



# An improved micropropagation system, ex vitro rooting and validation of genetic homogeneity in wild female *Momordica dioica*: an underutilized nutraceutical vegetable crop

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**Abstract** *Momordica dioica* Roxb. ex Willd., is a perennial and dioecious ( $2n = 28$ ) plant of family Cucurbitaceae. Conventional methods of propagation through seeds, stem cuttings and rhizomatous/tuberous roots are inadequate for its mass cultivation as a vegetable crop. This paper reports an improved and efficient micropropagation method for wild female *M. dioica* using nodal explants. Shoot amplification was achieved using subculturing of in vitro raised shoots on MS medium supplemented with various concentrations of 6-benzylaminopurine (BAP) alone or in combination with indole-3-acetic acid (IAA). The maximum number of shoots ( $45.30 \pm 3.83$ ) with an average length  $6.52 \pm 0.89$  cm were differentiated on MS medium containing  $0.5 \text{ mg L}^{-1}$  BAP,  $0.1 \text{ mg L}^{-1}$  IAA and additives ( $50 \text{ mg L}^{-1}$  ascorbic acid,  $25 \text{ mg L}^{-1}$  each of adenine sulphate, citric acid and L-arginine). The cloned shoots were rooted ex vitro. Each shoot treated with  $250 \text{ mg L}^{-1}$  IBA for 5 min produced  $12.3 \pm 1.33$  with a mean length  $5.4 \pm 0.73$  cm. More than 85% (46 plants) of ex vitro rooted plantlets were successfully hardened in a greenhouse with normal growth characteristics. In order to evaluate the genetic stability of micropropagated plants, the two PCR-based techniques, Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence

Repeats (ISSR) were used. The amplification patterns of the micropropagated and mother plant were monomorphic thus depicting genetic stability of the micropropagation system. This protocol could be effectively employed for the mass multiplication of wild female *M. dioica*, a popular summer vegetable crop.

**Keywords** Cucurbitaceae · Ex vitro rooting · Genetic homogeneity · Micropropagation · Spine gourd

## Abbreviations

BAP	6-benzylaminopurine
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
ISSR	Inter Simple Sequence Repeat
Kin	Kinetin
MS	Murashige and Skoog (1962)
NOA	Naphthoxyacetic acid
PCR	Polymerase chain reaction
PFD	Photon flux density
PGRs	Plant growth regulators
RAPD	Random Amplified Polymorphic DNA
RH	Relative humidity

## Introduction

*Momordica dioica* Roxb. ex Willd., is a nutraceutical plant (Nagarani et al. 2014; The Plant List 2016), commonly known as Spine gourd in English and Kakoda in Hindi (Sinha 1997). Fruits of this plant are known for the highest amount of carotene (162 mg/100 g of edible portion) amongst the cucurbits. Fruits are also rich in fiber, proteins, vitamin (ascorbic acid, thiamine, riboflavin and niacin), fatty acid (oleic acid and linoleic acid) and minerals such

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as calcium, magnesium, phosphorous, iron and iodine. (Ram et al. 2001; Bharathi et al. 2007). According to Salvi and Katewa (2015), the fruit of Spine gourd could be promoted as a vitamin and mineral source for cereal-based diets in poor rural societies. The fruits are available from August to February, and are used as a popular summer vegetable (Rana and Das 2016). Beside fruits, young twigs and leaves are also used as a vegetable (Bandyopadhyay and Mukherjee 2009). This plant is distributed throughout the Indian subcontinent including Myanmar, Java, Yunnan, and peninsular Malaysia (Catalogue of Life 2016). According to Bawara et al. (2010), Indo–Malayan region is the centre of origin of *M. dioica*.

Luo et al. (1998) isolated five compounds (three triterpenes and two steroids) from *M. dioica* roots, which exhibited anticancer activity on cancer cell line (L1210) in a pharmacological testing. Recently, Sukumar et al. (2016) suggested that the crude methanolic seed coat extract of spine gourd is a potential source of natural anti-cancerous agent. Extracts of plant parts of *M. dioica* are loaded with numerous medicinal properties that are antioxidant, analgesic, nephroprotective, neuroprotective, antiallergic, antiulcer, antimicrobial, antidiabetic, antimalarial, anti-inflammatory, hepatoprotective, antifertility, antiedemic, antifeedant, insecticidal, grain protectant, allelopathic (Talukdar and Hossain 2014), antipsychotic (Rakh and Chaudhari 2010), antilipid peroxidative (Rao and Mohan 2016), etc.

Traditionally this plant is propagated through the seeds, stem cuttings and rhizomatous/tuberous roots (Rai et al. 2012a). Dormancy and unpredictable sex ratio are two major problems associated with the propagation through seeds (Ali et al. 1991; Mondal et al. 2006). Stem cuttings are prone to transfer of diseases and are also not much successful as only 36% survival was observed by Ram et al. (2001). Owing to late accessibility of stem cuttings in fruiting season and insufficient availability of tuberous roots, profitable propagation are critically restricted (Rai et al. 2012a). Also, low multiplication rate (Mondal et al. 2006) and engaging the precious cultivable land (until next planting season; Ram et al. 2001; Nabi et al. 2002) are another hurdles in propagation through the tuberous roots. According to Thiruvengadam et al. (2012), male plants are dominant in a natural population and sex determination is possible only when the plants start to flower. Since female/fruitful plants are commercially important, it is desirable to have high ratio of female to male plant (Rasul et al. 2007). Recently, Mohanty et al. (2016) recognized *M. dioica* as a strategic plant for studying the early evolution of sex in angiosperms. An Sequence Tagged Site (STS) marker associated with male specific sex expression was identified by Mohanty et al. (2016) owing to the sex-specific economic and medicinal values of spine gourd. Moreover,

Patil et al. (2012) identified a RAPD based SCAR (Sequence Characterized Amplified Region) marker for sex identification in *M. dioica*.

As the traditional methods of propagation of wild female *M. dioica* have constraints, an efficient micropropagation system is prerequisite for the large scale multiplication, especially in case of sex specific plants (Thiruvengadam et al. 2012). During the last two decades, extensive efforts have been made on in vitro propagation of spine gourd using different explants and modes of regeneration (Hoque et al. 2000; Thiruvengadam and Jayabalan 2001; Thiruvengadam et al. 2006). Earlier, Hoque et al. (1995) reported shoot regeneration using hypocotyls explants (8.8 shoots). Nabi et al. (2002) evaluated four types of explants (node, shoot tip, leaf and cotyledon), out of which cotyledons showed the best response (25.33 shoots). Later, Shekhawat et al. (2011) achieved  $29.2 \pm 0.78$  shoots (per vessel) and Rai et al. (2012a) achieved 6.2 shoots per explant via axillary shoot proliferation of nodal segments. Thiruvengadam et al. (2012; 2013) reported 43 shoots per explant using petiole derived callus and a protocol for somatic embryogenesis via suspension cultures of leaf derived callus in *M. dioica*, respectively. In addition, Thiruvengadam et al. (2016) reported the induction of hairy roots in spine gourd by *Agrobacterium rhizogenes* mediated transformation and confirmed the greater potentiality of hairy roots than normal roots for the production of valuable phenolic compounds.

The aim of present study was to further improve the method for large-scale propagation of wild female *M. dioica* growing in Rajasthan. The multiplication rate obtained in the present study is higher than the all previous reports on the same taxon. For making the protocol cost and labor effective, efforts have been made to standardize the optimum conditions for concurrent ex vitro rooting and hardening. To evaluate the genetic homogeneity of micropropagated plants; two PCR-based fingerprinting techniques (RAPD and ISSR) were used. To the best of our knowledge, there is no any other report available pertaining to the detection of genetic homogeneity in micropropagated *M. dioica* using ISSR markers.

## Materials and methods

### Explant preparation and surface sterilization

Wild female plants of *M. dioica* were identified by presence of Kakoda fruits growing on undisturbed hedges of agricultural fields in Pali (a district of western Rajasthan, India). A plant with superior morphological characters was uprooted along with tuberous root and potted in greenhouse

of Biotechnology Unit, Jai Narain Vyas University, Jodhpur. This was maintained as a mother stock for source of explants. Nodal shoot segments (8–10 cm having 2–3 nodes) were prepared from fresh sprouts harvested from the greenhouse maintained plant. Prior to surface sterilization, the cut ends of shoot segments were sealed with molten wax (Alpha Chemika, Mumbai) by giving a quick dip. These shoot segments were initially treated with 90% ethanol for 30–40 s and then surface sterilized using 0.1% (w/v)  $\text{HgCl}_2$  (HiMedia<sup>®</sup>, Mumbai, India) followed by rinsing with autoclaved water (5–6 times). The ends of wax sealed shoot segments were removed (1 cm) and two explants (3–4 cm having 1–2 node) were made from each shoot segments under the laminar air flow hood.

### Culture initiation and culture conditions

The explants were vertically inoculated on agar gelled [0.8% (w/v) bacteriological grade, Qualigens Fine Chemicals, Mumbai, India] MS (Murashige and Skoog 1962) medium having sucrose 3% (w/v) and supplemented with 0.25, 0.5 or 1.0  $\text{mg L}^{-1}$  BAP alone or in combination with 0.1  $\text{mg L}^{-1}$  IAA (all PGRs were from HiMedia<sup>®</sup> Mumbai, India). In addition, additives namely 50  $\text{mg L}^{-1}$  ascorbic acid, 25  $\text{mg L}^{-1}$  each of adenine sulphate, citric acid and L-arginine (HiMedia<sup>®</sup>, Mumbai, India) were also incorporated in medium. The cultures were incubated in a culture room having temperature  $26 \pm 2$  °C, light intensity  $40\text{--}50 \mu\text{mol m}^{-2} \text{s}^{-1}$  photon flux density [PFD; provided by cool and white fluorescent tubes (Philips India Ltd, Mumbai)] for 12–14 h photoperiod and 55–60% relative humidity (RH).

### Shoot multiplication and maintenance of cultures

The cultures were multiplied by two methods: (1) repeated transfer of mother explants (after excising the first crop of sprouts) for three passages at an interval of 3–4 week each; (2) subculturing of in vitro raised shoots (after making a group of 4–5 shoots). In both methods, fresh MS medium with cytokinins (0.1, 0.25 or 0.5  $\text{mg L}^{-1}$  BAP or Kin) alone or in combinations with 0.1  $\text{mg L}^{-1}$  IAA and additives were used. The shoot cultures were maintained for 4 years (2012–16) in culture room of Biotechnology Unit, Jai Narain Vyas University, Jodhpur, Rajasthan.

### Ex vitro rooting and acclimatization

Microshoots (5–6 cm) were individually excised, rinsed carefully with sterile water and dipped in freshly prepared auxins (50, 100, 250 or 500  $\text{mg L}^{-1}$  IBA or NOA) for different time durations (1, 3, 5, 7 or 9 min). The auxin-treated shoots were planted in polycarbonate-capped glass

bottles (420 mL; 70 mm diameter  $\times$  130 mm height; Siddhivinayak Glass Concepts, Firozabad, India) containing steam sterilized ( $1.1 \text{ kg cm}^{-2}$ , 121 °C for 45 min) Soilrite<sup>®</sup> [a mix of expanded perlite (horticulture grade), Irish peat moss, and exfoliated vermiculite (1:1:1), Keltech Energies Limited, Bengaluru, India], and moistened with quarter strength of aqueous MS salts solution. The bottles were kept initially near pad section (RH 80–90% and temperature  $28 \pm 2$  °C) of the greenhouse for acclimatization.

### Hardening and soil transfer

The rooted plantlets were gradually acclimatized by loosening polycarbonate-caps of glass bottles over a period of 2–3 weeks and completely removed thereafter. Simultaneously, the bottles were also shifted from pad section to fan section (RH 40–50% and temperature  $36 \pm 2$  °C) of the greenhouse for continuous hardening. The hardened plants (15–20 cm long) were transplanted to earthen pots containing sandy habitat soil and farm yard manure (FYM) in 2:1 (v/v) ratio. These were kept near fan section of greenhouse for next 2–3 weeks and finally transferred to the nursery.

### Assessment of genetic stability of micropropagated plants

The genetic homogeneity of micropropagated plants and donor plant were analyzed using polymerase chain reaction (PCR)-based DNA primers, RAPD and ISSR. Young/juvenile leaves of fourteen randomly selected micropropagated plants and donor plant (maintained in nursery) were used for genomic DNA isolation using cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1990). The concentration of genomic DNA was quantified by A260/A280 absorbance ratio using spectrophotometer (SL 164 Double Beam; UV-VIS Spectrophotometer, Elico Hyderabad, India) and quality/purity estimation were done using a 0.8% submerged agarose gel electrophoresis (AE-6125/6133 ATTO, Japan). For genetic stability testing, 16 each of RAPD and ISSR primers were screened.

### RAPD analysis

RAPD amplification was performed in a reaction mixture of 15  $\mu\text{l}$  containing 2.5  $\mu\text{l}$  of template DNA (50–60 ng), 1.5  $\mu\text{l}$  of  $10 \times$  PCR buffer [100 mM Tris (pH 9.0), 500 mM KCl, and 1% Triton X-100 with 15 mM  $\text{MgCl}_2$ ; GeNei<sup>TM</sup>, Bangalore, India), 1.5  $\mu\text{l}$  of  $\text{MgCl}_2$  (2.5 mM; GeNei<sup>TM</sup>, Bangalore, India), 0.3  $\mu\text{l}$  of dNTPs (10 mM; GeNei<sup>TM</sup>, Bangalore, India), 1.2  $\mu\text{l}$  of RAPD (10  $\mu\text{M}$ ; Operon Technologies, Alameda, California), 0.3  $\mu\text{l}$  of Taq

polymerase (5 unit; GeNei™, Bangalore, India) and 7.7 µl sterile distilled water. The samples were amplified in a thermal cycler (Eppendorf 5331, Germany) using the program adopted by Rathore et al. (2014a).

### ISSR analysis

For ISSR (UBC series, University of British Columbia, Vancouver, Canada) amplification, PCR was performed in a 15 µl reaction mixture having the same composition as described for RAPD primers. For PCR amplification, thermal cycler was programmed as described by Agarwal et al. (2015).

The annealing temperature ( $T_a$ ) was kept 2 °C below the melting temperature ( $T_m$ ) of all the screened primers (RAPD or ISSR). The amplified samples were stored at 4 °C until further analysis. All the PCR products were separated electrophoretically on 1.4% agarose gel (A9539, Sigma, St Louis, MO, USA) using 1 × TBE (Tris buffer, Boric acid, EDTA) buffer and stained with ethidium bromide (0.25 µg ml<sup>-1</sup>). The size of the amplified bands was determined using 100 bp DNA ladder (GeNei™, Bangalore, India) and gels were picturized using a gel documentation system (Syngene Gel Doc, Syngene, Synoptics Ltd, UK).

### Experimental design and statistical analysis

All the experiments were designed in a randomized block design (RBD) for single factor analysis and repeated three times with a minimum of 20 replicates per treatment. The data were analyzed statistically using SPSS ver 17 (SPSS Inc., Chicago, USA) and the results were expressed as mean ± SD of three independent experiments. Observations were made on the basis of morphological characters like number, length and quality of shoots/roots per culture vessel over a period of 1–4 weeks as per the experiment designed. The significance of differences among mean values were carried out using Duncan's multiple range test (DMRT;  $P < 0.05$ ). For genetic stability testing, amplification with each primer was repeated two times for verifying the results. The PCR amplified bands were manually scored in form of "1" for presence and "0" for absence. Only clear/well resolved and reproducible bands ranging from 200 to 1200 bp were recorded.

## Results and discussion

### Establishment of axenic cultures

We established axenic cultures of wild female *M. dioica* using fresh/juvenile nodal explants. The explants collected

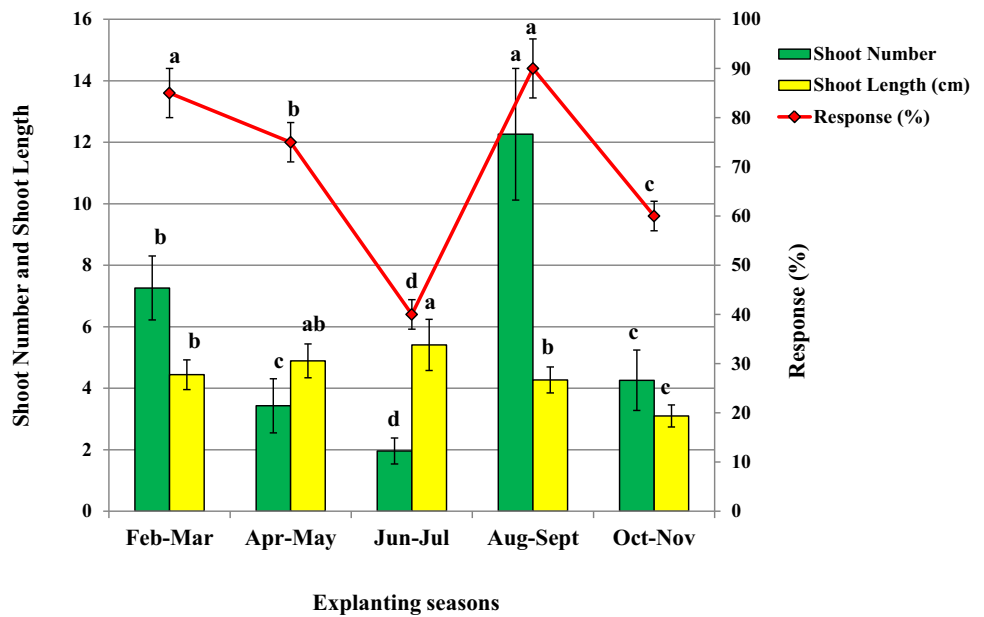
during the months of August–September 2011 (just after the monsoon season in Rajasthan, India) were the best and exhibited more than 90% bud breaking (Fig. 1). Nevertheless, the explants collected during the rest of season(s) also showed bud breaking but exhibited comparatively lower response. Phulwaria et al. (2011) described that during winter and summer seasons, certain growth arresting factors may accumulate in plants which are diluted/degraded in rainy season and results in a better culture initiation. Owing to the soft/delicate and herbaceous nature with large sized vessels, the stem was very sensitive to the physical and chemical injuries during sterilization. To overcome this problem, we carefully handled the shoot segments and sealed the cut ends with molten wax as method developed and described by Shekhawat et al. (2011) for surface sterilization of *M. dioica*.

### Shoot bud induction, multiplication and maintenance of cultures

Nodal explants obtained from greenhouse maintained wild female *M. dioica* sprouted within 4–5 days of inoculation. The shoot number/length varied with the type and concentration of PGRs tested. The explants inoculated on MS medium with 0.5 mg L<sup>-1</sup> BAP and 0.1 mg L<sup>-1</sup> IAA produced the highest number of shoots (12.26 ± 1.04) with a mean length 4.27 ± 0.26 cm, after 4 weeks of inoculation (Table 1 and Fig. 2a). Kinetin was not evaluated for shoot bud induction as many earlier reports suggests that BAP is a superior cytokinin for cucurbits such as *Momordica charantia* (Agarwal and Kamal 2004), *Colocynthis citrullus* (Ntui et al. 2009), *Citrullus lanatus* (Ganasan and Huyop 2010), etc. These results are in accordance with Moon et al. (2000), where the "Tongilwand" (an oriental melon cultivar) exhibited the optimum shoot bud induction response on 0.5 mg L<sup>-1</sup> BAP and 0.1 mg L<sup>-1</sup> IAA. Our results are in contrast with Shekhawat et al. (2011) on the same plant, where 2.0 mg L<sup>-1</sup> of BAP gave the optimum response. In the present study, we observed that BAP concentration beyond 0.5 mg L<sup>-1</sup> produced callus at the base of explants and decreased shoot number, as reported by Rai et al. (2012a) as well.

During shoot multiplication through repeated transfer of mother explants, the maximum number of shoots (5.1 ± 0.73) with mean length (4.6 ± 0.61 cm) was produced on MS medium containing 0.5 mg L<sup>-1</sup> BAP, 0.1 mg L<sup>-1</sup> IAA and additives, after the first repeated passage (Figs. 2b and 3). Although shoots were formed after the second and third passages, however their number decreased with each passage. This method of shoot multiplication has also been used in *Cadaba fruticosa* (Lodha et al. 2015) and *Blyttia spiralis* (Patel et al. 2016). In the second approach, in vitro raised shoots were multiplied through subculturing.

**Fig. 1** Effect of explanting seasons on culture establishment in terms of percentage response, number and length of shoots. Error bars indicate SD. Mean values sharing the same letter do not differ significantly ( $P < 0.05$ ) according to Duncan's multiple range test



**Table 1** Effect of plant growth regulators (PGRs) and their concentrations on bud breaking from nodal explants of wild female *M. dioica*

Concentrations of PGRs		Shoot number Mean ± SD	Shoot length (cm) Mean ± SD
BAP (mg L <sup>-1</sup> )	IAA (mg L <sup>-1</sup> )		
Control	0.0	1.33 ± 0.48 <sup>g</sup>	6.00 ± 0.20 <sup>a</sup>
0.25	0.0	4.13 ± 0.89 <sup>f</sup>	4.69 ± 0.35 <sup>b</sup>
0.5	0.0	6.93 ± 1.28 <sup>d</sup>	4.40 ± 0.26 <sup>b</sup>
1.0	0.0	8.86 ± 1.27 <sup>c</sup>	2.51 ± 0.18 <sup>e</sup>
0.25	0.1	5.93 ± 0.82 <sup>e</sup>	3.92 ± 0.44 <sup>d</sup>
0.5	0.1	12.26 ± 1.04 <sup>a</sup>	4.27 ± 0.26 <sup>c</sup>
1.0	0.1	10.16 ± 1.48 <sup>b</sup>	2.69 ± 0.15 <sup>e</sup>

Medium: MS + additives

The same letters within a column are not significantly different at  $P < 0.05$  according to DMRT

Out of the various concentrations and combinations of PGRs tested; MS medium containing 0.5 mg L<sup>-1</sup> BAP, 0.1 mg L<sup>-1</sup> IAA and additives proved to be the best. On this medium, the maximum number of shoots (45.30 ± 3.83) with a mean length (6.52 ± 0.89 cm) was produced, after 5 weeks (Table 2 and Fig. 2c). The shoot multiplication achieved in this study was superior to the all earlier reports on the same taxon (Hoque et al. 1995, 2000; Thiruvengadam and Jayabalan 2001; Nabi et al. 2002; Thiruvengadam et al. 2006; Shekhawat et al. 2011; Rai et al. 2012a; Thiruvengadam et al. 2012; Patel and Ishnava 2015).

In this study, we observed that incorporation of an auxin (IAA) at a lower concentration (0.1 mg L<sup>-1</sup>) significantly increased the number of shoots by promoting axillary

branching. There are several researches indicating that an appropriate auxin(s)-cytokinin(s) combination is mandatory for a better shoot proliferation/growth (Phulwaria et al. 2014; Masondo et al. 2015; Patel et al. 2016). Su and Zhang (2014) explained that plant regeneration is not individually regulated by either auxins or cytokinins alone but it is the product of a complex mechanism of inter-connecting hormonal signaling pathways. Shimizu-Sato et al. (2009) and Su et al. (2011) have also described auxins–cytokinins crosstalk mechanism depicting that cytokinins promote stem cell proliferation in shoot meristem and inhibit its differentiation, whereas auxin triggers organ primordium formation through suppressing cytokinin biosynthesis, and these in turn regulates shoot meristem development and shoot branching.

### Ex vitro rooting and acclimatization

Of the various treatments studied, the maximum ex vitro rooting response (90%) was achieved with 250 mg L<sup>-1</sup> IBA (treated for 5 min) on which 12.3 ± 1.33 roots of an average length 5.4 ± 0.73 cm were produced after 4 weeks (Fig. 2d and Table 3). IBA beyond 250 mg L<sup>-1</sup> resulted in callus induction at the base of shoots while lower concentration gave poor rooting response. Similarly, NOA was also found less efficient for ex vitro rooting in comparison to IBA. This study reveals the advantageous role of IBA in rooting and is in accordance with many preceding reports on different plants such as *Salvadora oleoides* (Phulwaria et al. 2014), *Cadaba fruticosa* (Lodha et al. 2015), *Blyttia spiralis* (Patel et al. 2016), etc. This is the first successful report of ex vitro rooting in *M. dioica* and probably in family Cucurbitaceae with a significant response rate (90%). However, a moderate success



**Fig. 2** In vitro propagation and ex vitro establishment of *M. dioica*. **a** Shoot bud induction from nodal explant on MS + BAP (0.5 mg L<sup>-1</sup>) + IAA (0.1 mg L<sup>-1</sup>) and additives. **b** Shoot multiplication by repeated transfer of mother explant on MS + BAP (0.5 mg L<sup>-1</sup>) + IAA (0.1 mg L<sup>-1</sup>) and additives. **c** Shoot multiplication by subculturing of in vitro raised shoot-clumps on MS + BAP (0.5 mg

L<sup>-1</sup>) + IAA (0.1 mg L<sup>-1</sup>) and additives. **d** Ex vitro rooted shoot in Soilrite® on pulse treatment with IBA (250 mg L<sup>-1</sup>) for 5 min. **e** Ex vitro rooted plantlets undergoing acclimatization in the greenhouse. **f** Successfully hardened plants of wild female *M. dioica* maintained in the nursery, formation of tuber in next year (inset)

(only 34%) has been achieved by Shekhawat et al. (2011). Use of quarter strength of aqueous MS salts (instead of full strength MS) provided starvation to develop roots, as updates are available on molecular determinants of low nitrogen availability-specific regulators that favor primary and lateral root growth/development (Kiba and Krapp 2016). Ex vitro rooting is more beneficial than in vitro rooting in terms of cost, time, labor and chemical consumption; and the resultant plants get concurrently acclimatized (Hatzilazarou et al. 2006; Agarwal et al. 2015; Kitto 2015; Bohra et al. 2016).

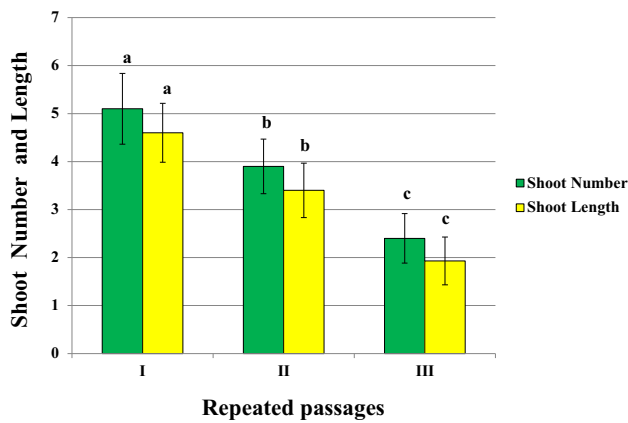
### Hardening and soil transfer

For a successful photoautotrophic acclimatization, a transitional environment (RH, temperature, light, etc.) is prerequisite for one to several weeks (Xiao and Kozai 2004;

Perveen et al. 2013). The differential regimes of RH and temperature between pad and fan section of the greenhouse coupled with gradual unscrewing of the bottles supported acclimatization (Fig. 2e). Above 85% (46 plants) of ex vitro rooted plantlets were successfully hardened and transplanted to earthen pots/polybags. The micropropagated plants (transferred to the nursery) were healthy and did not show any morphological defect in comparison to the mother plant (Fig. 2f). Also, tuber formation was recorded next year (Fig. 2f inset).

### Assessment of genetic homogeneity in micropropagated plants

During in vitro culture, genotype, the mode of regeneration, nature of explant, type and concentration of PGRs,



**Fig. 3** Effect of repeated transfer of mother explants on shoot number and shoot length. Medium: MS + BAP (0.5 mg L<sup>-1</sup>), IAA (0.1 mg L<sup>-1</sup>) and additives. Error bars indicate SD. Mean values sharing the same letter do not differ significantly (*P* < 0.05) according to Duncan’s multiple range test

**Table 2** Effect of plant growth regulators and their concentrations on shoot multiplication of *M. dioica*

Concentrations of PGRs			Shoot number Mean ± SD	Shoot length (cm) Mean ± SD
BAP	Kinetin	IAA		
Control	0.0	0.0	8.96 ± 1.24 <sup>k</sup>	6.79 ± 09 <sup>f</sup>
0.1	0.0	0.0	11.23 ± 1.19 <sup>j</sup>	10.59 ± 0.20 <sup>a</sup>
0.25	0.0	0.0	15.13 ± 1.10 <sup>i</sup>	6.45 ± 0.35 <sup>g</sup>
0.5	0.0	0.0	25.03 ± 1.80 <sup>g</sup>	4.46 ± 0.34 <sup>l</sup>
0.1	0.0	0.1	18.10 ± 1.72 <sup>h</sup>	7.43 ± 0.16 <sup>d</sup>
0.25	0.0	0.1	35.33 ± 2.78 <sup>e</sup>	5.52 ± 0.17 <sup>j</sup>
0.5	0.0	0.1	45.30 ± 3.83 <sup>a</sup>	6.52 ± 0.89 <sup>g</sup>
0.1	0.1	0.1	19.26 ± 2.75 <sup>g</sup>	10.56 ± 0.21 <sup>a</sup>
0.1	0.25	0.1	21.43 ± 2.52 <sup>f</sup>	8.51 ± 0.18 <sup>b</sup>
0.1	0.5	0.1	22.40 ± 3.02 <sup>f</sup>	7.57 ± 0.16 <sup>c</sup>
0.25	0.1	0.1	24.90 ± 0.71 <sup>e</sup>	7.22 ± 0.20 <sup>e</sup>
0.25	0.25	0.1	27.16 ± 2.84 <sup>d</sup>	6.71 ± 0.27 <sup>f</sup>
0.25	0.5	0.1	22.56 ± 1.30 <sup>c</sup>	6.17 ± 0.12 <sup>h</sup>
0.5	0.1	0.1	38.36 ± 1.79 <sup>a</sup>	5.22 ± 0.20 <sup>k</sup>
0.5	0.25	0.1	33.06 ± 0.78 <sup>b</sup>	5.76 ± 0.26 <sup>i</sup>
0.5	0.5	0.1	32.23 ± 1.50 <sup>b</sup>	6.21 ± 0.16 <sup>h</sup>

Medium: MS + additives

The same letters within a column are not significantly different at *P* < 0.05 according to DMRT

duration and number of subcultures, and culture conditions are some of the factors that determine the genetic variation in micropropagated plants (Bairu et al. 2011). Somaclonal variation, however, is an important source of genetic variability for breeding programs but it is undesirable in cloning as it reduces the trueness of selected genotype (Sebastiani and Ficcadenti 2016). Therefore, ascertaining the genetic integrity of micropropagated plants is a

**Table 3** Effect of auxins (IBA or NOA) on ex vitro rooting of micropropagated *M. dioica*

Auxins (mg L <sup>-1</sup> )		Response (%)	Root number Mean ± SD	Root length (cm) Mean ± SD
IBA	NOA			
0.0	0.0	30	1.2 ± 0.42 <sup>g</sup>	1.7 ± 0.29 <sup>g</sup>
50	–	45	4.1 ± 0.73 <sup>e</sup>	2.81 ± 0.33 <sup>de</sup>
100	–	70	7.2 ± 0.78 <sup>c</sup>	4.05 ± 0.68 <sup>bc</sup>
250	–	90	12.3 ± 1.33 <sup>a</sup>	5.4 ± 0.73 <sup>a</sup>
500	–	80	8.5 ± 0.84 <sup>b</sup>	4.65 ± 0.47 <sup>b</sup>
–	50	20	2.3 ± 0.67 <sup>f</sup>	1.98 ± 0.33 <sup>fg</sup>
–	100	35	5.4 ± 0.69 <sup>d</sup>	2.5 ± 0.47 <sup>ef</sup>
–	250	55	8.8 ± 0.78 <sup>b</sup>	3.7 ± 0.67 <sup>c</sup>
–	500	50	6.9 ± 0.73 <sup>c</sup>	3.43 ± 0.47 <sup>cd</sup>

Auxin treatment duration: 5 min

The same letters within a column are not significantly different at *P* < 0.05 according to DMRT

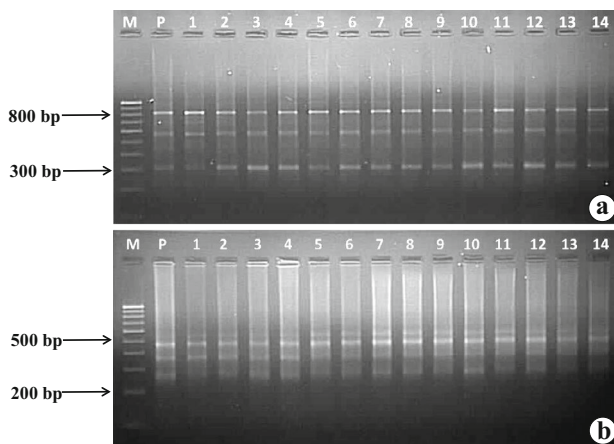
prerequisite to uphold the agronomic/horticultural trait(s) of an elite genotype (Rai et al. 2012b). To evaluate genetic integrity, we compared RAPD and ISSR patterns of fourteen randomly selected micropropagated plants generated after 4 years of in vitro multiplication and maintenance.

Out of 16 RAPD (decamer) primers screened initially, 10 primers produced a total number of 31 reproducible amplicons, with an average of 3.1 bands per primer, ranging from 200 to 1200 bp (Table 4). A maximum of four monomorphic bands among the micropropagated and the donor plant were achieved on RAPD primers [OPE-01, OPG-04 (Fig. 4a) and OPC-05]. RAPD primers have been extensively used in testing genetic homogeneity of micropropagated plants, such as *Cleome gynandra* (Rathore et al. 2014a), *Alhagi maurorum* (Agarwal et al. 2015), including few Cucurbits such as *M. dioica* (Rai et al. 2012a) and *Cucumis melo* L. var. *cantalupensis* (Sebastiani and Ficcadenti 2016) owing to its simplicity, arbitrary and dominant nature, and cost effectiveness (Asthana et al. 2011; Phulwaria et al. 2014).

After preliminary screening of 16 ISSR primers, 8 primers gave amplification results. These 8 primers developed a total of 26 reproducible amplicons with an average of 3.25 bands per primer. The number of amplicons for each primer ranged from 2 (UBC-816) to 4 [UBC-817, UBC-821 (Fig. 4b) and UBC-822; Table 4]. ISSR primers have also been widely used in genetic stability assessment of *Psidium guajava* (Rai et al. 2012b), *Jatropha curcas* (Mendel Soares et al. 2016), including few Cucurbits such as *Luffa acutangula* (Velivela et al. 2016), and *Citrullus lanatus* (Vinoth and Ravindhran 2016), owing to its higher reproducibility, more informative nature, no requirement of

**Table 4** RAPD and ISSR primers used in analysis of genetic homogeneity in *M. dioica* somatic clones

S. No.	Primer name	Primer Sequence (5'→3')	Total No. of bands
<i>RAPD primers</i>			
1	OPG-06	GTGCCTAACC	3
2	OPE-01	CCCAAGGTCC	4
3	OPA-05	AGGGGTCTTG	3
4	OPA-19	CAAACGTCGG	2
5	OPA-11	CAATCGCCGT	3
6	OPG-04	AGCGTGTCTG	4
7	OPA-09	GGGTAACGCC	2
8	OPH-05	AGTCGTCCCC	3
9	OPQ-17	GAAGCCCTTG	3
10	OPC-05	GATGACCGCC	4
<i>ISSR primers</i>			
1	UBC-816	(CA) <sub>8</sub> T	2
2	UBC-817	(CA) <sub>8</sub> A	4
3	UBC-818	(CA) <sub>8</sub> G	3
4	UBC-821	(GT) <sub>8</sub> T	4
5	UBC-822	(TC) <sub>8</sub> A	4
6	UBC-835	(AG) <sub>8</sub> YC	3
7	UBC-836	(AG) <sub>8</sub> YA	3
8	UBC-840	(GA) <sub>8</sub> YT	3

**Fig. 4** Validation of genetic homogeneity in micropropagated plants of wild female *M. dioica*. **a** DNA amplification pattern obtained with RAPD primer OPG-06. **b** DNA amplification pattern obtained with ISSR primer UBC-821. Lane M: 100 bp ladder, Lane P: Mother plant, Lanes 1 to 14: Micropropagated plants

prior sequence information and freeness from radioactivity hazards (Phulwaria et al. 2014).

Use of more than one type of marker has always been recommended (Rathore et al. 2014a, b; Saha et al. 2015; Singh et al. 2016) for a better analysis of genetic integrity, as different markers targets different regions in the genome (Venkatachalam et al. 2007). Therefore, we also used two types of markers (RAPD and ISSR) in our study. The amplification products obtained by all RAPD and ISSR

primers were homogeneous across all the samples. These results confirm the trueness of micropropagated plants and validate that *M. dioica* cultures could be maintained over a prolonged period (four years) without any somaclonal variation. This is probably due to regeneration of plants through organized/pre-existing meristems, which is supposed to maintain strict genotypic and phenotypic stability under culture conditions (Bennici et al. 2004; Rathore et al. 2014a). In addition, maintenance of shoot cultures on lower levels of PGRs also assists in upholding genetic uniformity (Venkatachalam et al. 2007).

## Conclusions

This study reports an improved micropropagation system for wild female *M. dioica*, a nutraceutically important and underutilized vegetable crop. The single step concurrent ex vitro rooting and hardening technique developed in this study could be useful for other cucurbits. This is the first report on accessing genetic homogeneity of *M. dioica* somatic clones using ISSR markers. As our protocol is genetically stable over a prolonged period (four years) as revealed by RAPD and ISSR markers, this could be employed for large scale propagation, germplasm conservation and genetic transformation studies in this important cucurbit.

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