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Adventitious Shoot Regeneration from Immature Embryo Explant Obtained from Female × Female *Momordica dioica*

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Abstract

Higher percentage of adventitious shoots regenerated from the immature embryo explant than that of immature cotyledon of the crosses made between female and female (pollen was collected from induced bisexual flower) of tetraploid kakrol (*Momordica dioica*) on MS induction medium supplemented with different concentrations and combinations of BAP, NAA and GA_3. The best response of shoot proliferation was obtained in embryo explants grown in supplemented with 10.8 mg/l BA, 1.08 mg/l NAA and 0.54 mg/l GA_3; whereas, shoot regeneration on cotyledon was achieved on a MS supplemented with 16.2 mg/l BA, 2.7 mg/l NAA and 0.54 mg/l GA_3. In both cases shoot primordia emerged continuously. This system exhibited a potential for repeated harvesting of the shoots from the original explant as the latter continued to expand and regenerate new shoots, upon repeated periodical subculturing on to fresh medium. Regenerated shoots were excised from both sources and rooted in MS supplemented with different concentrations of IAA. There was no significant difference of root proliferation rate than their sources. Rooted plantlets were acclimatized successfully and later established in the field for the production of female plant and also evaluation of somaclonal variation.

Introduction

Teasle gourd (*Momordica dioica*, vernacular: kakrol) is a cucurbitaceous popular summer vegetable in Bangladesh and its neighboring countries. The fruit contains a high amount of vitamin C (Bhuiya et al. 1977). Slices of unripe fruits are served in different types of curries and fried forms. The young twigs and leaves of this crop are also used as vegetable (Fakir et al. 1992). Its popularity in domestic as well as export market has increased sharply during the last decade. As a result commercial cultivation of kakrol has also been expanded. Improvement of this vegetable crop has not been attempted, perhaps because of its dioecious nature and its vegetative mode of propagation (Ali et al. 1991). Normal

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germination of its seeds is difficult (Rashid 1976) and time consuming, moreover it is not possible to predict the sex of the plants produced from seeds. For the commercial cultivation, male and female plants are grown together at a ratio of 1 male : 15 female (Rashid 1976). Conventional techniques of reproduction are often tedious and impractical on a large scale. From the seeds of female × female are the successful way to get a large number of female plants, but the normal rate of seed germination is very low.

Successful application of plant biotechnology for plant improvement or large scale propagation requires the development of an efficient shoot regeneration system from cultured cells or tissues. The application of tissue culture method for large scale propagation of cucurbitaceous vegetable taxa has been well demonstrated (Moreno and Roig 1990, Dong and Jia 1991, Debeaujon and Branchard 1992, Misra and Bhatnagar 1995, Islam et al. 1999). Shoot regeneration only from specific cotyledonary parts has been described in our previous paper (Hoque et al. 1995, Hoque et al. 2000). However, no shoot regeneration from either tetraploid kakrol embryo explants or crosses between female and female of kakrol has been reported until now.

The present paper describes a rapid, simple and comparatively efficient shoot regeneration system from immature embryo- and cotyledon explants from the seeds of female × female of kakrol, thus establishing a technique that can be commercially exploited for mass production of female kakrol plants. Somaclonal variants are likely to arise from a large micropropagated population providing material to breeders for further improvement of this popular vegetable.

Materials and Methods

Seeds from unripe fruits (18 days after anthesis) of the cross between female × female tetraploid kakrol [*Momordica dioica* (Roxb.) Willd.] were collected from the experimental field (Hoque et al. 2001a).

Seeds were washed with distilled water (200 ml) containing three drops of Tween 80 for five min to remove the outer mucilaginous sheath. Tween 80 was removed by washing five - six times with distilled water. Surface sterilization of seeds was carried out with 0.1% $HgCl_2$ (w/v) for ten min and subsequently the seeds were rinsed four times with sterile distilled water. The seed coat was removed and explants (6) consisting of embryo and fully grown cotyledons were cultured in Petri dishes on to a solid (30 ml, agar 0.6%) MS containing sucrose 3%, and without growth regulators led to the swelling of the explants, facilitated dissection of the embryos on the next day. After swelling the explants, embryo (2 mm) and cotyledon were dissected carefully and cultured separately on to the MS containing different concentrations and combinations of BA (5.4 - 16.2 mg/l), NAA (0.54 - 2.7 mg/l) and GA_3 (0.54 mg/l). For shoot multiplication, a part of

nodular callus (6 - 7 mm^3) which originated from both explants was cultured on to the selected regeneration media (30 ml) in conical flasks of 250 ml.

The pH of the medium was adjusted to 5.8 with 1N NaOH or 0.5N HCl solution prior to autoclaving. Initially, the medium (10 ml) was dispensed into glass tubes (2.5 × 15 cm), covered with non-absorbent cotton plugs, and steam sterilized by autoclaving the medium at 121˚C for 20 min at 1.0 kg cm^{-2} pressure. Cultures were maintained at 26 ± 1˚C under a 16-h photoperiod of 50 µmol $m^{-2}s^{-1}$ provided by white fluorescent tubes.

Rooting of *in vitro* derived shoots was accomplished on MS supplemented with IBA (0.54 - 21.6 mg/l). After root formation the cultures were maintained at room temperature (28 - 30˚C) for ten days. Thereafter, plantlets were removed carefully from the culture vials, washed thoroughly to remove all traces of the medium and planted in plastic pots containing a mixture of non-sterile soil and sand in the ratio of 2 : 1. Initially for four to five days, the pots were covered with polythene bags to maintain high humidity and after 30 days the potted plants were transferred to field.

Each experiment was randomized completely, with three replications per treatment and 18 explants per replication. Observations were recorded every week for shoot proliferation and rooting. The comparison of means was analyzed using DMRT (Duncan 1955) at $p < 0.05$.

Results and Discussion

Fully grown immature cotyledons and embryo axes were collected 18 days after anthesis and used for comparative study on the effect of different levels of BA, NAA and GA_3 supplements on adventitious shoot regeneration efficiency of seeds from female × female of kakrol. The results recorded in three - four weeks revealed that the frequency of adventitious shoot buds from immature embryo axes was greater in number than those obtained from immature cotyledonary explants (Table 1). Almost similar observations were also described in sainfoin (Özcan et al. 1996) and vetch (Sancak et al. 2000). On most immature embryo axes, early shoot development from pre-existing meristems inhibited callus formation. Initially most of the embryo explants developed shoots within five - seven days from the initiation of culture. After removal of these shoots, compact nodular morphogenic green callus developed after 12 - 15 days of culture. These nodular calli developed into adventitious shoot buds, subsequently giving rise to normal shoots within three weeks of culture (Fig. 1b). After seven days of culture, immature cotyledon explants became swollen and produced morphogenic callus at the wound site where the embryonic axis had been dissected out. Green adventitious shoot initials formed on the callus pieces

within 14 - 21 days subsequently developed into normal shoots four weeks after culture initiation (Fig. 1a).

Shoot regeneration from cotyledonary and embryo axes of many cucurbitaceous vegetable taxa and many other plants have been well demonstrated (Moreno and Roig 1990, Dong and Jia 1991, Özcan et al. 1996, Islam et al. 1999, Sancak et al. 2000, Hoque et al. 2000, Hoque et al. 2001b). The percentage of cotyledon and embryo axes producing shoots varied significantly depending on the concentration of growth regulators and summarized in Table 1.

Table 1 Shoot regeneration from immature embryo axes and cotyledons of kakrol after six weeks in culture on MS supplemented with various concentrations and combinations of BA, NAA and GA_3.[a]

Growth regulators (mg/l)			Embryos producing shoots (%)	Number of shoots per embryo[b]	% cotyledons producing shoots	Number of shoots per cotyledon[c]
BA	NAA	GA_3				
5.4	0.54	0	36.8 de	4.2 ± 0.6 def	28.4 de	3.3 ± 0.4 ef
10.8	0.54	0	42.6 de	5.7 ± 1.2 def	34.3 d	4.1 ± 0.9 ef
16.2	0.54	0	48.3 d	7.1 ± 1.3 g	39.2 d	6.4 ± 1.1 de
5.4	1.08	0	47.2 d	6.8 ± 0.9 g	34.6 d	3.7 ± 0.5 ef
10.8	1.08	0	52.3 c	7.3 ± 1.1 g	40.3 d	5.4 ± 0.8 def
16.2	1.08	0	55.6 c	9.4 ± 2.1 de	43.1 c	6.8 ± 1.3 de
5.4	2.7	0	41.4 de	7.5 ± 1.2 g	32.4 d	4.9 ± 1.1 def
10.8	2.7	0	56.8 c	9.7 ± 2.3 de	44.6 c	6.9 ± 1.2 de
16.2	2.7	0	66.3 b	12.1 ± 1.8 cd	48.3 b	9.4 ± 2.1 cd
5.4	1.08	0.54	53.7 c	17.3 ± 2.3 ab	39.3 d	13.2 ± 2.1 bc
10.8	1.08	0.54	76.4 a	22.4 ± 3.3 a	53.8 b	16.4 ± 2.4 a
16.2	1.08	0.54	72.3 a	19.5 ± 2.8 ab	51.4 b	15.2 ± 1.9 ab
5.4	2.7	0.54	49.6 d	13.2 ± 1.9 cd	41.3 c	11.5 ± 1.6 cd
10.8	2.7	0.54	57.1 c	16.3 ± 1.8 bc	49.6 b	15.4 ± 1.8 ab
16.2	2.7	0.54	68.2 b	18.2 ± 2.1 ab	61.9 a	17.3 ± 2.2 a

Each value is the mean of three replications each with 18 explants. [a]Values within a column followed by different letters are significantly different at the $p < 0.05$ level. [b]From immature embryo axes which regenerated shoots. [c]Regenerated shoots from immature cotyledons.

In both cases, shoot regeneration was influenced by BA, NAA and GA_3 levels. Considering both the percentage of shoot regeneration and the mean number of shoots per explant, the highest shoot regeneration ability was achieved on MS supplemented with 16.2 mg/l BA, 2.7 mg/l NAA and 0.54 mg/l GA_3 for cotyledonary explants; for embryo explants the maximum capacity was demonstrated with 10.8 mg/l BA, 1.08 mg/l NAA and 0.54 mg/l GA_3 (Fig. 1c). In general, rate of adventitious shoot regeneration was reduced in absence of GA_3 in the media.

The results obtained in the present work confirm that the embryo explants from crossed seeds, female × female kakrol flowers showed regeneration potential higher than cotyledon explants. In the present study, it was also found that adventitious shoot regeneration in cotyledons predominantly occurred at the wound site, resulting from the broken embryo axis. Similar results were also obtained from pea (Özcan et al. 1993), sainfoin (Özcan et al. 1996) and vetch (Sancak et al. 2000).

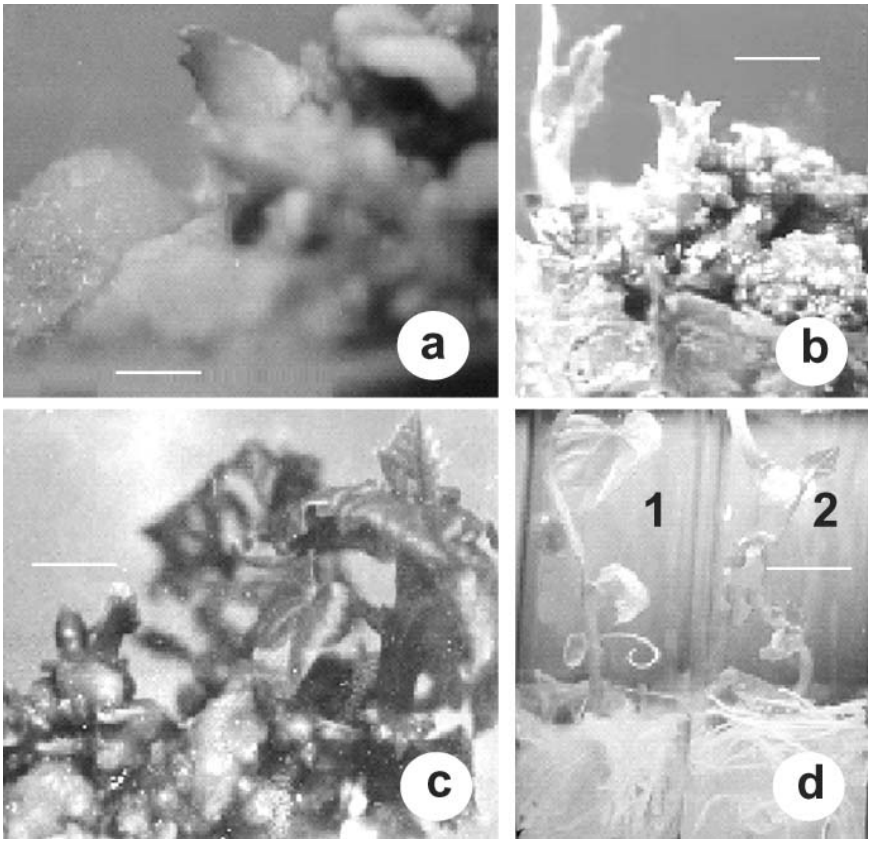
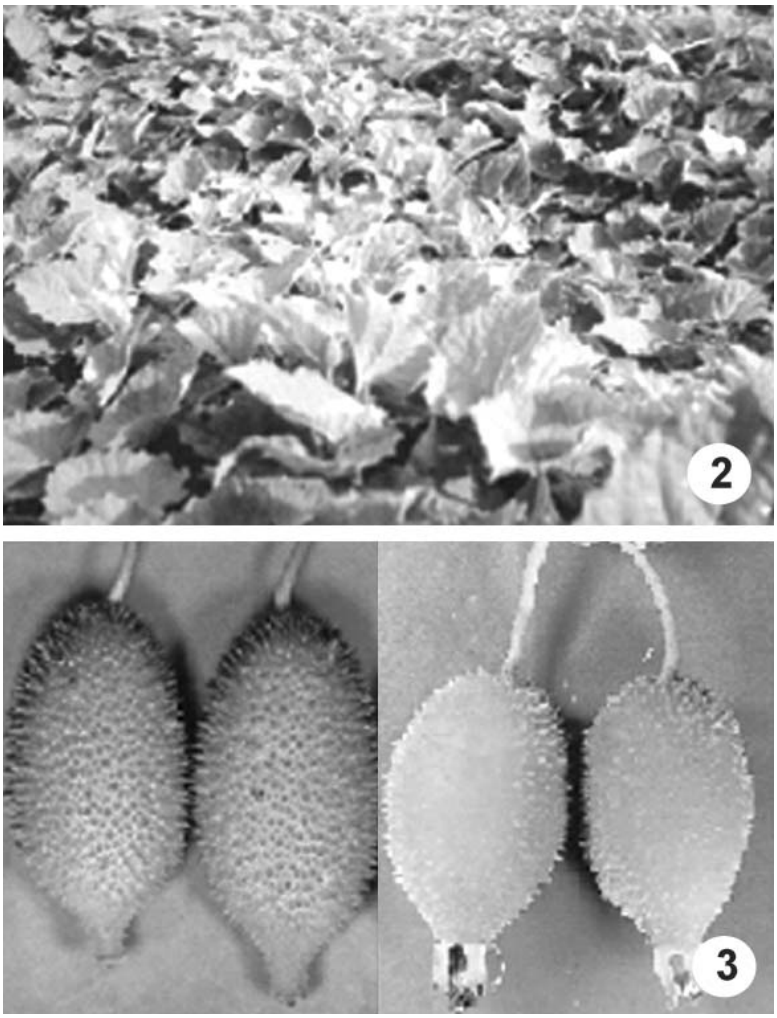


Fig. 1. Shoot regeneration from immature cotyledons and embryo axes of seeds from crosses between female and female of kakrol. (a) Adventitious shoot bud regeneration from cotyledon after four weeks in culture. (b) Adventitious shoot bud regenerated from nodular callus of embryo axes after four weeks in culture. (c) A part of nodular callus from an embryo explant producing multiple shoots after three weeks of subculture. (d) 1. Rooting with 10.8 mg/l IBA, 2. Rooting with 5.4 mg/l IBA, after two weeks in culture. Bar = 1 cm in a, b, c and d.

Regenerated shoots (3 - 4 cm in length) were excised and transferred to MS supplemented with different concentrations of IBA (Fig. $1d_2$). One hundred per cent of regenerated shoots rooted with 5.4 mg/l IBA within two weeks. In all cases branch roots were induced from the main root when growth regulator

concentrations were increased. After root formation the cultures were kept for ten days at room temperature (28 - 30˚C) for hardening. About 85% of these semi-hardened plantlets survived in non-sterile soil and sand mixture but direct transfer of plantlets reduced the survival rate to 60%. To maintain high humidity during the initial four to five days the pots were covered with polythene bags with perforations and after 30 days the plants were transferred to field, where they showed normal growth; and all regenerants were female (Fig. 2). Mature fruits are shown in Fig. 3



Figs. 2-3: 2. Regenerants grown in the field 60 days after transplantation. 3. Mature fruits.

In conclusion, to our knowledge this is the first report for *in vitro* adventitious shoot regeneration from embryo axes of seeds from female × female of tetraploid kakrol. Our method allows plant regeneration through adventitious

shoot bud formation in kakrol within four weeks, and the young plants can be transferred to soil seven - eight weeks thereafter. The protocol described here provides a rapid and prolific shoot regeneration system that has opened possibilities for commercial production of female kakrol plants and at the same time would provide somaclonal variants to plant breeders to come up with improved varieties through their selection.

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