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In vitro protocol optimization for mass propagation of pineapple (*Ananas comosus* L.) cv. smooth cayenne

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Abstract
Pineapple is the third most important tropical fruit in the world, after the banana and citrus. The fruits are important source of vitamin A, B and C and contain a protein digesting enzyme bromelain. Pineapple fruits are consumed fresh or processed into canned fruit, juice or jam. Micro propagation technique (Plant tissue culture) offers an opportunity for large scale production of uniform pineapple planting material in a relatively short period of time. The result showed that contamination, survival rate and brown of Mancozeb 80%WP at 0.6 g/L more clean, survival rate and less brown 8.4, 91.6 and 33.1% respectively, copper sulfate at 0.6 g/L less contamination, high survival rate and less brown 12.6 89.4 and 35.3% respectively and at 5% active chlorine had also effective to clean explants, less phenol and higher survival rate in the treatment combination of the experimental results at 30% (V/V) of NaOCl were 10.5, 49.8 and 92.5 % respectively. The highest shoot proliferation were recorded at 2.5 mg/l BA in combination with 0.5 mg/l KN 58.6 shoots/explant in full MS media and the highest root induction also recorded at 1 mg/l NAA 10.67 root /shootlet in half strength MS media. Pineapple was highly adapted for micro climate of greenhouse and the seedling were planted in the soil media of top soil, vermin compost and sand 0:1:2 ratios respectively have been acclimatized 99.6% in primary for a month. Thus, efficient protocol has been established for the mass propagation of the improved pineapple smooth cayenne cultivar.

Keywords: pineapple (*Ananas comosus* L.), micro propagation, axillary bud, acclimatization

Introduction

Ananas comosus, commonly known as pineapple, is an herbaceous perennial belonging to the order Bromeliales, family Bromelaceace and sub-family Bromelioideae (Bartholomew *et al*., 2002) [1]. According to Carlier *et al*. (2014), there are 56 genera of the pineapple which include 2921 species. Pineapples originated in South America and through travels and migration of different peoples, its cultivation spread to other parts of the world (Gene Technology Regulator, 2003). Pineapple is the third most important tropical fruit in the world, after the banana and citrus. The fruits are important source of vitamin A, B and C and contain a protein digesting enzyme bromelain. Pineapple fruits are consumed fresh or processed into canned fruit, juice or jam. Potentials exist for commercial production and processing of crop in Nigeria and other developing tropical countries. Conventionally, pineapple is propagated by the use of slips arising from the stalk below the fruit, suckers originated from leaf axils or leaves, crowns of the fruits or ratoons that arise from underground part of the stems. This conventional method of propagation is slow and allows for transfer of pineapple requires large volume of planting materials, which are hardly obtained by conventional method of propagation. Micro propagation technique (Plant tissue culture) offers an opportunity for large scale production of uniform pineapple planting material in a relatively short period of time (Ika and Mariska, 2003) [6]. Rapid multiplication of pineapple through axillary buds culture was reported (Zuraida A., *et al*., 2011) [10]. However, low multiplication rate and poor survival rates during acclimatization have been identified as some of the problems affecting the micro propagation technique (Escalona *et al*., 1999) [4]. In addition, the choice of medium used for tissue culture depends upon the species, cultivar and culture conditions and adjustments in growth medium are determined by experimentation (Usman *et al*., 2011) [9]. Therefore, this study was conducted with the primary objective of establishing an efficient rapid micro propagation protocol for large scale propagation of improved pineapple.

Materials and Methods

Mother Plant collection and establishment

Mother plants of selected variety were collected from Jimma Agricultural Research Center. The collected mother plants were established in a fully controlled greenhouse at Jimma plant biotechnology laboratory and finally used as sources of explants. Subsequently, morphologically good and healthy axillary bud from the matured seedlings has been collected. The explants have been washed under running tap water supplemented with detergents followed by surface sterilization of different treatment mancozeb, copper sulfate and sodium hypo chlorite supplemented with three drops of Tween 20 and rinsed with sterile water.

Fig 1: Propagules naturally produced by a pineapple plant, in this case cv. Smooth cayenne: A = Crown; B = Slips; C = Hapa; D = Indication of potential Sucker insertion sites.

Data analysis

Data were analyzed using the analysis of variance (ANOVA) using General leaner Model (GLM) of SAS statistical software version 9.3. The mean variability among treatments least significant difference (LSD) @ 5% level of significance used.

Results and Discussion

Explants initiation

Surface sterilized explants have been cultured aseptically on different strength MS medium (Murashige and Skoog 1962) supplemented with 30 g/L sucrose, Hormone free, solidified with (7 g/L) agar and the pH were adjusted at 5.8. Thus cultures were maintained at 25± 2°C, 16/8h photoperiod and light intensity of 40 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent lamps incubated for six weeks.

Table 1: The effect of Mancozeb 80% WP, $CuSO_4.2H_2O$ and 5% active chlorine NaOCl, for sterilization technique in the laminar flow hood aseptically on the contamination, survival and brown at six weeks

Treatments	Concentration	Contamination %	Survival %	Brown %
Mancozeb 80% WP % (W/V)	0	57.1^{a}	53.7^{c}	70.1^{a}
	0.3	48.6^{b}	51.4^{c}	49.3^{b}
	0.6	8.4^{d}	91.6^{a}	33.1^{c}
	0.9	25.9^{c}	74.1^{b}	47.3^{b}
	1.2	6.1^{d}	75.6^{b}	52.5^{b}
$CuSO_4.2H_2O$ % (W/V)	0	57.3^{a}	53.7^{c}	70.8^{a}
	0.3	29.8^{b}	70.2^{b}	50.1^{b}
	0.6	12.4^{d}	89.6^{a}	35.3^{c}
	0.9	46.6^{b}	54.4^{c}	49.5^{b}
	1.2	8.9^{d}	73.3^{b}	70.3^{a}
NaOCl % (V/V)	10	53.8^{a}	47.2^{d}	66.3^{a}
	20	51.1^{ab}	48.8^{cd}	68.8^{a}
	30	10.5^{d}	92.5^{a}	49.8^{b}
	40	25.6^{c}	73.4^{b}	55.6^{b}
	50	8.3^{d}	70.2^{b}	70.8^{a}
	Mean	37.58	66.25	61.35
	F-test	**	**	**
	LSD @ 5%	3.1	5.9	6.4
	CV %	6.36	7.01	9.4

** Significant at P<0.01, Means with same letter within a column are not significantly different at $p<0.05$ ANOVA at least significance difference (LSD). CV % = percent of coefficient variation V/V= volume pre volume and W/V = weight per volume

The analyzed result in (Table 1) and (Fig 1) revealed that contamination, survival rate and brown of Mancozeb 80%WP at 0.6 g/L more clean, survival rate and less brown 8.4, 91.6 and 33.1% respectively and also there is highly significance difference among mean treatment of copper sulfate at 0.6 g/L less contamination, high survival rate and less brown 12.6 89.4 and 35.3% respectively. In addition to that, 5% active chlorine had also effective to clean explants, less phenol and higher survival rate in the treatment combination of the experimental results at 30% (V/V) of NaOCl were 10.5, 49.8 and 92.5 % respectively. Hence, sodium hypochlorite (house bleach) was applied in the laminar air flow hood and final disinfectants of surfactants. These results were in agreement with (McDonald, D. B., *et al*., 1986 and Berihu M., 2018) that obtained high survival rate less contamination and low phenolic rate in 100 mg/L of copper sulfate and 1.5% (V/V) of sodium hypochlorite for 10 min soaked.

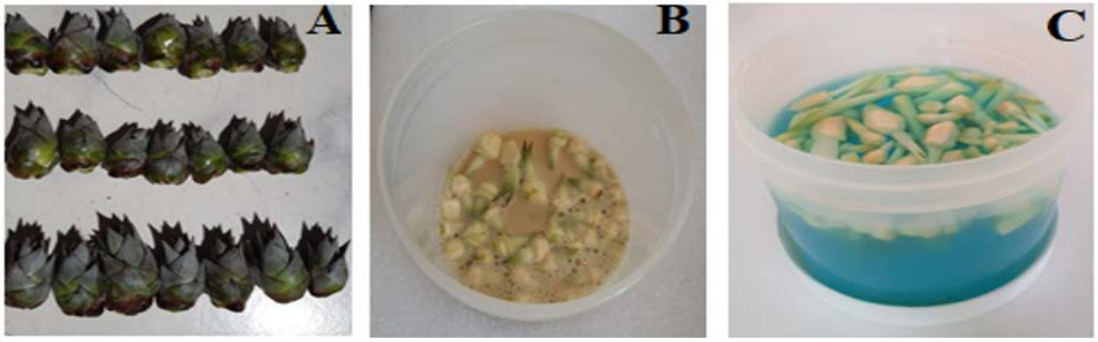

Fig 1: surface sterilization of pineapple cv. Smooth cayenne explants were often at Kumla, biotechnology laboratory A) Slips preparation from mother plant, B) Soaked of explants in Mancsa solution for 3 0 min and C) Soaked of explants in CuSO4.2 H20 solution for 30 minute

The effect of MS media strengths for shoot growth

Clean explants were incubated on four different concentrations of inorganic salts was observed in Table (2). The analyzed data showed that the explants cultured on full strength MS medium gave the best results of shoot number (3.3) as compared with 1/4 MS which scored (1.3) respectively. Moreover, full strength of MS salts were increase the growth morphology shoot length (4.1 cm) as compared with ¼ MS which scored (2.3 cm) respectively. In addition to that, full strength of MS salts concentration has been used shoot development and morphological proliferation of explants cultured while compared with others concentration in all parameters. According to Fayek *et al*. (2007), stated that shoot tip and nodal segment explants of three female jojoba clones were compared for its potentiality of *in vitro* clonal propagation on media strength of (Full MS, ¾ MS, ½ MS and ¼ MS) was considered at shoot proliferation. Therefore, these results revealed that pineapple explants had been needed high nutrient requirements to shoot development and proliferation.

Table 2: The effect of MS media strength on growth character of cv. Smooth cayenne *in vitro* culture

MS Strength	Number of shoot	Shoot length (cm)	Number of leaves	Root induction (%)
Full	3.3[a]	4.1[a]	12.3[a]	100.0[a]
¾	2.6[b]	3.2[b]	9.6[b]	63.6[b]
½	2.6[b]	3.0[b]	8.9[b]	63.6[b]
¼	1.3[c]	2.3[c]	6.4[c]	30.3[c]
Means	2.35	3.15	9.3	64.4
LSD@ 5%	0.45	0.39	2.2	42.2
F-test	**	*	**	*
CV %	5.6	7.8	7.3	11.4

** Significant at P<0.01 & * significant at P<0.05, Means with same letter within a column are not significantly different @ 5% least significance difference (LSD) results, CV % = Percent of coefficient variation.

Shoot proliferation

All concentrations of BA alone and along kin showed a realization of shoots oscillating from 1.0 to 58.6 as shown in (Table 3). Clean explants have been applied on MS basal medium and Plant growth regulators of 2.5 mg/L BA along with 0.5 mg/L KN produced the highest number of micro shoots per explant (58.6) followed by 2 mg/l plus 0.25 mg/L BA and KN (43.7) while the lowest micro shoot were obtained in control treatments. The additive BA at 2.5 mg/l with composition of 0.5 mg/l of KN also induced shoot elongation to the highest length 5.36 cm followed by BA at 2 mg/l and 0.25 mg/l KN (5.23 cm) while compared to treatment with 3.5 mg/L BA plus 1 mg/l KN concentration shoot length (2.41 cm). Moreover, the concentrations of 2.5 mg/L BA along with 0.5 mg/L KN supplemented into medium required with extended period of eight weeks to obtained (12.01) leaf number per explant. At the second, third, fourth and fifth subculture the growth rate have been more morphological improvement of shoot numbers, shoot length and leaves number. At the end of incubation period some verification symptoms were observed that returns to increment number of leaves that decrease or competitive some growth factor (*etc.* Light, salts).The highest concentration of BA in the medium was more stimulatory to callus development than the lowest concentration of this growth regulator. These results corroborate with those obtained by Be and Debergh (2006). They reported that more axillary callus was produced per inoculums when BA concentration was increased. Generally, cytokinins are known to stimulate cell division and axillary bud proliferation (Kyte and Kleyn, 1996), there by resulting to significant shoot formation at the expense of root development. According to Firoozabady and Gutterson (2003), addition of BA in MS medium was essential for the regeneration plantlets from shoot apices of pineapple. Zuraida *et al.* (2011) [10] working on Maspine pineapple by treated with BAP 0, 0.5, 1.0, 2.0 and 5.0 mg/l, they found that the highest number of shoots was observed on the medium containing 5 mg/l BA (7 plantlets). Therefore, Smooth cayenne pineapple could easily produce *in vitro* shoots from axillary buds at 2.5 mg/l BA along with 0.5 mg/l KN in composition could be produced the highest shoot number per explant among the treatment combinations. Those results correlated with MS medium supplemented with 3.0 mg/l BA were suitable for clonal propagation of *Ficu sbenjamina* vars. Natasja and Starlight (Rzepka-Plevnes and Kurek, 2001). Almeida *et al*., (2002) introduced the pineapple explants in MS medium supplemented with 2 mg/l BAP and then sub cultured for multiplication in solid and liquid MS medium with different BAP concentration (1.5 or 3 mg/l). To maximize the number of plantlets obtained by micro propagation, Danso *et al.* (2008) studied various combinations of N6-benzylaminopurine (BAP) and naphthalene acetic acid (NAA) in solid or liquid cultures. They reported that liquid cultures required 5.0 mg/l BAP to increase the multiplication rate. Khan *et al*. (2004) found that BAP 0.5 mg/l was better for the pineapple number and length of shoots/explant. Enrichment of media with 0.54 – 2.69 µm NAA and 0.44 µm BA was obtained for enhanced regeneration by using nodules regenerated shoots for the pineapple (Teng and Yu, 1996). Firoozabody and Gutterson (2003) used a combination of 1.5 mg/l BA and 0.5 mg/l NAA to produce the highest rate of shoot multiplication for pineapple explants.

Table 3: Effect of BA and KN concentration on shoot growth of cv. Smooth cayenne

Treatments		Shoot number	Shoot length (cm)	Leaf number
BA (mg/L)	KN (mg/L)			
0	0	1.0[j]	4.07[cd]	4.33[gh]
1.5	0	29.6[f]	3.71[e]	5.01[fg]
2	0	33.3[e]	3.23[f]	5.67[ef]
2.5	0	42.0[b]	4.92[b]	8.53[c]
3	0	20.3[h]	2.[81gh]	3.67[hij]
3.5	0	24.3[g]	2.86[gh]	3.33[ij]
0	0.12	36.3[d]	4.81[b]	4.33[gh]
0	0.25	38.0[cd]	4.13[cd]	5.66[ef]

0	0.5	30.0^{f}	3.67^{e}	7.67^{cd}
0	0.75	21.0^{h}	3.13^{fg}	5.33^{ef}
0	1	13.7^{i}	2.73^{h}	4.01^{hi}
1.5	0.12	37.0^{c}	4.23^{c}	6.01^{e}
2	0.25	43.7^{b}	5.23^{a}	9.33^{b}
2.5	0.5	58.6^{a}	5.36^{a}	12.01^{a}
3	0.75	36.0^{d}	3.86^{de}	7.01^{d}
3.5	1	30.0^{f}	2.41^{i}	3.01^{j}
Mean		30.3	3.8	5.92
F-test		**	**	**
LSD @ 5%		2.11	0.31	0.76
CV %		7.1	6.9	9.7

** Significant at P<0.01, Means with same letter within a column are not significantly different @ 5% least significance difference (LSD) results, CV % = Percent of coefficient variation.

Fig 2: *In vitro* growing of pineapple cv. Smooth cayenne A) Initiation in test tube, B) Shoot multiplication on solid MS media & C) Shoot multiplication in liquid MS media

Root Induction and elongation

According to the data in Table (4) all the explants treated with half MS media strength, 30g/L table sugar and different concentration treatments of plant growth regulators of IBA and NAA have been insignificant effect on root induction values along with root length and numbers. The effect of NAA on shoot elongation was observed in Table (4) and (Fig. 3A), which showed that applied NAA at 1mg/L gave the maximum root number and length of the explants 10.67 and 9.96cm respectively while compared to control and also the result followed in the treatment of IBA at 2.0 mg/l squired the root number and length 8.33 and 7.33cm respectively in two months of incubation period. On the other hand, IBA and NAA with high concentration gave a negative respond on root number and length by decreased the values to (2.92 and 2.99cm) and (2.2 and 0.67cm roots/micro shoots respectively. In this application there are many investigators discussed with this work, Pierik *et al.* (1984) and Danso *et al.* (2008) reported that *in vitro* rooting of pineapples can be enhanced by an addition of auxin such as NAA, IBA or combination of NAA and IBA in the medium. The marked improvement in the mean number of roots produced when NAA and IBA were applied in combination may have resulted from the fact that these hormones can act either in concert or synergistically for the induction of *in vitro* roots (Danso *et al.*, 2008).

Table 4: Effect of IBA and NAA concentration on root growth of cv. Smooth cayenne

Treatments		**Root number**	**Root length (cm)**
IBA (mg/L)	**NAA (mg/L)**		
0	0	0.0^{i}	0.0^{h}
1	0	6.01^{d}	5.83^{de}
2	0	8.33^{b}	7.33^{b}
3	0	5.67^{de}	5.23^{ef}
4	0	3.67^{fg}	3.76^{f}
5	0	2.92^{g}	2.99^{g}
0	1	10.67^{a}	9.96^{a}
0	2	8.17^{b}	6.33^{c}
0	3	4.57^{de}	5.36^{d}
0	4	3.01^{f}	3.76^{f}
0	5	2.2^{h}	0.67^{h}
Mean		4.82	4.64
F-test		**	**
LSD @ 5%		0.78	0.68
CV %		8.56	9.67

** Significant at P<0.01, Means with same letter within a column are not significantly different @ 5% least significance difference (LSD) results, CV % = Percent of coefficient variation.

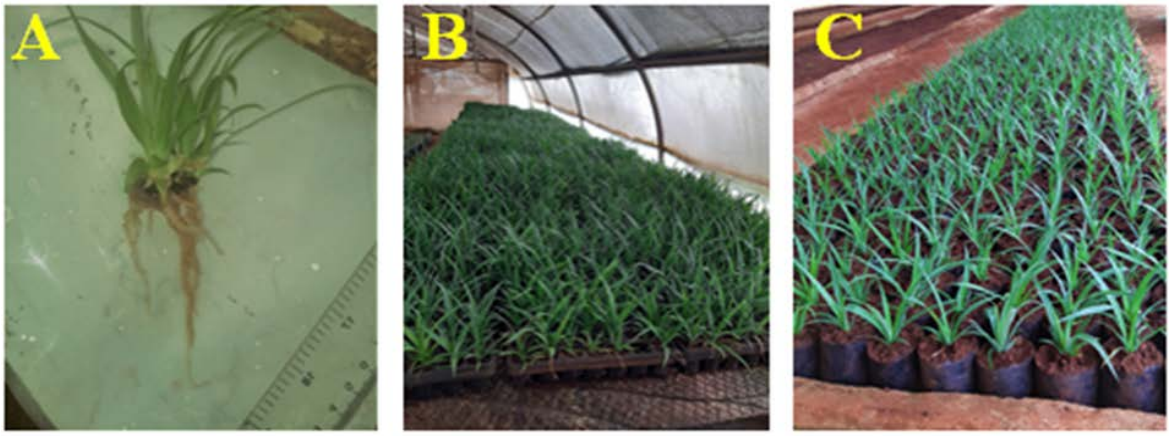

Fig 3: *In vitro* growing o f pine apple cv. Smooth cayenne A) Root inducing and elongation, B) Primary acclimatization on tray & C) Secondary acclimatization on polyethylene bag

Acclimatization

The acclimatized experiment has been performed using different substrates such as top soil, vermin compost and sand soil in different composition of unsterilized soil media prepared at Jimma agricultural research center. The treatments were used different treatment ratio of top soil, vermin compost, sand soil in A=1:1:1, B=1:1:2, C=1:1:0, D=2:1:0, E=0:1:1, F=0:1:2 and G=0:0:1 respectively had been planted on trays for a month. The result showed that soil media substrates containing top soil, vermin compost, sand soil in 0:1:2 ratio gave the highest survival percentage which was 99.6% after a month of transfer (fig.3 B) and heights scoured in all other parameters as compared to top soil, vermin compost, sand 2:1:0, 83% hardening off respectively in the primary acclimatization. The primary and secondary established seedling (fig.3 Band C) respectively had been exposed to an open field environment where they showed rapid growth after the acclimation period in greenhouse on nine months. This system is presently being introduced for the large-scale production of this improved pineapple cv. smooth cayenne. Thus, an efficient and viable protocol has been established for the mass propagation of the improved pineapple smooth cayenne cultivar.

Table 5: The effect of different ratio of (Top soil: Vermin compost: Sand; 1:1:1 = treatment **A**) respectively soil media composition for primary acclimatization of pineapple in four weeks.

Treatments	Number of leaf	Plant height cm	Root length cm	Root number	% acclimatized
A = 1:1:1	8.3^{cd}	6.1^{c}	5^{c}	2.9^{c}	94.3^{c}
B = 1:1:2	8.0^{d}	6.4^{bc}	5.3^{bc}	3.5^{abc}	93.3^{c}
C = 1:1:0	8.7^{cd}	7.5^{b}	5.3^{bc}	3.3^{bc}	89.7^{d}
D = 2:1:0	9.0^{bcd}	7.3^{b}	5.3^{bc}	2.8^{c}	82^{e}
E = 0:1:1	9.3^{abc}	7.0^{bc}	7.6^{ab}	3.1^{bc}	90^{d}
F = 0:1:2	10.3^{a}	9.1^{a}	8^{a}	4.2^{a}	99.6^{a}
G = 0:0:1	10^{ab}	7.2^{bc}	7.3^{abc}	3.8^{ab}	97^{b}
Mean	2.19	2.73	5.16	0.75	99.94
F- test	**	**	*	*	**
LSD @ 5%	1.2	1.18	2.6	0.8	2.02
CV %	7.6	9.3	13.8	12.5	3.3

** Significant at P<0.01 & * significant at P<0.05, Means with same letter within a column are not significantly different @ 5% least significance difference (LSD) results, CV % = Percent of coefficient variation.

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