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Large Scale Plant Regeneration *in vitro* from Leaf Derived Callus Cultures of Pineapple [*Ananas comosus* (L.) Merr. cv. Giant Kew]

M.N. Amin, M.M. Rahman, K.W. Rahman, R. Ahmed, M.S. Hossain and M.B. Ahmed
Department of Botany, University of Rajshahi, Rajshahi-6205, Bangladesh

Abstract: The leaf base explants from the *in vitro* established shoot cultures were induced to form callus and subsequently to differentiate into shoots on MS medium supplemented with different concentrations and combinations of cytokinins and auxins. The cultured explants produced calli from their cut margins within four weeks of incubation on media supplemented with 0.5-3.0 mg L^{-1} 2,4-D alone and in combination with 0.5-3.0 mg L^{-1} BA. Maximum number of shoot buds with optimum callus growth was observed on MS medium containing 1.0 mg L^{-1} BA and 0.1 mg L^{-1} NAA after six weeks of culture. Rooting was induced in the *in vitro* regenerated shoots on ½ MS medium with different concentrations and combinations of NAA, IBA or IAA. Rooting performance was best when the microshoots were rooted on ½ MS medium containing 0.2 mg L^{-1} IBA + 0.2 mg L^{-1} NAA. The regenerated plantlets were successfully transferred to soil and percentage of their survivability under ex vitro condition was almost ninety.

Key words: *In vitro*, plant regeneration, callus culture, pineapple

INTRODUCTION

The pineapple, *Ananas comosus* (L.) Merr. is a tufted stiff herb with reduced stem and warty skinned sweet fruits. It is an important fruit of Bangladesh belonging to the family Bromoliaceae. It is widely cultivated throughout the Bangladesh for its delicious juicy fruit, particularly in the hilly areas[1]. It is becoming an important item for the fruit processing and canning industries. Its fresh market value is high because it is a cheap source of the vitamins A, B, C, calcium, iron and enzyme bromelin.

Pineapple is a vegetatively propagated crop and cultivated in the tropic using suckers and crown tips as saplings. But the average rate of the production of suckers is very low. It takes several years to produce enough planting material for one hectare of land starting with a single plant. Hence, there is a shortage of planting material during planting time, specially for newly introduced cultivars. Moreover, the produced suckers are not uniform in size and age, resulting in erratic flowering and fruiting and bear many viral diseases from their mother plants. Tissue culture approach is an efficient method for rapid *in vitro* clonal propagation of pineapple, which was suggested long before by Mapes [2], Jones and Murashige[3] and Hosoki and Asagira[4]. It is also possible to produce disease free, uniform propagules at any time of the year by this method. Mathews[5] Mathews and Rangan[6], Zepeda and Sangawa[7], Aghion and Beauchesne[8] and Lakshmi *et al.*[9] have reported multiple shoot formation in lateral bud cultures of pineapple. Khatun *et al.*[10] and Rahman *et al.*[11] have also reported the micropropagation from crown tip explants of pineapple cultivars grown in Bangladesh.

However, callus culture technique has advantages over lateral bud and crown tip explant culture for producing plantlets in large number and generating variation using somaclones[12,13]. The present research was conducted to establish a suitable *in vitro* plant regeneration protocol for pineapple cv. Giant Kew through callus culture from leaf base explant.

MATERIALS AND METHODS

The creamy-white leaf base segments were excised aseptically from *in vitro* grown cultures, originally established from the crown tip explants of field grown mature plant (*cf.*) [11]. Excised leaves were cut into pieces (1-1.5 mm) from basal site and placed on the medium. The explants were initially cultured on MS medium[14] supplemented with various concentrations of either 2, 4-D alone or in combination with BA for callus induction. Four-week old calli were subcultured on MS medium containing different combinations of BA with NAA, IBA or IAA for shoot differentiation. The calli were subcultured at monthly intervals on same medium for growth, elongation and proliferation of shoots rapidly.

Corresponding Author: M.N. Amin, Professor, Department of Botany, University of Rajshahi, Rajshahi-6205, Bangladesh

Table 1: Effects of different concentrations of 2.4-D alone and in combination with BA for callus induction from leaf base explant. There were 20 explants in each treatment and data $\bar{x} \pm SE$ were collected after 4 weeks of culture

Growth regulators (mg L^{-1})	% of explants induced callus	Callus colour	Degree of callus formation
2,4-D			
0.5	45	Cr	+
1.0	50	Cr	+
1.5	55	Cr	+
2.0	70	Crw	+++
2.5	60	Crw	++
3.0	30	Cr	+
2,4-D+BA			
0.5+0.5	35	Cr	+
0.5+1.0	50	Cr	++
0.5+2.0	40	Cr	++
0.5+3.0	30	Cr	+
1.0+0.5	50	Cr	+
1.0+1.0	60	Crw	++
1.0+2.0	45	Cr	+
1.0+3.0	40	Cr	+
2.0±0.5	60	Crw	+++
2.0±1.0	80	Crw	+++
2.0±2.0	95	Crw	+++
2.0±3.0	55	Cr	+
3.0+0.5	45	Cr	+
3.0+1.0	50	Crw	++
3.0+2.0	45	Cr	+
3.0+3.0	30	Cr	+

(+) Slight callusing; (++) Considerable callusing and (+++) Profuse callusing Cr = Creamy; Crw = Creamy-white

Table 2: Effects of different concentrations and combinations of BA with auxins for shoots regeneration on MS medium from leaf base derived callus. There were 20 cultures in each treatment and data $\bar{x} \pm SE$ were collected after 6 week of culture

Growth regulators(mg L^{-1})	% of culture developed shoots	No. of shoots per culture	Length of the useable shoot(cm)
BA+NAA			
0.5+0.1	50	5.20±0.11	4.33±0.11
0.5+0.5	40	4.86±0.19	4.10±0.19
0.5+1.0	35	4.35±0.23	3.80±0.23
1.0+0.1	100	18.50±0.20	5.28±0.20
1.0+0.5	80	12.70±0.19	4.85±0.19
1.0+1.0	50	8.45±1.14	4.26±1.14
2.0+0.1	75	6.51±0.21	4.65±0.21
2.0+0.5	65	5.40±0.13	4.25±0.13
2.0+1.0	50	5.00±0.05	4.10±0.05
BA+IBA			
0.5+0.1	45	4.95±0.11	3.50±0.11
0.5+0.5	35	3.54±0.19	3.25±0.19
0.5+1.0	30	3.25±0.23	2.95±0.23
1.0+0.1	70	7.50±0.20	4.32±0.20
1.0+0.5	55	6.45±0.19	4.00±0.19
1.0+1.0	40	4.55±1.14	3.95±1.14
2.0+0.1	50	5.25±0.21	4.10±0.21
2.0+0.5	40	4.28±0.13	3.55±0.13
2.0+1.0	30	3.62±0.05	3.00±0.05
BA+IAA			
0.5+0.1	-	-	-
0.5+0.5	-	-	-
0.5+1.0	30	2.25±0.23	2.25±0.23
1.0+0.1	40	4.10±0.20	3.50±0.20
1.0+0.5	50	4.35±0.19	3.65±0.19
1.0+1.0	30	3.75±1.14	3.25±1.14
2.0+0.1	30	3.10±0.21	2.56±0.21
2.0+0.5	40	3.50±0.13	3.10±0.13
2.0+1.0	-	-	-

(-)Indicate no response

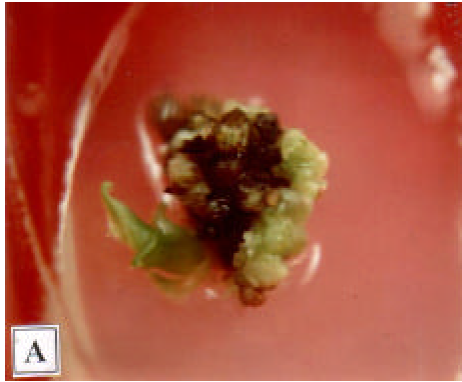
The shoots having more than 4 cm length (from base to leaf) were harvested from the proliferating cultures and cultured individually for rooting on ½ MS (half strength of both major and minor salts) medium supplemented with NAA, IBA, IAA.

The pH value of all media was adjusted to 5.7±0.1 before addition of agar and sterilized by autoclaving for 20 minutes at 1.1 Kg/cm^{-2} pressure at 121°C. For solidifying the medium, 7-8 gm L^{-1} agar was used as gelling agent. The tubes or flasks containing explants and proliferating cultures were incubated on culture racks in the growth chamber. The cultures were maintained at 25±2°C under the cool white fluorescent lights for 16 h photoperiod with a photon flux density of about 60 μ mol.m^{-2} s^{-1}.

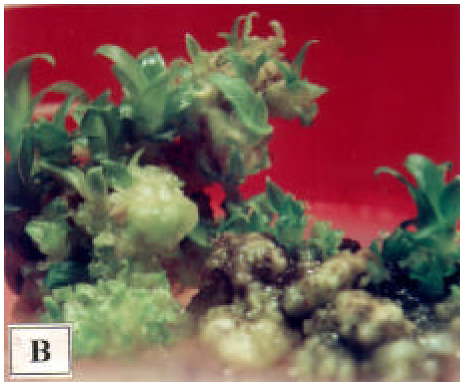
RESULTS AND DISCUSSION

Callus induction from the prepared leaf base segments was done on MS medium with various levels of 2,4-D alone and in combination with BA with a view to finding out optimum growth regulator concentration and combination. After 4 weeks of incubation the cultured explants produced creamy-white callus mass on medium having 2.0 mg L^{-1} 2,4-D alone, but these calli had slight regenerative potentialities. An increasing trend of callus development was noticed as the concentration of 2,4-D was increased in the basal medium from 0.5-2.0 mg L^{-1}. The highest concentration (2.0 mg L^{-1}) of 2,4-D produced the highest percentage (70%) of callus formation. These findings corroborated with that of Burba *et al.*[15] who reported callus formation in sugarcane when MS basal medium was supplemented with 2,4-D ranging from 0.5-3.0 mg L^{-1}. Callus inducing potentialities of the explant was found to differ depending upon the growth regulator supplements (Table 1). Among different combinations of auxins and cytokinins tried, 2,4-D with BA was found to be most effective for induction of callus. The callus thus produced was semihard in texture, faster in growth and creamy-white in colour. The highest frequency of shoot regenerable (caulogenic) callus induction (95%) was recorded at the end of 4 weeks on medium containing 2.0 mg L^{-1} 2,4-D with 2.0 mg L^{-1} BA (Fig. 1A). Similar combination of auxin with cytokinin for callus induction was reported in the past by Malamug *et al.*[16] in case of ginger and Vincent *et al.*[17] in case of *Kaempferia galanga.*

Different concentrations and combinations of cytokinin (BA) with auxins (NAA, IBA and IAA) were used for shoot differentiation from the regenerable calli (Table 2). It was noticed during the present investigation that shoot differentiation was highly influenced by

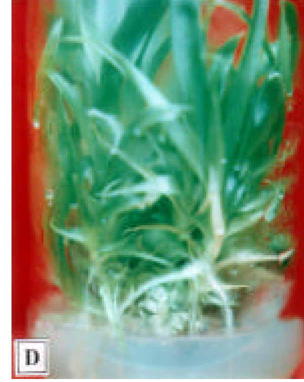


A. Callus induction from leaf explant on MS medium supplemented with 2.0 mg L^{-1} BA+2.0 mg L^{-1} 2,4-D after four weeks in culture



B. Differentiation of shoot bud primordia from callus on MS+1.0 mg L^{-1} BA+0.1 mg L^{-1} NAA medium after four weeks in subculture

C. Development and elongation of shoots on shoot regenerated medium after six weeks in culture



D. A cluster of Developed shoots on shoot regenerated medium after ten weeks in culture

E. Development of roots from the base of microshoot on ½ MS fortified with 0.2 mg L^{-1} IBA+0.2 mg L^{-1} NAA after six weeks in culture

F. Regenerated plantlet on the soil after two weeks of transfer under *in vitro* condition

Fig.1: A-F. Callus induction and shoot differentiation from callus in pineapple

Table 3: Effect of different concentrations and combinations of auxins on adventitious root formation from the *in vitro* grown microshoots cultured on ½ MS medium. There were 15-20 microshoots in each treatment. Data ($\bar{x} \pm SE$) were recorded after 6 weeks of culture

Different concentrations of auxins (mg L^{-1})	% of micros hoots rooted	Number of roots per micros hoots	Average length of the root (cm)
IBA			
0.1	40	3.85±0.12	2.95±0.21
0.2	45	4.12±0.21	3.00±0.46
0.5	50	4.26±0.15	3.24±0.18
NAA			
0.1	35	3.19±0.21	2.45±0.24
0.2	40	3.54±0.23	2.55±0.14
0.5	50	3.85±0.18	3.10±0.23
IAA			
0.1	-	-	-
0.2	25	2.40±0.26	2.30±0.16
0.5	30	2.54±0.21	2.40±0.13
IBA+NAA			
0.1+0.1	40	4.10±0.25	2.85±0.18
0.1+0.2	60	4.85±0.23	3.10±0.12
0.1+0.5	45	4.25±0.26	3.00±0.23
0.2+0.1	80	5.45±0.20	3.40±0.21
0.2+0.2	100	6.50±0.22	3.45±0.20
0.2+0.5	75	5.00±0.21	3.25±0.15

(-) Indicate no response

concentration and type of the growth regulators used in the caulogenic medium. Among the concentrations and combinations of BA with auxins, which were used for shoot regeneration, the best performance was showed in MS medium supplemented with 1.0 mg L^{-1} BA+ 0.1 mg L^{-1} NAA (Fig. 1B and C). On this combination, the percentage of explant produced shoots were 100 while the number of shoots and average length of the shoots were 18.50±0.20 and 5.28±0.20 cm per culture, respectively (Fig. 1D). Sinha and Roy[18] reported the positive effects of BA and NAA combination on shoot formation in gladiolus primulinus. A high level of cytokinin (1.0 mg L^{-1}) and low level of auxin (0.1 mg L^{-1}) combination seems to play an important role for differentiation of adventitious shoot, as suggested by Islam *et al.*[19] and Karim *et al.*[20]. Canlas and Javier[21] reported that the axillary bud explants (aerial suckers) from the pineapple cultivar 'Queen' produced callus on MS medium containing cytokinin with auxin in combination while shoots regenerated from the callus when they were transferred on MS medium without plant growth regulator.

Three auxins were used at different concentrations and combinations to induce and differentiate adventitious roots. Among the three auxins, IBA and NAA were found to show comparatively better response than IAA for production of roots (Table 3). Reasonably better rooting was observed on ½ MS medium having combined auxins than single auxin. The best rooting was observed in ½ MS medium supplemented with 0.2 mg L^{-1} IBA + 0.2 mg L^{-1} NAA where 100 percent of microshoots produced root and number of roots per microshoots was 6.50±0.22 (Fig. 1E). Unlike the present findings, Cabral *et al.*[22] reported that the regenerated microshoots of pineapple produced roots on MS medium supplemented with different types of auxins and a cytokinin, BA. However, there are some other reports on root induction of pineapple microshoots on half strength of MS medium supplemented with different auxins viz. IBA and NAA[23,10].

The complete plantlets thus produced from the *in vitro* regenerated microshoots were transferred to soil. On an average 90% survival of the regenerated plantlets of pineapple was achieved in the present study (Fig. 1F).

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