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Massive *in vitro* biomass and propagules production of pineapple (*Ananas comosus* L.Merr) cv Smooth cayenne

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Abstract

Smooth cayenne pineapple explants were cultured on full strength MS medium solidified with agar at 7 g/l, enriched with sucrose at 30 g/l and Benzylaminopurine (BAP) at 2.25 mg/l and subcultured every 60 days on the same but fresh medium for eight consecutive subcultures. Shoots formation per explant increased over the first five subcultures from 7 at the first to 16 shoots at the fifth then declined to 8 shoots over the next three subcultures. Total length and total weight of shoots per explant increased from 72 mm and 1.59 g at the first to reach a maximum of 149 mm and 2,58 g by the fifth subculture. Average length and average weight of shoot, on the contrary, decreased over subcultures from 11 mm and 0.24 g at the first to 7 mm and 0.09 g by the fifth subculture. However, the shoot resumed gaining weight and length after that to reach by the eighth subculture equal length and weight as that of the first subculture. Assuming that all of the *in vitro* produced shoots at each subculture were used on the next subculture, biomass weighting 25 tons and a total of 103 millions shoot could be produced after eight subcultures (16 months).

Key word: Pineapple; *Ananas comosus;* Micropropagation; Biomass; Subcultures.

Introduction

In vitro culture of pineapple has been suggested as an efficient and rapid method of pineapple propagation by several researchers. Various explants, medium states, hormones and systems were tested for *in vitro* culture of pineapple and different results of shoot formation rate and expected total of propagules were reported. Starting with single explant a total shoots production range from 200 (Bordoloi and Sarma, 1990), 280 (Delvi, *et al*, 1997), 300 (Dewald, *et a.,l* 1988), 5000 (Zepeda and Sagawa, 1981), 40000 (liu *et al.*, 1989), 80,000 (Kiss, *et al*, 1995) and 100000 shoots (Sripaoraya, *et al*, 2003) were expected per year. Other reported that a single explant could be used to produce 2013 shoots per 5 months (Almeida, *et al*, 2002), 16000 shoot per 6 months (Firoozabady and Gutterson, 2003), 10000 shoots per 6 months (Soneji, *et al*., 2002), 1.0 million shoots per 9 months (Vesco *et al.*, 2001), 121125 shoots (Hamad and Taha, 2008) per 10 months and 15000 shoots per 10.5 months (Bhatia and Ashwath, 2002). Pannetier *et al* (1976), on the other hand, expected production of 2 million shoots per two years of repeated culturing of a single explant. Starting with 80 (Almeida *et al*., 2002), 42 (Perez *et al*., 2009), 40 (Fitchet, 1990), 22 (Firoozabady and Gutterson, 2003), 10 (Drew, 1980), 8 (Firoozabady and Gutterson, 2003) and 8 explants (Escalona, *et al.*, 1999) production of 161080, 24768, 30000, 15757, 1.2 million, 6000 and 11000 shoots were expected per 8, 6, 6, 7, 4, 6 and 4 months respectively. From axillaries of 10 to 20 suckers, Bhatia and Ashwath (2002) expected 1.0 million per year. Using of 50 clusters of three shoots in a temporary immersion bioreactor, Escalona, *et al.* (1999) obtained 2412 shoots after one month and half and 1969 shoots after one month.

These differences on the reported expected total shoots could be attributed to different hormone treatments, medium state, length of incubation period, cultivars as well as method of calculation of the expected total shoots. Nevertheless, in spite of these discrepancy of expected total shoots, these figures indicated *in vitro* culture potential as propagation method to overcome the shortages of pineapple planting materials. Furthermore, pineapple has high bromelian content, a very important compound with multi industrial and medical uses. Hence, beside mass production of propagules, *in vitro* pineapple culture could be used for huge production of biomass for forage, medical and biogas industry. The objective of this study were firstly to monitor the effect of eight subcultures on the *in vitro* shoot formation, shoot weight and length. Secondly to use the results for computation of total shoots and total weight that could be produced over eight 60-day long subcultures.

Materials and Methods

Explants and medium

In vitro obtained shoots from four months old stock pineapple culture were used in this study. Nine shoots was cultured individually each one in a glass jar (5x 15 cm) containing 20 ml of MS (Murashige and Skoog, 1962) medium supplemented with sucrose at 30 g/l, BAP at 2.25 mg/l, adjusted to pH 5.7 and solidified with agar at 7 g/l. After 60 days of incubation under 16 and 8 hours of light and darkness and a constant temperature of 25 C^0, one shoot from each jar was re-cultured again on the same but fresh medium and kept under the same incubation conditions. The rest of the shoots of each jar were weighted (total weight per explant) then separated into individual shoots to count the shoots and measure their length. Average weight per shoot were computed by dividing the weight of shoots per jar (total weight per explant) by the number of shoots per jar (shoots per explant). Summation of the shoot length of the shoots produced from one explant was considered as the total shoot length per explant and used for computation of average shoot length by dividing by number of shoot produced per explant. Eight consecutive subcultures were conducted and at each subculture the same data were recorded. Data obtained from each three of the nine individually cultured explants was averaged out and considered as a replicate. The data of each parameter; average shoot formation, shoot length and shoot weight and total shoot length and total shoot weight per explant over all of the eight subcultures were presented in separate tables each table consisted of three replicates.

Calculations of expected total shoots and weight

Table of average shoot formation per explant and average shoot weight per explant were used to estimate the expected total shoots and total weight that could be obtained over consecutive subcultures. The expected total shoots were estimated using four approaches. "Replicate approach" in which the total shoots produced at the first subculture were actually counted (sum of the average shoot formation of the three replicates of the first subculture divided by three). For estimation of the total shoots after the first two subcultures, the total shoots produced at the first subculture multiplied by the average shoot formation obtained at each replicate of the second subculture. Then the sum of the total shoots of replicates divided by three gave the total shoots that could be obtained after two subcultures. The total shoots after two subcultures

multiplied by average shoots formation obtained at each replicate of the third subculture and the sum of total shoots of replicates divided by three gave estimate total shoots after three subcultures. The same procedures used to estimate the total shoots that could be produced after four, five, six, seven and eight subcultures. "Subculture approach" in which the total shoots estimated by multiplying of average shoot formation of each subculture (Sub1 x Sub2 …… x Subn). "Overall subculture approach" in which the total shoots estimated by average shoots overall subcultures to the power of actual subcultures (average shoot formation over subcultures) $^{\text{Actual subcultures}}$. "Extrapolation approach" in which total shoot per year estimated by average shoots formation over any consecutive subcultures to the power of expected subcultures per year (average shoot formation over any consecutive subcultures)[6]. Table for the total shoots estimation over consecutive subcultures using replicate approach and table for average shoot weight for each subculture were used for estimation of total weight (biomass production) of shoots produced over 8 consecutive subcultures. The average shoot weight obtained at each subculture multiplied by the expected total shoots of each replicate of that subculture and dividing the sum of replicates by three to obtain the total weight in grams produced after any number of consecutive subcultures. The obtained data of weight converted to kilograms by dividing by 1000. Data of total shoots and total weight over subcultures were first transformed using natural log and then used for statistical analysis. ANOVA analysis and means separation of all parameters were conducted using SAS statistical package.

Results

The *in vitro* shoot formation capability increased over the first three subcultures (7, 10 and 15 shoots respectively) and reached a steady state (15 and 16 shoots) for the next two subcultures (the fourth and fifth subculture). Then the shoot formation declined from 16 at the fifth to 7, 8 and 8 shoots at the sixth, seventh and the eighth subculture (Table 1). The expected total shoots and weight that could be produced after consecutive subcultures based on "replicate approach" is presented in table 1. Starting by one explants, the total shoots over consecutive subcultures increased as the number of subcultures increase to reach a reasonable total (15,063 shoots) after four subcultures. The total shoots increased to reach 103 million by the eighth subculture. Different method of estimation of total shoots gave different figures. Estimation of total shoots after 4 and 6 subcultures using replicate approach (Table, 1) and subcultures approach (Table, 2) gave almost equal expectation of 15,063 and 15,750 shoots and 1,767,392 and 1,764,000 shoots respectively while estimated total shoots per year using extrapolation approach were 2,631,644 and 2,512,993 shoots. If the subcultures continued up to 8 subcultures, the different in the expected total shoots between replicate and subculture approach as well as overall subcultures approach become very big. The estimated total after 8 subcultures based on replicate, subculture and overall subcultures approach reached 103.9; 112.9 and 178.3 million respectively while total shoots based on extrapolation approach declined to 1.54 million shoots.

Average length and weight of the shoots decreased over the first six subcultures. The length decreased from 11 mm at the first subculture to 7 mm at the sixth subculture. The weight decreased from 0.24 g at the first subculture to 0.09 g at the six subculture. After the sixth subculture the shoots weight and length started to increased. By the eighth subculture the shoot length and weight was the same as that obtained after the first subculture (11 mm, 0.24 g respectively). Contradictory to the average length and weight, the total length and weight per explant (one explant/culture) was increasing over the first four subcultures to a maximum of

149 mm and 2.58 g. Then, both decreased to a minimum of 50 mm and 0.96 g by the sixth subculture. During the seventh and eighth subculture, the cultures resumed some weight gaining and shoot elongation capability. The total length and total weight per culture after the eighth subculture (87 mm, 1.87 g) was longer and heavier than those obtained during first and second subculture. The biomass production (total weight) expected after four consecutive subcultures, on the other hand, were 2.7 kg. A reasonable biomass production (2839.6 Kg) obtained after seven subcultures. The total weight increased to reach 25383.7 kg after eight subcultures.

Discussion

The three growth parameters, shoot formation, shoot weight and shoot length of Smooth cayenne pineapple responded differently to repeated subcultures. The shoot formation capability was enhanced over the first four subcultures and retard afterward. After the fifth subculture, there was 50 % loss in shoot formation capability. The shoot formation decreased from 16 to 8 shoots (Table, 1). This indicated that the commonly using of one treatment over several subculture is not proper practice. It simples the procedures but did not optimize the shoot formation. To obtain the highest shoot formation per explants over eight subcultures, three different treatment should be developed. One to improve the shoot formation at the first two, one to maintained the highest rate of the third up to fifth and one to prevent decline of shoot formation after the fifth subculture. The low shoot formation at the first two subcultures could due to improper medium strength, type, state or pH adjustment and the decline in shoot formation after the fifth subculture could be due to hormone residual effect, length of incubation or few axilary buds on the explants . If so, using higher medium strength (1.5 and 2.0x), optimizing the different components of medium (macro, micro, amino acid and organic components) or liquid medium may improve the shoot formation at the first two subcultures. Using of low concentration of hormone, different hormone, longer incubation and longer explants may minimize the decline of shoot formation after the fifth subculture. The decline in shoot formation over subcultures has been observed for pineapple as well as other plants. However, the subculture at which the decline started varied from third (Delvi, *et al.*, 1997; Fitchet, 1990), fourth (Hamad and Taha, 2008), fifth (Singh and Manual, 2002) to seventh subculture (Kofi and Adachi, 1993). The difference subculture at which the decline occurred may due to difference in age of the shoots they used, hormone and incubation period.

The response of the other two growth process, shoot length and weight, over subcultures was of opposite nature to that of shoot formation. Both of the shoot length and weight decreased over the first six subcultures and then started to increase. Such decrease indicated that shoot formation process was dominating over shoot growth and development. That is optimizing of either one will be at the expense of the other. Compared to first subculture, the most marked reduction in shoot length occurred during the second and the fifth subculture. Each resulted in loss of 5 and 3 mm in average shoot length. Similar, the lightest shoot weight was obtained after the sixth subculture. However, the greatest loss on average shoot weight occurred during second, third and sixth subcultures. Each respectively caused 0.06, 0.08 and 0.11 g loss in shoot weight. After that, the shoots started to gain some weight and increase in length. By the eighth subculture, the *in vitro* capability to gain weight and length was completely recovered. The shoot length and weight after the eighth was the same as that obtained after the first subculture

(11 mm, 0.24 g respectively). Subcultures which increased the shoot formation decreased the weight of individual shoot. If this decrease in shoot weight over subcultures could be overcome and on the same time highest shoot formation maintained, the system would be optimum for biomass production.

Over the first six subcultures the expected total shoots from single explant estimated by "replicate approach" (Table, 1) and "subculture approach" (Table, 2) was in general on the range of the expected total reported in the previous studies (1000 to 1.3 million). However, after the seven subcultures not only the difference between our estimation of expected total shoots and the reported ones was too huge but also the difference between the different approaches of calculations was too big. Using either one of these approaches, the total shoots after seven subcultures (14 months) was over 13 million and after eight subcultures (16 months) was over 100 million. Using replicate (Table, 1) and subculture approach (Table, 2) for estimation of total shoots after 4 subcultures gave almost equal expectation of 1,767,392 and 1,764,000 shoots respectively while according to extrapolation approach the expected total reached 4 million shoots (Table, 2). If the subculture continued up to 8 subcultures, the different between replicate, subculture and overall subcultures approach of calculation become very big. The expected total reached 103.9; 112.9 and 178.3 million shoots respectively. Of all of these approaches, replicate always resulted in lower expected total than the others. Subculture approach was higher than replicate but less than overall subculture approach. The subculture and replicate approach was based on actual shoot formation and so the expected total would be very close to the total that could be actually obtained. Overall subcultures approach used one fixed average for all subcultures (shoot formation average overall subcultures). In reality different subcultures had different shoot formation average (Table, 1). The shoot formation was higher at some subcultures and lower at others. Hence, using of one fixed shoot formation average would lead to different total shoots estimate from that using replicate and subculture approach. Extrapolation approach resulted in higher total than the other as long as the shoot formation average of six or less subcultures was used for calculation. However, if shoot formation average of more than six subcultures was used, the total shoots according to extrapolation approach were less than one hundredth of any of the other approaches (Table, 2). Extrapolation approach is based on assumption that if sub-culturing were continued for more than the actually carried ones, shoot formation average per explants would remain the same. This is not true at least for the first five subcultures. It is based on theoretical rather than actually conducted subcultures.

Biomass production would very soon become one of the important goals of pineapple tissue culture. There is a great possibility of using the pineapple *in vitro* mass for forage, extraction of bromelian and for biogas. Table (2) showed that the system has to run for 7 to 8 subcultures before a substantial total weight (2838.6 and 25383.6 kg respectively) could be obtained. Biomass production is the result of total shoots multiplied by average weight of single shoot. If the goal is mass production of propagules, then increasing the shoot formation is priority. However, if the goal is biomass production, then the choice depend on whether the high shoot formation would compensate for light weight of shoots or the heaviest weight of shoot would compensate for low rate of shoot formation. The best treatment is that which result in shoot rate and weight that produce the highest biomass. Reasonable total shoots (15063 shoots per 8 months) and highest shoot formation (15 shoots) obtained after four subcultures but the total weight was only 2.7 kg. For highest total weight (25383.7 kg) the culture should be repeated

for eight times (16 months). Taking in consideration that the calculation was based on a single explant as starting point, higher production could be obtained over few subcultures (4 subcultures) by using of large number of explants. If 10 or 100 explants were used instead of one, by the fourth subculture the expected total shoots and total weight would be 150630 and 1506300 shoots and 270 and 2700 kg per 8 months respectively. If the system continue for six subcultures about 2300 and 23000 kg of green forage is expected to be produced per one year. Other approach to obtain highest biomass is to develop two regimes one for obtaining and maintain highest shoot formation at each subculture and highest total shoots over consecutive subcultures. Other for increasing the shoot weight and used at the last subculture. To shorten the time for highest total shoots and highest biomass production to few subcultures three problem need to be solved. Identify and optimize the physical and chemical factors that could induce highest shoot formation just after the explants being established. Overcome shoot formation and shoot weight decline over subcultures and maintain highest shoot formation and shoot weight over subcultures.

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Table 1. *In vitro* shoots formation (shoots/explant), growth (shoot length and weight/explants) and (total shoot length and weight /explant) and expected total shoots and total weight over consecutive subcultures.

Subcutures	Shoots per explant			Total per explant		Total shoots and weight (Repl. approach)*	
No	No	Length (mm)	Wieght (g)	length (mm)	Weight (g)	No	Weight (kg)
1	7 b	11 a	0.24 a	72 bc	1.59	7 h	0.002 h
2	10 ab	8 bc	0.18 abc	81 abc	1.88	70 g	0.01 g
3	15 ab	9 ab	0.1 c	133 ab	1.51	1027 f	0.10 f
4	15 ab	10 ab	0.2 ab	149 a	2.58	15063 e	2.65 e
5	16 a	7 c	0.09 c	119 abc	1.33	241008 d	20.03 d
6	7 b	7 c	0.13 bc	50 c	0.96	1767392 c	230.56 c
7	8 b	10 ab	0.21 ab	77 abc	1.61	13550005 b	2839.61 b
8	8 b	11 a	0.24 a	87 abc	1.87	103883372 a	25383.67 a

Data represent means of 9 shoots individually cultured in 20 ml of agar solidified (7 g/l) full MS medium containing 30 g/ l of sucrose, enriched with BAP at 2.25 mg/l and adjusted to pH (5.7) for 60 days under 16/8 hours of light and dark and constant temperature of 25 C^0

Calculation was according to the steps stated in Materails and Methods using replicate average approach.

Table 2. Estimation of expected total shoots by subculture, overall subculture and extrapolation approaches

Subcultures No.	Shoots per explants Each sub	Overall Sub	Expected total shoots Subculture*	Overall sub**	Extrapolation***
1	7				
2	10	8.5	70	72	377,150
3	15	10.66	1,050	1,211	1,467,382
4	15	11.75	15,750	19,061	2,631,644
5	16	12.6	252,000	317,580	4,001,504
6	7	11.66	1,764,000	2,512,993	2,512,993
7	8	11.14	14,112,000	21,291,014	1,911,222
8	8	10.75	112,896,000	178,347,783	1,543,301

* Expected total shoots estimated by multiplying of average of shoot obtained at each subculture (sub1x sub2 x sub3 x…..subn).

** Expected total shoots estimated by (average shoot formation over consecutive subcultures) **Sub No**

*** Expected total after one year based on shoot formation average of any consecutive subcultures (average shoot formation over any consecutive subcultures) [6]