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ORIGINAL ARTICLE

High-frequency shoot regeneration from leaf explants through organogenesis in bitter melon (*Momordica charantia* L.)

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Abstract An efficient protocol for in vitro organogenesis was achieved from callus-derived immature and mature leaf explants of *Momordica charantia*, a very important vegetable and medicinal plant. Calluses were induced from immature leaf explants excised from in vitro (15-day-old seedlings) mature leaf explants of vivo plants (45 days old). The explants were grown on Murashige and Skoog (MS) medium with Gamborg (B5) vitamins containing 30 g l^{-1} sucrose, 2.2 g l^{-1} Gelrite, and 7.7 μM naphthalene acetic acid (NAA) with 2.2 μM thidiazuron (TDZ). Regeneration of adventitious shoots from callus (30–40 shoots per explant) was achieved on MS medium containing 5.5 μM TDZ, 2.2 μM NAA, and 3.3 μM silver nitrate ($AgNO_3$). The shoots (1.0 cm length) were excised from callus and elongated in MS medium fortified with 3.5 μM gibberellic acid (GA_3). The elongated shoots were rooted in MS medium supplemented with 4.0 μM indole 3-butyric acid (IBA). Rooted plants were acclimatized in the greenhouse and subsequently established in soil with a survival rate of 90%. This protocol yielded an average of 40 plants per leaf explant with a culture period of 98 days.

Keywords Adventitious shoots · Growth regulators · Organogenic callus · Hardening

Abbreviations

$AgNO_3$	Silver nitrate
2,4-D	2,4-Dichlorophenoxyacetic acid
BAP	6-Benzylaminopurine
FAA	Formaldehyde acetic acid alcohol
GA_3	Gibberellic acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
MS	Murashige and Skoog medium
NAA	α-Naphthaleneacetic acid
TDZ	Thiadiazuron

Introduction

Bitter melon (*Momordica charantia* L.) grows in tropical areas of Asia, Amazon, east Africa, and the Caribbean and is cultivated throughout the world for use as a vegetable and medicine. Bitter melon plants contains high levels of iron, beta carotene, calcium, potassium, vitamins, phosphorus, and good dietary fiber (Sultana and Bari Miah 2003). They also contain an array of biologically active proteins, namely, momordin, α- and β-momorcharin, cucurbitacin, and MAP30, that have shown to have highly effective anti-human immunodeficiency (HIV), anti-tumor,

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anti-diabetic, and anti-rheumatic properties and to function as febrifuge medicine for jaundice, hepatitis, leprosy, hemorrhoids, psoriasis, snakebite, and vaginal discharge (Singh et al. 1998; Bourinbaiar and Lee-Huang 1996; Beloin et al. 2005). To date, the genetic improvement of bitter melon has been mainly achieved by conventional plant breeding methods, but more recent techniques in plant genetic engineering have advanced and opened a new avenue for crop improvement. The availability of an efficient in vitro regeneration system is the primary requirement for the genetic transformation of most plants (Thiruvengadam and Yang 2009), and plant regeneration protocol from individual cells or explants is essential for the application of genetic engineering to bitter melon. Only limited success has been obtained in regenerating *M. charantia* from different explants of nodal and shoot tips (Wang et al. 2001; Sultana and Bari Miah 2003; Huda and Sikdar 2006; Malik et al. 2007), cotyledons (Islam et al. 1994), shoot apices, and nodal and internodal explants (Agarwal and Kamal 2004). In vitro plant regeneration from axillary bud and nodal and shoot-tip explants have been reported in *Momordica dioica* (Thiruvengadam and Jayabalan 2001; Thiruvengadam et al. 2006a). In previous reports, we have described an efficient system for somatic embryogenesis via cell suspension cultures using leaf and petiole embryogenic callus in *M. charantia* and *M. dioica* (Thiruvengadam et al. 2006b, 2007). Paul et al. (2009) investigated the effect of exogenous polyamines (PAs) on enhancing somatic embryogenesis in *M. charantia*. Malik et al. (2007) reported that various explants of leaf, stem, and cotyledon induced different types of callus in *M. charantia* but that none of these produced any shoots. *Agrobacterium*-mediated *β*-glucuronidase expression was detected in explants of immature cotyledonary nodes in *M. charantia* (Sikdar et al. 2005). To the best of our knowledge, however, there has been no report on bitter melon regeneration via organogenesis from immature and mature leaves. The purpose of this study was, therefore, to establish an efficient reproducible protocol for high-frequency regeneration via organogenesis from mature and immature leaves of bitter melon. We believe that our findings may facilitate genetic transformation in this important cucurbit and may also be applicable to other related plant species.

Materials and methods

Seed material and explants preparation

Seeds of bitter melon (*M. charantia* L. cv. Coimbatore-1) were obtained from Arignar Anna farm, Kudimianmalai, Pudukkottai, Tamil Nadu, India. The seeds were surface-sterilized first with 70% (v/v) ethanol for 1 min and then with 25% (v/v) commercial bleach (with sodium hypochlorite as the active agent) containing 0.05% (w/v) of Tween-20 (polyethylene sorbitan monooleate; Nutritional Biochemical, Cleveland, OH) for 20 min and then rinsed thoroughly (three times) with sterile distilled water. Disinfected seeds were germinated in the dark for 48 h in a jar containing agar-solidified half-strength Murashige and Skoog (1962) MS basal salts (1/2MS) plus B_5 vitamins (Gamborg et al. 1968) without growth regulators at a density of 12 seeds per jar. The seedlings were grown at 25 ± 2°C under a 16/8-h (light/dark) photoperiod with light provided by white fluorescent lamps at an intensity of 30 μmol m^{-2} s^{-1}. The immature leaves were excised from 15-day-old seedlings, and mature leaves were excised from highly proliferating (45-day-old) plants growing in a growth chamber [25 ± 2°C, 16/8-h (light/dark) photoperiod and 45 μmol m^{-2} s^{-1} irradiance]. Mature leaves were rinsed thoroughly in running tap water for 2 h. The leaves were then surface sterilized by agitation in 5% laboline and 0.1% $HgCl_2$ for 10 min and rinsed five to seven times with sterile distilled water (Thiruvengadam et al. 2006b).

Callus induction and shoot regeneration

After sterilization, the explants were cut into approximately 1-cm^2 sections that included the midrib portion and cultured in vitro with their adaxial sides against semi-solid callus induction medium in culture tubes (25 × 150 mm; LCM, Lake Charles, LA) plugged with non-absorbent cotton plugs. The callus induction medium comprised MSB_5 (MS salts + B_5 vitamins) plus 30 g l^{-1} sucrose, 2.2 g l^{-1} gelrite (Sigma–Aldrich, St. Louis, MO) and different concentrations of auxins [0.0–8.8 μM; 2,4-dichlorophenoxyacetic acid (2,4-D) naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA)] in combination with different concentrations of cytokinins [0.0–4.5 μM; 6-benzylaminopurine (BAP), thiadiazuron (TDZ)], adjusted to pH 5.8 prior autoclaving. All cultures were maintained at 24 ± 2°C and a relative humidity (RH) of 60–65% under a 16/8-h (light/dark) photoperiod with light provided by 40-W cool-white fluorescent lamps (Sylvania, Danvers, MA) at a photosynthetic photon flux density (PPFD) of 45 μmol m^{-2} s^{-1}. After 3 weeks of culture, well-developed calluses were produced from the cut ends of the leaf. These calluses (1 g fresh mass) were transferred to MS medium with 30 g l^{-1} sucrose, 2.2 g l^{-1} gelrite and different concentrations of TDZ (0.0–6.6 μM), NAA (0.0–5.5 μM), and silver nitrate ($AgNO_3$; 0.0–4.4 μM). TDZ and $AgNO_3$ were filter sterilized (filter pore size 0.22 μm; Micron Separations, Westboro, MA) and then added to the autoclaved media under aseptic conditions. Callus with regenerating adventitious buds was subcultured twice at 15-day intervals in the same medium.

Shoot elongation and rooting

For elongation, regenerated shoots were harvested and transferred to shoot elongation medium containing MS basal salts plus B_5 vitamins supplemented with gibberellic acid (GA_3; 0.0–4.5 μM), 30 g l^{-1} sucrose, and 2.2 g l^{-1} gelrite. The elongated shoots were excised individually and transferred to rooting medium supplied with IAA, IBA, and NAA (0.0–6.0 μM) for 3 weeks. All compounds and plant growth regulators were from Sigma–Aldrich. The medium was adjusted to pH 5.8 before autoclaving at 121°C for 15 min. Cultures were maintained as described above.

Hardening and acclimatization

Plantlets with well-developed roots were removed from the culture medium. The roots were gently washed under running tap water to remove the adhering medium, and plantlets were transferred to plastic cups (diameter 6 cm) containing sterilized soil, sand, and vermiculite mixture (4:3:3, v/v/v). The potted plants were then covered with polyethylene to maintain a condition of high humidity (85% RH) and grown for 2 weeks at a PPFD of 25 μmol m^{-2} s^{-1} before planting to the greenhouse. After the development of new leaves, the covers were removed, and hardened plants were transferred to earthen pots (diameter 18 cm) filled with soil mix (peat, perlite, and vermiculite in equal proportions: 1:1:1, v/v/v) and grown to maturity. The survival percentage was calculated after 5 weeks in the greenhouse.

Histology

A standard histological procedure (Berlyn and Miksche 1976) was followed for fixation in formaldehyde acetic acid alcohol (FAA; 0.5:0.5:9.0, v/v/v), embedding in paraffin, and sectioning. Microtome sections (10 μm) were collected on glass slides coated with Haupt's adhesive, air dried overnight, and stained with 0.1% aqueous toluidine blue for 10 min without removing the paraffin. Following several rinses in distilled water, the slides were air dried for 2 days and stored in desiccator until mounting. Prior to mounting with Permount (Fisher Scientific, Fair Lawn, NJ), the paraffin was removed by rinsing the slide twice (5 min each time) in fresh xylene. Although the rehydration and dehydration steps common to conventional staining methods are eliminated from this staining procedure, the sections obtained are equally well-suited for light photomicrography.

Experimental design and data analysis

The data were collected 3 weeks after the initiation of callus induction, after 4 weeks of shoot regeneration culture, after 2 weeks of elongation culture, and after 3 weeks in the rooting experiments. All experiments were conducted with a minimum of three replicates per treatment. The data were analyzed statistically using SPSS ver. 14 (SPSS, Chicago, IL). The significance of differences among means was carried out using Duncan's multiple range test at $P = 0.05$. The results are expressed as the mean ± standard error (SE) of three experiments.

Results and discussion

Callus induction

The callus was initiated from the cut ends of leaf segments in MS medium containing either auxin alone or auxin combined with cytokinin. The quality of the callus was assessed after 3 weeks of culture. Among the three auxins tested individually, 2,4-D produced yellowish friable callus, NAA produced greenish friable callus, and IAA produced brownish friable callus, and all of the callus produced was non-organogenic (data not shown). The combination of 7.7 μM NAA with 2.2 μM TDZ produced greenish compact callus (Fig. 1a; Table 1) with a callusing response from mature leaves (73.4%) and immature leaves (94.4%). Agarwal and Kamal (2004) and Malik et al. (2007) observed a callogenic response in shoot-tip, nodal, internodal, leaf, stem, and cotyledon explants of *M. charantia* cultured in medium supplemented with BAP and either NAA or 2,4-D, but the callogenic percentage was low and the calluses were also non-organogenic. In contrast, Nabi et al. (2002) and Devendra et al. (2009) found that the combination of BAP and NAA or 2,4-D produced organogenic callus in *M. dioica*. Auxin and cytokinin combinations have been found to induce organogenic calluses from different explants of cucumber (Ziv and Gadasi 1986; Handley and Chambliss 1979; Punja et al. 1990; Nishibayashi et al. 1996; Seo et al. 2000). Leaf explants were not callogenic when cultured on hormone-free MS medium. Similar results were observed in *M. charantia* (Malik et al. 2007).

Adventitious shoot formation

Three-week-old nodular greenish compact callus was obtained from leaf explants cultured in MS medium with 7.7 μM NAA and 2.2 μM TDZ. The nodular greenish compact callus was transferred to MS medium containing different concentrations of TDZ and combinations of NAA (2.2 μM) with $AgNO_3$ (3.3 μM) (Fig. 2). The nodular greenish compact callus induced shoot initiation in MS medium containing TDZ (5.5 μM), NAA (2.2 μM), and

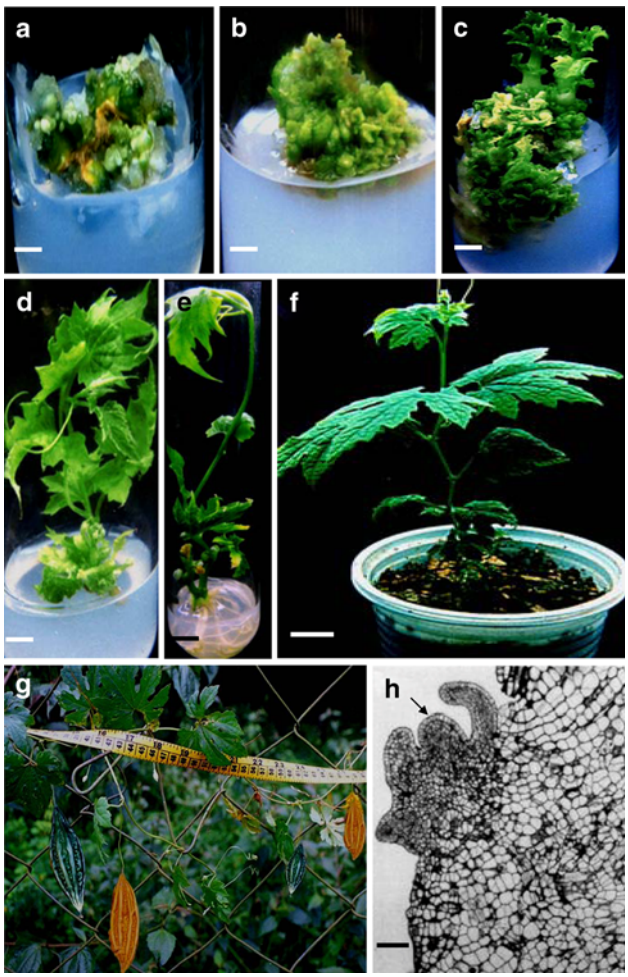

Fig. 1 Regeneration of adventitious shoots from immature leaf-derived callus of *Momordica charantia* L. **a** Greenish compact callus (7.7 μM NAA, 2.2 μM TDZ). *Bar*: 10 mm. **b** Initiation of adventitious shoots from greenish compact nodular callus (5.5 μM TDZ, 2.2 μM NAA, 3.3 μM $AgNO_3$). *Bar*: 10 mm. **c** Proliferation of adventitious shoots (5.5 μM TDZ, 2.2 μM NAA, 3.3 μM $AgNO_3$). *Bar*: 10 mm. **d** Elongation of shoots (3.5 μM GA_3). *Bar*: 10 mm. **e** In vitro rooting of shoots (4.0 μM IBA). *Bar*: 10 mm. **f** Hardened plants. *Bar* 3.0 cm. **g** Plant acclimated in the field with fruits (close-up view of reproductive stage). **h** Longitudinal section of immature leaf-derived callus showing the meristematic zone. *Bar* 50 mm. *Arrows* indicate emerging shoot primordial. *NAA* α-Naphthaleneacetic acid, *TDZ* thiadiazuron, *$AgNO_3$*silver nitrate, *IBA* indole-3-acetic acid, *GA_3* gibberellic acid

$AgNO_3$ (3.3 μM) within 2 weeks (Fig. 1b). In comparison, TDZ induced a larger number of shoots in *Cucurbita pepo* (Pal et al. 2007), *Cucumis sativus* (Zhang and Cui 2001; Selvaraj et al. 2006a), and *Melothria maderaspatana* (Baskaran et al. 2009) and a fewer number of shoots in watermelon (Krug et al. 2005) and cucumber (Zhang and Cui 2001). BAP combined with NAA resulted in a much lower shoot regeneration frequency in bottle gourd (Han et al. 2004), while Seo et al. (2000) reported that 5.0 μM NAA combined with 2.5 μM BAP produced more shoots in cucumber. Ntui et al. (2009) stated that BAP alone resulted in shoot induction and elongation in Egusi melon. We obtained maximum shoot bud differentiation (30–40 shoots per explant) within 4 weeks of culture initiation on MS medium containing TDZ (5.5 μM), NAA (2.2 μM), and $AgNO_3$ (3.3 μM) (Fig. 1c). This result is supported by earlier results in which shoot bud in cotton was induced with the combination of TDZ, NAA, and $AgNO_3$ (Ouma et al. 2004). Han et al. (2004) reported that maximum shoot regeneration in bottle gourd was obtained on medium containing BAP (3.0 mg l^{-1}) and $AgNO_3$ (0.5 mg l^{-1}). In our study, shoot regeneration frequency and number of shoots per explant progressively decreased with increasing levels of $AgNO_3$, with shoot regeneration completely inhibited at $AgNO_3$ concentrations >4.0 μM (Table 2). The beneficial effects of $AgNO_3$ on shoot organogenesis have been reported in cucurbits (Yadav et al. 1996; Mohiuddin et al. 1997; Shyamali and Hattori 2007). Our study on bitter melon showed that the enhancement of in vitro multiple shoot bud induction can be achieved by using $AgNO_3$ (Table 2). We found that the application of TDZ together with NAA and $AgNO_3$ was significantly beneficial to bud formation from leaves of bitter melon. In addition, those explants cultured in the dark did not respond to shoot formation, and seed germination was significantly effective in the dark pretreatment. In melon, light is required for the regeneration of cotyledon explants (Leshem et al. 1995), whereas other explants do not require light (Curuk et al. 2003). Mature and immature leaves were tested for their organogenic potential for adventitious shoot induction. In our study, callus induction and shoot and root induction frequency in immature leaves was higher than that in matures leaves (Tables 1, 2; Figs. 2, 3, 4). Lee et al. (2003) stated that the shoot induction rate in immature seedlings was higher than that in mature seedlings in winter squash. Dong and Jia (1991) suggested that immature explants are physiologically very active and easily affected by environmental factors, such as exogenous hormones.

Shoot elongation and rooting

The regenerated shoots (height 1.5 cm) harvested from calluses were transferred to shoot elongation medium containing 3.5 μM GA_3 (Fig. 3). The elongated shoots attained an average height of 9.2 cm within 2 weeks (Fig. 1d). These results corroborate the findings of Selvaraj et al. (2006b) and Ananthakrishnan et al. (2003) on *Cucumis sativus* and *Cucurbita pepo*, respectively. However, they are not in agreement with other results on the cucurbitaceae *Cucumis melo* (Kathal et al. 1988), *C. sativus* (Handley and Chambliss 1979), and *Citullus vulgaris* (Srivastava et al. 1989). The elongated shoots

Table 1 Comparative effect of various concentrations of α-naphthaleneacetic acid (NAA) with 2.2 μM thiadiazuron on callus induction from mature and immature leaves of bitter melon *Momordica charantia* L.

PGR (μM)	Percentage of callus initiation		Nature of callus	
	Mature leaves	Immature leaves	Mature leaves	Immature leaves
NAA				
1.1	36.2 ± 1.2^{h}	51.4 ± 1.0^{h}	YF	YF
2.2	43.6 ± 1.0^{g}	58.2 ± 1.4^{g}	YF	YF
3.3	55.4 ± 1.5^{f}	$64.4 \pm 1.2^{e,f}$	YBF	YGF
4.4	$61.8 \pm 2.0^{d,e}$	69.2 ± 2.0^{e}	YBC	YGC
5.5	65.2 ± 1.0^{d}	75.4 ± 1.8^{d}	YGC	YGC
6.6	$70.6 \pm 1.4^{a,b}$	80.2 ± 1.5^{c}	YGC	YGC
7.7	73.4 ± 1.6^{a}	94.4 ± 1.2^{a}	GC	GC
8.8	69.2 ± 1.2^{c}	$83.6 \pm 1.2^{a,b}$	YBC	YBC

Values are given as the mean ± standard error (SE) of three replicates per treatment. Values followed by different letters indicate a significant difference between means ($P < 0.05$) according to Duncan's multiple range test (DMRT). The data were recorded after 3 weeks of culture

YF Yellowish friable, *YBF* yellowish-brown friable, *YBC* yellowish-brown compact, *YGC* yellowish-green compact, *GC* green compact

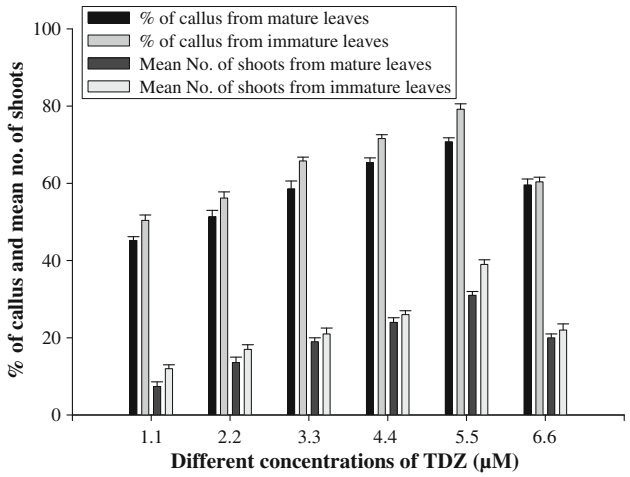

Fig. 2 Effect of different concentrations of TDZ in combination with NAA (2.2 μM) and $AgNO_3$ (3.3 μM) on adventitious shoot regeneration from mature and immature leaves of bitter melon. The results are expressed as the mean ± standard error (SE) from three replicates per treatment. The data were recorded after 4 weeks of culture

Table 2 Effect of different concentrations of silver nitrate ($AgNO_3$) with NAA (2.2 μM) and thiadiazuron (5.5 μM) on adventitious shoot bud induction from mature and immature leaves of bitter melon

Concentration of $AgNO_3$ (μM)	Number of shoots per explant	
	Mature leaves	Immature leaves
0.0	6.0 ± 0.5^{d}	12.0 ± 0.6^{c}
1.1	13.0 ± 0.8^{c}	$19.0 \pm 0.8^{b,c}$
2.2	16.0 ± 1.0^{b}	21.0 ± 1.0^{b}
3.3	32.0 ± 1.0^{a}	40.0 ± 1.2^{a}
4.4	$4.0 \pm 0.5^{d,e}$	7.0 ± 1.0^{d}

Values are the mean ± standard error (SE) of three replicates per treatment. Values followed by different letters indicate significant a difference between means ($P < 0.05$) according to DMRT. The data were recorded after 4 weeks of culture

appeared to be normal and healthy, and no hyperhydricity was observed. For rooting, the elongated shoots were transferred to MS basal medium containing different concentrations of IAA, IBA, and NAA (data not shown). Three weeks after inoculation, root formation was observed from the cut portion of the shoot. Of the three auxins used, IBA was the best for root induction (Fig. 4). The effectiveness of IBA in rooting has been reported in *Momordica dioica* (Hoque et al. 1995; Nabi et al. 2002) and *Citrullus lanatus* (Ganasan and Huyop 2010). In contrast, NAA and IAA have been found to be effective in inducing roots in *Momordica charantia* (Islam et al. 1994; Sultana and Bari Miah 2003; Malik et al. 2007) and *M. dioica* (Thiruvengadam et al. 2006a). The highest number (13.0) of roots per shoot was recorded for regenerated shoots cultured at 4.0 μM IBA (Fig. 1e); NAA and IAA had a poor effect in terms of inducing the production of weak and slender roots (data not shown).

Hardening and acclimatization

The rooted plants were gently removed from the vessels, washed initially to remove adhered agar and traces of the medium to avoid contamination, and then washed for 10 min in distilled water (Thiruvengadam et al. 2006a). They were then transferred to plastic vessels containing a sterile soil, sand, and vermiculite mixture (Fig. 1f), and after

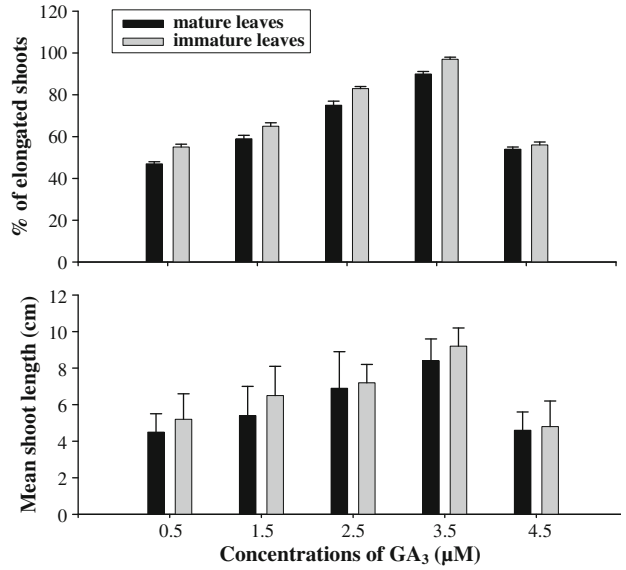

Fig. 3 Effect of GA_3 on shoot elongation from regenerated shoots cultured on MS medium. The results are expressed as the mean ± SE of three replicates per treatment. The data were recorded after 2 weeks of culture

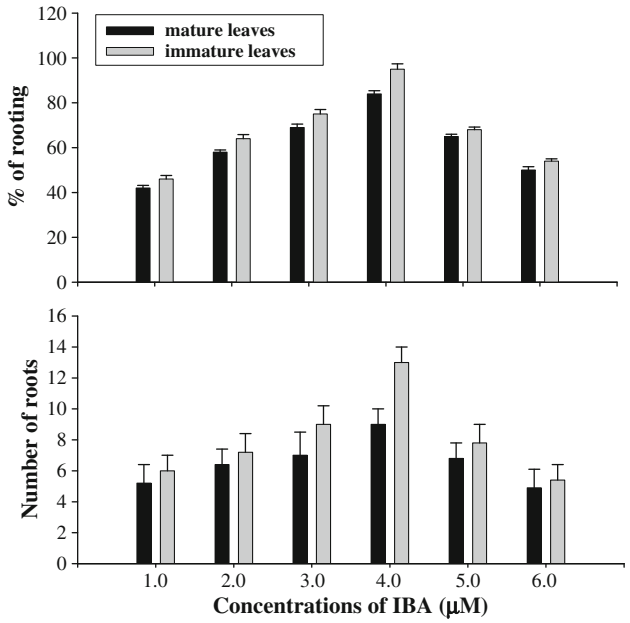

Fig. 4 Effect of IBA on root induction of bitter melon. The results are expressed as the mean ± SE from three replicates per treatment. The data were recorded after 3 weeks of culture

2 weeks, they were transferred to pots. The hardening of potted plants for 15 days in a growth chamber was found to be essential. We had a 90% survival rate of plants derived from mature and immature leaf explants when rooted plantlets were transferred from pots to field conditions (Table 3). Regenerated plants transferred to the field became fully established and grew well (Fig. 1g) and were similar to the parental plants in their morphology. We observed that the flowers and fruits of regenerated plants were higher than wild-type plants (Table 3). Seeds were obtained from these plants that grow normally and fertile (Fig. 1g).

Histology of organogenic callus

Sections of immature leaf-derived organogenic callus (3 week-old) were examined. The section revealed meristematic activity at the sub-epidermal region in the organogenic callus. The meristematic cells were distinguishable from other cells by their small size and deeply stained cytoplasm. These cells produced a homogenous mass of densely stained cells, delimited distally by a single layer of cells to form shoot primordia which led to the organization of shoot meristem and a pair of leaf primordia (Fig. 1h). The meristemoids from callus started after 3 weeks of culture. Adventitious shoot buds surrounded by leaf primordial originated from these meristemoids. The shoot buds subsequently developed into shoots.

Conclusion

In recent years, many research groups have been involved in establishing reliable regeneration procedures for agronomically important plants primarily because such protocols are both primary and essential steps for facilitating gene introduction and crop improvement. In this study, we demonstrated an efficient plant regeneration system using immature and mature leaves of bitter melon via organogenesis. This protocol yielded about 40 plants per explant within a period of 98 days. We believe that this regeneration system could be used in the production of transgenic bitter melon plants.

Table 3 The frequency of ex vitro survival and flowering of acclimatized from leaf derived plants of bitter melon

Number of plants transferred to hardening		Number of acclimatized plants		Number of flowers per plant		Number of fruits per plant		Number of fertile plants		Number of sterile plants	
Wt	Tcp	Wt	Tcp	Wt	Tcp	Wt	Tcp	Wt	Tcp	Wt	Tcp
20	20	20	18	44	47	39	41	18	18	0	0

Wt Wild type, *Tcp* tissue culture plants

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