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Callus Induction and High-frequency Plant Regeneration of Pineapple (*Ananas comosus* (L.) Merr.)

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Abstract

Meristem tips of the crown of *Ananas comosus,* cultured on MS supplemented with 1.5 mg/l NAA and 1.0 mg/l Kn, callused within three weeks. This callus, when subcultured on MS with 1.5 mg/l Kn + 0.5 mg/l NAA produced a large number of shoots. After four weeks, shoots were excised and implanted in shoot elongation medium consisting of MS basal salts fortified with 15% coconut water only. After harvesting shoots, the old callus was transferred to the fresh medium of the same constituents, where the old callus expanded and many new buds also emerged. Shoots rooted well within two weeks, when they were excised individually and implanted in half strength MS with 2.0 mg/l IBA. Eighty per cent plantlets survived when transferred to open field. Regenerated plants were morphologically uniform with normal leaf shape and growth pattern.

Introduction

In vitro culture techniques constitute an important component of biotechnology and have the potential not only to improve the existing cultivars, but also for the generation of novel plants in a comparatively short time compared to conventional breeding.

Fruit is the oldest human food and its history of cultivation goes back to the earliest known human civilization (Singh 1985). It has already been proved that *in vitro* cloning is efficient and a reliable horticultural technique for mass propagation of a number of fruits. Numerous tropical and subtropical fruit plants have been propagated successfully *in vitro*, but actual commercial production is probably still limited to only a few of them.

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Pineapple (*Ananas comosuss*) is one of the most popular and delicious triploid fruit and is esteemed for its pronounced flavor and nutritive elements. The fresh fruit is a good source of vitamins A and B and is rich in vitamin C. It also contains some minerals, such as iron and phosphorus. It has low sugar and fat contents. Pineapple is propagated vegetatively through suckers, arising from buds below the ground level, slips, which are borne on the peduncle, just below or at the base of the fruit or crowns on top of the fruit.

Direct micropropagation system of pineapple through enhanced axillary bud development and organogenesis from different explants were reported by many authors (Lakshmi Sita et al. 1974, Mathews and Rangan 1979, Zepeda and Sangawa 1981, Carbral et al. 1984, De Wald 1988, Cote et al. 1991, Daquinta et al. 1994, Benega et al. 1995, Khatun 1997, Rahman et al. 2001). Roy et al. (2000) described a protocol for large-scale propagation of pineapple on a commercial basis, directly from dormant axillary buds excised from the crown. There is hardly any report on regeneration of pineapple from callus culture (Canals and Javier 1994) and as such the present investigation was undertaken to develop a protocol for regeneration of pineapple from callus initiated from explants. Plantlet production can be scaled up manyfold through the intervening callus phase, compared to direct regeneration (Sha Valli Khan et al. 2002). This process also causes somaclonal variation, presenting an opportunity to select plants with desirable characters (Larkin and Scowcroft 1981, Cuenca et al. 2000).

Materials and Methods

Explants were taken from the crowns of *Ananas comosus* (L.) Merr. (Bromeliaceae) cv. Madhupur. They were collected in April - May from Madhupur pineapple fields of Tangail district in Bangladesh. Explants were prepared for further experiments according to the procedure described by Roy et al. (2000). After surface sterilization apices were excised at the base and divided into pieces of explants each containing 2 - 3 nodes.

The basal medium used for all experiments was MS mineral formulation containing standard salts and vitamins, 30 g/l sucrose and 8 g/l agar (BDH, England). The media were variously supplemented with 2,4-D, BA, Kn alone or in combinations with NAA or IBA and coconut water (5 - 15%). The pH was adjusted to 5.8 ± 0.05 before adding agar, and the media were autoclaved at 1.1 kg cm^{-2} for 20 min at 120_C.

Cultures were incubated at 25 ± 1_C with a photoperiod of 16 h/day at 30 µmol s^{-1},m^{-2} light intensity by cool white fluorescent light. Cultures were initiated in 150 ∇ 25 mm glass tubes containing 12.5 ml of medium. The cultures

were regularly subcultured at four weeks intervals on fresh medium in 100 ml conical flasks or juice bottles containing 40 ml of medium. Observations were recorded every five days following implanting and subculturing. All experiments were repeated twice with at least 12 cultures per treatment.

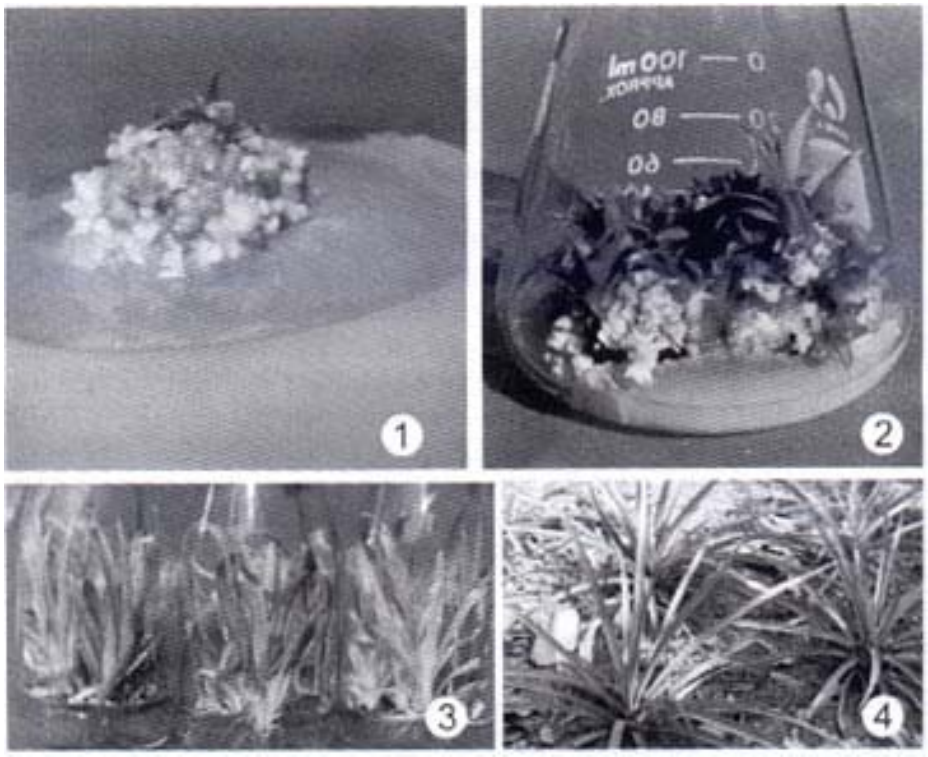
One g (approx.) of each sample (fresh weight) of callus was placed on shoot induction medium. Each treatment consisted of five callus samples. The medium for shoot induction was MS supplemented with Kn or BA alone or in combination with NAA. The regenerated shoots were separated from the base and subcultured for elongation in MS basal medium without growth regulators and organic supplements or only with coconut milk (CM) (5 - 15%). Multiplication was repeated at regular intervals. The optimum hormone levels and other supplements in the culture medium essential to maximize production of uniform plantlets were selected. Shoots about 3 - 6 cm in length obtained from the elongation media were separated aseptically from the culture vessels and transferred to rooting media, containing different combinations of auxins. Following generation of sufficient root systems the regenerated plantlets were considered ready for transplantation. For hardening, the vessels containing rooted shoots were kept at normal room temperature and light for 14 days. Thereafter, the rooted shoots were removed from the culture, washed thoroughly to remove remnants of agar from roots and transplanted to small pots containing garden soil, compost and sand (1 : 1: 1 v/v). Plants were well covered with polythene sheet for three weeks to ensure high humidity while irrigating regularly. After three months the plantlets were transferred to open field.

Results and Discussion

Cut ends of *in vitro* cultured explants callused approximately three weeks after the explants were placed on callus induction medium. The explants callused on MS supplemented with either 1.5 mg/l NAA + 1.0 mg/l Kn or 2.0 mg/l 2,4-D alone or 1.5 mg/l 2,4-D + 1.0 mg/l NAA. The callus, initiated on media supplemented with the above hormonal combinations, was compact. The proliferated calli were subcultured (Fig. 1). Callus was maintained on callus inducing medium and aliquots of approximately one g (fresh weight) were taken and cultured on the regeneration medium.

For shoot induction one g (fresh weight) of callus, maintained in callus initiating medium, was cultured on the regeneration medium. The results show (Table 1) that the highest number of shoots (Fig. 2) was found to regenerate in the medium fortified with 1.5 mg/l Kn + 0.5 mg/l NAA. BA and Kn alone. BA-NAA combination was not so efficient to initiate shoot regeneration from callus

as Kn-NAA combination. The percentage of calli forming shoots, and the number of regenerated shoots per callus varied according to the growth regulator treatment (Table 1). MS containing Kn alone resulted in shoot differentiation, more efficiently than that observed in BA, but at a lower frequency compared to NAA supplemented media. Similarly, the need to adjust auxin and cytokininlevels in the media to maximize the production of



Figs. **1 - 4** : Plant regeneration from callus culture of pineapple. **1**. Two-month-old callus initiated from the cut ends of shoot/apices of crown cultured on MS with 1.5 mg/l NAA + 1.0 mg/l Kn. **2**. Shoot regeneration from callus cultured on MS with 1.5 mg/l Kn + 0.5 mg/l NAA. **3**. Rooted shoots in half strength MS with 2.0 mg/l IBA. **4**. One-year-old regenerated plants established in the field.

adventitious shoots has been well documented for *Carica papaya* (Mondal et al. 1994), *Hybanthus ennea-spermus* (Prakash et al. 1999), *Salvia selarea* (Liu et al. 2000) and *Bixa orellana* (Sha Villa Khan et al. 2002). After excision of shoots, callus was recultured for four weeks on MS fortified with 1.5 mg/l Kn + 0.5 mg/l NAA to harvest shoots for the second time. The differentiated shoots were excised for shoot elongation and the calli were repeatedly subcultured at four-week intervals to increase the stock propagules. The shoot

differentiation potential of callus remained unchanged even after 12 subcultures. The sustained production of shoots from callus by repeated subcultures was also reported in *Sapium sebiferum* (Sirial and Dhar 1996) and *Bixa orellana* (Sha Villa Khan et al. 2002). For elongation the shoots were excised and cultured separately. The 15% CM supplemented media supported shoot elongation more efficiently than other supplements.

Table 1. Effect of cytokinin-auxin on shoot regenerating ability of the callus of *Ananas comosus* cv. Madhupur. Data were taken two months after inoculation.

Growth regulator (mg/l)	Culture with regenerated shoot (%)	No. of shoot/ culture
Kn		
0.5	15 (2.4)	4 (1.2)
1.0	20 (3.1)	4 (1.6)
1.5	30 (5.6)	6 (1.9)
2.0	18 (3.9)	4 (0.9)
2.5	10 (1.3)	3 (0.8)
Kn + NAA		
1.0 + 0.5	75 (8.6)	15 (3.4)
1.5 + 0.5	100	35 (5.7)
1.5 + 1.0	78 (6.9)	16 (3.2)
2.0 + 1.0	35 (4.7)	12 (3.1)
BA		
0.5	00	00
1.0	00	00
1.5	12 (1.7)	3 (0.7)
2.0	10 (0.8)	3 (1.5)
2.5	00	00
BA + NAA		
1.0 + 0.5	34 (4.6)	5 (1.3)
1.5 + 0.5	68 (8.6)	8 (1.8)
1.5 + 1.0	45 (6.5)	8 (2.1)
2.0 + 1.0	25 (3.8)	7 (2.3)

Standard error in parenthesis.

For rooting on excised shoots, either single or a combination of two or three auxins are used routinely. In the present experiment 2.0 mg/l IBA was found to be the best for root induction (Table 2, Fig. 3). Root formation was not observed when shoots were cultured on a medium lacking auxin. In contrast, root formation was observed when the regenerated shoots were cultured on half strength MS supplemented with auxin (IBA and/or NAA). The rooting response varied with the type and concentration of auxin (Table 2). Plants that were transferred directly to the field did not survive. After three weeks of

hardening, about 85% of plantlets survived in pots. All hardened plants were successfully established in field, where the plants appeared morphologically uniform with normal leaf form, shape and growth pattern (Fig. 4).

Table 2. Effect of auxins in half strength MS medium on root formation in regenerated shoots of *Ananas comosus*. Data were recorded 35 days after culture.

Growth regulators (mg/l)	Rooted shoots (%)	Days required for root induction
IBA		
1.0	0	0
1.5	42.5 (3.2)	26 (1.8)
2.0	100	12 (0.9)
2.5	73.5 (5.2)	20 (1.3)
NAA		
1.0	0	0
1.5	0	0
2.0	0	0
2.5	0	0
IBA + NAA		
2.0 + 1.0	52.5 (5.2)	17 (1.3)
2.0 + 1.5	59.3 (6.1)	16 (1.0)
2.0 + 2.0	41.7 (4.2)	19 (1.2)
2.0 + 2.5	42.1 (3.9)	18 (1.5)

Standard errors in parenthesis.

The technique described here appears to be a promising method of propagation of pineapple. As the potentiality of shoot multiplication from callus continued for a long time, regenerants may be characterized by somaclonal variation. Several species of *Dubosia* (Lin and Griffin 1992), *Cuphea* (Millam et al. 1997), *Amaranthus* (Bennici et al. 1997) and *Salvia* (Liu et al. 2000) produced regenerants through callus-mediated adventitious shoot differentiation. Such regenerants may prove to be a potential source of somaclonal variants, giving birth to traits of agronomic importance. The regenerated plants of pineapple are currently being screened for agronomically useful genetic variants.

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