

*Full Length Research Paper*

## Influence of medium type and growth regulators on *in vitro* micropropagation of pineapple (*Ananas comosus* (L.), var. Smooth cayenne)

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Aseptic cultures of pineapple 'smooth cayenne' were established from shoot tip explants. They were initiated on Murashige and Skoog (MS) basal medium with vitamins supplemented with various combinations of 6-benzylaminopurine (BAP) (0, 0.1, 0.2, 0.3 and 0.4 mg L<sup>-1</sup>) and Gelrite [1.0 g L<sup>-1</sup> (semi-liquid), 1.5 g L<sup>-1</sup> (semi-solid) and 2.0 g L<sup>-1</sup> (solid)]. Explants were transferred five weeks after *in vitro* initiation to semi-liquid medium containing higher concentrations of BAP (0, 0.5, 1.0, 1.5 and 2.0 mg L<sup>-1</sup>) for proliferation. Four sub-cultures were made at five weeks interval for twenty weeks in the proliferation medium. Established shoots were introduced into rooting medium containing either full (4.4 g L<sup>-1</sup>) or half (2.2 g L<sup>-1</sup>) strength MS basal medium with vitamins supplemented with 0, 0.5 mg L<sup>-1</sup> BAP alone or in combination with 0, 0.9 and 1.8 mg L<sup>-1</sup>  $\alpha$ -naphthaleneacetic acid (NAA). Semi-liquid MS basal medium supplemented with 0.1 and 0.3 mg L<sup>-1</sup> BAP gave the best regeneration results, producing the highest average shoot length (34.6 mm) and average number of shoot buds (2.4) per explants. Significant difference ( $p \leq 0.05$ ) in average shoot number per explant was observed with semi-liquid MS basal medium supplemented with 1.5 mg L<sup>-1</sup> BAP, producing the highest average shoot number (6.1) per explant at 5 weeks and this increased to 167.7 after 20 weeks. The half-strength MS basal medium without growth regulators or with 0.9 mg L<sup>-1</sup> NAA gave the highest average root length (29.3 mm) and root number per shoot (7.9). The semi-liquid MS basal medium supplemented with low BAP (1.5 mg L<sup>-1</sup>) is a cost-effective method for *in vitro* propagation of pineapple. Half strength MS basal medium without hormones or with low NAA are also cost-effective methods for inducing roots in pineapple.

**Key words:** Gelrite, growth regulators, medium type, pineapple, propagation.

### INTRODUCTION

Pineapple [*Ananas comosus* (L.) Merr] is a tropical fruit of great economic importance with a lot of health benefits (Duval et al. 2001). It is considered to be an exotic fruit,

used for dessert due to its attractive flavor and nutritive value (Khan et al., 2004). Pineapples are commonly consumed as fresh fruits or processed into canned fruit,

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juice and jam for export (Roostika and Mariska, 2003). Nigeria is the largest producer of pineapples in Africa and the 8th largest producer in the world; with 'smooth cayenne' being the most commonly cultivated variety in Nigeria (Oduote, 2013).

Large number of healthy pineapple planting materials are required (planting density of 60,000 per hectare) in order to meet the demand of both processing and an expanding fresh-market sector (Danso et al., 2008). Conventionally, pineapple is propagated vegetatively through suckers, slips or crowns (Khan et al., 2004), but this has limitations such as low multiplication rate, transmission of diseases and lack of uniformity (Lieu et al. 2004). *In vitro* propagation of pineapple shoots is proposed as a means of addressing these problems because it allows for efficient and rapid multiplication of disease-free pineapple plantlets in a relatively shorter period independent of the season (Firoozabady and Gutterson, 2003; Sether et al., 2001). The success of an *in vitro* or a micropropagation procedure with regards to the survival rate and performance of the plants depends on several factors including explants source, type of growth hormones, gelling agent, carbon source, pH of the medium and condition of the growth room during the *in vitro* growth process (Zuraida et al., 2011).

Micropropagation of shoot tips and buds from crown have been reported in pineapple (Al-Saif et al., 2011; Hammad and Taha, 2008; Kiss et al., 1995). A commonly used method was a combination of 6-benzylaminopurine (BAP) and naphthalene acetic acid (NAA) (Firoozabady and Gutterson, 2003), indole acetic acid (IAA) (Hamad and Taha, 2008), indole butyric acid (IBA) (Boxus et al. 1991) or 2,4-dichlorophenoxy acetic acid (2,4-D) (Liu et al., 1989). Although *in vitro* micropropagation of pineapple with BAP alone has been reported (Almeida et al., 2002; Be and Debergh, 2006), the use of BAP alone or at low concentrations for rapid *in vitro* multiplication of pineapple will be a cost-effective method.

Liquid culture medium is known to promote faster rates of growth of *in vitro* plantlets because there is rapid absorption of the nutrients by the plantlets during continuous agitation of the medium on a shaker (Danso et al., 2008; Firoozabady and Gutterson, 2003). The cost of production using this technique can be high particularly in developing countries like Nigeria with unstable power supply, which depend on alternative power backup. Testing the culture medium for regeneration and proliferation of pineapple using different of levels Gelrite (gelling agent) in combination with BAP and or NAA may result in the production of a cost-effective and efficient method for *in vitro* multiplication of pineapple. This study was therefore carried out to determine the concentration(s) of Gelrite and BAP that promotes maximum rate of multiplication of pineapple var. smooth cayenne with minimal input of chemical materials.

The effect of full and half strength MS supplemented

with or without selected growth regulators on root induction was also studied.

## MATERIALS AND METHODS

### Plant material and sterilization

Crowns from freshly harvested pineapple 'smooth cayenne' were used as the explant source. Leaves were removed from crowns by gentle peeling, leaving about five primordial leaves and the base intact (Khan et al., 2004). Afterwards, the peeled crowns were thoroughly washed under running tap water for 60 min before transferring to the Laminar Flow Hood for surface sterilization. Surface sterilization was done by sequential immersion in 70% (v/v) ethanol for 5 min with gentle swirling, 20% sodium hypochlorite solution (v/v) is containing three drops of Tween 20 for 10 min and finally, 15% sodium hypochlorite solution (v/v) containing 3 drops of Tween 20 for 15 min. This was followed by three rinses in sterile distilled water to remove all traces of sodium hypochlorite and other chemicals.

### Regeneration medium and culture conditions

Under aseptic conditions, the shoot tip with one to two leaf primordial (approximately 1 cm<sup>3</sup>), was inoculated into freshly prepared Murashige and Skoog (MS), (1962) basal medium with vitamins in a test tube. This medium was supplemented with 30.0 g L<sup>-1</sup> sucrose, BAP at 0, 0.1, 0.2, 0.3, and 0.4 mg L<sup>-1</sup> and Gelrite at 1.0, 1.5, and 2.0 g L<sup>-1</sup>; which constitutes semi-liquid, semi-solid and solid media; respectively. Prior to use of Gelrite, the pH of the medium was adjusted to 5.8 using 0.1 M sodium hydroxide (NaOH) or 0.1 M hydrochloric acid (HCl). The medium was dispensed at 15 ml to each test tube of size (24 x 145 mm) and sterilized by autoclaving at 15 psi and 121°C for 15 min. The cultures were maintained in the growth room at 26 ± 2°C under white fluorescent bulbs controlled with automatic timer to supply 16 h photoperiod (40 µmoles photons m<sup>-2</sup> s<sup>-1</sup>). A treatment consisted of eight test tubes arranged in a completely randomized design with three replications according to Snedecor and Cochran (1980). The best regeneration medium was used in subsequent studies.

Data were collected on the number of shoot buds, and the shoot length (mm) per tube for a successive period of 5 weeks.

### Multiplication medium

Regenerated shoots obtained from the shoot-tip cultures were used as explants for this experiment. These were planted on freshly prepared multiplication medium. The multiplication medium consisted of MS basal salt with vitamins supplemented with 3% sucrose, BAP (0.5, 1.0, 1.5 and 2.0 mg L<sup>-1</sup>) and Gelrite (1.0 g L<sup>-1</sup>). A treatment consisted of eight culture jars arranged in a completely randomized design with three replications. Successive subcultures were carried out for up to 4 cycles with 5-weeks interval per cycle. The cultures were maintained in the growth room under the same condition as the regeneration stage. The number of shoots produced per initial explants in each jar was counted at 5 weeks interval for 20 weeks.

### Rooting medium

Proliferated shoots were separated, and each single shoot was

**Table 1.** Effect of different combinations of Gelrite and BAP concentrations on *in vitro* regeneration of shoot-tips of pineapple after 5 weeks of initiation<sup>a</sup>.

Gelrite (g L <sup>-1</sup> )	BAP (mg L <sup>-1</sup> )	Average no. of shoot buds/explants	Average shoot length (mm) explants
1.00	0.0	1.0 ± 0.0	24.3 ± 2.2
	0.1	2.0 ± 0.3	34.6 ± 2.7
	0.2	1.8 ± 0.3	30.4 ± 1.6
	0.3	2.4 ± 0.3	32.3 ± 2.4
	0.4	1.9 ± 0.3	28.0 ± 2.7
1.50	0.0	1.0 ± 0.0	21.0 ± 1.6
	0.1	1.8 ± 0.3	23.5 ± 1.6
	0.2	1.8 ± 0.3	24.8 ± 2.8
	0.3	1.5 ± 0.2	27.6 ± 1.4
	0.4	1.6 ± 0.3	26.8 ± 2.3
2.00	0.0	1.0 ± 0.0	20.9 ± 1.8
	0.1	1.0 ± 0.0	23.4 ± 1.7
	0.2	1.0 ± 0.0	28.1 ± 2.9
	0.3	1.0 ± 0.0	24.5 ± 2.4
	0.4	1.0 ± 0.0	29.9 ± 1.1
LSD		0.6	5.4

<sup>a</sup>Results are mean values ± standard error.

transferred to a rooting medium in test tube, for root induction and elongation. The rooting medium was composed of full-strength (4.4 g L<sup>-1</sup>) or half-strength (2.2 g L<sup>-1</sup>) MS basal medium with vitamins, supplemented with or without growth hormones. Medium supplemented with hormones had varying concentrations; BAP (0, and 0.5 mg L<sup>-1</sup>) and NAA (0, 0.9 and 1.8 mg L<sup>-1</sup>). The hormones were added alone and in combination.

Data on number of roots per shoot, number of days to first root emergence and root length were recorded for six weeks. Rooted plantlets were subsequently rinsed free of culture medium and transferred to black polythene bags containing sterile top soil. These were maintained under shade for 40 days before they were transferred to the field.

### Statistical analysis

Data were subjected to analysis of variance (ANOVA) using the GLM procedure of SAS (Statistical Analysis Software, 2008). Means were separated using Fisher's LSD at  $p \leq 0.05$ .

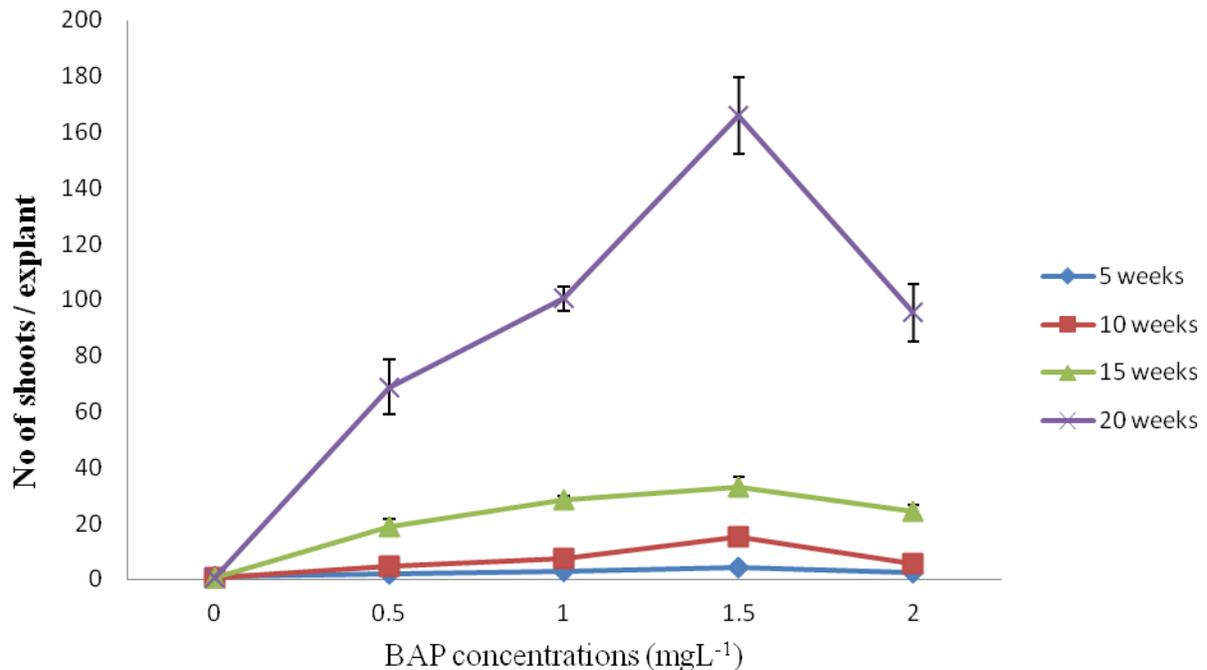
## RESULTS AND DISCUSSION

### Shoot regeneration

Experiments were carried out to determine the best medium composition for shoot regeneration from shoot-tip cultures under controlled environment. The results showed that all the medium solidification types supplemented with BAP promoted regeneration of shoot buds but the semi-liquid medium (1.0 g L<sup>-1</sup> Gelrite) gave

the best regeneration in terms of the average number of shoot buds produced per explant (2.4) and average shoot length (34.6 mm) (Table 1). The highest average shoot length (34.6 mm) was observed in the medium with 0.1 mg L<sup>-1</sup> BAP and the highest average number of shoot buds (2.4) was observed in 0.3 mg L<sup>-1</sup> BAP. This result agrees with previous report (Firoozabady and Gutterson, 2003), which shows that BAP (0.1 to 0.5 mg L<sup>-1</sup>) added to MS medium is essential for the regeneration of plants from shoot apices of pineapple. Paiva et al. (1998) obtained best results in the shoot induction of pineapple, cv. Skay, with either 1.0 mg L<sup>-1</sup> BAP or 0.1 mg L<sup>-1</sup> TDZ. This is different from results of this study in which lower concentrations of BAP (0.1 and 0.3 mg L<sup>-1</sup>) gave best results for shoot induction.

The differences between both results may be due to genotype effect. Al-Saif et al. (2011) reported that 2.0 mg L<sup>-1</sup> BAP in solid medium (7.0 g L<sup>-1</sup> solidified with agar) gave shoot length of 9.5 mm. Result of this study showed that lower concentrations of BAP (0.1 to 0.4 mg L<sup>-1</sup>) in solid medium produced higher shoot length (20.9 to 29.9 mm) (Table 1). With the exception of 0.3 mg L<sup>-1</sup> BAP, an increase in BAP concentrations from 0 to 0.4 mg L<sup>-1</sup> in the solid medium (2.0 g L<sup>-1</sup> gelrite) resulted in increased shoot length. This is not in agreement with report of Hamad and Taha (2009) who observed a decrease in shoot length as BAP concentration increased up to 0.5 mg L<sup>-1</sup> in solid medium. The highest average number of shoot buds per explant and shoot length obtained in



**Figure 1.** Effect of BAP concentrations on average shoot number of pineapple produced in a semi-liquid MS basal medium with vitamins  $\pm$ SE.

semi-liquid medium (solidified with 1.0 g L<sup>-1</sup> Gelrite) in this study maybe due to increased intake of the nutrients made possible by free movement of compounds or nutrients from the culture medium into the plant tissues. Gupta et al. (1981) showed that the use of liquid medium facilitates the nutrient uptake by the plant due to better distribution in the culture medium. Similar higher regeneration rates in liquid medium when compared with solid medium have been reported by Alvard et al. (1993).

### Shoot proliferation

After the determination of the optimal Gelrite concentration for regeneration, the BAP concentration was increased from 0.5 to 2.0 mg L<sup>-1</sup> with the aim of inducing more shoots in the semi-liquid medium (Figure 1). After five weeks of culture, the basal portions of pineapples consisting of proliferating buds (clumped explant) were observed to produce more shoots (Plate 1A and B). Medium supplemented with 1.5 mg L<sup>-1</sup> BAP produced most average number of shoots (6.1) and this was significantly higher ( $p \leq 0.05$ ) than that of other BAP concentrations evaluated (Figure 1). Zuraida et al. (2011) reported that explants cultured on liquid medium supplemented with 1.0 mg L<sup>-1</sup> BAP produced the highest number of shoots (31) after 4 weeks when compared with 5 mg L<sup>-1</sup> BAP which produced 14 shoots after four weeks of culture. This confirms the assertion that liquid culture

explants usually display a higher frequency of growth rate when compared with solid or semi-liquid cultures (Gupta et al., 1981), and maybe as a result of continuous shaking of liquid cultures thus promoting increased intake of the nutrients in the medium. After a second sub-culture, a 3-fold increase in the average number of shoots (18.3) was recorded for 1.5 mg L<sup>-1</sup> BAP at 10 weeks; and at 20 weeks a 28-fold increase was observed (167.6) (Figure 1, Plate 1C). Result of this study is similar to report of Almeida et al. (2002) in which MS medium supplemented with 1.5 mg L<sup>-1</sup> BAP gave the best treatment at proliferation stage, producing an average of 701.1 shoots per explant in liquid medium after five subcultures. Hamad and Taha (2008) also reported that in 4 cycles of culture, shoot number was highest in BAP at 1.5 mg L<sup>-1</sup>.

The proliferation induced by BAP corroborates report of Kyte and Kleyn (1996) that a cytokinin is very crucial for cell division and axillary bud multiplication in plants. In this study, BAP (1.5 mg L<sup>-1</sup>) alone produced average shoot number of 18.3 per clumped explant. This is higher than the average shoot number of 12.0 per explant observed in solid medium supplemented with BAP (2.0 mg L<sup>-1</sup>) (Al-Saif et al., 2011); 9.0 shoots per explant obtained in response to 1.0 mg L<sup>-1</sup> BAP (Be and Debergh, 2006). Barboza and Caldas (2001) used etiolated nodal segments for micropropagation of the pineapple hybrid PE X SC-52, and observed that BAP (2.0 mg L<sup>-1</sup>) promoted the highest number of plants per



**Plate 1.** Different stages of *in vitro* propagation of smooth cayenne in semi-liquid medium. A = Regeneration stage with bud formation. B = Initial multiplication stage; C = advanced multiplication stage; D = rooting in half-strength MS basal medium with 0.9 NAA mg L<sup>-1</sup>. E = rooting in half-strength MS basal medium without growth hormones.

shoot culture (10.4) and per nodal segment, when compared with kinetin (5.0 mg L<sup>-1</sup>) or a combination of BAP (2.0 mg L<sup>-1</sup>) and NAA (1.86 mg L<sup>-1</sup>). Alvard et al. (1993) and George and Sherrington (1984) both reported higher rates of growth in liquid cultures when compared with solid medium. This may be attributed to the exposure to greater surface of the explants to a medium with uniform distribution of nutrients, and enhanced nutrient uptake. Medium containing BAP 1.0 mg L<sup>-1</sup> which gave the second highest average number of shoots per explants (4.6) was observed to have a 2-fold increase in the average number of shoots per explant (9.5) after 10 weeks and a 25-fold increase in the average number of shoots (102.4) per explant after 20 weeks (Figure 1). Further increase in BAP concentration (2.0 mg L<sup>-1</sup>) did not result in appreciable increase in the number of

shoots. This may be due to inherent cytokinin already present in the plant resulting to habituation decline.

### Root induction

Medium with half-strength MS basal medium with vitamins (2.2 g L<sup>-1</sup>) supplemented with 0.5 mg L<sup>-1</sup> BAP + 0.9 mg L<sup>-1</sup> NAA gave the lowest average days to root emergence (1.2) and the lowest average number of roots per shoot (approx. 0.3) (Table 2). This was followed by half-strength MS basal medium with vitamins supplemented with 0.9 mg L<sup>-1</sup> NAA alone which gave average days to root emergence of (approximately 7.0) and the highest mean number of roots per shoot (approximately 7.9). There was no significant difference ( $p \leq 0.05$ ) in

**Table 2.** Influence of MS strength and different growth regulator concentrations on root induction of pineapple cultured for 6 weeks<sup>a</sup>.

MS strength	Plant growth regulators (mg L <sup>-1</sup> )		Average days to root emergence	Average no of roots/shoot	Average root length (mm)	Rooted plantlet (%)
	BAP	NAA				
Half strength	0.00	0.00	7.33 <sup>b</sup> ± 0.23	6.25 <sup>b</sup> ± 0.29	29.3 <sup>a</sup> ± 1.0	100 <sup>a</sup> ± 0.0
	0.00	0.90	6.96 <sup>b</sup> ± 0.19	7.88 <sup>a</sup> ± 0.54	26.5 <sup>b</sup> ± 0.8	100 <sup>a</sup> ± 0.0
	0.00	1.80	7.46 <sup>b</sup> ± 0.19	7.79 <sup>a</sup> ± 0.40	28.7 <sup>a</sup> ± 1.1	100 <sup>a</sup> ± 0.0
	0.50	0.00	-	-	-	-
	0.50	0.90	1.17 <sup>c</sup> ± 0.81	0.29 <sup>d</sup> ± 0.20	1.0 <sup>d</sup> ± 0.7	8 <sup>c</sup> ± 0.06
	0.50	1.80	-	-	-	-
Full strength	0.00	0.00	-	-	-	-
	0.00	0.90	-	-	-	-
	0.00	1.80	10.71 <sup>a</sup> ± 0.3	3.46 <sup>c</sup> ± 0.42	6.4 <sup>c</sup> ± 0.6	92 <sup>b</sup> ± 0.06
	0.50	0.00	-	-	-	-
	0.50	0.90	-	-	-	-
	0.50	1.80	-	-	-	-

<sup>a</sup>Means with same letter in the same column are not significantly different at  $P \leq 0.05$  according to L.S.D.

the number of roots induced when NAA concentration was doubled from 0.9 NAA (7.9) to 1.80 mg L<sup>-1</sup> NAA (7.8) in half strength MS medium. Contrary to result of this study, Hamad et al. (2013) reported that in liquid medium, shoots failed to form roots in half-strength MS basal medium containing NAA. Danso et al. (2008) reported that shoots cultured on 7.5 mg L<sup>-1</sup> BAP and NAA concentrations (7.5 to 15.0 mg L<sup>-1</sup>) did not result in any root formation in MD2 pineapple per shoot. Firoozabady and Gutterson (2003) obtained roots from liquid cultures of pineapple cultured on MS medium supplemented with 0.5 mg L<sup>-1</sup> NAA and 0.5 mg L<sup>-1</sup> IBA. NAA and IBA are root inducing growth regulators and have been used either alone or in combination for root induction in many cultures (Be and Debergh, 2006; Gupta et al., 1981). The average number of roots per shoot obtained with medium supplemented with 0.9 mg L<sup>-1</sup> NAA (7.9) (Plate 1D) or 1.8 mg L<sup>-1</sup> NAA (7.8) are higher than those obtained in medium supplemented with 1.0 mg L<sup>-1</sup> IBA (5.0) (Khan et al, 2004); and when IBA was substituted by 1.0 mg L<sup>-1</sup> NAA (3.6); this took between 8-15 days for induction of root.

The full or half-strength MS basal medium supplemented with 0.5 mg L<sup>-1</sup> BAP did not produce any roots. This result agrees with the reports of Be and Debergh (2006), Danso et al. (2008) and Gupta et al. (1981) which showed that NAA, as an auxin, is important for root induction and regulation in plants. The half-strength MS basal medium containing no growth regulators had the highest root length (29.3 mm) (Table 2, Plate 1E) and the third highest mean number of roots (6.3). Almeida et al. (2002) also recorded success in rooting when shoots were transferred to MS medium with half the concen-

tration of salts and no growth regulators for 30 days. The half-strength MS supplemented with 1.8 mg L<sup>-1</sup> NAA gave the second highest average roots' length (28.7 mm, Table 2). Shoots cultured on half strength MS basal medium with vitamins and no growth regulators, 0.9 mg L<sup>-1</sup> NAA or 1.8 mg L<sup>-1</sup> NAA recorded 100% rooting. The full-strength MS medium with no growth hormones did not produce any root. Only full-strength MS supplemented with 1.8 mg L<sup>-1</sup> NAA produced roots; the mean number of roots per explants and root lengths were 3.46 and 6.4 mm, respectively. These were significantly lower ( $p \leq 0.05$ ) than results obtained from shoots cultured on half-strength MS medium.

Nearly all plantlets were successfully hardened in black polythene bags (survival rate of 85%) containing sterile top soil and placed under shade.

The results of this study demonstrates the efficient use of semi-liquid medium with low concentrations of BAP for *in vitro* regeneration and multiplication of pineapple, as well as the use of half-strength MS basal medium with vitamins and no growth hormones or low NAA concentration for root induction. The minimal use of materials and chemicals in tissue culturing of pineapple, directly translates to low cost of production. This economic approach to micro propagation enhances the availability and affordability of *in vitro* derived pineapple cv. smooth cayenne as quality planting materials to meet the ever increasing demand by farmers.

### Conflict of interest

Authors declare that there is no conflict of interest.

whether financial or relevant interest that has influenced this study.

### Conflict of Interests

The author(s) have not declared any conflict of interests.

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