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Effect of exogenous polyamines enhances somatic embryogenesis via suspension cultures of spine gourd (*Momordica dioica* Roxb. ex. Willd.)

M. Thiruvengadam[1], K.T. Rekha[2], N. Jayabalan[3], N. Praveen[1], E.H. Kim[1], I.M. Chung[1]*

[1]Department of Applied Life Science, Konkuk University, Seoul 143-701, South Korea
[2]Department of Environmental and Herbal Science, Tamil University, Thanjavur-613005, Tamil Nadu, India
[3]Department of Plant Science, Bharathidasan University, Tiruchirappalli – 620024, Tamil Nadu, India

***Corresponding author: imcim@konkuk.ac.kr**

Abstract

An efficient method of somatic embryogenesis using exogenous polyamines through suspension culture protocol was developed from leaf derived callus of spine gourd (*Momordica dioica* Roxb. ex. Willd.). Embryogenic callus was originated from cut slices of leaf explants on MS solid medium supplemented with 4.4 µM 2,4-D with addition of polyamines (putrescine, spermidine and spermine). Putrescine at a concentration of 1.0 µM showed maximum increase in fresh weights of embryogenic calli. The maximum frequency of somatic embryos (30.3%) was observed on MS medium supplemented with 3% (w/v) sucrose and 3.3 µM 2,4-D for three weeks of culture. The MS liquid medium augmented with 3.3 µM 2,4-D and 0.5 µM putrescine was effective to achieve high frequency of somatic embryo induction (59.0%). Moreover addition of PAs to the embryogenic media resulted in lowering of endogenous free PA level of 21-day-old embryogenic calli. Thus, when the media was supplemented with exogenous PAs a positive correlation was found to exist between somatic embryogenesis enhancement and decrease in endogenous free PA levels. Sustained cell division resulted in the formation of cell aggregates and then progressed to globular, heart and further to torpedo and cotyledonary stages within 5 weeks. Transfer of individual embryos on to a fresh MS basal medium with no plant growth regulators was able to achieve complete maturation. Conversion of embryos into plants was achieved on 1/2 strength MS semi solid medium containing 0.5 µM gibberellic acid (GA_3) and 1.5% sucrose. Twenty two percent of somatic embryos were converted into true-to-type fertile plants. Regenerated plantlets were successfully hardened, with a survival rate of approximately 76%, and established in the field.

Keywords: *Momordica dioica*, Putrescine, Somatic embryogenesis, Spermidine, Spermine, Suspension culture.
Abbreviations: PAs: Polyamines; Put: Putrescine; Spd: Spermidine; Spm: Spermine; 2,4-D: 2 4-dichlorophenoxyacetic acid.

Introduction

Spine gourd (*Momordica dioica* Roxb. ex. Willd) is a perennial, dioceous climbing creeper belonging to family Cucurbitaceae. It is distributed in Bangladesh, China, India, Malaysia, Nepal, Myanmar, Pakistan and Sri Lanka (Rakh and Chaudhari, 2010). Immature green fruits are cooked as vegetable and young leaves and flowers are also consumed. Fruits contain highest amount of protein, calcium, phosphorous, iron and highest amount of carotene amongst the cucurbitaceous vegetables (Ram et al., 2001). The plant was reported to possess anti-diabetic (Reddy et al., 2006), anti-cancer (Luo et al., 1998), analgesic, postcoital anti-fertility (Shreedhar et al., 2001), nematocidal, anti-allergic, anti-malarial, anti-feedant, anti-bacterial (Nabi et al., 2002), anti-oxidants, hepatoprotective (Jain et al., 2008), jaundice and bleeding pile properties (Deokule, 2006). This popular vegetable has high demand in market but still remain underutilized and unexploited, due to vegetative mode of propagation and dioecious nature (Ali et al., 1991). The improvement of *M. dioica* through conventional breeding techniques has several limitations because of its dioecious nature and difficulty in seed germination (Rashid, 1976; Ali et al., 1991). Propagation by tuberous roots is limited due to the low multiplication rate (Mondal et al., 2006). Improvement of plant species via biotechnological approaches depends on the tissue culture response particularly plant regeneration through somatic embryogenesis. The establishment of an efficient regeneration system through somatic embryos will allow for crop improvement through a programme that includes somatic hybridization, synthetic seed production, *in vitro* selection and genetic transformation. Only few reports on direct organogenesis in *M. dioica* are currently available (Nabi et al., 2002; Thiruvengadam et al., 2006b; Shekhawat et al., 2011). In our earlier studies, we have reported regeneration of *M. dioica* through somatic embryogenesis from petiole derived callus (Thiruvengadam et al., 2007). Polyamines are small, aliphatic amines that are present in all plant cells. Putrescine, spermidine and spermine are the most common PAs found in higher plants (Galston, 1983). Polyamines have been implicated in various plant growth and developmental processes. These include stimulation of cell division, response to environmental stresses, regulation of rhizogenesis, embryogenesis, senescence, floral development and fruit ripening (Evans and Malmberg, 1989; Kakkar and Rai, 1993). There are few reports which suggests that polyamines are important for somatic embryogenesis in *Panax ginseng* (Kevers et al., 2000), *Gossypium hirsutum* (Sakhanokho et al., 2005), *Araucaria angustifolia* (Steiner et al., 2007), *Coffea canephora* (Clelia et al., 2008) and *Citrus sinensis* (Wu et al., 2009). Exogenous application of Put has been successfully used to enhance salinity (Chattopadhayay et al., 2002), cold (Nayyar et al., 2004), drought (Zeid et al., 2006), water logging (Arbona et al., 2008) and flooding tolerance of plants (Yiu et al., 2009). Ornithine decarboxylase (ODC) and arginine decarboxylase (ADC) are the two important enzymes responsible for putrescine

biosynthesis (Paul et al., 2009). Recent studies have also indicated that polyamine metabolism might be involved in the competence of explants for somatic embryogenesis in many plant species, with both total endogenous levels and the ratios of individual polyamines being important for the early stages of somatic embryogenesis in *Solanum melongena* (Yadav and Rajam, 1998), *Medicago sativa* (Huang et al., 2001), *Oryza sativa* (Shoeb et al., 2001) and *P. ginseng* (Monteiro et al., 2002). To our knowledge, there are no reports on exogenous polyamines supplement for spine gourd regeneration via somatic embryogenesis from leaf explants. This work described an effect of polyamines on high frequency induction of somatic embryogenesis in spine gourd.

Results and discussion

Callus induction

Green-yellow friable calli were induced on MS solid medium containing 4.4 µM 2,4-D and 1.0 µM Put with culture duration of 3 weeks (Fig. 1a). Putrescine at a concentration of 1.0 µM showed maximum six fold increase in fresh weights of embryogenic calli (Fig. 2). Similar results were reported in *M. charantia* (Paul et al., 2009) and *C. canephora* (Kumar et al., 2008). The most commonly used growth regulator to induce somatic embryogenesis is 2,4-D. For instance, MS medium supplemented with 2,4-D was effective for the induction of somatic embryogenesis from leaf and stem explants of *Cucumis sativus* (Malepszy et al., 1982; Malepszy and Nadolska-Orczyk, 1983) and *M. charantia* (Thiruvengadam et al., 2006a). In contrast, 2,4-D with KIN was responsible for inducing somatic embryos in *C. melo* (Oridate and Oosawa, 1986) and *Cucurbito pepo* (Kintzios et al., 2002). The callus showed dense cytoplasm, small vacuoles and large nuclei with deeply stained nucleoli when observed under a compound microscope. Such potentially embryogenic calli were suitable for suspension cultures. The maximum efficiency of callus and typical embryogenic cells were noted on MS medium containing 4.4 µM 2,4-D and 1.0 µM Put.

Suspension culture of somatic embryos

The green-yellow friable embryogenic calli were suspended in MS liquid medium containing various concentrations of 2,4-D (1.1 - 4.4 µM) along with PAs (0.1 - 1.0 µM Put, Spd, Spm) to establish the somatic embryogenesis. The MS liquid medium augmented with 3.3 µM 2,4-D and 0.5 µM putrescine was effective to achieve high frequency of somatic embryo induction (59.0%).

Maximum number of somatic embryos produced per 0.2 g of callus was twofold higher than without putresine (Fig. 3 and 4). Similar results were reported in *G.hirsutum* (Sakhanokho et al., 2005), *C. canephora* (Kumar et al., 2008) and *M. charantia* (Paul et al., 2009). Auxins were absolutely necessary for somatic embryo induction but were often omitted in the period of maturation and germination (Mohamed et al., 2005; Thiruvengadam et al., 2006a). Active division and growth of cells were observed in 3.3 µM 2,4-D until the 7th and 8th day of culture. Initially, the calli exhibited highly vacuolated cells. After 6 days, the callus showed two morphologically distinct kinds of cells, namely, spherical and elongated cells, both with visible cytoplasm and nucleus. Each spherical embryogenic cell transversely divided into two cells, four cells and subsequently into a group of cells that was considered to be the proembryo (Fig. 1b - f). The proembryo further divided to form globular embryos (Fig. 1g) and heart shaped embryos (Fig. 1h) within a period of two weeks, which later developed into torpedo and cotyledonary embryos (Fig. 1i) within a week. On the same MS medium with 2,4-D, the torpedo and cotyledonary embryos did not develop, but some abnormal structures appeared such as stunted embryo polarity, poorly developed cotyledons, trumpet with leafy structure, which later turned to green calli (data not shown). Thus torpedo shaped embryos recallused on 2,4-D containing medium. The presence of 2,4-D in the culture medium inhibited embryo development of squash beyond the globular stage (Jelaska et al., 1985). The complete removal of 2,4-D at later stages of culture stimulated further embryo development into the heart and torpedo stages in *M. charantia* (Thiruvengadam et al., 2006a). The histological examinations revealed that the globular stage somatic embryos were individuals and develop into bipolar structures showing clearly well-developed shoot and root meristems, which consist of small cells with a dense cytoplasm (Fig. 1k). Our histological report clearly confirmed the formation of bipolar structures in spine gourd. Globular embryos consisting of small cells with dense cytoplasm and large nuclei were observed in regal geranium (Wilson et al., 1994).

Maturation and plantlet formation of somatic embryos

Cotyledonary-shaped embryos, when transferred to MS medium without 2,4-D, greatly improved maturation within 7 days of culture (Fig. 1j), whereas in the same system, the presence of 2,4-D resulted in malformed embryo structures (data not shown), which later developed into friable callus. In *C. sativus*, hormone- free MS basal medium was found to be optimal for maturation and further development (Rajasekaran et al., 1983; Wyszogrodzka and Shahin, 1985). In our study, the complete removal of 2,4-D and PAs from the culture medium improved embryo maturation. In contrast, PAs enhanced the somatic embryo maturation in spruce (Amarasinghe et al., 1996). Based on our experiment, after maturation, the embryos had the potential capacity to synthesize their own hormones for successive function during further development and conversion. Consequently, the matured somatic embryos produced shoots and roots from the poles that enhanced extension of hypocotyl and plantlet development on solidified half strength MS medium with 1.5% of sucrose, 0.5 µM gibberellic acid (GA_3) and 0.2% gelrite within 14 days (Fig. 1l). The average frequency of germinating embryos into tiny plantlets (22%) was achieved. This result was in agreement with *Macrotyloma uniflorum* (Mohamed et al., 2005) for somatic embryo germination induced by ABA. These plantlets were transplanted into plastic cup containing red soil, perlite and vermiculite (3:1:1) mixture and after 2 weeks (Fig. 1m), they were transferred to pots. A total of 2 months was required for the production of plantlets that were ready to be potted. Regenerated plantlets were successfully hardened, with a survival rate of approximately 76%, and established in the field.

Effects of exogenous PAs

PAs are important for cellular differentiation to somatic embryogenesis (Chi et al., 1994; Bajaj and Rajam, 1996; Rajam, 1997) and have been suggested as regulators of somatic embryos (Montague et al., 1979). Putrescine enhanced somatic embryogenesis in several plant species such as *P.ginseng* (Kevers et al., 2002), *G.hirsutum* (Sakhanokho et al., 2005) and *M.charantia* (Paul et al.,

Table 1. Total individual endogenous free PA levels and individual PA ratios (Put/Spd, Put/Spm) with respect to time (days) of callus culture *in vitro*.

Time in culture (days)	Endogenous free Put content (nmol g^{-1} FW)	Endogenous free Spd content (nmol g^{-1} FW)	Endogenous free Spm content (nmol g^{-1} FW)	Total endogenous free PA concentation (nmol g^{-1} FW)	Put/Spd	Put/Spm
0	7200.11±31.11^{a}	360.23±11.0^{cd}	150.15±3.05^{c}	7680.49±45.16^{a}	19.98^{a}	47.95^{a}
14	277.41±5.25^{c}	353.35±25.2^{d}	81.24±2.44^{de}	712.00±32.69^{de}	0.785^{c}	3.41^{c}
21	1795.67±145.1^{b}	847.73±45.4^{a}	169.33±5.30^{a}	2812.73±65.80^{b}	2.120^{b}	10.6^{b}
28	423.75±45.4^{bc}	403.21±11.0^{c}	89.04±4.12^{d}	916.00±50.52^{cd}	1.05b^{c}	4.75^{bc}
35	243.34±3.10^{cd}	425.1±27.3^{bc}	157.12±7.2^{b}	925.61±37.60^{c}	0.57^{cd}	1.54^{cd}
42	175.65±12.5^{d}	441.21±18.7^{b}	152.2±12.1^{bc}	769.12±43.1^{d}	0.39^{d}	1.15^{d}

The data were statistically analyzed using Duncan's multiple range test. Values are mean ± SE.. In the same *column*, significant differences according to the least significant difference (LSD) at the $P \leq 0.5$ level are indicated by different letters.

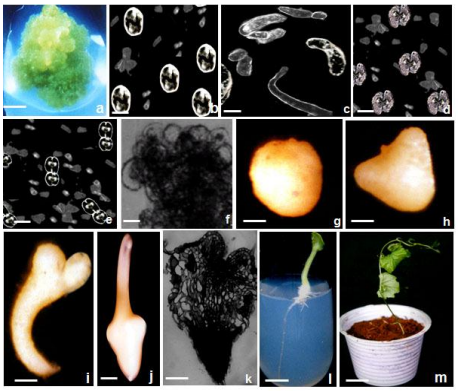

Fig 1. Effects of PAs on somatic embryogenesis from leaf explants through suspension cultures of spine gourd (*Momordica dioica*). (a) Leaf derived embryogenic callus (MS+ 4.4 µM 2,4-D, 1.0 µM KIN and 1.0 µM Put). (b) Spherical embryogenic cells. (c) Elongated cells. (d) Two cell stages. (e) Four cell stages. (f) Proembryos. (g) Globular embryo. (h) Heart-shaped embryo. (i) Torpedo shaped embryo (MS+ 3.3 µM 2,4-D and 0.5 µM Put). (j) Matured cotyledonary shaped embryo with shoot primordia and root primordial (MS basal liquid medium). (k) Longitudinal sections of heart shaped embryo. (l) Germination of somatic embryos (1/2 MS + 2% sucrose and 0.5 µM GA_3). (m) Hardened plant. Bar represents 0.4 mm (a-f); 0.5 mm (g-j) 0.05 mm (k); 0.5 mm (l); 3.0 cm (m).

2009). However, in suspension cultures of *Pinus taeda* (Silveira et al., 2004) and the callus of *Nicotiana tabacum* (Rastogi and Davies, 2000), high levels of endogenous Put was associated with cellular growth reduction. Young leaves showed abundance of Put followed by Spd and Spm (Table 1). When cultured in embryogenic medium for 14 days, callogenesis was observed from the leaf explants along with a decrease in total free PA level (Table 1). A positive correlation exists between PA synthesis and abundance with cell division in young leaves of tobacco (Paschalidis et al., 2005). After 21 days of culture during induction of SE, there was an increase in free PA level, 6 fold increase in case of free Put and approximately 2 fold increase in case of free Spd and Spm (Table 1). Compared with calli in culture at 21 days, during induction of SE, there were high levels of endogenous free Put as compared to free Spd and Spm. After embryo induction there was a decline in the total free PA level as embryos began to develop. Amongst the PAs, decline in free Put level was most prominent as compared to free Spd and Spm levels as evidenced by Put/Spd and Put/Spm ratios which decreased after 21 days of culture and became < 1 and < 2, respectively (Table 1). PA synthesis was triggered during somatic embryo induction in 21-day-old calli. The effects of exogenous PAs on fresh weights and number of somatic embryos of 21-day-old calli grown on PA enriched and PA deprived media was observed whether exogenous PAs affected the growth of early induced somatic embryogenic callus. When exogenous PAs, Put (0.1, 0.5, 1.0 µM), Spd (0.1, 0.5, 1.0 µM) and Spm (0.1, 0.5, 1.0 µM) were added individually to the embryogenic medium, their fresh weight as well as number of somatic embryos of 21-day old calli increased by varying folds as compared to the embryogenic calli growing in media deprived of PA supplementation. Amongst PAs, Put at a concentration of 1.0 µM, (Fig. 2) was found to be most effective in increasing the fresh weights of 21-day old embryogenic callus (5.3 fold). Spd and Spm at a concentration of 0.1 µM were also found to be effective in increasing the fresh weights of 21-day old embryogenic callus (3.0 fold) and (3.5 fold) respectively (Fig. 2). Amongst the three PAs namely Put, Spd and Spm; Put, at a concentration of 0.5 µM was found to be most effective in increasing the number of somatic embryos produced per 0.2 g of 21-day old embryogenic callus (2.5 fold). Spd and Spm at a concentration of 0.5 µM were also found to be effective in increasing the number of somatic embryos produced per 0.2 g of 21-day old embryogenic callus (1.8 fold) and (1.5 fold) respectively (Fig. 4). Exogenously added PAs have been widely used for studying the function of PAs in cell growth and differentiation, and can be an interesting strategy for regulation of endogenous PAs pools, which have been correlated with embryogenic competence in many plant species (Takeda et al., 2002). In the present study, an increase in the endogenous levels of total free PAs (Fig. 5) was observed when a PGR medium culture was supplemented with Put (1.0 µM), as compared to the control. Not only PA levels have been associated with embryogenic competence, but differences in the levels of

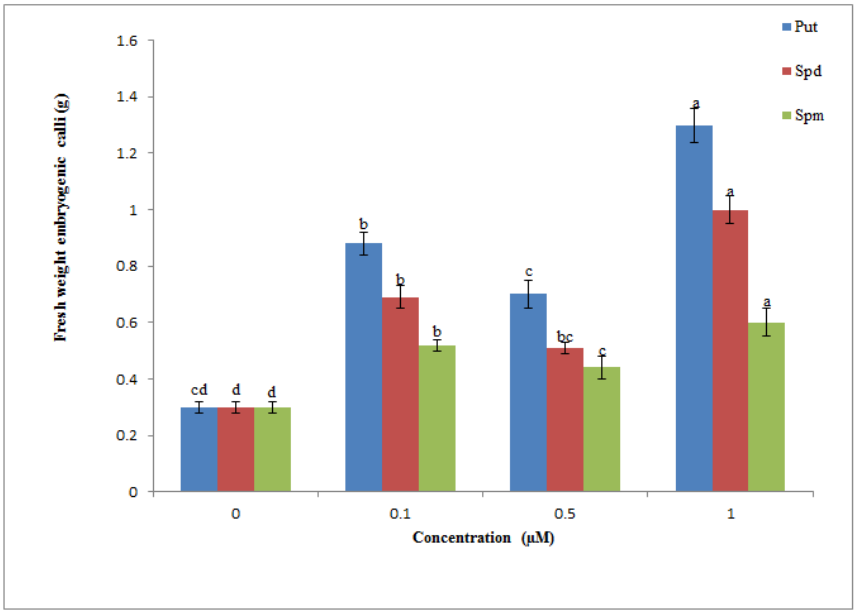

Fig 2. Effects of 4.4 µM 2,4-D with exogenous PAs; Put, Spd and Spm on fresh weights of three week-old embryogenic calli. The data were statistically analyzed using Duncan's multiple range test. Values are mean ± SE. In the same *column*, significant differences according to the least significant difference (LSD) at the $P \leq 0.5$ level are indicated by different letters.

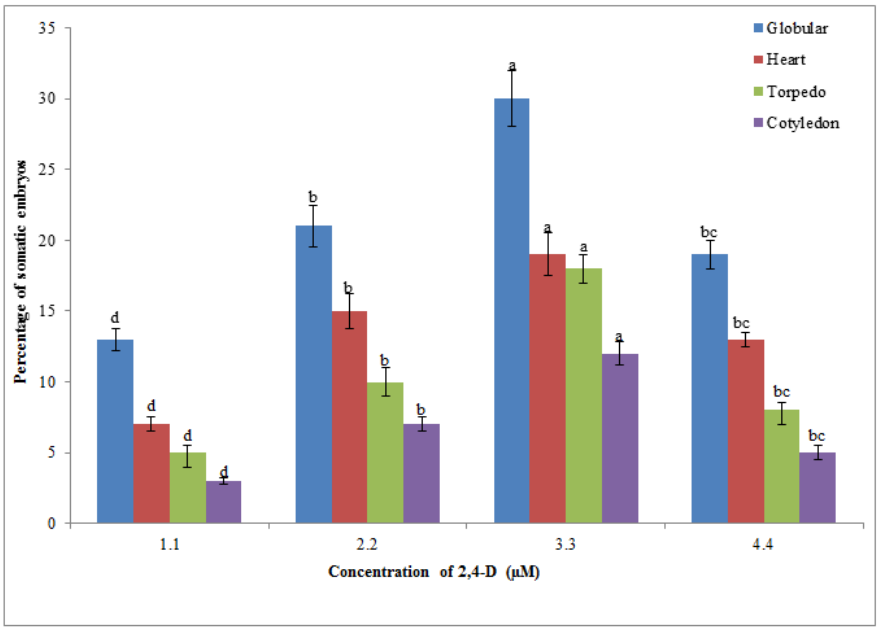

Fig 3. Influence of 2,4-D on differentiation of somatic embryos from leaf callus of spine gourd. The data were statistically analyzed using Duncan's multiple range test. Values are mean ± SE. In the same *column*, significant differences according to the least significant difference (LSD) at the $P \leq 0.5$ level are indicated by different letters.

specific PAs have also been implicated (Li and Burritt, 2003).

Materials and methods

Plant material and explants preparation

Tubers of *Momordica dioica* Roxb. ex. Willd (one-year-old) were collected from the Semmalai hills, Western Ghats (altitude 300 – 600 m) and raised in the Botanical Field Evaluation Garden at Kulathur, Tamil Nadu, India. Leaf explants were collected and washed in running tap water for 5 min and surface sterilized in 70% (v/v) ethanol for 1 min. Further, explants were treated with 1.0% (v/v) sodium hypochlorite solution for 10 min with occasional agitation. Finally, the leaves were washed thoroughly in sterilized distilled water to ensure that the last traces of ethanol and sodium hypochlorite were removed and cut into small pieces of 0.3-0.5 cm^2 in size.

Callus induction

Leaf explants were placed in MS medium (Murashige and Skoog, 1962), containing 3% sucrose, 0.2% gelrite (Sigma, St. Louis, USA) with different concentrations of 2,4-D (2.2 - 5.5 µM) alone and in combination with putrescine (Put) [0.1,

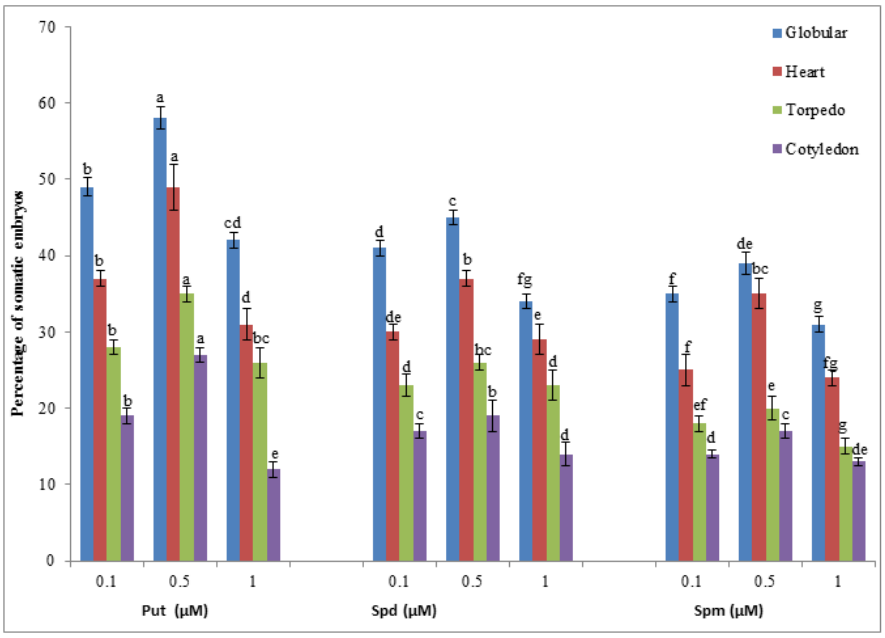

Fig 4. Effects of 3.3 µM 2,4-D with exogenous PAs; Put, Spd and Spm on percentage of somatic embryos produced per 0.2 g of three-weak old embryogenic callus. The data were statistically analyzed using Duncan's multiple range test. Values are mean ± SE. In the same *column*, significant differences according to the least significant difference (LSD) at the $P \leq 0.5$ level are indicated by different letters.

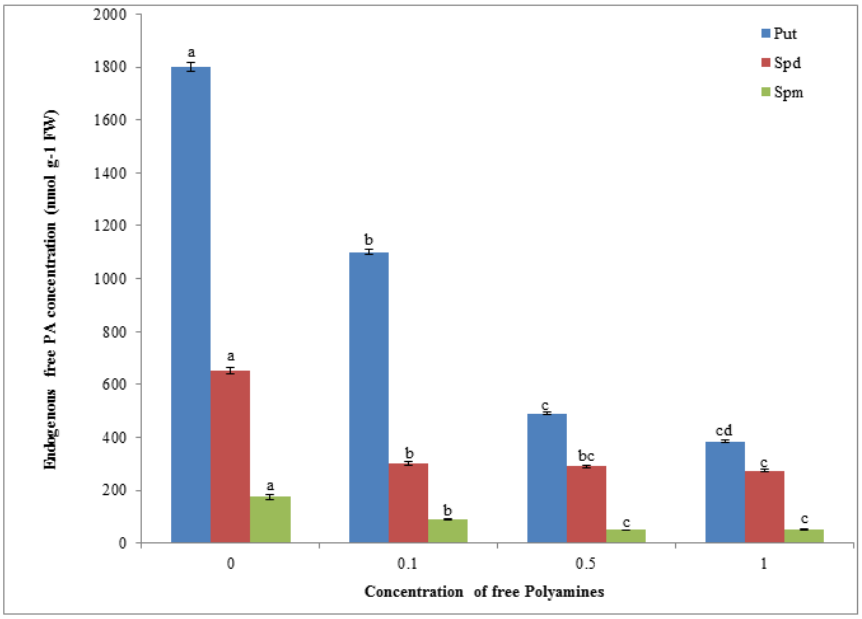

Fig 5. Effects of exogenous PAs on endogenous free PA concentration of 21-day old embryogenic calli. The data were statistically analyzed using Duncan's multiple range test. Values are mean ± SE. In the same *column*, significant differences according to the least significant difference (LSD) at the $P \leq 0.5$ level are indicated by different letters.

0.5, 1.0 µM], spermidine (Spd) [0.1, 0.5, 1.0 µM] and spermine (Spm) [0.1, 0.5, 1.0 µM] were added for callus induction. The incubation condition for culture, unless stated otherwise, was maintained at 25 ± 1°C and 16/8-h (light/dark) photoperiod of cool white fluorescent light (45 µmol m^{-2} s^{-1}). Two transfers were made at an interval of 11 days in the same induction medium.

Suspension culture

For initiation of cell suspension cultures, two-week-old green yellow, friable calli (> 500 mg fresh weight each) were aseptically transferred to 100 ml Erlenmeyer flasks (Pyrex, USA) containing 25 ml of liquid MS medium supplemented with various concentrations of 2,4-D (1.1 - 4.4 µM) and agitated on a gyratory shaker (110 rpm) maintained at 25 ± 1°C and 16/8-h (light/dark) photoperiod of cool white fluorescent light (45 µmol m^{-2} s^{-1}). Suspensions were subcultured every 7 days with concentration of 2,4-D and in combination with putrescine [0.1, 0.5, 1.0 µM], spermidine [0.1, 0.5, 1.0 µM] and spermine [0.1, 0.5, 1.0 µM]. At the end of the second subculture (2 weeks), the cells were filtered through 150 µM stainless steel sieves to separate possible embryogenic cells and small clumps of cells. Cells from the suspension were observed under a microscope during the culture period. A PAs free MS with 2,4-D medium was used

as control. Embryos at different stages of development were separated manually and subcultured in MS liquid medium with different 2,4-D concentrations (1.1 - 4.4 µM) and PAs (Put, Spd and Spm 0.1, 0.5, 1.0 µM). After fourth subculture (2 weeks), globular, heart, torpedo and cotyledonary shaped embryos were formed.

Maturation and germination

Maturation of embryos did not progress in MS liquid medium with 2,4-D and PAs, hence cotyledonary-shaped embryos were transferred into 100 ml Erlenmeyer flasks containing 25 ml of hormone-free MS liquid medium for maturation and further development. For germination, the mature embryos were cultured on MS medium, which contained 1.5% sucrose, 0.5 µM GA_3 and 0.2% gelrite for 14 days. The cultures were maintained at 25 ± 1°C and 16/8-h (light/dark) photoperiod of cool white fluorescent light (45 µmol m^{-2} s^{-1}) and plantlet conversion frequency was observed.

Hardening

The germinated plants were removed from the culture tubes and washed in running tap water. They were transferred to plastic cup containing red soil, perlite and vermiculite mixture (3:1:1) and placed in green house. The plants were watered daily with Hoagland's nutrient solution (Hoagland and Arnon, 1950). The potted plants were then covered with polyethylene bag to maintain a condition of high humidity (85% RH) and grown for 2 weeks at a photosynthetic photon flux density (PPFD) of 25 µmol m^{-2} s^{-1} before planting in the greenhouse. After the development of new leaves, the covers were removed and hardened plants were transferred to earthen pots (diameter 18 cm) filled with soil mix (peat, perlite and vermiculite in equal proportions: 1:1:1) and grown to maturity. The survival percentage was calculated after 4 weeks in the greenhouse.

Histology

The somatic embryos were fixed in 100 ml solution of FAA (formalin, alcohol and acetic acid) which contains 5.4 ml formalin (37%), 65.6 ml alcohol (96%), 5.0 ml acetic acid and 24 ml distilled water (Gerlach, 1984). Samples were cut and embedded in hydroxyethylmethacrylate (Histo-Technique-Set Technovit 7100; Kulzer, Wehrhein, Germany). At the beginning of this process, the specimens were dehydrated for 2 h through a graded series of alcohol (70%, 90%, 96% and 100%). The samples were then pre-infiltrated overnight with a mixture of equal parts of 100% ethanol and Technovit 7100 base liquid. The somatic embryos were then transferred into an infiltration solution of 100 ml Technovit 7100 base liquid and 1 g hardener I for 1 day. A vacuum was established for 30 min at the start of the last two processes. The cultures were embedded in Teflon moulds with a mixture of 15 parts of infiltration solution and one part of hardener II. The samples were polymerized for 1 h at 37°C and further for 6 h at 37°C. Specimens were mounted on block-holders with Technovit 3040. Slices (6 µm) were cut at 37°C using a Jung CM 1800 microtome equipped with type 818 disposable microtome blades (both from Leica Instruments, Nussloch, Germany). Slices were stretched on a bath of distilled water and mounted on slides. They were then stained with 0.05% toluidine blue O (Seva, Heidelberg, Germany) dissolved in 1% sodium tetraborate decahydrate buffer rinsed in distilled water, dried and covered with Entellan (Merck, Darmstadt, Germany) and a coverslip. Microscopic analysis was performed using a Leitz DMR microscope (Leica, Wetzlar, Germany) with a Wild MPS 48/52 camera (Leica, Heerbrugg, Switzerland).

Polyamine extraction

Polyamines were extracted using the method of Smith (1991) with minor modifications. The callus were frozen in liquid nitrogen and ground to a fine powder using a pre-chilled mortar and pestle. The frozen powder (100 mg) was homogenized in cold 5% perchloric acid (PCA) (0.1 g tissue/ ml PCA) and the homogenates were maintained at 4°C for 60 min. The extracts were centrifuged for 20 min at 13000 rpm, and the supernatants were removed and used for the determination of free polyamines. The above extraction procedure was repeated three times.

Dansylation of PAs and TLC analysis

After extraction sodium bicarbonate (50 mg) was added to an aliquot (100 µl) of extract, followed by addition of dansyl chloride in acetone (10 mg/ml). The mixture was incubated at 26°C for 16 h in the dark. Dansylated PAs were extracted with 0.2 ml toluene by vortexing for 1 min and centrifugation for 10 min at 10,000 rpm. The toluene phase was removed and the PAs were analysed using the method of Torrigiani et al., (1987) with minor modifications. Pre-coated plates of silica gel 60 F254 (Merck, Darmstadt, Germany) were used and run with ethylacetate: cyclohexane (2:3, v/v) as the eluent. Spots were visualized under UV radiation and those corresponding to Put, Spd and Spm were identified by comparison with dansylated standards. They were scraped off the TLC plate, the powder eluted with acetone and their relative fluorescence was measured using a spectrofluorometer (F-4500, Hitachi Ltd., Tokyo, Japan) with an excitation at 360 nm and emission at 506 nm.

Statistical analysis

For callus induction, at least 50 explants were used and each experiment was repeated three times. Samples of suspension cultures were taken randomly at the end of each subculture and the number of embryos were counted under a microscope. Counts were made from 10 different independent samples and percentage of embryos were calculated on the basis of the total number of proembryogenic cells present in the field. Each experiment on fresh weight and number of somatic embryos of 21-day old embryogenic calli growing in PA free or PA enriched media was carried out with five replicates and each experiment was repeated twice. Each experiment on PA analysis was carried out with three replicates and each experiment was repeated twice. All the experiments were carried out in completely randomized design. Mean ± SE values of results are presented. The analysis of variance (ANOVA) was carried out to detect the significance of differences among the treatment means. The treatment means were compared using Duncan's multiple range test at $P \leq 0.5\%$ level by SPSS software version (SPSS 14 for windows, SPSS, Chicago, IL, USA).

Conclusion

Polyamines had an enhancement effect during early stages of embryogenic callus growth, in terms of weight and number of somatic embryos in *M. dioica*. Moreover exogenous polyamines when supplemented *in vitro* resulted in lowering

of endogenous PAs level probably by inhibiting the activity of ornithine decarboxylase. Thus, the present study on *in vitro* induction and enhancement of somatic embryos using exogenous polyamines could give better utilization of spine gourd embryos towards plant genetic improvement.

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