	0.92	6.75	41.31	248.16
Cost per shoot (USA $)					
4	1	0.053	0.062	0.040	0.050
	2	0.027	0.039	0.028	0.026
	3	0.019	0.018	0.017	0.021
	4	0.014	0.016	0.016	0.016
6	1	0.039	0.076	0.038	0.051
	2	0.020	0.038	0.024	0.022
	3	0.014	0.021	0.016	0.017
	4	0.011	0.019	0.014	0.016
8	1	0.040	0.063	0.037	0.052
	2	0.021	0.032	0.028	0.023
	3	0.014	0.018	0.018	0.018
	4	0.011	0.013	0.014	0.016

Each full strength, liquid stationary MS supplemented with sucrose at 20 g/l and BAP and adjusted to pH 5.0.

Table (3). Estimation of amount and percentage of various cost items during multiplication of Moris pineapple over four subcultures (using four explants in 8 ml of medium per culture).

		Subcultures				Cost	
	Cost items	1	2	3	4	Total	%
		Cost per subculture (USA $)					
C.tubes	Tubes	0.138	0.969	5.815	34.892	41.815	16.80
Medium	MS	0.059	0.413	2.481	14.887	17.841	7.19
	Sucrose	0.013	0.091	0.546	3.275	3.925	1.60
	BAP	0.001	0.008	0.050	0.298	0.357	0.15
Electricity	Autoclave	0.009	0.065	0.388	2.326	2.788	1.12
	Laminar	0.135	0.365	1.711	9.788	12.000	4.84
	Incubation	0.369	2.585	15.508	93.046	111.508	44.94
Labor	Mprep.	0.012	0.086	0.517	3.101	3.717	1.50
	Mdisp	0.026	0.179	1.077	6.461	7.743	3.12
	Culture	0.154	1.077	6.461	38.769	46.461	18.72
Total cost per sub.		0.916	5.839	34.554	206.846	248.156	100
Total shoots		84	504	3024	15120		
Total cost over sub.		0.916	6.756	41.31	248.156		
Cost per shoot		0.011	0.013	0.014	0.016		

Cost estimation based in total shoot production at each subculture starting with 12 shoots.
The medium was full strength, liquid stationary MS supplemented with sucrose at 20 g/l and BAP at 2.0 mg/l adjusted to pH 5.0.

Discussion

Use of one explant and 8 ml per culture resulted in the highest shoot formation (6.75 shoots) and highest total shoots (22680 shoots) but at highest total cost (USA $ 1175.25) and highest cost per shoot (5.2 cents). If analysis of cost was not conducted, this combination of explants density and volume of medium would be recommended as the best treatment. Compared to density of one explant and 8 ml of medium per culture, increasing the density to four and keeping the medium at 8 ml per culture reduced the total cost by six times (USA $ 248.16) and the cost per shoot by three times (1.6 cents) but resulted in lower rate (6 shoots) and loss of 33 % of total shoots (15120 shoots). Using of four explants in 6 ml of medium reduced the total cost seven times (USA $ 162.92), cost per shoot three times (1.6 cents), but resulted in loss of 56 % of the total shoots (10080 shoots).

Using of four explants on 4 ml of medium reduced the total cost 10 times (USA $ 120.98), maintained the cost per shoot at (1.6 cents) but resulted in lower rate (5 shoots) and loss of 67 % of the total shoots (7500 shoots). Other combinations (three explants in 6 and 8 ml) reduced the total cost four times (USA $ 306.33 and 330.75) and maintained the cost per shoot at 1.6 cents while the loss in total shoots was only 21 % (17640 and 18144 shoots). On other word, comparing the total shoots, total cost of the explants density and medium volume combinations which resulted in lowest cost per shoot (1.6 cents) indicated that a compromise between saving of USA $ 85.24 (248.16- 162.92) and extra producing of 5040 shoots (15120- 10080) and saving USA $127.18 (248.16- 120.98) and extra production of 7620 shoots (15120- 7500) has to be made based on budget available and amount of propagules demanded by prospective client.

If the target production is less than 7000 shoots in 8 months period, using of four explants in 4 ml would achieve the target at the lowest total cost (USA $ 120.98). Moreover, table, 1 and 2 showed that using of one and three explants in 6 ml of medium resulted in statistically equal total shoots (18144 and 17640 shoots) but at different total cost (USA $ 918.22 and 306.33).That is using of one instead of three explants in 6 ml of medium is just wasting of about USA $ 611.89. Similar, using one explant in 4 ml of medium and four explants in 8 ml of medium resulted in equal shoot rate and total shoots (14400 and 15120 shoots), but in term of total cost using of one explant in 4 ml instead of four explants in 8 ml of medium is just wasting of USA $ 466.51 (714.67- 248.16)

Cost is always emphysis as the main obstacle of commercial micro-propagation of pineapple, yet in all pineapple tissue culture studies neither the total cost nor the cost per shoot were itemized. Developing of a system for commercial production requires not only cost estimation but also assortment of the cost into different items and determination of contribution of each cost item into the final total cost. Estimation of cost (Table, 2) showed that at any medium volumes per culture and subculture, the lowest cost per shoot when one explant was used per culture was always above 5.0 cents and using of two explants per culture did not reduce the cost below 2.0 cents. For the cost to reach manageable level (1.6 cents) the explants density per culture have to be not less than three explants. In most reported studies the explants density was always less than three explants and medium volume higher than 15 ml, little wonder that the cost was always considered as the main obstacle of micro-propagation. Itemization of cost (Table3) showed that the major four items of total cost and cost per shoot were electricity of incubation (44.94 %), labor wages (23.34 %), culture tubes (16.8 %) and medium components (8.9 %). Minimizing the cost could be obtained by focusing on the items that caused the highest percentage of the total cost. In tropical countries and during the summer in temperate zone area, 45% of the total cost (electricity cost of incubation) could be saved by outdoor incubation under polyethylene or saran net house [16]. The culture tubes could be reused by decanting the old medium and dispensing new one under laminar cabinet. However, saving the cost by reusing of culture tubes would increase the laminar operation time (electricity cost for laminar operating) and working hours. In general, the electricity cost, price of culture tubes and labor wages varied in different countries and within country. In global market, project which would not be feasible in certain countries could be very profitable on others.

Using of liquid culture [17], commercial sugar [18] and temporary immersion system [9, 15 and 18] have been suggested for increasing multiplication rate and minimizing the medium cost of micro-propagation. This study demonstrated that using three times less amount of medium per culture (6 and 8 ml) than the commonly used volume (20 to 25 ml) resulted in comparable shoot formation rate and total shoots. That is reducing the cost of medium by three times.

Further reduction in medium cost could be obtained by using of low medium strength, optimum combination of explants density and medium volume per culture at each subculture. *In vitro* shoots formation of Moris pineapple varied over subcultures and at each subculture there were different optimum combination of explants density and medium volume per culture (Table, 1). Using of one fixed medium volume and explants density per culture at all subcultures simplify the procedures of tissue culture but obtaining of highest total shoots at lower total cost and cost per shoot requires optimizing the medium and explants density at each subculture. Using of different medium volumes and explants density at different subcultures have not been reported, but it worth trying. It may result in higher shoot formation rate and total shoot than fixed medium volume and explants density per culture at all subcultures.

Table 1 showed that using four explants during the first and second subculture, one explant during the third and two explants during the fourth subculture and 8 ml of medium per culture resulted in highest shoot formation (7, 6, 9 and 7 shoots). If these rate is maintained when the different explants density per culture is applied at different subculture, it would lead to substantial increase in the total shoots (31752 shoots). Testing of different explants density per culture at each subculture are suggested for future investigation.

References:

1) Zepeda, C. and Sagawa, Y. (1981). *In-vitro* propagation of pineapple. *Hort. Sci.*16 (4); 495
2) Kiss, E., Kiss, J. Gyulai, G. and Heszky, L. E. (1995). A novel method for rapid micro propagation of pineapple. *Hort. Sci.* 30(1), 127-129.
3) Sripaoraya, S., Marchant, R. Power, J. B. and Davey, M. R. (2003). Plant regeneration by somatic embryogenesis and organogenesis in commercial pineapple (*Ananas comosus* L). *In Vitro Cell. Devl. Biol. Plants,* 39 (5): 450-454.
4) Hamad, A. M. Taha, R. M . (2008). The effect of different hormones and incubation periods on *in vitro* proliferation of pineapple (*Ananas comosus* L. Merr cv. Smooth Cayenne) shoot-tip culture. Pak. J.Biol. Sci. 11: 386-391.

5) Vesco, L. L. D., Pinto, A. A. Zaffari, G. R. Nodari, R. O. Reis, M. S. and Guerra, M. P. (2001). Improving pineapple micropropagation protocol through explant size and medium composition manipulation. *Fruits Paris*, 56: 143- 154.
6) Almeida, W. A., Santana, B., Rodriguez, G. S., and Costa, M. A., (2002). **Optimization of a protocol for the micropropagation of pineapple.** *Rev. Brasil. Fruticul*. 24 (2): 296-300.
7) Perez, G., Yanes, E. Isidron, M. and Lorenzo, J. C. (2009). Phenotypic and AFLP characterization of two new pineapple somaclones derived from *in vitro* culture. *Plant Cell Tiss.Org. Cult*. 96: 113- 116.
8) Fitchet, M. (1990) . Clonal propagation of Queen and Smooth cayenne pineapples. *Acta. Hort*. 275: 261- 266.
9) Firoozabady, E. and Gutterson, N. (2003). Cost effective *in vitro* propagation methods for pineapple. *Plant Cell Report.* 21; 844- 850.
10) Drew, R. A. (1980). Pineapple tissue culture. Un-quarted for rapid multiplication. *Queen Land Agri. J.* 106; 447-451.
11) Perez, G., Yanez, E., Mblogholi, A., Valle, B., Sagarra, F., Yabor, L., Aragon, C., Gonzalez, J., Isidron, M. and Lorenzo, J. C. (2012). New pineapple somaclonal variants: P3R5 and dwarf. *American J. of Plant Sciences*, 3: 1- 11.
12) Bhatia, P. and Ashwath, N., (2002). Development of a rapid method for micropropagation of a new pineapple (*Ananas comosus* (L) Merr. Clone Yeppoon gold. *Acta Hort*.575: 125- 131.
13) Soneji, J. R., Rao, P. S. and Mhatre, M.(2002). Somaclonal variation in micropropagated dormant axillary buds of pineapple (*Ananas-comosus* L., Merr.). *J. Hort. Sci. Biotech*. 77 (1): 28-32.
14) Murashige T and Skoog, F. (1962). A revised medium for rapid growth and bioassay with tobacco tissue. *Physiol. Plant.* 15: 473- 497.
15) Escalona, M., Lorenzo, J. C. Gonzalez, B. Daquinta, M. Gonzalez, J. L. Desjardins, Y. and Borroto, C. G. (1999). Pineapple (*Ananas comosus* L. Merr) micropropagation in temporary immersion systems. *Plant Cell Report*.18 (9): 743-748.
16) Be, L. V. and Debergh, P. C., (2006). Potential low cost micropropagation of pineapple (*Ananas comosus*). *S. Afr. J. Bot.* 72: 191- 194.
17) Zuraida, A. R., Nurul Shahnadz, A. H. Horteeni, A., Roowi S., Che Radziah, C. M. Z and Sreeramanan, S. (2011). A novel approach for rapid micropropagation of maspine pineapple (*Ananas comosus* L.) shoot using liquid shake culture system. *African J. of Biotechnology*, 10 (9):3859- 3866
18) Dutta, I., Bhadra, J., Ghosh, P., Saha, B. and Datta, S. (2013). An efficient and cost effective protocol for in vitro propagation of pineapple. J. of Ornamental Plants, 3 (4): 229-234.