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Development of an efficient protocol for micropropagation of pineapple (*Ananas comosus* L. var. smooth cayenne)

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The aim of this study was to establish an efficient micropropagation protocol for pineapple. Axillary buds were excised from the crown and inoculated on a liquid basal culture Murashige and Skoog (MS) medium supplemented with sucrose (3%), benzylaminopurine (BA) (2.5 µM) and naphthaleneacetic acid (NAA) (0.62 µM) for shoot induction. Shoot multiplication and elongation was on MS basal medium supplemented with 3% sucrose, 0.8% agar and different concentrations of BA (5, 7.5, 10 and 12.5 µM) and NAA (2, 2.5 and 3 µM). Result showed that MS supplemented with BA (5 µM) and NAA (3 µM) gave the highest number of plantlets of 11.5 and 14.4 and the highest mean plant height at shoot elongation of 5.8 and 7.6 cm, respectively. Regenerated plantlets were hardened on different media. Non-acid washed riverside sand gave the highest recovery rate of 87.4%. Use of riverside sand as substrate for hardening will serve as cost-effective substitute for perlite or vermiculite.

Key words: pineapple, micropropagation, benzylaminopurine (BA), naphthaleneacetic acid (NAA), acclimatization.

INTRODUCTION

Pineapple is the third most important tropical fruit in the world, after the banana and citrus. The fruits are important source of vitamin A and B_1 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 deveolping 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. Micropropagation technique (Plant tissue culture) offers an opportunity for large scale production of uniform pineapple planting material in a relatively short period of time (Escalona et al., 1999; Ika and Mariska, 2003). Rapid multiplication of pineapple through axillary buds culture was reported (Dal Vesco et al., 2001; Be and Debergh, 2006; Danso et al., 2008; Zuraida et al., 2011; Yapo et al., 2011). However, low multiplication rate and poor survival rates during acclimatization have been identified as some of the problems affecting the micropropagation technique (Escalona et al., 1999)

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Table 1. Optimizing *in vitro* shoot proliferation in pineapple using different combinations of Benzyl-6-aminopurine (BA) and Napthaleneacetic acid (NAA).

Parameter	Crop vigour score (1-5) at shoot elongation	Mean plantlet height at shoot elongation (cm)	Mean plantlet height at shoot proliferation (cm)	Number of plantlets at multiplication
BA (µM)				
5.00	4.0[a]	5.8[a]	2.2[b]	11.5[a]
7.50	3.7[b]	5.8[a]	2. 9[a]	11.1[a]
10.00	3.7 [b]	7.0[a]	2.5[b]	11.0[a]
12.50	2.5[c]	5.0[a]	1.7[c]	7.8[b]
SE(±)	0.08	0.72	0.09	0.95
CV (%)	6.70	36.7	12.1	27.50
NAA (µM)				
2.00	4.1[a]	5.1[b]	3.1[a]	7.6[b]
2.50	2.3[b]	4.9[b]	1.9[b]	9.1[b]
3.00	4.0[a]	7.6[a]	1.9[b]	14.4[a]
SE (±)	0.07	0.62	0.08	0.82
CV (%)	6.70	36.70	12.10	27.5
BA x NAA	**	*	**	**

Means with the same letter in a column are not significantly different using Lysergic acid diethylamide (LSD) (*significant at P<0.005, **significant at P<0.001).

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). Therefore, this study was conducted with the primary objective of establishing an efficient rapid micropropagation protocol for large scale propagation of pineapple.

MATERIALS AND METHODS

The study was carried out in the Biotechnology Laboratory of Department of Plant Science, Ahmadu Bello University Zaria, Nigeria. The plant material of pineapple (*Ananas comosos* L. var. smooth ceyanne) was obtained from a fruit market in Zaria. Crowns of young fruits were taken as a source of explant. The leaves were then removed and the crown was thoroughly washed with detergent under running tap water. The crown was surface sterilized by sequential treatment for 5 min in 70% alcohol, 20 min in 10% NaOCl (commercial bleach) plus 2 to 3 drops of tween 20, rinsed thrice with sterile distilled water, 10 min in 5% NaOCl plus 2 to 3 drops of tween 20 washed three times with sterile distilled water and with occasional stirring. All sterilization work was done under laminar air-flow cabinets.

Axillary buds were excised from the crown and inoculated in test tubes over paper bridges on a liquid basal culture Murashige and Skoog (1962) medium supplemented with sucrose (3%), BA (2.5 µM) and NAA (0.6 µM) for shoot induction. Media was adjusted to pH 5.8 before autoclaving for 15 min at 12°C and 15 psi. At every stage the cultures were kept in the growth room at a temperature of 27 ± 2°C, with light provided by cool white florescent tube lights for a 16 h photoperiod. After four weeks of inoculation, shoots were subcultured in kidney jars on MS basal medium plus 3% sucrose, solidified with 0.8% agar and supplemented with different concentrations of growth regulators; BA (5, 7.5, 10 and 12.5 µM) and NAA (2, 2.5 and 3 µM) for shoot multiplication.

The shoots multiplied from each treatment were then separated and rooted on hormone free MS basal medium. Subsequently, the rooted plantlets were acclimatized in the screen house during which they were grown on different media that is, acid washed river side sand and non acid river side sand to evaluate hardening response. Data were recorded on percentage of plantlets showing transplanting shock sign at 1 week after transplanting (WAT) and fully recovered plantlets at 4 WAT. The experimental design used was completely randomized design replicated three times. For the multiplication study a 4 x 3 factorial experiment was used. Data collected were subjected to analysis of variance and means were separated using Lysergic acid diethylamide (LSD) (SAS Institute, 1988).

RESULTS AND DISCUSSION

Experiments using phytohormones has for long established the importance of relative ratio of cytokinin and auxin in plant development. Regeneration of shoots and roots from tissues and cells culture can be induced by increasing or decreasing the relative cytokinin-to-auxin ratio in the culture mediun. Since crop species have different response to treatment with exogenous phytohormones, determintion of optimum cytokinin-to-auxin ratio that will induce maximum level of morphogenesis is essentail in the establishment of efficient micropropagation system. The effects of different concentrations of benzylaminopurine (BA) and naphthaleneacetic acid (NAA) on *in vitro* morphogenesis and plantlets development in pineapple are presented on Table 1. The presence of exogenous BA and NAA in culture medium was found to promote morphogenesis and plantlets development. Supplementing Murashige and Skoog (1962)

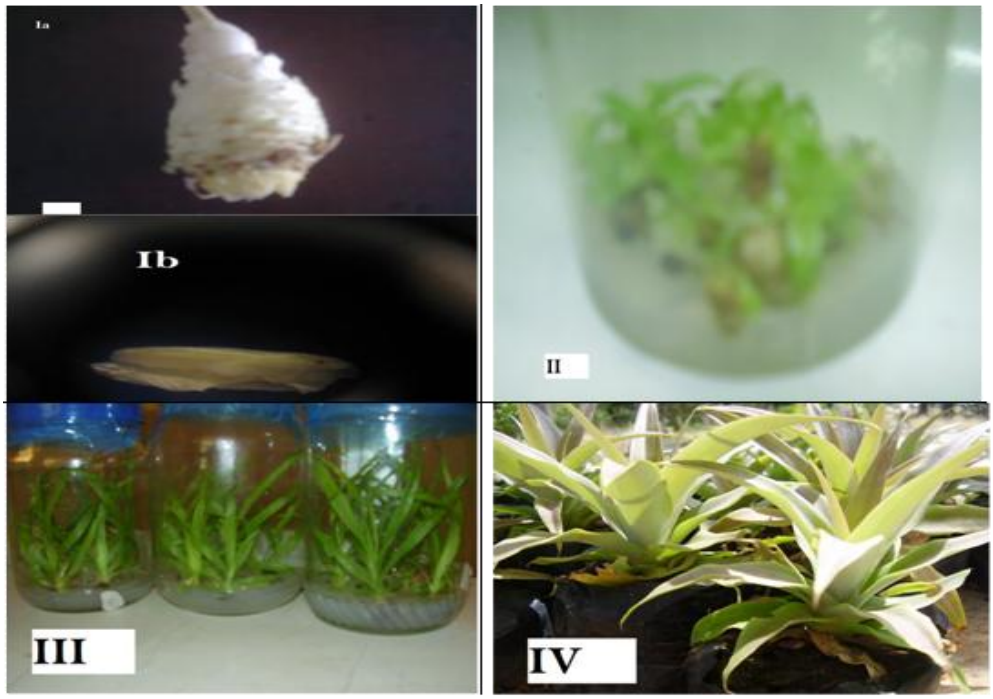

Figure 1. I to IV; I: IA = pineapple crown and IB = excised bud, II: multiplication on ms medium portified with 5.0 µm BA, III: plantlets elongation on ms supplented with 5.0 µm naa, IV: acclimatized pineapple seedlings ready for transplanting.

with 5.0 µM BA proved to provide the best condition for morhpogenesis. MS portified with 5.0 µM BA provided the highest number of plantlets (11.5) compared to other concentrations used in this study.

Our observation is consistent with the optimum range (4.0 to 5.0 µM BA) earlier reported for pineapple (Dal Vesco et al., 2001; Danso et al., 2008; Zuraida et al., 2011; Yapo et al., 2011). Further increase in BA concentration from 5.0 µM BA resulted in a progressive decline in the number of plantlets. However, the decline become statistically significant only when the concentration of BA was increased to 12.5 µM. The effect of cytokinin in apical dominance is antagonistic to that of auxin. While auxin are known to promote apical dominanace by suppressing the activity of auxiliary buds, elevated levels of cytokinin in the auxiliary buds were reported to release them from apical dominance (Shimizu-Sato et al., 2009). Increase in the ratio of BA-to-auxin in the medium portified with BA correlated with prolific induction of shoots. This could be as the result of direct absorption and subsquent accumulation of BA in the auxiliary buds of the cultured plantlets. Over production of cytokinin in nicotiana and cucumber tissues transformed with 35S-ipt resulted in development of stunted plantlets and proliferation of undifferenciated cells (Smigocki and Owens, 1989). It could therefore be possible that 5.0 µM of BA is plateau for *in vitro* proliferation in pineapple and any increase in BA concentration will result in the decline in proliferation of shoots until it reachs a point at which the effect become detrimental to the cell's ability to differenciate.

The ability of different concentrations levels (2.0, 2.5 and 3.0 µM) of NAA to induce plantlets proliferation *in vitro* was also evaluated in this study. Application of exogenous NAA was observed to significantly influenced *in vitro* morphogenesis in pineapple (Figure 1). The highest plantlets number (14.4) was obtained when MS was fortified with 3.00 µM NAA. Similar observation was also reported by Dal Vesco et al. (2001). Reduction in the concentration of NAA to 2.5 µM resulted in significant decrease in the number of plantlets. Plantlets height at elongation was also influenced by NAA concentration. Supplementing MS with 3.0 µM NAA gave the highest plantlets height (7.6 cm) at elongation phase. There was a significant decrease in the plantlets height with decrease in the concentration of NAA. Auxins are generally known for thier ability to induce rhizogenesis at low concentration and callogenesis at rised concentration.

However, NAA has been widely used to induce direct shoots formation and embryogenesis under *in vitro* condition. The ability of NAA to induce *in vitro* morhpogenesis has been reported in pineapple (Dal Vesco et al., 2001; Danso et al., 2008; Zuraida et al., 2011; Al-Saif et al., 2011; Yapo et al., 2011) and other species (Garcia et al., 2007; Ebrahimie et al., 2007; Usman et al., 2011; Sani et al., 2012).

The effect of exogenous NAA on *in vitro* morphogenesis

Table 2. Hardening response of pineapple plantlets grown on different media.

Treatment	Plantlets showing transplanting shock signs at 1 WAT (%)	Fully recovered plantlets (%) at 4 WAT
Acid washed river side sand	14.7	15.1
Non-acid washed river side sand	11.3	87.4
LSD (5%)	7.31	22.47

Means with the same letter in a column are not significantly different using Lysergic acid diethylamide (LSD) at 5% probability level.

seems to be an indirect one. Application of NAA was reported to influence morphogenesis via alternative pathways, by increasing the endogenous ctokinin level or increasing the levels of endogenous NAA (Ebrahimie at el., 2006). Both cytokinin and NAA were reported to actively participate in determining the pattern of differenciation in plants. Auxin are known for their ability to induce cell elongation and could be the possible reason for efficient plantlets elongation in the NAA portified media used in this study. The BA and NAA interraction was significant for both Number of plantlet at multiplication, plant hieght at elongation and crop vigor (factor of nuber of leaves and general greeness of the plantlets) during elongation phase. Interraction however, was not significant for plantlets height during proliferation (Plates I to IV).

The response to hardening of pineapple plantlets was also evaluated on two different harening media (Table 2). No significant difference was observed among the media in terms of plantlets showing transplanting shock signs at 1 wk after transplanting (1 WAT). However, non-acid washed river side sand significantly produced more fully recovered plantlets (87.4%) at 4 wks after transplanting (4 WAT). The poor recovery observed by the acid-washed river sand could be attributed to the pH level of the soil.

Conclusion

The result obtained in this study suggests that an efficient micropropagation protocol has been achieved. BA (5 µM) and NAA (3 µM) could be use for plantlets multiplication, shoot elongation and good crop vigor. While hardening of plantlets during acclimatization can be, done using non-acid washed riverside sand. The relatively very high recovery rate of 87.4% obtained with riverside sand is conceived as a cost saving measure that could substitute for the more expensive and difficult to source substrates like vermiculite, perlite and ghyphy under our laboratory conditions.

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