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In vitro Regeneration of Sweetgourd (*Cucurbita moschata* Duch.)

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Abstract

Cotyledon explants of sweetgourd (*Cucurbita moschata* Duch.) were cultured on MS supplemented with different concentrations of BAP alone and in combination with NAA, TDZ and 2,4-D. High shooting frequency (94.4%) was obtained from BARI Mistikumra-1 when cotyledon segments were cultured on MS with 4.44 μM BAP alone. The highest number (8.9) of shoot development and minimum days (14.7) required for shoot induction were also observed in 4.44 μM BAP treatment for BARI Mistikumra-1. In contrast, 83.3% shooting frequency were observed from BARI Mistikumra-2 producing 11.9 shoots in 8.4 days on 8.88 μM BAP. The regenerated shoots of both varieties were rooted on half strength of MS and MS supplemented with 0.53 μM NAA and 2.46 μM IBA. Rooting was observed in BARI Mistikumra-1 with 92.6% frequency in 12.3 days whereas it took 8.6 days with 93.3% rooting frequency in BARI Mistikumra-2 when shoots were cultured on MS supplemented with 0.53 μM NAA. Well-developed rooted plantlets were transferred to pots containing sterile soil and covered with polythene bags in the greenhouse for hardening. The acclimated plants were planted in the field after three weeks.

Introduction

The *Cucurbitaceae* is a large family with a number of valuable edible vegetable crops in the tropics, subtropics and many other regions around the world. Among them sweetgourd (*Cucurbita moschata* Duch.) is the most accepted one for a number of reasons ranging from agricultural purposes to commercial and ornamental sales. Besides the edible fruits, immature fleshy green leaves, tender

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shoots and male flowers are used as vegetables and contain appreciable amounts of vitamins and minerals (Robinson and Walters 1997). Thus, it has the potentiality to contribute substantially in solving the malnutrition problems and thus, improvement of the sweetgourd crop and development of new varieties are necessary. However, there are many agricultural problems in the cultivation of sweetgourd for which conventional breeding often does not have ready answers and the current partial solution is the increasing use of agrochemicals against pests. The crop is affected by several viruses such as - Zucchini yellow mosaic virus (ZYMV), Watermelon mosaic virus (WMV), Papaya ringspot virus W (PRSV-W), and Cucumber mosaic virus (CMV) (Zitter et al. 1996) along with some fungal diseases. Development of this crop to resist various pests and diseases while improving its growth and vigor would be of considerable commercial value. Biotechnology may play a key role in helping researchers by solving certain breeding problems in this crop. Plant genetic transformation is a viable way for crop improvement but its use requires a reliable and efficient *in vitro* culture system (Debeaujon and Branchard 1993). Despite its importence, this species has not been subjected to sufficient genetic or biotechnological investigations.

Plant regeneration of *Cucurbita* species has been reported previously through direct and indirect organogenesis. Carol et al. (1995) also reported the formation of somatic embryos in six squash cultivars (*C. pepo*) through the use of cotyledon segments. Similarly, squashes (*C. pepo* and *C. maxima*) could be regenerated *via* direct organogenesis using cotyledons (Ananthakrishnan et al. 2003, Lee et al. 2003). But reports on *in vitro* regeneration of sweetgourd (*C. moschata*) through tissue culture are rare (Zhang 2008, Zhao 1999, Hegazi 1999). Lack of protocol for effective plant regeneration of this crop may be one of the reasons behind very limited study on its successful stable transformation. Hence, the objective of this study was to establish an efficient regeneration protocol using two popular sweetgourd cultivars aiming at future genetic transformation.

Materials and Methods

Fresh, uniform and healthy seeds of sweetgourd cultivars BARI Mistikumra 1 and BARI Mistikumra-2 were collected and sterilized for 12 - 15 minutes in 1% (v/v) sodium hypochlorite containing 3 - 4 drops Tween 20 in a laminar air flow cabinet. Afterwards, they were rinsed 4 times with sterile distilled water at 5 minutes interval and placed in test tubes containing half strength of MS with 30 g/l sucrose, vitamins and 8.5 g/l agar. The pH of the medium was adjusted to 5.8 and autoclaved at 121°C, 15 psi for 20 min. Initially the cultures were kept in

dark for germination and after two days, transferred into a growth chamber maintained at 24 ± 2°C under a 16/8 hrs (light/dark) photoperiod.

Cotyledons were carefully excised from *in vitro* grown seedlings and placed on sterile Petri dishes or hard papers. Each cotyledon was cut off close to the hypocotyl and then cut across in half with the distal parts discarded as the distal end of cotyledons was not found to produce any shoots in a previous study (Ananthakrishnan et al. 2003). The proximal parts were cut in half with each part having a part of the stalk attached. The apical bud of the seedlings was removed carefully from the explants. Explants of both the cultivars were placed on MS without growth regulators (control) and with different concentrations of BAP (2.22, 4.44, 6.66 and 8.88 μM), BAP and NAA in combinations (4.44 + 0.53, 8.88 + 0.53, 22.20 + 0.53 and 0.44 + 5.30 μM), TDZ (0.23, 0.45, 2.25 and 4.50 μM) and 2,4-D (2.26, 4.52, 6.78 and 9.04 μM), respectively. The explants were subcultured in MS having five different concentrations of BAP for further multiplication. Four explants were placed on each Petri dish (15 × 90 mm) containing about 30 ml medium. The Petri dishes were sealed with Parafilm and kept at 25 ± 1°C under a 16/8 hrs (day/night) photoperiod with a light intensity of 1500 lux and sub-cultured every 14 days on the same medium and kept under the same conditions. When shoots attained a height of 2.0 - 2.5 cm, they were cleaned, excised and transferred to half strength of MS, MS supplemented with 2.46 μM IBA and 0.53 μM NAA for root induction. Well developed rooted shoots were transferred to pots in sterile soil and enclosed with polythene bags to maintain high humidity. The plantlets were kept in the greenhouse and watered once or twice in a week while keeping in covered conditions. After 2 - 3 weeks, bags were removed and the plantlets were transferred to larger pots for further growth.

The experiment was set up in a CRD with three independent replicates. The analysis of variance for different parameters was performed and the means were compared by R programme using STAR software at 5% level of significance.

Results and Discussion

The study was conducted by culturing cotyledon explants obtained from 8-10 days old *in vitro* grown seedlings. Shoots initiated directly from the joint between hypocotyl and cotyledon within 25 days. Multiple shoots originated solely from the proximal part of cotyledon and this result indicated that organogenic competent cells were restricted to this part of the cotyledon. Similar observations had also been reported for other species of *Cucurbita* (Gambley and Dodd 1990, Compton 2000, Lee et al. 2003, Ananthakrishnan et al. 2003).

Experiments were carried out to study the effects of MS with different concentrations of growth regulators on regeneration of cotyledon explants (Table 1). The results showed that the explants cultured on MS with BAP and TDZ produced adventitious shoots whereas medium supplemented with 2,4-D and BAP in combination with NAA produced only callus. Explants on control medium did not show any response and a lower percentage of shooting was observed from BAP in combination with NAA in both varieties (Table 1). The results showed that the shooting response was highly variable in different concentrations of BAP and TDZ (Table 1). Previously, Abrei and Staden (2001) observed that TDZ did not improve the shoot production in cucurbits. It has

Table 1. Response of sweetgourd cotyledons on media supplemented with different concentrations of plant growth regulators (mean values).

Conc.		BARI Mistikumra-1		BARI Mistikumra-2	
(μM)		% of response	Type of response	% of response	Type of response
Control		0.0	-	0.0	-
BAP					
2.22		79.2	S	66. 7	S
4.44		89.9	S	75.0	S
6.66		76.4	S	100.0	S
8.88		68.6	S	100.0	S
BAP	**NAA**				
4.44	0.53	100.0	C	100.0	C
8.88	0.53	100.0	C	25.0	CS
22.20	0.53	50.0	CS	12.5	CS
0.44	5.30	75.0	C	50.0	C
TDZ					
0.23		52.8	S	75.0	S
0.45		63.9	S	50.0	S
2.25		72.2	S	41.7	S
4.50		44.4	S	50.0	S
2,4-D					
2.26		88.9	C	88.9	C
4.52		100.0	C	100.0	C
6.78		100.0	C	88.9	C
9.04		100.0	C	100.0	C

S - shoot, C - callus, CS - callus with shoots.

been also reported that the use of high doses of TDZ had negative effects on organogenesis (Pal et al. 2007). On the other hand, the use of either TDZ or the combination of BAP and NAA caused a drastic decrease in the frequency of explants with shoot buds of watermelon (Krug et al. 2005). The importance of BAP for organogenesis had been studied in cucurbits (Curuk et al. 2002, Lee et al.

2003) and it was mentioned as an exclusive requirement for shoot formation. BAP was also reported superior to TDZ (Ficcadenti and Rotino 1995) for melon (*Cucumis melo*). Therefore, four different concentrations of BAP were used in this study to further optimize the regeneration protocol of sweetgourd.

Regeneration from both the varieties were significantly influenced by different concentrations of BAP. The highest shoot induction rate was obtained from 4.44 μM BAP followed by 2.22 μM BAP for BARI Mistikumra-1 (Table 2 and Fig. 1A). In contrast, the highest percentage of regeneration with minimum days to shoot induction was observed in 8.88 μM BAP for BARI Mistikumra-2 (Table 2, Fig. 2A). Krug et al. (2005) reported *in vitro* organogenesis of watermelon with higher efficiency, when cotyledon segments from the proximal region collected from 3-day-old seedlings were cultivated in MS with 1 mg/l BAP (4.44 μM) and coconut water (10%). But Zhao (1999) observed that the explants of cotyledonary base from 4-day-old seedlings of *C. moschata* incubated on medium containing 4.0 mg/l BAP (17.76 μM) and 0.4 mg/l IAA (2.28 μM) showed the best shoot induction (50%) which was lower than that observed in the present study. The highest frequency (63.7%) and the average time (11.0±1.03 days) for shoot

Table 2. Effect of BAP on shoot induction from cotyledon explants of sweetgourd (mean values).

BAP (μM)	BARI Mistikumra-1			BARI Mistikumra-2		
	% of shoot induction	Days for shoot induction	No. of shoots/ explant	% of shoot induction	Days for shoot induction	No. of shoots/ explant
Control	0.00e	0.00e	0.00c	0.00c	0.00c	0.00c
2.22	86.7b	17.3c	6.4b	58.3ab	8.5b	7.5ab
4.44	94.4a	14.7d	8.9a	61.1ab	10.7a	8.4ab
6.66	76.4c	20.1b	6.5b	50.0b	10.4a	6.1b
8.88	68.6d	23.8a	5.9b	83.3a	8.4b	11.9a

Means followed by different letters within a column are significantly different at $p < 0.05$.

regeneration in pumpkin (*C. moschata*) were reported earlier from seven-day-old cotyledon explants cultured on MS containing 0.5 mg/l BAP (2.22 μM) (Zhang et al. 2008). This might have caused due to differences in cultivars and culture conditions. The highest number of shoots (8.9) per explant in the present study was also observed in 4.44 μM BAP which was statistically different than other concentrations of BAP for BARI Mistikumra-1. In contrast, the highest number of shoots (11.9) was recorded in 8.88 μM BAP for BARI Mistikumra-2 (Table 2, Figs 1B, 2B). But Ahmad and Anis (2005) observed similar number of shoots (8.80 ± 0.27) in MS with 1.0 μM BA + CH 200 mg/l from nodal explants of cucumber.

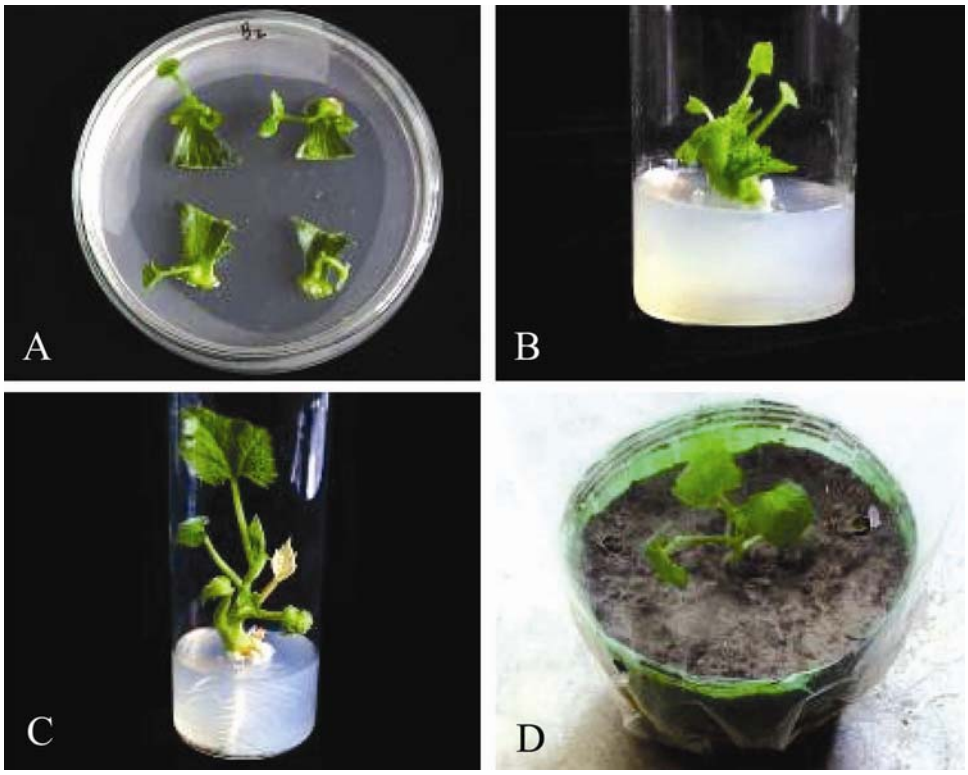

Fig. 1A-D. Regeneration of BARI Mistikumra-1. (A) Shoot initiation on 4.44 μM BAP, (B) multiple shoot formation on 4.44 μM BAP, (C) well developed rooted plantlet on 0.53 μM NAA and (D) hardening of plantlets in greenhouse.

Root growth was observed within 3 weeks after the placement of elongated shoots (2.0 - 2.5 cm) on rooting medium. Among the three different rooting media the percentage of rooting was variable for both the varieties. The highest percentage of rooting was observed in MS supplemented with 0.53 μM NAA followed by half-strength MS for both BARI Mistikumra 1 and BARI Mistikumra 2, respectively while the lowest rooting frequency was observed in 2.46 μM IBA for both BARI Mistikumra 1 and BARI Mistikumra 2, respectively (Table 3). Wei et al. (2006) and Sultana et al. (2004) found similar results in watermelon (*Citrullus lanatus* Thumb.) where the highest rooting frequency (95%) was recorded in 0.1 mg/l NAA (0.53 μM). Pal et al. (2007) also observed 59.33% rooting frequency in 0.5 mg/l IBA (2.46 μM) of summer squash (*C. pepo*).

Akther et al. (2007) observed 100% of rooting in MS without growth regulators for sweetgourd. Our results also showed better rooting frequency in growth regulator free medium than that obtained from the medium supplemented with IBA (Table 3). Rooting in plant growth regulator-free

medium during organogenesis has been reported earlier in *C. pepo* (Ananthakrishnan et al. 2003) and (Lee et al. 2003).

Table 3. Effect of different concentrations of plant growth regulators on root induction of sweetgourd (mean values).

Growth regulators (μM)	BARI Mistikumra-1		BARI Mistikumra-2	
	% of rooting frequency	Days for root induction	% of rooting frequency	Days for root induction
½ MS	87.7b	14.2b	80.0b	14.4a
0.53 NAA	92.6a	12.3c	93.3a	8.6b
2.46 IBA	70.3c	18.4a	76.7c	10.2b

Means followed by different letters within a column are significantly different at $p < 0.05$.

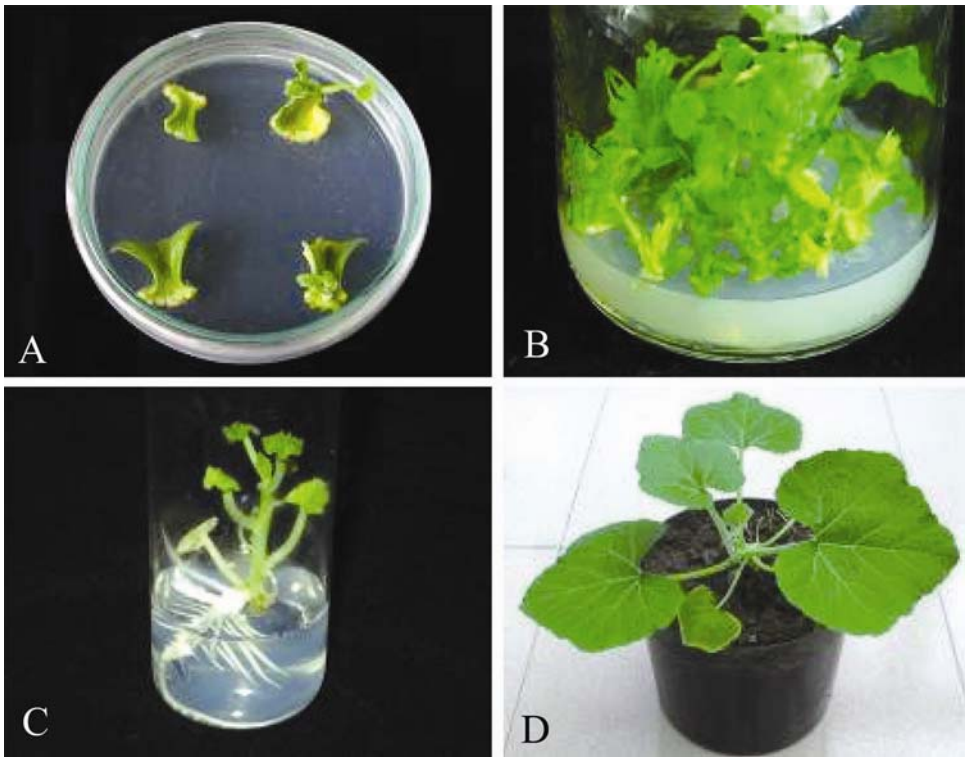

Fig. 2A-D. Regeneration of BARI Mistikumra-2. (A) Shoot initiation on 8.88 μM BAP, (B) multiple shoot formation on 8.88 μM BAP, (C) well developed rooted plantlet on 0.53 μM NAA and (D) hardened plantlet in greenhouse.

The duration for root formation was delayed in the rooting medium without any auxin. Minimum number of days required for rooting was 12.3 in BARI Mistikumra-1 whereas it was 8.6 in BARI Mistikumra-2 when the shoots were

cultured on 0.53 μM NAA (Table 3, Fig.1C, 2C). Akther et al. (2007) showed the minimum number of days required for rooting of *C. moschata* was 6.63 in MS without growth regulators. Zhang et al. (2008) found that the least number of days required for root induction was 6 ± 0.8 in MS without growth regulators. Previously it was reported that rooting response may vary with different genotypes, different conditions of shoots used, variation in medium, number of subcultures and culture conditions (Shakti et al. 2007). After rooting, the healthy rooted plantlets were transferred to greenhouse for hardening (Figs 1D, 2D). Following 2 - 3 weeks of acclimation, the plantlets were transferred to the field for further growth and development.

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