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MOLECULAR CONFIRMATION OF SEX IN REGENERATED PLANTLETS OF SPINE GOURD (*Momordica dioica* Roxb. Ex. WILD) BY USING RAPD MARKERS

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KEYWORDS

Embryogenic callus

Regenerated plantlets

Sex determination

RAPD primer

ABSTRACT

Plant tissue culture techniques offer a great opportunity to overcome the limitations associated with the large scale cultivation of spine gourd. Present study was carried out to formulate the best possible media for large scale production of spine gourd and result of the study revealed that highest percentage (85%) of embryogenic callus was obtained from MS medium supplemented with 2.0 mg/L each of 2, 4-Dichlorophenoxyacetic acid (2, 4-D) and 6- Benzylamino Purine (BAP) in leaf explants of spine gourd. Maximum number of shoots (12.15 ± 1.51 shoots) were observed on MS medium augmented with BAP (4.0 mg/L) in combination with L-glutamine (2.0 mg/L) from leaf derived embryogenic callus of spine gourd. Identification of sex by using morphological characters in the newly regenerated plantlets of spine gourd at fourth leaf stage is another problem for large scale propagation of female plants. PCR based molecular marker OPA-15, a Random Amplified Polymorphic DNA (RAPD) primer can be used as a differential marker to identify female plants from male plants at pre-flowering stage in newly regenerated plantlets (*in vitro*) and as well as in field plants (*in vivo*) of spine gourd. A unique amplification band (700 bp) in size appeared only in female samples, but not in male samples of spine gourd.

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1 Introduction

Spine gourd (*Momordica dioica* Roxb. ex. wild) is a perennial, rhizomatous, dioecious climber belongs to cucurbitaceae family. This is a highly demandable seasonal vegetable and medicinal plant in Asian countries. This species is indigenous to India; because of its higher medicinal importance it is demandable vegetation throughout the world (De Wilde & Duyfjes, 2002; Joseph, 2005; Joseph & Antony, 2008). Spine gourd had various vernacular names in different regional languages of India viz; *Akakara* and *Bodakakara* in telugu, *Kakor*, *Parora* and *Golbandra* in Hindi, *Dharkarela* in Panjabi, *Batkarila* in Assami, *Banzakartoli* in Marathi, *Kartoli* in Bengali, *Aegaravalli*, *Tolloopavai* and *Palupaharkai* in Tamil, *Erimapasel*, *Venpaval* in Malayalam, *Adavihagal* and *Madahagala-Kayi* in Kannada and *Vahisi* in Sanskrit (Bawara et al., 2010). Fruit of spine gourd are free from cholesterol and are highly energetic (45.74 Kcal.) with adequate amount of water (84%), protein (3.1 g) and important minerals and vitamins (Gopalan et al., 1994; Ram et al., 2004; Aberoumand & Deokule, 2009). The medicinal importances of spine gourd are sex-specific and only female plants have medicinal values (Sastri et al., 1962). Kumar & Prajapati (2003) reported that the fruit giving plants have its own value in preparation of medicines and leaves of female spine gourd are used as an aphrodisiac, to eliminate the parasites present in the human intestine, cure fever and respiratory disorders. Furthermore, Kumar & Prajapati (2003) stated that root tubers are used for the treatment of headaches, kidney stones and jaundice. Medicinal value of this plant was also reported by Jain & Singhai (2010) also these researchers reported that fruits are useful in the treatment of asthma, leprosy, fever, tumors, urinary discharges, excessive salivation, and heart disease. Furthermore, Jain & Singhai (2010) also noticed that fruit powder is used to induce sneezing, leading to nasal clearing.

Vegetative mode of propagation through root tubers, availability of root tubers to the farmers, very low percentage of seed germination (10%), hard seed coat and seed prolonged dormancy (4 to 5 months) are the major hurdles for large scale cultivation of spine gourd. By using plant tissue culture technique it can be propagate rapidly and in large quantity within a short period of time. But there is another problem associated with the newly regenerated plantlets of spine gourd, that the identification of sex at very early stage of growth. RAPD - PCR technique offers a great opportunity to identify the sex of the newly regenerated plantlets. Therefore present study has been carried out for the formulation of effective tissue culture media for the better and rapid growth of spine gourd tissue. Furthermore, an effort of female plantlets identification at early stage of growth with the help of RAPD – PCR was also carried out in present study.

2 Materials and Methods

2.1 Plant materials

Male and female plants of spine gourd were collected from different geographical locations viz Warangal, Karimnagar and Khammam districts of Telangana State and were grown in the research field of the Department of Botany, Kakatiya University, Warangal.

2.2 Micropropagation

Young and well matured leaves obtained from *M. dioica* plants are used in present investigation. Explants were sterilized with 0.1 % HgCl₂ for 3-4 minutes then rinsed with double distilled water and inoculated on MS medium (Murashige & Skoog, 1962) augmented with various concentrations of 2, 4 -D in combination with BAP (0.5 to 3.0 mg/L each). The culture vessels were incubated and maintained at 25 ± 2° C temperature and 16 hour light (3000 lux light intensity). After 3 weeks embryogenic compact callus with green spots was developed from the cut portion of the explant. These calli were sub cultured on MS medium fortified with BAP (1.0 to 5.0 mg/L) in combination with L-glutamine (2.0 mg/L). Newly regenerated micro shoots were transferred to IBA (1.0 mg/L) rooting medium, after 4 weeks of the cultures. The regenerated plantlets were acclimatized and transferred to field conditions.

2.3 Molecular Confirmation of Sex in Regenerated Plantlets by using RAPD markers:

By using RAPD – PCR technique the sex of the field grown plants (*in vivo*) had determined and these amplification results were comparing with the amplification results of *in vitro* developed plantlets and sex of newly regenerated plantlets (*in vitro*) of spine gourd had confirmed.

Known sex (male and female) type leaves of the mother plant and randomly selected *in vitro* regenerated plants at 4th leaf stage of the plant were collected and used for RAPD – PCR amplification. Genomic DNA was isolated from juvenile leaves of the female and male mother plants (*in vivo*) and regenerated plants (*in vitro*) by using the protocol of Doyle & Doyle (1990). The concentration of DNA was determined by a UV-Vis spectrophotometer and quality of genomic DNA was checked following electrophoresis on 0.8 % agarose gel. RAPD assay was performed using 4 RAPD primers (Bioserve, Mumbai, India) (Table 3). PCR was performed in a volume of 21 µl reaction mixture containing 2.0 µl genomic DNA (80 ng), 2.0 µl Taq buffer, 2.0 µl dNTPs, 2.0 µl MgCl₂, 2.0 µl random primer (10 pM), 1.5 µl Taq DNA polymerase and 8.5 µl PCR grade water. DNA amplification was carried out in a DNA thermal cycler (My gene, USA). The PCR program was setup with an initial denaturation, denaturation, annealing, extension and final extension at 94° C for 3 min, 94° C for 45 seconds, 32° C for 30 sec, 72° C for 2 min and 72° C for 7 min, respectively (Table 4). The amplified products of DNA were analyzed by gel Agarose gel electrophoresis, λ DNA used as a molecular marker and visualized by staining with ethidium bromide and cyanolxylol and recorded using a gel documentation unit.

Table 1 *In vitro* callus induction in leaf explants on MS medium supplemented with different concentrations of 2, 4 - D (0.5 – 2.5 mg/L), either alone or in combination BAP (0.5 – 2.5 mg/L) in *M. dioica* Roxb. after 4 weeks of culture.

Ms medium with plant growth regulators (mg/L)		% explants responded	Fresh Weight of callus (g) (Mean $\pm$ S.E)	Nature of callus
2,4 D	BAP			
0.5	--	36	0.48 $\pm$ 0.04 ^a	Soft White
1.0	--	48	0.62 $\pm$ 0.06 ^b	Soft White
1.5	--	66	0.96 $\pm$ 0.01 ^c	Soft White
2.0	--	70	1.68 $\pm$ 0.04 ^f	Soft White
2.5	--	60	1.26 $\pm$ 0.02 ^d	Soft White
0.5	0.5	45	0.49 $\pm$ 0.01 ^a	Friable White
1.0	1.0	55	0.89 $\pm$ 0.04 ^c	Friable White
1.5	1.5	78	1.42 $\pm$ 0.07 ^e	Friable White
2.0	2.0	94	1.80 $\pm$ 0.06 ^h	Friable Green
2.5	2.5	85	1.62 $\pm$ 0.08 ^f	Friable White

(Mean $\pm$ SE) Five replicates per treatment; repeated thrice. Means in each column followed by same letters are not significantly different according to DMRT at $P < 0.05$.

Table 2 *In vitro* shoot regeneration via indirect organogenesis in leaf calli cultured on MS+ BAP (1.0 – 5.0 mg/L) either alone or in combination with L- glutamine (2.0 mg/L) in *M. dioica* Roxb. after 4 weeks of culture.

MS medium with growth regulators (mg/L)		Leaf calli	
BAP	L-glutamine	No. of shoots/explant (Mean $\pm$ S.E)	Shoot length(cm) (Mean $\pm$ S.E)
1.0	--	2.15 $\pm$ 0.28 ^a	3.60 $\pm$ 1.42 ^j
2.0	--	4.56 $\pm$ 0.64 ^c	3.10 $\pm$ 0.82 ^h
3.0	--	5.85 $\pm$ 0.82 ^d	2.75 $\pm$ 0.71 ^g
4.0	--	9.15 $\pm$ 1.86 ^g	0.90 $\pm$ 0.08 ^a
5.0	--	6.42 $\pm$ 0.99 ^c	2.80 $\pm$ 0.65 ^g
1.0	2.0	3.46 $\pm$ 0.78 ^b	2.24 $\pm$ 0.18 ^f
2.0	2.0	5.84 $\pm$ 0.98 ^d	2.16 $\pm$ 0.17 ^e
3.0	2.0	8.99 $\pm$ 1.08 ^f	1.47 $\pm$ 0.14 ^c
4.0	2.0	12.15 $\pm$ 1.51 ^h	1.02 $\pm$ 0.08 ^b
5.0	2.0	9.75 $\pm$ 0.97 ^g	1.92 $\pm$ 0.13 ^d

(Mean $\pm$ SE) Five replicates per treatment; repeated thrice. Means in each column followed by same letters are not significantly different according to DMRT at $P < 0.05$.

Table 3 List of RAPD primers.

S.NO	RAPD Primer	Sequence of the primer
1	OPA -15	5'- TTCCGAACCC - 3'
2	OPA -16	5'- AGCCAGCGAA- 3'
3	OPA -17	5'- GACCGCTTGT- 3'
4	OPA -18	5'- AGGTGACCGT- 3'

Table 4 Thermocycler programming conditions.

Steps	Temp ($^{\circ}$ C)	Duration	Cycle (No.)
Initial denaturation	94	3.0 min	1.0
Denaturation	94	45 sec	45.0
Annealing	32	30 sec	45.0
Extension	72	2.0 min	45.0
Final extension	72	7 min	1.0

Table 5 Number and size of DNA bands in the RAPD sex determination profile of *M. dioica* by using OPA – 15 primer.

RAPD Primer	Male		Female	
	No. of DNA bands	Size of the DNA band	No. of DNA bands	Size of the DNA band
OPA – 15 (5'-TTCCGAACCC-3')	3	1500 bp 1300 bp 800 bp	4	1500 bp 1300 bp 800 bp 700 bp

3 Results and discussion

3.1 Micropropagation

Maximum amount of mean fresh weight (1.68 ± 0.04 g) of callus was observed in leaf explants cultured on MS medium supplemented with 2, 4-D (2.0 mg/L). The callus that developed from leaf explants was smooth, friable white, whereas the amount of callus was increased from leaf explants cultured on MS medium supplemented with 2, 4-D (2.0 mg/L) and in combination with BAP (2.0 mg/L) and more over that green compact callus was formed.

Maximum amount of embryogenic green compact callus with a mean fresh weight (1.80 ± 0.06 g) was observed in leaf explants cultured on MS medium supplemented with 2, 4-D (2.0 mg/L) + BAP (2.0 mg/L) (figure 1- a). Maximum mean number of adventitious shoots (12.15 ± 1.51), with a mean length (cm) of shoots (1.02 ± 0.08) was observed in leaf derived embryogenic calli subcultured on MS medium supplemented with BAP (4.0 mg/L) and L- glutamic acid (2.0 mg/L) (Table-1, figure 1- a, c). After 4 weeks of subculture these regenerated plantlets were transferred to rooting medium. Maximum number of roots (30) was observed on MS medium supplemented with IBA 3.0 mg/L. After 4 weeks the regenerated plantlets were acclimatized and transferred to field conditions (Table-2, figure 1- d).

The callus that developed from leaf explants was smooth, friable white, whereas the amount of callus was increased from these explants cultured on MS medium supplemented with 2, 4-D (2.0 mg/L) and in combination with BAP (2.0 mg/L) and more over that green compact callus was formed. Similar observation were made in leaf explants (Devendra et al., 2009; Thiruvengadam & Chung, 2011; Mustafa et al., 2012), petiole explants of *M. dioica* (Thiruvengadam et al., 2012) leaf explants of *Melothria maderaspatana* (Baskaran et al., 2009), nodal explants of *M. charantia* (Al Munsur et al., 2009).

Auxins in combination with cytokinins were found to be more efficient for the induction of green compact callus. Similar observations were made in leaf explants of *M. dioica* (Hoque et al., 2000; Nabi et al., 2002); root and leaf explants of *Momordica charantia* (Agarwal & Kamal, 2004).

The callus generated from leaf explants have more regeneration capacity than the calli generated from node, internode and tendril explants of *M. dioica*. Mustafa et al. (2012) reported that callus derived from leaf and petiole explants had higher regeneration potential than callus derived from leaf and nodal explants. Highest number of shoots (12.15) regenerated on MS medium supplemented with BAP (4.0 mg/L) in combination with L-glutamine (2.0 mg/L) in leaf callus of *M. dioica*. Similarly, highest number of shoots (11.15) regenerated from nodal calli cultured on MS medium supplemented with BAP (4.0 mg/L) + L-glutamine (2.0 mg/L). Although cultured cells are normally capable to synthesizing all the required amino acids, the addition of certain amino acids or amino acid mixtures may be used to further stimulate cell growth. The use of amino acids is particularly important for establishing cell cultures. Amino acids provide plant cells with an immediately available source of nitrogen, which generally can be taken up by the cells more rapidly than inorganic nitrogen.

Addition of non toxic glutamine to the culture medium maintains a high growth rate of cells for longer period (Gamborg et al., 1968). The full strength MS medium containing PVP (50 mg/L) and glutamine (40 mg/L) was effective to achieve a high frequency of somatic embryo induction, maturation and further development of *M. charantia* (Thiruvengadam et al., 2006). Highest number of shoots (48 shoots) was produced on MS medium containing TDZ (4.0 μ M), 2, 4-D (1.5 μ M) in combination with L-glutamine (0.07mM) in internode derived callus of *M. charantia* L (Thiruvengadam et al., 2012). The addition of L-glutamine (0.5 μ M) resulted in the great improvement (44.5 %) of embryogenic frequency and development in suspension culture of *Cucumis anguria* L. (Thiruvengadam et al., 2013).

Selvaraj et al., (2007) reported that MS medium with BAP (8.88 μ M), NAA (1.34 μ M), Zeatin (0.91 μ M) together with L-glutamine (136.85 μ M) produced large number of shoots in Cucumber. Addition of L- glutamic acid in adventitious shoot regeneration medium greatly increased the production of shoots from callus.

Figure 1 *In vitro* plantlet regeneration of spine gourd (*Momordica dioica* Roxb. Ex. Wild)

[(a) Induction of callus from leaf explant cultured on MS + 2, 4-D (2.0mg/L) + BAP (2.0mg/L), after two weeks of culture. (b) Multiple shoot bud induction from leaf derived callus on MS + BAP (4.0 mg/L) + L-glutamine (2.0 mg/L), after three weeks of the culture. (c) Shoot elongation on MS basal medium, after 2 weeks of culture (d) Rooting of shoot on MS + IBA (3.0 mg/L), after two weeks of the culture (e) Hardening of the plantlet in a plastic cup. (f) Acclimatization of plantlet in a pot].

This is in agreement with the findings of Selvarj et al., (2002) for *Cucumis sativus*. Maximum number of adventitious shoots regenerated on MS medium fortified with BA (2.0 μ M), TDZ (4.0 μ M), 10% v/v coconut water, and in combination with L-glutamine (0.08mM) in petiole derived callus of *Melothria maderaspatana* (Baskaran et al., 2009).

Devendra et al. (2009) reported that highest shoot regeneration (12.33 shoots) was observed on MS medium supplemented with BAP (1.5 mg/L) in combination with Kn (1.5 mg/L) in leaf derived calli of *M. dioica*. According to Debnath et al., (2013) requirement of high cytokinin (BAP 4.0 mg/L) and coconut milk (15% v/v) in nodal callus culture clearly indicate that the differential morphogenetic potentiality of the three explants. The reported result is significant in terms of high

shoot (86 ± 3.44 shoot buds) was found on the 126 days of culture on the same inductive medium for further multiplication and growth.

3.2 Molecular Confirmation of Sex in Regenerated Plantlets of Spine gourd

3.2.1 RAPD – PCR amplification analysis:

Four RAPD primers namely OPA-15, OPA-16, OPA-17, OPA-18 were utilized for PCR amplification of genomic DNA isolated from leaf tissue of male and female spine gourd *in vivo* and randomly selected *in vitro* regenerated plants. Out of four primers used, OPA-15 produced distinct bands in male and female plants.

Figure 2 RAPD profile for the confirmation of sex in *in vitro* regenerated plantlets of *M. dioica* by comparing *in vivo* male and female mother plants amplified with OPA-15 primer.

[Lane: M: Marker - Lane 1: Male mother plant (*in vivo*), Lane 2: Female mother plant (*in vivo*), Lane 3, 4 & 6: Male regenerated plant (*In vitro*), Lane 5 & 7: Female regenerated plant (*In vitro*)]

3.2.2 Primer:

OPA-15 primer with the primer sequence 5'-TTCCGAACCC-3' was amplified genomic DNA samples of *in vivo* and *in vitro* developed male and female plants of *M. dioica*. Three DNA bands, sized 1500 bp, 1300 bp and 800 bp in male samples, whereas, four DNA bands, sized 1500 bp, 1300 bp, 800 bp and 700 bp in female samples was observed, respectively (Table 5, figure 2).

The family Cucurbitaceae have different types of sex forms i.e. bisexuality, monoecious and dioecious types. Particularly in dioecious plants identification of sex in seedling stages of plant is very important for selection of medicinally important female plants for large scale cultivation. This could be very useful for the farmer that they need not to wait until flowering stage occur to select the staminate and pistillate plants.

According to Renner & Ricklefs (1995) nearly 6% angiosperms are dioecious throughout the world. In Cucurbitaceae, the sex of an individual is difficult to determine at the early stages of development, particularly before flowering. This is very important for breeding programmers to develop new varieties.

In present study it was observed that among all the four RAPD primers OPA-15 primer (5'-TTCCGAACCC-3') produced a unique band of 700 bp in size appeared only in female samples (Pic-2, lanes 2, 4 and 6), while it is absent in male samples (Pic-2, lanes 1, 3 and 5). This unique 700 bp DNA band was highly reproducible under a broad range of amplification conditions. Therefore OPA – 15 primer can be used as a differential marker to identify female plants from male plants at pre flowering stage.

Similar type of effort for sex determination in *M. dioica* using RAPD marker OPA-15 had done earlier by Patil et al. (2012) and Baratakke et al. (2013). They reported the presence of 1500 bp unique band in male while a 900 bp unique band in female samples. But in present investigation this unique band was reported at 700 bp in female samples of *M. dioica* by using the same OPA -15 primer. The variation in the results may be due to biotype variation, environmental and ecological difference between Telangana region and north Indian region.

Baratakke et al. (2013) used the SCAR marker for the identification sex in *M. dioica* at pre-flowering stage. In cucurbits female sex- associated RAPD marker in pointed gourd (*Trichosanthes dioica* Roxb.) has been also reported by Singh et al. (2002).

There are several reports on sex determination in dioecious plants. Sex determination in papaya by using PCR based RAPD molecular marker was reported by several authors from various countries like in Japan (Ursaki et al., 2002), Hawaii (Deputy et al., 2002), Brazil (Lemos et al., 2002) and India (Parasnis et al., 1999). In *Salix viminalis* (Alstrom-Rapaport et al., 1998), *Encephalartos natalensis* (Prakash & Staden, 2006), *Borassus flabellifer* (George et al., 2007), *Simmondsia chinensis* (Agrawal et al., 2007) and *Pandanus fascicularis* (Vinod et al., 2007) also sex is determined by using PCR based RAPD molecular marker.

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Conflict of interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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