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Full Length Research Paper

An efficient regeneration from petiole derived callus of male and female spine gourd (*Momordica dioica* Roxb. ex. Willd.)

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Spine gourd (*Momordica dioica* Roxb. ex. Willd.) is a delicious vegetable of south Asia. Its commercial cultivation is limited by vegetative mode of propagation and dioecious nature. An efficient *in vitro* regeneration protocol was developed from petiole derived callus of male and female plants. About 88.2% male and 95.5% of female petiole explants derived from one year old *in vivo* grown plants produced green, compact organogenic callus in MS medium containing 6.0 µM 2,4-D and 2.0 µM BAP after two successive subculture at 11 days interval. Adventitious shoots were produced from the organogenic callus when it was transferred to MS medium supplemented with, 6.0 µM TDZ and 1.0 µM 2,4-D with shoot induction frequency (male 87.5% and female 93.0%) and regeneration (male 38 shoots and female 43 shoots per explant). Shoot proliferation occurred when callus with emerging shoots were transferred in the same medium at an interval of 15 days. The regenerated shoots were elongated in MS medium augmented with 3.0 µM GA_3. The elongated shoots were rooted in MS medium supplemented with 1.5 µM IBA. Rooted plants were acclimatized in green-house and subsequently established in soil with a survival rate of 95%. The survival percentage differed with seasonal variations.

Key words: Adventitious shoots, dioecious, hardening, organogenic callus, *Momordica dioica*.

INTRODUCTION

Spine gourd or teasle gourd (*Momordica dioica* Roxb. ex. Willd.) is a dioecious, perennial cucurbitaceous climber and originated from the Indo-Malayan region. It has been highly cultivated in India, China, Nepal, Bangladesh, Myanmar, Pakistan and Sri Lanka (Trivedi and Roy, 1972; Rakh and Chaudhari, 2010). Immature green fruits are cooked as vegetable and young leaves, tuberous roots and flowers are also consumed. Fruits contain high amounts of protein, calcium, phosphorous, iron, and highest amount of carotene amongst the cucurbitaceous vegetables (Bharathi et al., 2007). In addition, this species is valued for several medicinal and curative properties such as anti-diabetic, anticancer, anti-fertility abortificiant, anti-inflammatory, antioxidant activity, snake bites, scorpion sting, jaundice and bleeding pile properties (Luo et al., 1998; Ram et al., 2001; Reddy et al., 2006; Deokule, 2006; Jain et al., 2008; Bawara et al., 2010).

Plantation is done at beginning of the summer when monsoon starts, flowering starts in May to July, and fruiting ends in September to November. The plants remain dormant in winter (Rasul et al., 2007). Under optimum crop management fruit yield of 75-100 q ha^{-1} can be achieved (Bharathi et al., 2007). This popular vegetable has high demand in market but still remains as underutilized and underexploited (Bharathi et al., 2007; Ali et al., 1991) due to vegetative mode of propagation and dioecious nature.

Commercial propagation of spine gourd is largely depending on tuberous roots, followed by stem cuttings and seeds (Nabi et al., 2002). Propagation by tuberous roots is limited due to the low multiplication rate (Mondal et al., 2006) and occupies the valuable cultivation land until next planting season (Ram et al., 2001; Nabi et al.,

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Abbreviations: **BAP**, 6-benzylaminopurine; **GA_3**, Gibberellic acid; **TDZ**, Thiadiazuron; **NAA**, α-Naphthaleneacetic acid; **2,4-D**, 2,4-dichlorophenoxyacetic acid; **IBA**, indole 3-butyric acid.

2002). Stem cuttings containing 2-3 nodes from dark green vines of 2-3 months old plants are planted but only 36% of the plants sprout and survive (Ram et al., 2001), and may transmit diseases. Difficulty in propagation by seeds is unpredictable sex ratio in seedling progenies and dormancy (Ali et al., 1991; Mondal et al., 2006). Male plants dominate natural populations and determination of sex is possible only when the plants start flowering. Female plants, however, are commercially more important, and being a dioecious crop accommodating only 5-10% male plants in the field is imperative for good fruit set.

As the conventional methods of spine gourd propagation impose several limitations for large scale propagation of sex specific plants, an efficient clonal propagation method has been sought. During past years, considerable efforts have been made for *in vitro* plant regeneration of this important plant through direct organogenesis (Thiruvengadam and Jayabalan, 2001; Nabi et al., 2002; Bhosle and Paratkar, 2005; Thiruvengadam et al., 2006; Shekhawat et al., 2011). In our earlier studies, we have reported plant regeneration of somatic embryogenesis from petiole derived callus (Thiruvengadam et al., 2007). Recently, we have reported *Agrobacterium tumefaciens*-mediated leaf disc transformation of spine gourd (Thiruvengadam and Chung, 2011).

Moreover, there has not been any specific study on regeneration of male and female spine gourd separately. Dioecious species represent an interesting plant group suitable for studies on hormonal regulation of sex expression. Such studies may entail either analyses of endogenous hormone content or application of exogenous growth regulators (Chailakhyan and Khryanin, 1982).

Differences in the endogenous levels of cytokinins have been reported in male and female *Mercurialis annua* plants (Dauphin-Guerin et al., 1980). Chaturvedi and Sharma (1989) reported that both male and female explants of jojoba behaved alike morphogenically under similar growth regulator and culture conditions. In contradiction, the differential morphogenic response of nodal explants of male and female jojoba clones on media supplemented with different cytokinins has been reported (Agrawal et al., 1999; Prakash et al., 2003). Culafic et al. (1987) stated that male plant cotyledons, hypocotyls and shoot tip explants had higher response compared to female plant of *Rumex acetosella* and *R. acetosa*. Mehra and Cheema (1985) reported that female explants responded better at the lower levels of growth regulators in *Populus deltoids*.

To the best of our knowledge, there has been no report on plant regeneration from petiole derived callus of male and female plants of spine gourd. The objective of this study was to develop an efficient method for the high efficient *in vitro* regeneration of spine gourd, to obtain clones of known sex and characteristics using petiole explants from *in vivo* raised plants of female and male genotypes. We made an attempt to develop a protocol for a hardening from *in vitro*-derived shoots of *M. dioica* and also evaluate the survival percentage in the summer (March - June) and winter (September - December) seasons.

MATERIALS AND METHODS

Plant materials

Male and female tubers of *M. dioica* Roxb. ex. Willd (one year old) were collected from the Semmalai hills, Tamil Nadu and the plants were raised in the Botanical Field Evaluation Garden at Kulathur in Tamil Nadu, India. Petiole explants were collected and washed in running tap water for 5 min and surface sterilized in 70% (v/v) ethanol for 1 min.

Further, petiole explants were treated in 1.0% (v/v) sodium hypochlorite solution for 10 min with occasional agitation. Finally, petiole explants were rinsed four times with sterile double distilled water, blotted dry and trimmed from both ends to obtain about 0.6-0.7 mm.

Callus induction and adventitious shoot regeneration

The callus induction media consisted of MS salts (Murashige and Skoog, 1962), B_5 vitamins (Gamborg et al., 1968) supplemented with 3% sucrose, 0.8% phyto agar and different concentrations (1.0 - 8.0 µM) of 2,4-dichlorophenoxy acetic acid (2,4-D) either alone and in combination with (1.0 - 4.0 µM) benzylaminopurine (BAP) for callus induction.

The medium was adjusted with pH 5.8 prior to autoclaving at 121°C for 15 min. After 3 weeks of culture, well-developed callus were produced from the cut ends of the petiole. These callus (1 g fresh mass) were transferred to MS medium supplemented with 30 g l^{-1} sucrose, 8.0 g l^{-1} agar and different concentrations of BAP and TDZ (1.0 - 8.0 µM) alone and in combination with 2,4-D (1.0 µM) for adventitious bud induction. Callus with regenerating adventitious buds were subcultured twice at 12-day intervals in the same induction medium. The cultures were maintained at 25 ±2°C under 16 h light/ 8 h dark photoperiod with light intensity of 150 µmol m^{-2} s^{-1}.

Shoot elongation and root induction

The regenerated shoots were cultured in a test tube containing 15 ml of MS medium supplemented with different concentrations (1.0 - 4.0 µM) of gibberellic acid (GA_3) for shoot elongation. The elongated shoots were excised individually and transferred to rooting medium supplied with various concentrations (0.5 - 2.0 µM) of indole 3-butyric acid (IBA) for 3 weeks. The medium was adjusted to pH 5.8 before autoclaving at 121°C for 15 min. Cultures were maintained as described above.

Transplantation and acclimatization

Rooted plantlets were washed thoroughly with tap water to remove agar and transplanted to plastic pots containing a mixture of autoclaved sand, soil, and vermiculite (1:1:1 v/v/v). Potted plants were grown in a growth chamber (Sanyo, Tokyo, Japan) at 85% relative humidity for 2-3 weeks, and then moved to greenhouse for 3 weeks before transferring to the field. Initially, plants were covered with polyethylene bags to maintain high humidity (80%)

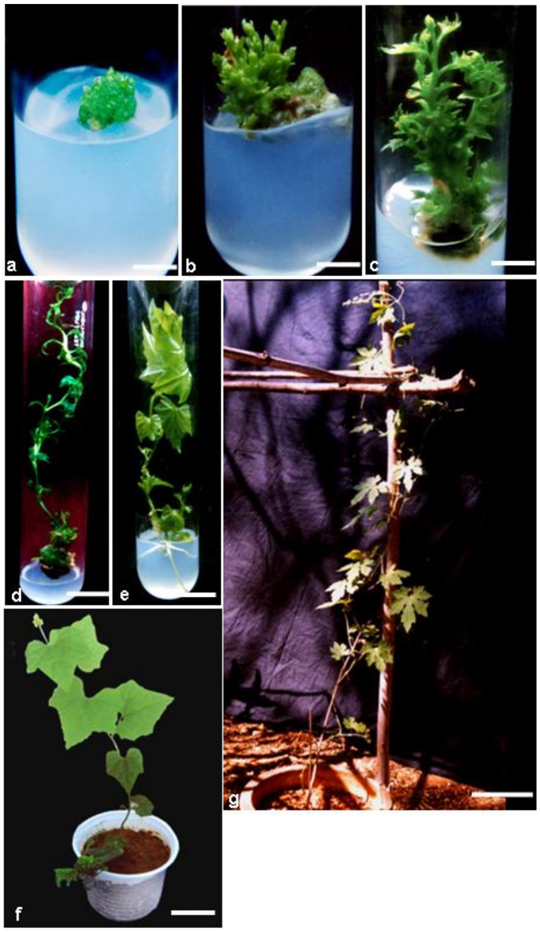

Figure 1. *In vitro* plant regeneration from petiole derived callus of female *Momordica dioica* Roxb. ex. Willd (a) Greenish compact organogenic callus (6.0 µM 2,4-D and 2.0 µM BAP), Bar: 10 mm; (b, c) Adventitious shoot regeneration and proliferation (6.0 µM TDZ and 1.0 µM 2,4-D), Bar: 10 mm; (d) Elongation of shoots (3.0 µM GA_3), Bar: 10 mm; (e) *In vitro* rooting of shoots (1.5 µM IBA). Bar: 10 mm, f. Hardened plants, Bar 3.0 cm; g. Hardened plants transferred to field (6 months old plant), Bar 5.0 cm.

and supplied daily with Hoagland nutrient solution (Hoagland and Arnon, 1950).

The polyethylene bags were gradually removed when the plants has been acclimatized. These acclimatized plantlets were kept in standing position by a wood stick which gives support for climbing of the plant and for better growth and survival. The survival percentage was calculated after 4 weeks in the greenhouse. After, 4 weeks acclimatization, plants were transplanted to garden. The survival percentage was evaluated in summer (March - June, 36 - 43°C) and winter (September - December, 25 - 28°C) seasons in the field.

Statistical analysis

The data were collected after 3 weeks from the initiation of callus induction, after 3 weeks of shoot regeneration culture, 1 week of elongation and after 3 weeks in the rooting experiments. All experiments were conducted with a minimum of ten 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 = 0.5. The results are expressed as the mean ± standard error (SE) of three experiments.

Table 1. Effect of 2,4-D and BAP on organogenic callus induction from petiole explants from male and female plants of *M. dioica*.

PGR (µM)		Callus induction (%)		Nature of callus	
		Male	Female	Male	Female
2,4-D					
2.0		40.4g	48.6f	YF	YF
4.0		48.5ef	54.0ef	YF	YF
6.0		51.6e	59.5e	YF	YF
8.0		63.0d	72.8d	BF	BF
2,4-D	BAP				
2.0	2.0	74.2c	80.5c	YF	YBF
4.0	2.0	82.5b	89.0b	YBF	GC
6.0	2.0	88.2a	95.5a	GC	GC
8.0	2.0	80.6bc	87.0bc	YBC	YBC

Each value represents the mean ± SE of 10 replicates per treatment. The data were statistically analyzed using Duncan's multiple range test (DMRT). In the same column, significant differences according to the least significant difference (LSD) at the P = 0.5 level are indicated by different letters. The data were recorded after 3 weeks of culture. YF Yellowish friable, BF Brownish friable, YBF yellowish-brown friable, YBC yellowish-brown compact, YGC yellowish-green compact, GC green compact.

RESULTS AND DISCUSSION

Organogenic callus induction

Petiole explants from *in vivo*-grown plants of spine gourd were cultured on MS medium supplemented with 2,4-D and in combination with BAP for induction of callus. After 3 weeks of culture incubation, MS medium containing 6.0 µM 2,4-D and 2.0 µM BAP produced greenish compact callus (Figure 1a) with a callusing response of male (88.2%) and female plants (95.5%) (Table 1). Similarly, 2,4-D along with BAP induced greenish nodular calluses in *M. dioica* and *Cucumis sativus* (Nabi et al., 2002; Selvaraj et al., 2006b). Punja et al. (1990) and Seo et al. (2000) reported callus formation in cucumber cultivars in combination of NAA and BAP from petiole and leaf explants respectively.

The combination of 7.7 µM NAA with 2.2 µM TDZ produced greenish compact callus in *M. charantia* (Thiruvengadam et al., 2010). Thiruvengadam et al. (2007) reported that 2,4-D (4.5 µM) and CM (10%) produced green-yellow friable calli in *M. dioica*. In our

Table 2. Effect of different concentrations of BAP and TDZ in combination with 2,4-D (1.0 µM) on adventitious shoot regeneration from petiole derived callus of *M. dioica*.

Plant growth regulators (µM)	% of calluses regenerating into shoots		Mean number of regenerated shoots per explant		Mean shoot length (cm)	
	Male	Female	Male	Female	Male	Female
BAP						
2.0	45.6f	49.2f	9.0g	12.5g	1.4f	2.0f
4.0	59.0d	65.8de	15.5ef	22.0ef	2.2e	3.4d
6.0	72.5b	80.5bc	21.2cd	30.4c	3.1cd	4.0cd
8.0	57.4e	61.0e	13.8f	20.5f	2.0ef	2.8de
TDZ						
2.0	58.0de	67.0d	16.0e	23.0e	3.2c	4.4c
4.0	70.0bc	84.0b	29.4b	36.6b	4.0b	5.2b
6.0	87.5a	93.0a	38.0a	43.0a	5.1a	6.5a
8.0	69.6c	77.0c	22.0c	30.0cd	3.5bc	5.0bc

Each value represents the mean ± SE of 10 replicates per treatment. The data were statistically analyzed using Duncan's multiple range test (DMRT). In the same column, significant differences according to the least significant difference (LSD) at the P = 0.5 level are indicated by different letters. Data were recorded after 3 weeks of culture.

present investigation, female plants produced higher levels of callus induction from petiole explants. Similar results were reported in Chinese gooseberry (Gui, 1979). In contrast, male plants produced more calli when compared to female in *Rumex acetosella* and *R. acetosa* (Culafic et al., 1987).

Adventitious shoot regeneration and elongation

Green compact callus was transferred to MS medium containing different concentrations of cytokinins (BAP and TDZ) and combination with 1.0 µM 2,4-D for adventitious shoot induction (Table 2). The greenish compact callus induced shoot initiation in MS medium containing 6.0 µM TDZ and 1.0 µM 2,4-D (Figure 1b and c). Similarly, TDZ and 2,4-D induced shoots in *Panicum virgatum* (Gupta and Conger, 1998). MS medium supplemented with 6.0 µM TDZ and 1.0 µM 2,4-D produced 38.0 shoots from male explant cultures and 43.0 shoots from female explant cultures after three weeks (Table 2). MS medium supplemented with 6.0 µM BAP and 1.0 µM 2,4-D produced 21.2 and 30.4 shoots from male and female derived cultures respectively. In the present study, TDZ was found to be more effective in shoot regeneration compared to BAP.

The effectiveness of TDZ over other cytokinins has also been reported in other cucurbits such as *Cucurbita pepo* (Pal et al., 2007), *Cucumis sativus* (Zhang and Cui, 2001; Selvaraj et al., 2006a) and *M. charantia* (Thiruvengadam et al., 2010). In the present investigation, female plants produced more shoots compared to male plants. Similar results were observed in other dioecious plants such as *Simmondsia chinensis* (Agrawal et al., 1999; Prakash et al., 2003), *Populus* sp. (Mehra and Cheema, 1985), and *Carica papaya* (De Winnaar, 1988).

The regenerated shoots when cultured in MS medium containing 3.0 µM GA_3 favoured shoot elongation after 1 week culture (Figure 1d and Figure 2). Likewise, GA_3 showed a better response for shoot elongation in *Cucumis sativus* (Selvaraj et al., 2006b), *Melothria maderaspatana* (Baskaran et al., 2009), *Momordica charantia* (Thiruvengadam et al., 2010) and *Trichosanthes anguina* (Ambetkar et al., 2012).

Rooting and hardening

The elongated shoots were transferred to the MS medium supplemented with different concentrations (0.5 - 2.0 µM) of IBA for rooting. A maximum of 12 roots per shoot were obtained in MS medium with 1.5 µM IBA after three weeks culture period (Figure 1e and Figure 3). The effectiveness of IBA in rooting has been reported in *M. dioica* (Hoque et al., 1995; Hoque et al., 2007; Nabi et al., 2002; Thiruvengadam et al., 2006), *Melothria maderaspatana* (Baskaran et al., 2009) *M. charantia* (Thiruvengadam et al., 2010) and *Citrullus colocynthis* (Meena et al., 2010). In contrast, NAA and IBA have been used successfully for *in vitro* rooting of wild as well as cultivated *Cucumis* species (Compton et al., 2001; Selvaraj et al., 2002). 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., 2006). They were then

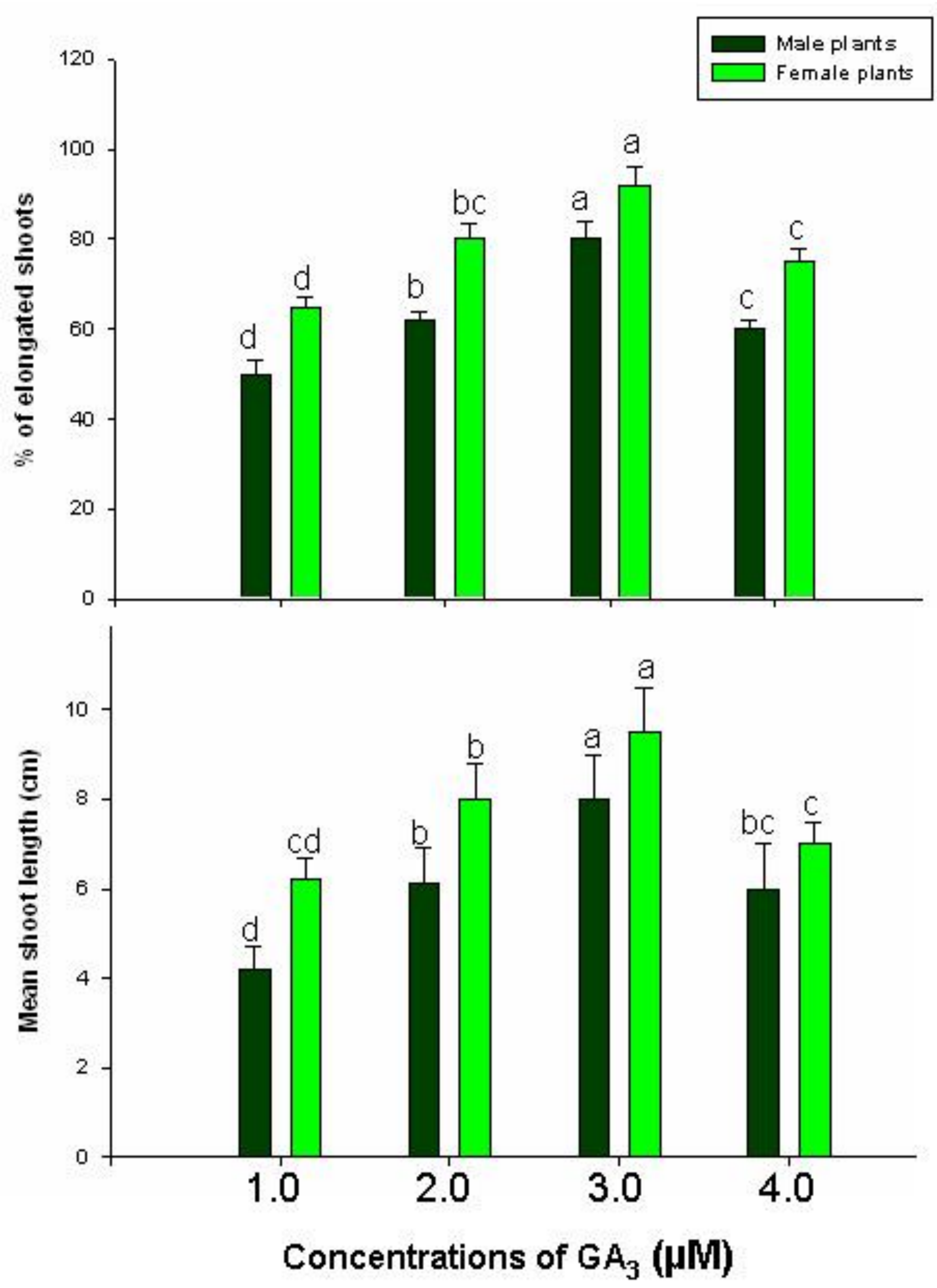

Figure 2. Effect of GA_3 on shoot elongation from regenerated shoots of *M. dioica*. Each value represents the mean ± SE of 10 replicates per treatment. The data were statistically analyzed using Duncan's multiple range test (DMRT). In the same column, significant differences according to the least significant difference (LSD) at the P = 0.5 level are indicated by different letters. The data were recorded after 3 weeks of culture

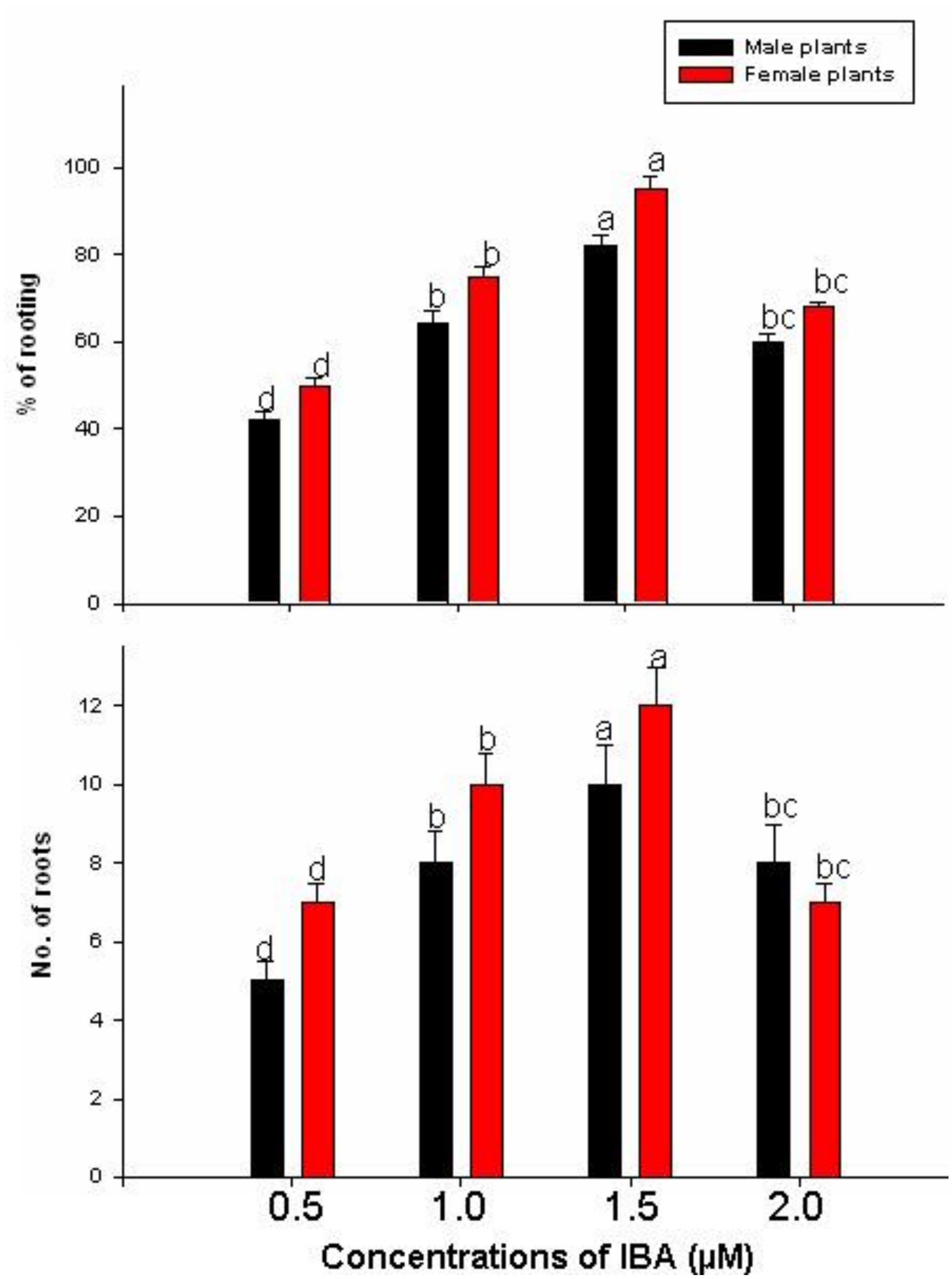

Figure 3 Effect of IBA on root induction of *M. dioica*. Each value represents the mean ± SE of 10 replicates per treatment. The data were statistically analyzed using Duncan's multiple range test (DMRT). In the same column, significant differences according to the least significant difference (LSD) at the P = 0.5 level are indicated by different letters. The data were recorded after 3 weeks of culture.

transferred to plastic vessels containing a sterile soil, sand, and vermiculite mixture (Figure 1f), and after 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 observed 95% survival rate of plants derived from petiole explants when rooted plantlets were transferred from pots to field conditions (Figure 1g). Hoque et al. (2007) reported that 85% survival of female x female clones of *M. dioica*. About 100 plants were tested for the survival rates in different seasons. The survival rate during summer (March - June) was 45 - 50%, whereas in winter (September - December), it was 85%. High temperatures (36 - 43°C) could be unfavorable for the establishment of plantlets in the field, whereas low temperatures (25 - 28°C) during winter could be favorable for establishment. The study therefore suggests that for

M. dioica, hardened micropropagated plants should be transferred to the field only during winter conditions for the best survival rate (95%). Shekhawat et al. (2011) reported that hardening of micropropagated plantlets of *M. dioica* has been the most difficult and require special treatments for hardening/acclimatization, while the plants are hardened, they need physical support (a wood stick was used for this purpose) and require habitat soil to develop a tuberous root system that is prerequisite for survival of the plants in field conditions. Nabi et al. (2002) also observed that the addition of habitat soil during hardening could increase the survival chances in the field.

Regenerated plants transferred to the field became fully established and grew well and were similar to the parental plants in their morphology. In our present investigation, female petiole explants produced higher level of callus induction, adventitious shoot regeneration; shoot elongation, rooting and acclimation of plant survival when compared to male plants. Similar results were reported in other dioecious taxa, namely *Actinidia deliciosa* (Gui, 1979), *Simmondsia chinensis* (Agrawal et al., 1999; Prakash et al., 2003), *Populus* sp. (Mehra and Cheema, 1985), and *Carica papaya* (De Winnaar, 1988). In conclusion, high frequency regeneration of shoots was achieved using petiole explants of spine gourd via indirect organogenesis. MS medium containing 6.0 μM 2,4-D and 2.0 μM BAP favoured organogenic callus induction, 6.0 μM TDZ and 1.0 μM 2,4-D combination induced adventitious shoots from organogenic callus.

About 43 shoots were produced per petiole explants in culture duration of 80 days.

We believe that this regeneration system could be used in the production of transgenic spine gourd plants by *Agrobacterium*- mediated genetic transformation as the protocol would yield higher number of shoots and the chance of recovering transformed plants at a higher frequency may be possible. This protocol thus provides a prolific, rapid and sex specific *M. dioica* propagation system that has opened possibilities for commercial production of female and male plants separately, and *ex situ* conservation of spine gourd.

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