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In vitro regeneration of *Momordica dioica* (Roxb.)

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Abstract: *Momordica dioica,* Roxb. (Family: Cucurbitaceae) commonly called as Kartoli, is an important medicinal plant, which has remained unexplored from the commercial point of view. Considering its scarce availability and the medicinal importance, *in vitro* cultures were established. Traditionally, *M. dioica* has been propagated mainly through its tuberous roots and less commonly by seeds. Germination through seeds is very difficult or impossible because of hard seed coat. As an alternative to traditional methods tissue culture offers an efficient method for propagation of *M. dioica.* Mature seeds were used for the regeneration of *M. dioica*. The decoated seeds of *M. dioica* were cultured on Murashige and Skoog basal medium (MS medium) supplemented with various combinations of Auxins (á – naphthaleneacetic acid) and Cytokinins (N^6 - benzyl adenine). MS basal medium supplemented with 4.44 µM and 8.88µM N^6 - benzyl adenine (BA) gave rise to maximum number of shoots in 7-8 weeks. *In vitro* grown shoots were sub cultured on MS medium supplemented with different concentrations of indole-3-butyric acid (IBA) for root initiation. MS medium with 0.049mM indole-3-butyric acid (IBA) showed rooting in 45 days. The regenerated plantlets were successfully hardened in vermiculite.

Keywords: Hardening, *Momordica dioica,* Multiplication, Rooting, Seed Explants, Shoot Initiation

INTRODUCTION

Momordica dioica, Roxb. (Family: Cucurbitaceae) is an unexploited and nutritionally rich fruit vegetable, commonly called as 'Kartoli' (Kirtikar and Basu, 1918; Sawant, 1993). It is dioecious perennial climber. Tuberous root is a one of the important characteristic feature of *M. dioica*. The plant grows only in the rainy season and its cultivation during the off seasons is still a major challenge. Alkaloids and Triterpenoids are the major components of the plant, which shows antimicrobial (Sadyojatha and Vaidya, 1995), antimalerial (Misra *et al.*, 1991), antitumour and anticancer (Luo *et al.*, 1998) properties, beside these fruits are rich source of protein and carbohydrates. The plant subsists an importance in Ayurvedic medicines as it is used against asthma, leprosy, tumor, jaundice and heart diseases (Kirtikar and Basu, 1918). Traditionally, Kartoli has been propagated mainly through its tuberous roots and less commonly by seeds. Germination through seeds is very difficult or impossible because of hard seed coat (Rashid, 1976). As an alternative to traditional methods tissue culture offers an efficient method for propagation of Kartoli, as it requires only small amount of propagating material and has the potential to provide the large number of cloned plants thus tissue culture offers a viable tool for the *in vitro* propagation of the same species. Considering the medicinal importance of *M. dioica* and its scarce availability, *in vitro* cultures were established. The present study was aimed to establish the standard protocol for organogenesis of the plant *M. dioica*.

MATERIALS AND METHODS

Plant material: The tuberous roots of *M. dioica* collected from Dr. Balasaheb Sawant Krishi Vidyapeeth, Dapoli, were grown in experimental green house of KET's Scientific Research Centre, Mulund. The fresh fruits were used for further tissue culture experiments.

Surface sterilization: Fresh fruits were selected as explants. The fruits were washed thoroughly under running tap water and treated with 2% (w/v) systemic fungicide Bavistin® (BASF India Ltd, India) for 10 min; followed by the wash of water. The same fruits were treated with 3-4 drops of tween-80 for 10 min. The fruits were washed with water and surface sterilized in laminar air flow cabinet by immersing in alcohol flaming. The fruits were cut open aseptically and the seeds are exploited. The seed were decoated and treated with antibiotic Ciplox® (Ahlcon Parenterals India Ltd, India) for 60 sec. The treated are inserted in the medium for shoot development.

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Media and culture conditions for shoot induction: The sterile seed explants were cultured on MS basal medium (Murashige and Skoog, 1962) supplemented with different concentrations and combinations of plant growth regulators: 0.54 µM, 1.08 µM α– naphthalene acetic acid (NAA) and 4.44 µM, 8.88 µM, 13.32 µM, 17.76 µM N^6– benzyl adenine (BA) and 3% (w/v) sucrose (Qualigens, Mumbai). The pH of the media was adjusted to 5.7 prior to the addition of 0.8% (w/v) agar (Bacteriological grade, Qualigens, Mumbai) and media was autoclaved at 121°C, 1.05 kg/cm^2 Pa for 15 min. The cultures were maintained at 24 ± 2 °C under 16 h photoperiod provided by the cool-white fluorescent tubes.

Media and culture conditions for root induction: *In vitro* elongated shoots were individually transferred on MS basal salts supplemented with 3% (w/v) sucrose, 0.8% (w/v) agar and 0.049, 0.148, 0.246, 0.344, 0.443, 0.49 µM IBA, 0.057, 0.171, 0.285, 0.399, 0.514, 0.571 µM IAA. The cultures were incubated at 24 ± 2°C under 16 hr photoperiod provided by the cool - white fluorescent tubes (3000 Lux).

Acclimatization: The plants were removed carefully from culture tubes, washed with water to remove traces of agar. The plantlets were dipped in 2% Diathane® for 10 min and transferred to the pots containing sterile vermiculite. The pots were covered with plastic caps to maintain constant humidity (100%), and allowed to acclimatize in the hardening room at around 24 - 26^0 C temperature for 14hrs photoperiod. During hardening procedures plastic cover was gradually perforated after 10 days followed by the removal of plastic cover after one month. During first 10 days of acclimatization no watering or any fertigation was provided to plants. After the plastic bags are removed, the water was provided to the plant every morning and evening by sprayer only.

The well hardened plants were transferred to the green house for further acclimatization. After 3 months from weaning, the plants were repotted in vermiculite, sand and soil (7:2:1) for further observation.

RESULTS AND DISCUSSION

Shoot initiation: A range of combinations of plant growth regulators was tried to obtain multiple shoot from seed explants of *M. dioica*. The significant number of shoots was obtained from different combination of NAA and BA. Preliminary, it was observed that the embryonic shoot developed first and at the same time it showed bulging at the base. Further, multiple shoot initiation was observed from the bulged portions within 25 days (Fig: 1a). Rao (Rao *et al.*, 1982) obtained shoot regeneration from embryonic axis of *Cucumis melo*. In present investigation maximum shoot initiation was observed on MS medium supplemented with BA (Fig: 1b – 1e) and combination of BA and NAA showed shoot as well as callus initiation (Fig: 1f). Shekhawat (Shekhawat and Shekhawat, 2000) obtained multiple shoot initiation and elongation from stem and nodal explants of *M. dioica* on MS medium with BA+NAA and BA+IAA in addition of adenine sulphate. Hoque (Hoque *et al.*, 1995) found that a combination of 1.5 mg/l BA and 0.1 mg/l NAA was more suitable combination for adventitious multiple shoots formation of *M. dioica*. Whereas in present investigation BA (8.88µM) was found to be most suitable concentration for multiple shoot initiation. The shoot number was calculated from second subculture onwards. MS basal medium supplemented with BA (8.88 µM) gave maximum shoot initiation (93.33%) as well as shoot number (mean - 40.25) (Table 1).

Further increase in the concentration of BAP failed to achieve multiple shoots. The media used for shoot multiplication favored shoot elongation. Hoque (Hoque *et al.*, 1998) failed to elongate the shoot buds induced on the medium when BA and Kn alone were used. There are some reports on shoot elongation in closely related species. The best shoot elongation was observed by Hossain (Hossain *et al.*, 1997) on pointed gourd (*T. dioica*

Table 1. Effect of BA and NAA on shoot initiation from seed explants of *M. dioica*.

No.	Growth regulators/Lit.		Response	% Shoot induction	Mean No. of Shoots ± SE
	NAAµM	BAPµM			
1.	0	4.44	S	83.33	36.06 ± 0.86
2.	0	8.88	S	93.33	40.26 ± 1.00
3.	0	13.32	S	80	29.13 ± 0.69
4.	0	17.76	S	73.33	25.16 ± 0.69
5.	0.54	8.88	C and S	70	20.06 ± 0.75
6.	0.54	13.32	C and S	66.66	15.13 ± 0.70
7.	1.08	4.44	C and S	76.66	19.30 ± 0.44
8.	1.08	8.88	C and S	56.66	12.30 ± 0.55
9.	1.08	13.32	C and S	63.33	13.36 ± 0.64
10.	1.08	17.76	C and S	66.66	18.06 ± 0.63

C and S – Callus and shoots, S - Shoots

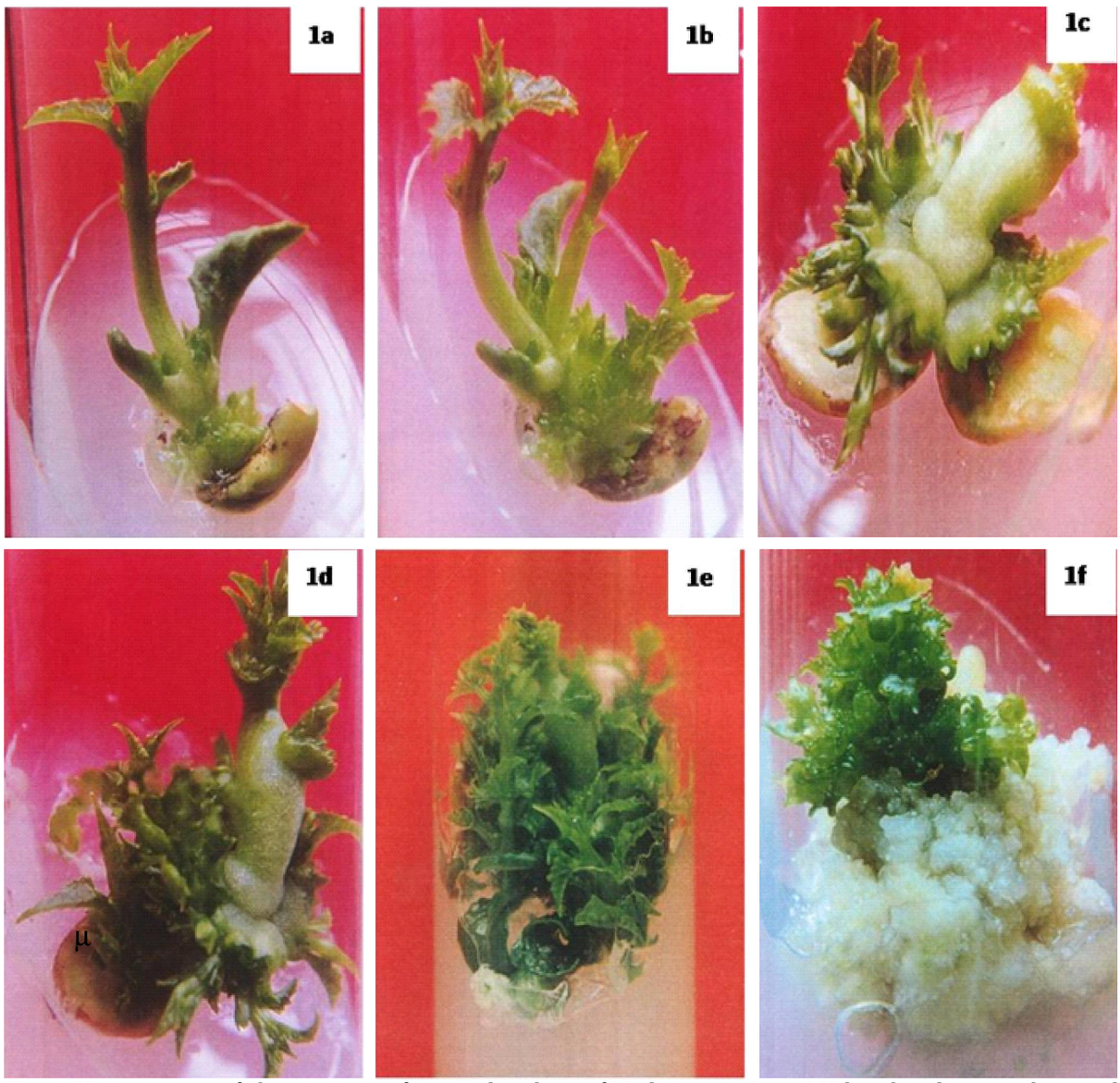

Fig. 1. *Various stages of Shoot initiation from seed explants of M. dioica. 1a & 1c: MS basal medium supplemented with 8.88 µM BA after 1st subculture. 1b & 1d: MS basal medium supplemented with 8.88 µM BA after 2nd subculture. 1e: MS basal medium supplemented with 8.88 µM BA after 3rd subculture 1f: MS basal medium fortified with 8.88 µM BA + 1.08 µM NAA after 1st subculture.*

Roxb) in MS supplemented with 1.0 mg/l BA, 0.1mg/l NAA and 10 mg/l adenine sulphate. Islam (Islam *et al.*, 1994) obtained the higest frequency of shoot formation (78%) with 7.9 shoots per explants in MS supplemented with 2.0 mg/l BA. There are some reports on several related species. Multiple shoot regeneration of *C. melo* using shoot tips as explants in 2.5 mg/l NAA and 1.0 mg/l BA was obtained by Moreno Moreno (Moreno *et al.*, 1985). Halder (Halder and Gadgril, 1982) was able to produce callus from cotyledons and embryo axis in squash (*Cucumis melo*) in MS supplemented with 2.0 mg/l NAA, and 15 % coconut milk and adventitious shoot and roots from 1mg/l NAA. Raut (Raut and Heble, 2001) observed multiple shoots with callus at the base from *P. amarus* on MS medium with BA (4.44 µM) + NAA (0.54). The callus initiation at the base of multiple shoots was avoided by elimination of NAA and the shoot cultures were maintained on the MS with BA (4.44 µM). Where as in present findings MS medium in combination with BA and NAA showed callus and shoot initiation while MS medium with BA showed only shoot initiation. Jerzy (Jerzy and Lubomski, 1991) and Guo (Guo and Goi, 1998) obtained shoot initiation of *Gerbera* and *Gloriosa* on MS medium supplemented with BA (13.32-17.76 µM).

Fig. 2. 2a-2c. *Effect of plant Growth Regulators on rooting of M. dioica.2d-2f: Hardening of in vitro plantlets of M. dioica.*

Thus the present investigation reveals that 8.88 µM BA is the best combination for the shoot multiplication and elongation obtain from seeds of *M. dioica*.

Root induction: *In vitro* elongated shoots were transferred on MS basal medium with different concentrations of IBA (0.049, 0.148, 0.246, 0.344, 0.443, 0.49 µM) and IAA (0.057, 0.171, 0.285, 0.399, 0.514, 0.571 µM) showed root induction (Table 2). MS with 0.443, 0.49 µM IBA and 0.514, 0.571 µM IAA showed optimum rooting 84.37%, 86.87%, 83.12% and 85% respectively but it failed to achieve tuberous root formation. Whereas MS basal medium with decreased concentration of IBA (0.246, 0.344µM) and IAA (0.285, 0.399 µM) showed significant rooting 85%, 82.5% and 83.75%, 81.25% respectively with tuberous root formation. Further reduction in the concentration of IBA (0.049, 0.148 µM) and IAA (0.057, 0.171µM) gave 64 - 67% rooting but it failed to achieve the tuberous root formation. Among the various concentrations of IBA and IAA, MS with IBA 0.246 µM (1.9 mean no. of root) and IAA 0.285 µM

Table 2. Effect of plant growth regulators on *in vitro* root initiation from elongated shoots of *M. dioica*.

Media (MS basal medium)		% of root induction	Days required for root induction	Mean no. of root ± S.C.
IBA µM/Lit	IAA µM/ Lit			
0.049	-	64.37	45	1.25 ± 0.07654
0.148	-	66.25	37	1.43 ± 0.08769
0.246*	-	85.00	32	1.90 ± 0.11144
0.344*	-	82.50	29	1.75 ± 0.10825
0.443	-	84.37	23	1.71 ± 0.101108
0.49	-	86.87	19	1.81 ± 0.120522
-	0.057	65	52	1.31 ± 0.094583
-	0.171	66.87	48	1.18 ± 0.0701
-	0.285*	83.75	35	1.84 ± 0.11971
-	0.399*	81.25	31	1.68 ± 0.1047
-	0.514	83.12	29	1.71 ± 0.11211
-	0.571	85	21	1.56 ± 0.11826

(* Indicate that plant let grown on the medium showed formation of tuberous root after hardening)

Table 3. Effect of various potting mixtures on hardening of *in vitro* rooted plantlets of *M. dioica* on IBA and IAA.

Medium	Potting Mixture	% Hardening	Days required for tuber root formation	Increase in shoot length after 2 months
MS+ IBA 0.246 µM/Lit	Vermiculite + Soil + Sand	62	-	2.32
	Vermiculite + Soil	46	-	1.68
	Vermiculite	72	55-65	2.84
MS+IAA 0.285 µM/Lit	Vermiculite + Soil + Sand	58	-	2.30
	Vermiculite + Soil	40	-	2.13
	Vermiculite	68	70-80	2.63

Table 4. Effect of plant growth regulators on hardening of the rooted plantlets of *M. dioica.*

Medium/ Lit		% Hardening	Days required for 1st leaf formation	Days required for tuber root formation
IBAµM	IAAµM			
0.246	-	73.3	20	62
0.344	-	46.6	28	81
-	0.285	66.6	24	74
-	0.399	53.3	32	87

(1.84 mean no. of root) proved to be the best media combination for most favorable root initiation (Fig. 2a – 2c). A similar effect of IBA was observed in *Zingiber officinale* (Rout *et al.*, 2001a), *Lawsonia inermis* (Rout *et al.*, 2001b), *Anisomeles indica* L. (Britto *et al.*, 2001), *Ceropegia bulbosa* Roxb. (Britto *et al.*, 2003).

Hoque (Hoque *et al.*, 1998) obtained 7 – 11 roots from *in vitro* shoots of *M. dioica* on ½ strength MS supplemented with 1.0mg/l IBA within 15-20 days. Nabi (Nabi *et al.*, 2002) obtained highest number of roots (2.80) from *in vitro* shoots of *M. dioica* on MS supplemented with 0.3mg/l IBA. The experimentation showed that IBA is better than IAA in terms of root initiation ability. The present investigation reveals the same observation but it was observed that the concentrations of IBA as well as IAA were less that is IBA [0.049 - 0.49 µM (0.01-0.1mg/l)] and IAA [0.057 - 0.571 µM (0.01-0.1 mg/l)]. The average number of roots observed on MS with IBA is 1.53 as well as on IAA is 1.46. The variation in root number could be due to the genotypic variation of the explants along with the cultural and Environmental conditions.

Acclimatization: *In vitro* plants were hardened successfully in 100% vermiculite. During the hardening process initially the plants were kept under plastic cover in which relative humidity was nearly 100% for 10 days. After 2 weeks, humidity was gradually lowered down by

perforating the plastic caps of *in vitro* plants. Similar process of maintaining humidity was practiced for hardening of Banana (Jasrai *et al.*, 1999) and *Alpinia* (Rolf and Ricardo, 1995). During the hardening process plants developed an efficient root system, fully expanded leaves and become photosynthetically active (Fig 2d-2f).

In present investigation it was observed that fully grown leaf pair developed after 20 days of hardening of *M. dioica*. The hardened plantlets were showed tuberous root formation after 1½ - 2 months. The repotting of the plant showed healthy growth in vermiculite, sand and soil (7:2:1).

Rooted plantlets of *M. dioica* on MS medium with IBA 0.246µM and IAA 0.285µM were transferred in different potting mixtures. Among the three potting mixtures utilized for hardening procedure, the better survival percentage was found in vermiculite (68 - 72%) as compared to vermiculite + Soil 1:1 and vermiculite + soil + sand 7:1:2 (40 - 46% and 58 - 62% respectively) (Table 3). In vermiculite the new leaf pair was formed after 20 - 24 days, contrary to this in vermiculite + Soil (1:1) and vermiculite + Soil + Sand (3:1:1) the new leaf pair appeared after about 27 - 35 days.

Patel (Patel *et al.*, 2000) found 95% survival of *in vitro* plants of *M. dioica* in soil: soilrite (3:1) mixture. On the other hand Parulekar, 1994 used different potting mixture for hardening of *M. dioica* like soil, Farm Yard Manure (FYM), sand, soil + FYM, soil + sand, soil + vermiculite, soil + sand + FYM. He observed maximum 63% survival in vermiculite. In the present investigation vermiculite proved to be the suitable mixture for hardening of rooted plantlets of *M. dioica.*

Further, the rooted plantlets on MS medium with IBA (0.246, 0.344µM) and IAA (0.285, 0.399µM) were transferred to vermiculite for hardening. All the plants were hardened properly. The rooted plantlets on IBA 0.246 µM showed tuberous root formation after 62 days. Whereas IBA 0.344µM, IAA 0.285µM and IAA 0.399µM showed tuberous root formation after 81, 74 and 87 days respectively (Table 4). Britto (Britto *et al.*,2003) obtained good results of hardening of *Ceropegia bulbosa* in vermiculite. About 20 microtubers were harvested from shoot weighted approximately 600 mg. Wala and Jasrai, (Wala and Jasrai, 2003) used vermiculite in combination with soil (1:1) for hardening of *Curculigo orchioides*. Saha (Shaha *et al.*, 2003) obtained tuberous root formation from *in vitro* hardened plants of *Hemidesmus indicus*. *In vitro* shoots were rooted on MS medium with IBA. Root formation occurred from cut ends of the shoots. It was observed that the tuberous roots were developed after one month.

Conclusion

Plant tissue culture has been viewed as a key technology to enhance the capacity for the production of large qualities of planting material of elite varieties and the technology is also being exploited for the production of secondary metabolites from elite plant material.

The *seeds* obtained from fresh fruit of *M. dioica* showed good source of planting material for the *in vitro* multiplication. Ms medium with 4.44 µM and 8.88 µM BA showed shoot initiation and increased concentration of BA showed shoot initiation with callus formation at the base of explants whereas the combination of NAA and BA showed shoot as well as callus initiation simultaneously. MS 8.88 µM BA showed maximum multiple shoot initiation of 93.33% and shoot number obtained from seed was maximum i. e. (40.25).

The multiple shoots obtained from seed explants of *M. dioica* were transferred on rooting medium for root initiation. MS medium in combination with Auxins, IBA and IAA proved that IBA showed maximum percentage of root induction (85%) than that of IAA (83.75%). The rooted plantlets were transferred to the different potting mixtures for hardening. During this process plant develops its root system. Vermiculite is found to be the suitable potting mixture for the hardening of *in vitro* rooted plantlets of *M. dioica*. The rooted plantlets obtained on IBA 0.246 µM were hardened rapidly as compared to the plantlets obtained on other media combinations. The first leaf occurred after 20 days of hardening, and the tuberous root formation took place after 62 days.

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