Journal of Ornamental Plants
Available online on: www.jornamental.com
ISSN (Print): 2251-6433 ISSN (Online): 2251-6441

An Efficient and Cost Effective Protocol for *In Vitro* Propagation of Pineapple

Ipsita Dutta, Joyita Bhadra, Pritha Ghosh, Babita Saha and Siraj Datta*
Department of Biotechnology, Haldia Institute of Technology, Haldia, West Bengal, India. Pin: 721657.

Received: 07 August 2013 Accepted: 28 October 2013
*Corresponding author's email: dattasiraj@gmail.com

Abstract

An efficient and cost effective protocol for *in vitro* propagation of Pineapple (*Ananas comosus* var. *Queen*) has been developed. In the proliferation stage, agar based Murashige and Skoog (MS) media was supplemented with 3.0 mg/l benzyleaminopurine (BAP), 0.5 mg/l indole acetic acid (IAA) and 50 mg/l adenine sulphate as RBC design experiment. Two approaches were taken to reduce the chemical cost of micropropagation media. Analytical grade sucrose was successfully replaced by commercial sugar, completely during proliferation stage and up to 66% during rooting stage. Again during the rooting stage, agar based solid media was replaced by liquid media (MS-media). Bio-degradable Coir and Luffa were used as supporting matrix. As supporting matrix in rooting media, Luffa was found to be more effective. The clonal fidelity of *in vitro* raised plantlets was confirmed by RAPD technique.

Keywords: Coir, Commercial sugar, Luffa, Micropropagation, Pineapple (*Ananas comosus*).

INTRODUCTION

Pineapple is one of the major economically important fruit crop in tropical zone. Comparing its annual world production which exceeds 15 billion kg per year, India produces nearly 1.3 billion kg per year (Economic Research Service, United State Department of Agriculture, 2012). But the seeds of these plants are very slow to germinate and therefore are not used for commercial purposes. For vegetative propagation of the plants, crowns and slips have been successfully used over years (United State Department of Agriculture, 2013). In case of industrial scale production suckers and heaps are also used (Firoozabady and Gutterson, 2003).

The tissue culture method of pineapple comes into the play to increase the selectivity of the desired traits coupled with a high multiplication rate. Following the standard tissue culture method (Mathews and Rangan, 1979; Zepeda and Segawa, 1981; Fitchet, 1990; Fitchet-Purnell, 1993; Kiss *et al.,* 1995; Gangopadhyay *et al.,* 2005) much higher rate of multiplication (40 to 85 fold in a 13 month period) was obtained. But the major requirement for an economical crop to remain industrially viable is that it should have a considerably low production cost; the condition which is not supported by the current tissue culture method of pineapple. Hence there has been a continuous effort among researchers to decrease the production cost of culture. But even a 3000-4000 fold multiplication in a 6 month period *in vitro* method, both solid and liquid culture approach (Escalona *et al.,* 1999; Firoozabady and Gutterson, 2003) did not provide a considerable cost reduction when compared to field- propagation method.

The present investigation involves approaches to reduce the cost of pineapple micropropagation by replacing some of the costly components by readily available and much cheaper substitutes. Two attempts had been taken. Sucrose, a major and yet costly ingredient of the media was attempted to be replaced by commercial sugar. Again liquid media is commercially cheaper than solid media as it eliminates the cost of agar, though the major problem there is vitrification (Bhojwani and Razdan, 2005). In earlier attempts, enhanced rate of multiplication and rooting were obtained through the use of Coir and Luffa sponge in *Gladiolus* and *Philodendron* respectively (Roy *et al.,* 2006; Gangopadhyay *et al.*, 2004). In the current project both 'Luffa' and 'Coir' had been tested as supporting bases in liquid media during rooting stage. Luffa was found to be more suitable as supporting matrix in this approach. Commercial sugar may contain some toxic material that can cause some genetic (somaclonal) variation. Then RAPD was performed to test the clonal fidelity of the tissue culture raised plantlets.

MATERIALS AND METHODS

Plant material

Aseptic culture of the 'Queen' variety of pineapple, originally procured from Manipur, was collected from the experimental garden, Department of Agricultural and Food Engineering, Indian Institute of Technology, Kharagpur. This particular variety of pineapples is small in size, but has its unique taste and flavor.

Aseptic techniques

The shoot apical meristems were first washed in tap water for at least 15 minutes followed by stirring with 20% Bavistin (20 minutes). After that, the explants were disinfected with Tween 20 and 0.5% Sodium hypochlorite solution (15 minutes) followed by five times washing in CA water (solution of 0.25% citric acid and 0.5% ascorbic acid).

Culture media and growth condition

The sterilized shoot apical meristems were established in MS media (Murashige and Skoog, 1962) with BAP (6-benzylaminopurine) 3 mg l^{-1}, IAA (indole acetic acid) 0.5 mg/l and adenine sulphate 50 mg/l supplementation (Datta *et al*., paper communicated). The competency of sucrose supplementation

by commercial sugar (at 16.67% (PA-0.5), 33% (PA-1.0), 50% (PA-1.5), 66% (PA-2.0) and 100% (PA-3.0)) in solid media was examined (Table1). The media was gelled with 0.8% agar and emerging of multiple shoots from the cut ends was observed within one month. The shoot buds from the matured plantlets were separated and placed in the same media for four more subcultures.

In rooting media, MS composition along with kinetin, 0.5 mg/l and IBA (indole butyric acid) and 2 mg/l were used (Gangopadhyay *et al.,* 2002). The plantlets were subsequently placed in culture tubes containing liquid media with sterile coir as supporting matrix or in culture jars containing either liquid media with sterile luffa or solid agar media. The effectiveness of sucrose supplementation by commercial sugar (at percentages, 16.67%, 33%, 50%, 66%, 100%) in rooting media (both solid and liquid), was tested (Fig. 1A, Table 1). After the rooting stage, the plantlets were transferred to soilrite for hardening.

RAPD

DNA was extracted from the explants by CTAB method (Rogers and Bendich, 1998). To amplify the obtained amount, PCR was done by adjusting the DNA concentration to 25µg ml^{-1}. The method of Williams *et al.* (1990) was followed using decamer primers, OPA 01- OPA 05 and OPB 01- OPB 05 (Operon Tech., Alameda, USA). The reaction mixture consisted of 1X buffer, 0.2 mM dATP, dTTp, dCTP, dGTP, 2 mM MgCl2, 0.2µm primer, 100ng of template DNA and 1 unit of Taq DNA polymerase (Roche). A thermal cycler (Eppendorf, Germany) was used for the amplification of DNA with an initial denaturation temperature of 94º C for two minutes. The reaction continued for 45 cycles, the temperature profile for each of the cycle was- denaturation at 94º C for one minute, annealing at 35º C for one minute and extension at 72º C for two minutes. The reaction was followed by five minutes hold at 72º C for ensuring complete extension of the primers.

Statistical analysis

RBD-ANOVA test was done in order to check whether any significant difference was present in the number of multiple shoots in solid media or in root lengths of the plantlets maintained in agar, coir and luffa medium. Test of significance was carried out by 'Student's t-test' method (Datta, 2006).

RESULTS AND DISCUSSION

Reduction of the cost of proliferation media

The proliferated plantlets responded readily in culture medium and a considerable number of adventitious shoots emerged from each of the responding explants. In the propagation media 100% supplementation of sucrose by commercial sugar was found to be successful. The multiplication rate was almost similar in both supplemented media and control. No significant difference in proliferation rate was observed between the control and treatment (RBD-ANOVA Test, at level

Table 1. Supplementation of sucrose by commercial sugar in different culture media.

Sl. No.	Culture Media	Sucrose concentration in media (%)	Sugar supplementation (%)	Commercial sugar concentration in media (%)	Cost reduction (%)
1	#PA-0	3.00	0.00	0.00	0.00
2	PA-0.5	2.50	16.67	0.50	2.34
3	PA-1.0	2.00	33.00	1.00	4.69
4	PA-1.5	1.50	50.00	1.50	7.03
5	PA-2.0	1.00	66.00	2.00	10.41
6	PA-3.0	0.00	100.00	3.00	15.12

#Control

Table 2. Mean root lengths of the plantlets with their standard error in different media.

Sl. No.	Culture Media	Agar (mean root length) ± SE	Luffa (mean root length) ± SE	Coir (mean root length) ± SE
1	#PA-0	1.045±0.125	2.423±0.265	1.360±0.161
2	PA-0.5	1.045±0.188	2.407±0.394	1.346±0.154
3	PA-1.0	1.042±0.093	2.400±0.261	1.290±0.176
4	PA-1.5	1.037±0.576	2.250±0.434	1.190±0.137
5	PA-2.0	1.020±0.112	2.200±0.356	1.090±0.108
6	PA-3.0	0.700±0.157	2.000±0.329	0.900±0.151

of p=0.001). Reduction of chemical cost was achieved by 15% (Table 1), giving its immense importance from industrial point of view.

Reduction of the cost of rooting media

Sing alternative matrix in liquid media

During rooting stage, maximum root lengths were observed in liquid media (no sucrose supplementation) using Luffa as supporting matrix, followed by coir and agar (Table 2). Cost reduction was maximum in coir matrix (62.83%), followed by luffa (52.5%) with respect to conventional agar media (Figure 1B).

Liquid media has many advantages over solid media such as efficient nutrient uptake, lower cost and dilution of excreted material (Smith and Spoomer, 1995; Aitcken-Christie *et al*. 1995). When the liquid media is supported by solid, biodegradable, fibrous matrices, the nutrients can diffuse easily through it and vitrification can be prevented (Gangopadhyay *et al.*, 2002). In the present investigation, symptoms of vitrification were not observed.

RBD-ANOVA test revealed significant difference among the root lengths of the explants in three different supporting matrices (at p=0.001, calculated value of 'f'(13.79) is greater than its Table value (~8.56)). The value of the treatment mean showed that the mean root length of agar media was lowest (1.0455 cm) and media with luffa was highest (2.423 cm). Also, the fact that the observed 't' value (3.2933) was higher than its Table value (at p= 0.01, t^{table} =2.82) indicated that the difference in root length between the highest and the lowest was highly significant.

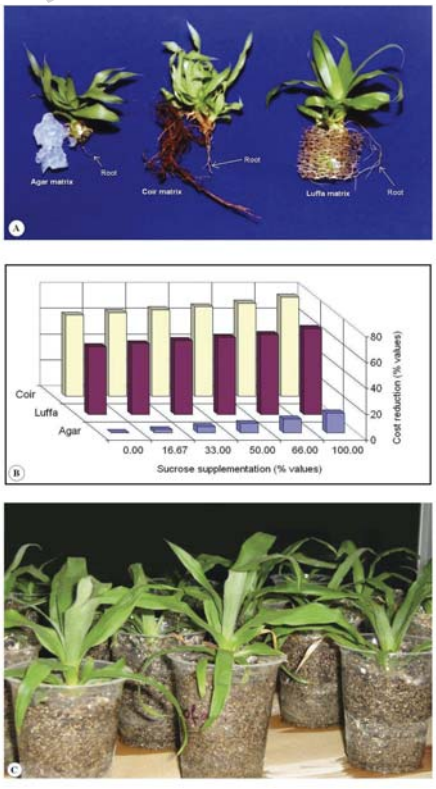


Fig. 1. A) Rooted plants grown on different matrices for rooting, just after transplantation. B) Relative cost reduction by sucrose supplementation (at different percentages) using different matrices in liquid media. C) Transplanted plants into the soilrite during hardening

Replacing sucrose with commercial sugar

When replacing sucrose with commercial sugar in rooting media significant root lengths were observed up to 66% supplementation (again better results were observed with Luffa). Reduction of cost due to the usage of Luffa matrix and 66% sucrose supplementation and is 61.92% (Fig. 1B).

From ANOVA test it was confirmed that supplementation of sucrose by commercial sugar is possible up to 66%, as no significant difference (at level p=0.001) results between the mean root lengths of control and treatment. But with 100% supplementation, there was significant difference (at level p=0.001) between the treatment and the control.

Clonal fidelity and hardening

Clonal fidelity is one of the most important factors that should be tested before commercialization. To test the clonal fidelity of micropropagated plant, RAPD was done taking randomly selected *in vitro* raised plantlets. Ten RAPD primers were used in this experiment (*In vitro* raised plantlets were selected randomly). The result of the respective profile of ten samples (Fig. 2) is being presented because of the identical profiles obtained in all the samples tested with each primer.

After adequate rooting, the *in vitro* grown plants were transplanted into the soilrite in a humidity tent for hardening (Figure 1C). The plants grown in Coir or Luffa were successfully transplanted in pots with 99% survivality over 78% successful survival rate of the plants grown in agar-gelled medium. It was found to be easier to take out the rooted plants from coir and luffa without damaging the plantlets.

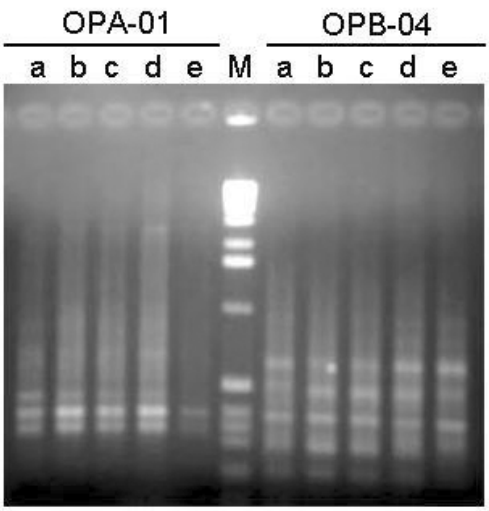
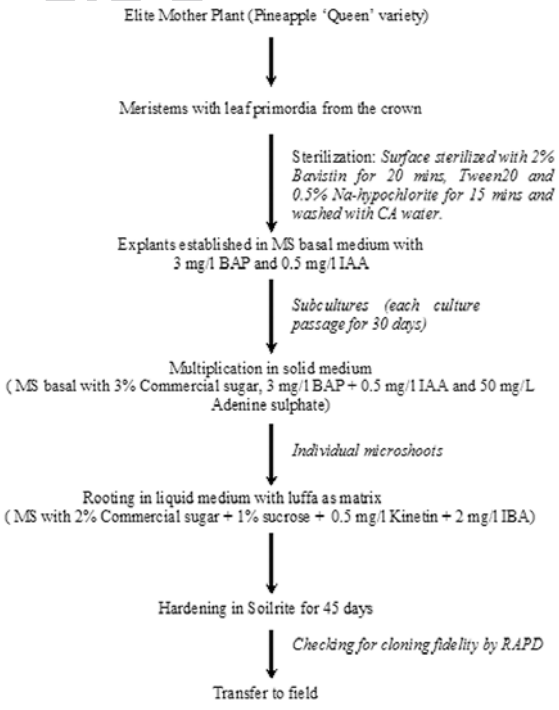


Fig. 2. RAPD profiles of randomly selected *in vitro* raised plantlets (a-j) using OPB-4 primer (Operon Tech., Alameda, USA). M: DNA molecular weight marker x, 0.07-12.2 kbp, Bochringer Mannheim, GmbH,Germany.

CONCLUSION

Conventionally agar and sucrose is used in multiplication and rooting stages. The result of the present investigation clearly indicates that 100% sucrose supplementation during proliferation reduces the chemical cost by 15.12% without causing any significant difference among the number of multiple shoots. During rooting no significant difference was observed up to 66% sucrose supplementation, and liquid media can be used with Luffa as supporting matrix, which reduced the chemical cost by 61.92%. Thus present experiment ensure almost 42.5% of total chemical cost reduction and an efficient micropropagation protocol (Scheme: 1) of pineapple for ready and commercial use.



Scheme 1: Presentation of the established cost-effective protocol of pineapple micropropagation.

ACKNOWLEDGEMENTS

We thank Prof. B. C. Ghosh, IIT Kharagpur for providing the plant material for our project.

Literature Cited

Aitken-Christie, J., Kozai, T. and Takayama, S. 1995. Automation in plant tissue cultures-general introduction and overview. p. 1-18. In :Aitken –Christie J., T. Kozai, M.A.L. Smith (eds) Automation and environmental control in plant tissue culture. Kluwer Academy Publication, Dordreeht.

Bhojwani, S.S. and Razdan, M.K. 2005. Plant tissue culture: Theory and practice, a revised Edition, Reed Elsevier India Pvt. Ltd.

Datta, A.K. 2006. Basic biostatistics and its application, New Central Book Agency (P) Ltd.

Economic Research Service, United State Department of Agriculture. 2012. www.ers.usda.gov/ Briefing/ FruitAndTreeNuts/ fruitnutpdf/ pineapple.pdf

Escalona, M., Lorenzo, J.C., Gonzalez, B., Daquinta, M., Gonzalez, J.L., Desjardins, Y., and Borroto, C.G. 1999. Pineapple micropropagation in temporary immersion system. Plant Cell Reproduction. 18: 743-748.

Firoozabady, E. and Gutterson, N. 2003. Cost effective *in vitro* propagation method for pineapple. Plant Cell Reproduction. 21: 844-850.

Fitchet, M. 1990. Clonal propagation of Queen and Smooth Cayenne pineapple. Acta Horticulturae. 275:261-266.

Fitchet-Purnell, M. 1993. Maximum utilization of pineapple crowns for micropropagation. Acta Horticulturae. 334: 325-330.

Gangopadhyay, G., Bandyopadhyay, T., Basu Gangopadhyay, S. and Mukherjee, K.K. 2004. Luffa sponge-a unique matrix for tissue culture of philodendron. Current Science. 86: 315-319.

Gangopadhyay, G., Bandyopadhyay, T., Poddar, R., Basu Gangopadhyay, S. and Mukherjee, K.K. 2005. Encapsulation of pineapple microshoots in alginate beads for temporary storage. Current Science. 88: 972-977.

Gangopadhyay, G., Das, S., Mitra, S.K., Poddar, R., Modak, B.K. and Mukherjee, K.K. 2002. Enhanced rate of multiplication and rooting through the use of coir in aseptic culture media. Plant Cell, Tissue and Organ Culture. 68: 301-310.

Kiss, E., Kiss, J., Gyulai, G. and Heszky, L.E. 1995. A novel method for rapid micropropagation of pineapple. Horticulture Science. 30: 127-129.

Mathews, H.V. and Rangan, T.S. 1979. Multiple plantlets in lateral bud and leaf explant *in vitro* cultures of pineapple. Scientia Horticlturae. 11: 319-328.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiology Plant. 15: 473-497.

Rogers, S.O. and Bendich, A.J. 1998. Plant molecular biology manual (eds Gelvin, S.P. and Schilperoort, R.A.) Kluyyer Publishers, Dordrecht, The Netherlands.

Roy, S.K., Gangopadhyay, G., Bandyopadhyay, T., Modok, B.K., Datta, S. and Mukherjee, K.K. 2006. Enhancement of *in vitro* microcorm production in Gladiolus using alternative matrix. African Journal of Biotechnology. 12: 1204 -1209.

Smith, M.A.L. and Spoomar, L.A. 1995. Vessels, gels, liquid media and support systems. P. 371-405. In: Aitken–Christie J, T. Kozai, M.A.L. Smith (eds) Automation and environmental control in plant tissue culture. Kluwer Academy Publication, Dordreeht.

Williams, J.G.K., Kubelik, A.R, Livak, K.J., Rafalski, J.A., and Tingey, S.V. 1990. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research. 18:6531 -6535.

Zepeda, C. and Sagawa, Y. 1981. *In vitro* propagation of Pineapple. Horticulture Science. 16:495.

United State Department of Agriculture. 2013. www.uga.edu/fruit/pinapple.htm.