African Journal of Biotechnology Vol. 10(19), pp. 3859-3866, 9 May, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1349
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

A novel approach for rapid micropropagation of maspine pineapple (*Ananas comosus* L.) shoots using liquid shake culture system

Zuraida A. R.[1], Nurul Shahnadz A. H.[2], Harteeni A.[2], Roowi S.[3], Che Radziah C. M. Z.[2] and Sreeramanan S.[4]

[1]Biotechnology Research Centre, Malaysian Agricultural Research and Development Institute (MARDI), G.P.O Box 12301, 50774, Kuala Lumpur, Malaysia.
[2]School of Biosciences and Biotechnology, Universiti Kebangsaan Malaysia (UKM), 436000 Bangi, Selangor Darul Ehsan, Malaysia.
[3]Food Technology Research Centre, Malaysian Agricultural Research and Development Institute (MARDI), G.P.O Box 12301, 50774, Kuala Lumpur, Malaysia.
[4]School of Biological Sciences, Universiti Sains Malaysia (USM), Minden Heights, 11800 Penang, Malaysia.

Accepted 14 March, 2011

Maspine (*Ananas comosus* L.) is currently the most preferred pineapple variety in Malaysia due to its pleasant aroma and applicability in caning. Large quantities of plant materials are needed to fulfill the market demand which could not be obtained from the conventional breeding method. Hence, *in vitro* procedure was developed as an alternative method to improve the multiplication rate of this special variety. Sterilized explants were cultured on solidified Murashige and Skoog (MS) medium supplemented with various combinations of 6-benzylaminopurine (BAP) (1 to 5 mg/l) and α-naphthaleneacetic acid (NAA) (1 to 5 mg/l) hormones. Pineapple plant cultures required 5 mg/l BAP to significantly increase the shoot development during the *in vitro* stage. In addition, explants were subsequently sub-cultured on medium with 1 mg/l BAP which produced highest number of proliferated *in vitro* plantlets. The optimization of the conditions for shoot propagation was carried out in both liquid and solid medium by supplementing with 1 or 5 mg/l of BAP. MS liquid medium supplemented with 1 mg/l BAP produced the highest number of shoots (31) after 4 weeks. The number of shoots formed was increased to 204 after third sub-culture in liquid medium. Shoot proliferation was increased up to nine-fold in liquid medium when compared to the cultures maintained on solid medium. This improved method of Maspine *in vitro* multiplication will serve as an alternative source of planting materials of this cultivar for subsistence and large-scale pineapple farmers.

Key words: Pineapple, *in vitro*, 6-benzylaminopurine, α-naphthaleneacetic acid, liquid medium.

INTRODUCTION

Pineapple is one of the most economically important tropical fruits (Duval et al., 2001). This bromeliad is routinely propagated vegetatively by means of lateral shoots, basal suckers or crowns. Pineapple micropagation can be considered to be easy, but the multiplication rate is low and it would take 8 years to obtain enough propagules from one mother plant (Almeida et al., 2002). In conventional breeding, clonal selection is tedious and requires several generations of backcrossing in order to develop pineapple varieties with desired traits. Being a vegetatively propagated plant, conventional hybridization techniques for the generation of better pineapple varieties are cumbersome and time consuming (Mhatre, 2007).

*Corresponding author. E-mail: azuraida@mardi.gov.my.

Abbreviations: MS, Murashige and Skoog; **BAP,** 6-benzylaminopurine; **NAA,** α-naphthaleneacetic acid; **RPM,** rotation per minute; **IAA,** indole acetic acid.

Hence, the need to improve the multiplication rates of selected elite genotypes led to the development of tissue culture techniques for the *Ananas comosus* (L. Merr) pineapple (Almeida et al., 2002). *In vitro* micropropagation of pineapple plantlets has many advantages over conventional methods of vegetative propagation. For instance, this technique allows an efficient and rapid increase of selected elite pineapple varieties. Many authors have reported successful production of pineapple via micropropagation system during the last few years (Firooozabady and Gutterson, 2003; Be and Debergh, 2006; Danso et al., 2008). According to Drew (1980), starting with 30 explants, it is possible to produced 1,250,000 pineapple plantlets within eight months of *in vitro* multiplication. Perhaps, the micropropagation protocol has now become fairly standardized for most of the important pineapple cultivars and minimum changes can be applied to newly derived varieties. In addition, *in vitro* pineapple plantlets would help to minimize the problem of 'natural flowering' which is rampant in pineapple cultivation industry.

The success of the micropropagation procedure depends on several factors, which could be observed during the *in vitro* growth process. Generally, most pineapples are produced restrictively by using dormant axillary buds from crowns (Soneji et al., 2002a; Sripaoraya et al., 2003) and multiple shoot induction via *in vitro* produced leaf bases (Soneji et al., 2000b). Almeida et al. (1997) reported that 3.0 mg/l 6-benzylaminopurine (BAP) combined with 2.0 mg/l indole acetic acid (IAA) produced the best results for the production of pineapple plantlets. According to Danso et al. (2008), the optimal conditions for MD2 pineapple plantlets production in both liquid and solid medium was determined by using Murashige and Skoog (MS) medium supplemented with 7.5 mg/l BAP and 2 mg/l α-naphthaleneacetic acid (NAA), which resulted in the production of 28.5 and 16.1 plantlets, respectively, during the first subculture. Generally, liquid cultures provide faster rates of *in vitro* growth of plantlets (Alvard et al., 1993; Firoozabady and Gutterson, 2003; Danso et al., 2008). In addition, Danso et al. (2008) reported that a lower concentration of BAP (5.0 mg/l) in liquid medium produced significantly more MD2 pineapple plantlets than in solid medium (7.5 mg/l).

The objective of the present work is to optimize a protocol for the micropropagation of pineapple cv. Maspine, by the manipulation of different BAP and NAA concentrations, and type of culture medium, aiming to achieve a maximum rate of multiplication and, therefore, improve the agronomical utilization of this cultivar.

MATERIALS AND METHODS

Plant material

Pineapple's crowns and suckers of Maspine were obtained from Malaysian Agriculture and Research Institute's (MARDI) station in Pontian, Johor, Malaysia. The leaves were removed, thoroughly washed in detergent using tap water and, subsequently, immersed in fungicide for one hour. Pineapple crown were sterilized with 50% Clorox [sodium hypochlorite 5.2% (15 min)], 20% Clorox (10 min) and rinsed once with sterile distilled water.

Shoot development

Sterilized crowns were cultured on MS medium (1962) supplemented with 30 mg/l sucrose, varying concentration of BAP (0 to 5 mg/l) and/or NAA (0 to 5 mg/l). Solidified MS medium with 0.7% agar was used for culture experiments. The development of shoots was monitored every week. Percentages of explants that developed into shoots and number of shoots developed were counted after 8 weeks of culture. The experiment followed a completely randomized design and 20 cultures were raised for each treatment and all experiments were repeated thrice.

Micropropagation of shoots

Initiated shoots obtained from previous experiment and supplemented with 1 and 5 mg/l BAP were sub-cultured into MS media containing different concentrations of BAP (1 to 5 mg/l). The number of regenerated plantlets were recorded after the first (4 weeks) and third (12 weeks) sub-cultures. The average of plant height and intensity of root formation were determined in this experiment.

Liquid cultures

Pineapple crown was sterilized with 50% Clorox (15 min), 20% Clorox (10 min) and rinse one time in sterile distilled water and, subsequently, cultured on solid MS medium for 4 weeks. Shoots obtained were transferred into 250 ml Erlenmeyer flask containing 50 ml of liquid or solid (addition of 0.9% agar) MS medium. The medium was supplemented with 1 or 5 mg/l BAP. Liquid cultures were incubated on rotary shaker at 100 rpm. All cultures were maintained in the growth room at 25°C in light (12 h day/night) provided by cool-white florescent lamp (40 µmol.m12.s^{-1} light intensity). The experiments followed a completely randomized design and ten flasks were raised for each treatment, each flask containing ten shoots. All experiments were repeated thrice. The cultures were subcultured approximately every 4 weeks for a total of 12 weeks. The regenerated pineapple shoots were determined after 4 (first sub-culture) and 12 weeks (third sub-culture) of culture.

Pineapple plantlets with actively growing roots were carefully removed from culture flasks, washed with tap water and transferred to paper cups containing autoclaved soil-rite. All plantlets were transferred to soil in polythene bags after 4 weeks and maintained in the greenhouse for acclimation before being transferred to the field. After 7 to 8 weeks, these plantlets were transferred to the field for further analysis on growth, flowering and fruiting.

Statistical analysis

All statistic analysis were done using the Statistical Package for the Social Sciences (SPSS).

RESULTS AND DISCUSSION

Shoots development

All combinations of BAP and NAA showed formation of

Table 1. Effect of different concentrations of BAP and NAA on shoot development and mean number of shoots produced per explant. Values followed in a column by the same letter are not significantly different at P ≤ 0.05 of Duncan multiple range test.

Plant growth regulator (mg/l)		Development of shoots (weeks)	Percentage of explants that generate shoots (%)	Number of shoots generated per explant
BAP	NAA			
	0	3-4	56^{ced}	1.0^{igh}
	0.5	2-4	52^{gcfed}	0.9^{ijh}
	1.0	2-3	44^{ghfe}	0.66^{ikjh}
0	2.0	6	32^{hi}	0.44^{ikj}
	5.0	-	0^{j}	0^{k}
	0	4-5	56^{ced}	2.26^{e}
	0.5	3-4	48^{gfed}	0.84^{ijh}
	1.0	2-3	48^{gfed}	1.02^{igh}
0.5	2.0	6-7	38^{gh}	0.48^{ikj}
	5.0	8	22^{i}	0.30^{kj}
	0	2-3	58^{ced}	2.28^{e}
	0.5	2-3	54^{cfed}	1.8^{fe}
	1.0	2-3	52^{gcfed}	0.92^{ijh}
1.0	2.0	7	32^{hi}	0.82^{ijh}
	5.0	8	24^{i}	0.5^{ikj}
	0	2-3	54^{cfed}	1.76^{fe}
	0.5	2-3	52^{gcfed}	1.68^{fe}
	1.0	2-3	52^{gcfed}	1.32^{fgh}
2.0	2.0	3	44^{ghfe}	1.6^{fg}
	5.0	6	40^{ghf}	1.04^{igh}
	0	2-3	86^{a}	6.98^{a}
	0.5	2-3	72^{b}	3.62^{cd}
	1.0	2-3	66^{cb}	3.38^{d}
5.0	2.0	2-3	62^{cbd}	4.0^{cb}
	5.0	2-3	64^{cb}	4.5^{b}

shoots with the exception of the treatment with 5 mg/l NAA alone (Table 1). MS medium supplemented with 5 mg/l BAP in the absence of NAA produced the highest percentage of shoot development (86%). The combination between 5 mg/l BAP and 0.5 to 5 mg/l NAA also induced shoot development between 62 and 72%. Other combinations of BAP (0.5 to 2 mg/l) and NAA (0 to 5 mg/l) induced shoot development in 22 to 58% of the explants. The treatment with 5 mg/l NAA and 0 mg/l BAP did not result in shoot formation. Moreover, higher concentrations of NAA (2 to 5 mg/l) supplemented into medium required extended time (6 to 7 weeks) to generate more *in vitro* shoots.

The highest concentration of BAP in the medium was more stimulatory to shoot 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 shoots were produced per inoculums when BAP concentration was increased. Generally, cytokinins are known to stimulate cell division and axillary bud proliferation (Kyte and Kleyn, 1996), thereby resulting to significant shoot formation at the expense of root development. According to Firoozabady and Gutterson (2003), addition of BAP in MS medium was essential for the regeneration plantlets from shoot apices of pineapple. In contrast, the medium supplemented with 5 mg/l NAA did not produce any shoot due to high concentration of this auxin. NAA and IBA are known as root inducing growth regulators and have been used either alone or in combination for root initiation in many cultures (Gupta et al., 1981; Be and Debergh, 2006; Danso et al., 2008).

The number of regenerated plantlets on different medium supplemented with various BAP and NAA concentration is shown in Table 1. All treatments analyzed produced shoots ranging from 0.5 to 7, except the treatment containing 5 mg/l NAA. The highest number of shoots was observed on the medium containing 5 mg/l BAP (7 plantlets). Therefore, Maspine pineapple could easily produce *in vitro* shoots from crown at 5 mg/l BAP (Table 2). Cytokinin alone in the culture medium induces shoot formation in many plants. MS medium supplemented with 3.0 mg/l BA was suitable for micropropagation of *Ficus Benjamina* vars. Natasja and

Table 2. Effect of various BAP concentrations on regenerated plants and rooting after first and third sub-culture.

Initial culture BAP (mg/l)	Number of plantlets after sub-culture			Average plantlets height (cm)	Induced Rooting	Description
	BAP (mg/l)	First sub-culture	Third sub-culture			
5.0	0	21[a]	49[b]	5-7	+++	Satisfactory growth
	1.0	19[a]	64[a]	3-7	++	Satisfactory growth
	5.0	11[b]	36[b]	1.5-3	+	Retarded growth
1.0	0	15[ba]	21[c]	5-7	+++	Satisfactory growth
	1.0	23[a]	38[b]	3-4	++	Limited growth
	5.0	16[ba]	29[bc]	1.5-4	+	Retarded growth

Initial explants were obtained from previous experiment (satisfatory growth, +++; limited growth, ++; no rooting, +). Values in a column followed by the same letter are not significantly different at $P \leq 0.05$ of Duncan multiple range test.

Starlight (Rzepka-Plevnes and Kurek, 2001). Jain (1997) reported that micropropagated *Saintpaulia ionantha* could be obtained by culturing leaf disks on MS medium containing 0.22 to 0.50 µM BA. The combination of auxins and cytokinins is essential for shoot induction and multiplication which depends on the plant genotypes. In *Petunia hybrida*, massive shoot multiplication was achieved by using MS medium amended with 2.2 µM BA and 5.7 µM IAA within 4 weeks of culture (Sharma and Mitra, 1976). High concentration of cytokinins is unsuitable for shoot formation from leaf or petiole explants in some ornamental pot plants. Takayama and Misawa (1982) used 1.3 µM BAP or 4.6 µM kinetin in combination with 5.4 µM NAA for shoot bud regeneration from leaf, petiole or inflorescence segments of *Begonia* species. Lower concentration of hormone is suitable for pineapple since it allows the production of healthy plants of desirable size in the growth chamber as well as under the natural environment of a glasshouse (Be and Debergh, 2006). Furthermore, lower concentration of cytokinins also provided high rates of shoot bud regeneration of flower peduncle segments of *Begonia* x *hiemalis* (Appelgren, 1985). However, some plants require NAA, as reported by Thengane et al. (2006). They stated that the application of NAA (5.3 to 10.7 µM) can originate germinating embryos with well-developed shoots.

Micropropagation of shoots

Shoots cultured on 5 mg/l BAP showed growth arrest when continuously sub-cultured at same concentration. Therefore, some modifications on the first sub-cultured media were needed in order to propagate the explants. After determining the optimum hormonal concentration for shoot development, shoots of cultures from the initial culture treated with 1 or 5 mg/l BAP were selected for the next stage by subculturing onto medium containing either 0.1 or 5 mg/l BAP for further shoot development. The plant cultures were sub-cultured every 4 weeks for 12 weeks on the same fresh medium aiming to increase the number of plantlets and formation. The results obtained are shown in Table 2. The initial cultures treated with 5 mg/l BAP and sub-cultured onto 1 mg/l BAP produced the highest number of plantlets at the third sub-culture (64 plantlets). Pineapple plant cultures showed a high growth rate with the plant height average between 3 to 7 cm and proper healthy root formation. A total of 36 regenerated plantlets were produced continuously on medium with 5 mg/l BAP. However, most of the plantlets showed growth arrestment and were small in size (Table 2).

Hence, the best initial microshoot response was observed when the explants were cultured on the medium containing 5 mg/l BAP. Hence, microshoots have to be transferred to medium with 1 mg/l BAP for better propagation rate at least for a few cycles. The important role of BAP hormone for shoot proliferation was also reported for other plants of the Asteraceae family, such as *Artemisia annua* (Usha and Swamy, 1998), *Wedelia calendulacea* (Emmanuel et al., 2000), *Echinacea purpurea* (Korach et al., 2002) and *Eclipta alba* (Dhaka and Kothari, 2005). All cultured plant shoots developed roots within two weeks after the third sub-cultured. However, the rooting was reduced when the concentration of BAP in the medium was increased, indicating that higher concentrations of BAP is detrimental to root development (Table 2). Pierik et al. (1984) and Danso et al. (2008) reported that *in vitro* rooting of pineapples can be enhanced by an addition of auxins 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).

Liquid cultures

In vitro shoots of Maspine pineapple were incubated in

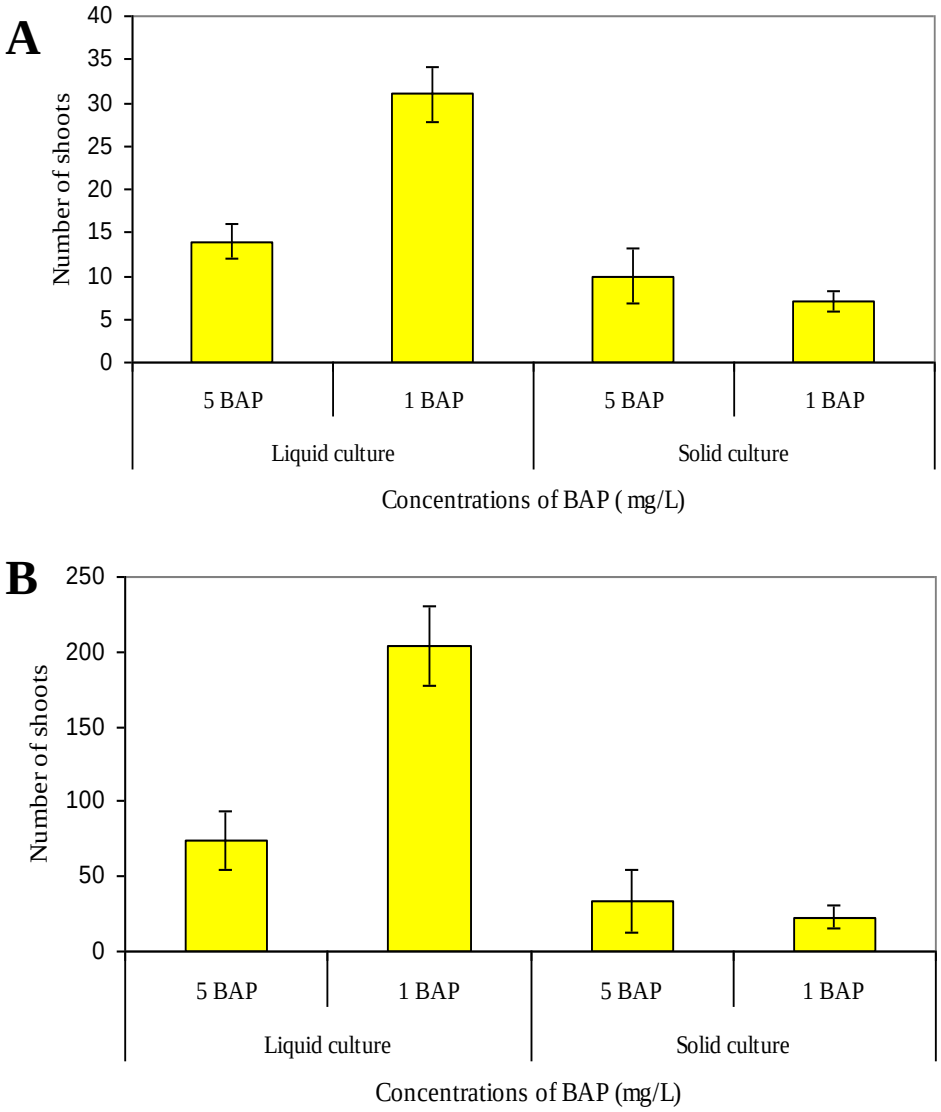

Figure 1. Effect of two different BAP concentrations on number of shoot production in liquid and solid medium after 4 weeks (A) and 12 weeks (B) of culture.

liquid and solid (0.7% agar) MS medium for a period of 4 and 12 weeks to compare the effect of these medium on shoots proliferation. After 4 weeks of culture, adventitious buds and shoots were regenerated. We observed that those explants cultured in liquid medium supplemented with 1 mg/l BAP produced the highest number of shoots (31) (Figure 1). In addition, the number of shoots was increased after 12 weeks of culture, achieving a total number of 204. On liquid medium supplemented with 5 mg/l BAP, only 14 and 74 shoots were regenerated after 4 and 12 weeks of culture, respectively. There was a rapid shoot proliferation in solid medium cultures but with no proper root development (Figure 2). We have observed a 9- and 5-fold increase in number of shoots when they were cultured in liquid medium containing 1 and 5 mg/l of BAP when compared with solid medium. The number of shoots obtained on solid medium supplemented with 1 and 5 mg/l BAP were 7 and 10 shoots which were increased to 23 and 24 shoots after 12 weeks in culture, respectively (Figure 1).

It was noticed that more shoots were produced when grown in liquid shake culture when compared with solid medium (Figure 1, 2 and 3). Liquid culture leads to the production of more shoots but these were lower in size than those obtained by using solid medium (Figure 3). The results obtained here confirmed the assertion that liquid culture explants usually display a higher frequency of growth rate when compared to solid cultures (Gupta et al., 1981). The higher rate of growth in liquid cultures may be attributed to the exposure of greater surface of the explants to the medium, therefore enhancing nutrient uptake due to its uniform distribution (George and

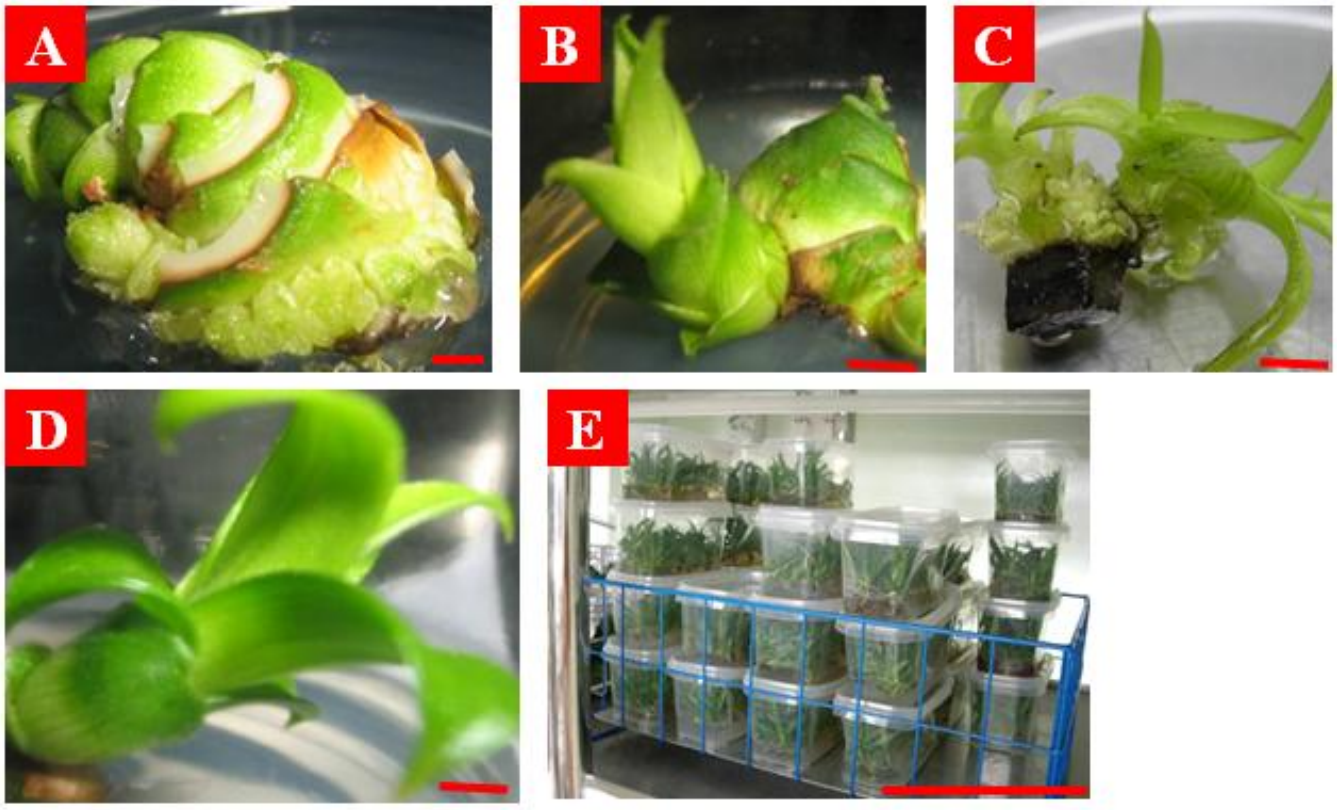

Figure 2. (A and B): Formation of microshoots from crowns after sterilization, (C): micropropagation of shoots on medium with 5 mg/l BAP, (D): development of shoot and (E): regenerated plantlets after sub-cultured on medium with 1 mg/l BAP. Bars represent 1 cm : 2 cm.

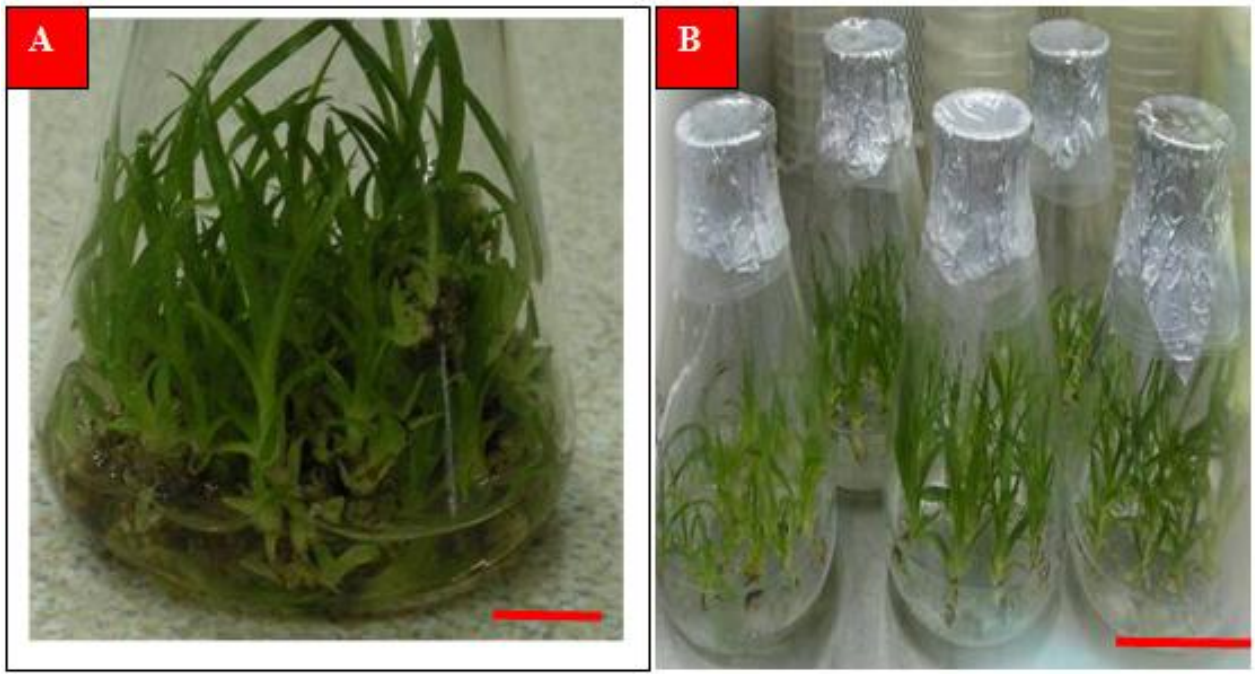

Figure 3. Shoot proliferation of Maspine pineapple in liquid (A) and on solid MS medium (B) after 12 weeks supplementation with 5 mg/l BAP. Bars represent 1 cm : 2 cm.

Figure 4. Production of *ex-vitro* pineapple plants. (A) Plants acclimatization in the greenhouse; (B) Three-month-old plants in the field. Bars represent 1 cm : 5 cm.

Sherrington, 1984; Alvard et al., 1993). It has also been shown that liquid medium disperses phenolic exudates from the explants, consequently, resulting in faster growth rate (Kyte and Kleyn, 1996).

Transfer of pineapple plantlets with sterile roots to the greenhouse conditions showed almost 100% of survival success (Figure 4a). The established plants were exposed to an open field environment where they showed rapid growth after the acclimation period in greenhouse (Figure 4b). This system is presently being introduced for the large-scale production of this specific pineapple cultivar. Most of the obstacles currently found in conventional pineapples micropropagation have been overcome. Thus, an efficient and viable protocol was established for the mass propagation of Maspine pineapple.

REFERENCES

Almeida WAB, de Matos AP, de Sousa A, da S (1997). Effect of benzylaminopurine (BAP) on *in vitro* proliferation of pineapple (*Ananas comosus* (L) Merr). Acta. Hortic. 425: 242-245.

Almeida WAB, Santana GS, Rodriguez APM, Costa MA, de C (2002). Optimisationof a protocol for micropropagation of pineapples. Rev. Bras. Fruct. 2: 296-300.

Alvard D, Cote F, Teisson C (1993). Comparison of methods of liquid medium cultures for banana micropropagation. Effect of temporary immersion of explants. Plant Cell. Tissues. Organ. Cult. 32: 555-60.

Appelgren M (1985). Effect of supplementary light to mother plants on adventitious shoot formation in flower peduncle segments of *Begonia×hiemalis*. Sci. Hortic. 25: 77-83.

Be LV, Debergh PC (2006). Potential low-cost micropropagation of pineapple (*Ananas comosus*). South Afr. J. Bot. 72: 191-194.

Danso KE, Ayeh KO, Oduro V, Amiteye S, Amoatey HM (2008). Effect of 6-Benzylaminopurine and Naphthalene acetic acid on *in vitro* production of MD2 pineapple planting materials. World. Appl. Sci. J. 3(4): 614-619

Dhaka N, Kothari SL (2005). Micropropagation of *Eclipta alba* (L.) Hassk-an important medicinal plant. *In Vitro.* Cell. Dev. Biol. Plant, 41: 658-661.

Drew RA (1980). Pineapple tissue culture unequalled for rapid multiplication. Queensland Agr. J. Brisbane, 106 (5): 447-451.

Duval MF, Noyer JL, Perrier X, Coppens d'Eeckenbrugge G, Hamon P (2001). Molecular diversity in pineapple assessed by RFLP markers. Theor. Appl. Genet. 102: 83-90.

Emmanuel S, Ignacimuthu S, Kathiravan K (2000). Micropropagation of *Wedelia calendulacea Less.* a medicinal plant. Phytomorphol. 50:195-200.

Firoozabady E, Gutterson N (2003). Cost effective *in vitro* propagation methods for pineapple. Plant Cell. Rep. 21: 844-850

George F, Sherrington PD (1984). Plant propagation by tissue culture. Exegetics Limited, pp. 284-330.

Gupta PK, Mascarenhas AF, Jagannathan, V, Benjamin DA (1981). Minimum growth conditions for Tissue culture of trees: clonal propagation of in *vitro* conservation of pineapple germplasm. In: 5th mature trees of *Eucalyptus citriodora* Hook, by tissue International Pineapple Symposium, 5, 2005, Port culture. Plant Sci. Lett. 20: 195-201.

Jain SM (1997). Micropropagation of selected somaclones of *Begonia* and *Saintpaulia*. J. BioSci. 22(5): 585-592.

Korach A, Juliani H, Kapteyn J, Simon JE (2002). *In vitro* regeneration of *Echinacea purpurea* from leaf explants. Plant Cell. Tissue Org. Cult. 69: 79-83.

Kyte L, Kleyn J (1996). Plants from test tubes: An introduction to micropropagation. Third edition, Daquinta JH, Gonzalez Y, Desjardins CG. p. 82.

Mhatre M (2007). Micropropagation of pineapple, *Ananas comosus* (L.) merr. In: Jain SM and Haggman H (eds.). Protocol for micropropagation of Woody Trees and Fruits. pp. 499-508.

Murashige T, Skoog F (1962). A revised medium for rapid growth and

bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.
Pierik RLM, Steegmans HH, Hendriks J (1984). The influence of naphtaleneacetic acid on the growth of *in vitro* cultivated seedling of *Bromeliaceae.* Sci. Hortic. 24: 193-199.
Rzepka-Plevnes D, Kurek J (2001). The influence of media composition on the proliferation and morphology of *Ficus benjamina* plantlets. Acta. Hortic. 560: 473-476.
Sharma AK, Mitra GC (1976). *In vitro* culture of shoot apical meristem of *Petunia hybrida* for mass production of plants. Indian J. Exp. Biol. 14: 348-350.
Soneji JR, Rao PS, Mhatre M (2000a). Somaclonal variation in micropropagated dormant axillary buds of pineapple (*Ananas comosus* L., Mer). J. Hortic. Sci. Biotech. 77: 28-32.
Soneji JR, Rao PS, Mhatre M (2000b). *In vitro* regeneration from leaf explants of pineapple (*Ananas comosus* L., Mer). J. Plant Biochem. Biot. 11: 117-119.
Sripaoraya S, Marchant R, Power JB, Davey MR (2003). Plant regeneration of somatic embryogenesis and organogenesis in commercial pineapple (*Ananas comosus* L.). *In Vitro*. Cell Dev. Biol. Plant, 39: 450-454.
Takayama S, Misawa M (1982). Factors affecting differentiation *in vitro* and a mass-propagation scheme for *Begonia×hiemalis.* Sci. Hortic. 16: 65-75.
Thengane SR, Deodhar SR, Bhosle SV, Rawak SK (2006). Repetative somatic embryogenesis and plant regeneration in *Garcinia indica* Choiss. *In Vitro*. Cell. Dev. Biol. Plant, 42: 256-261.
Usha R, Swamy PM (1998). *In vitro* micropropagation of sweet wormwood (*Artemisia annua* L.). Phytomorphol. 48: 149-154.