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RESEARCH NOTE

Assessment of the efficacy of amino acids and polyamines on regeneration of watermelon (*Citrullus lanatus* Thunb.) and analysis of genetic fidelity of regenerated plants by SCoT and RAPD markers

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Abstract A simple and efficient regeneration protocol was developed for watermelon from cotyledonary node explants excised from 7-day-old in vitro grown seedlings. This study describes the effect of amino acids and polyamines (PAs) along with plant growth regulators (PGRs) on multiple shoot induction and rooting. The highest number of multiple shoots (46.43 shoots/explant) was obtained from cotyledonary node and they were also elongated (6.3 cm/shoot) on MS medium supplemented with 1 mg l^{-1} N^6–Benzyladenine (BA), 5 mg l^{-1} leucine, and 10 mg l^{-1} spermidine. The elongated shoots developed profuse roots (23.03 roots/shoot) in MS medium containing 1 mg l^{-1} indole-3-butyric acid (IBA), 5 mg l^{-1} isoleucine, and 10 mg l^{-1} putrescine. All the rooted plantlets were successfully hardened and acclimatized in the greenhouse with a survival rate of 98%. The present study described an efficient method to obtain a 1.5-fold increase in the number of shoots, compared with the available regeneration protocols for watermelon. The plants developed in this study showed fivefold higher photosynthetic pigments compared to the control plants. The genetic fidelity of the regenerated plants was evaluated by SCoT and RAPD marker analyses, and banding patterns confirmed the true-to-type nature of in vitro regenerated plants.

Keywords Amino acids · Genetic fidelity · Molecular markers · Polyamines · Shoot multiplication · Watermelon

Citrullus lanatus (Thunb.), commonly known as watermelon, belonging to the *Cucurbitaceae* family, is an economically important crop. Watermelon productivity is greatly reduced due to many abiotic and biotic stresses. Genetic engineering and plant transformation are attractive and promising approaches for the production of elite cultivars. However, plant transformation suffers from poor regeneration potential of certain crops, including watermelon. Though, there are few reports available for the in vitro regeneration of watermelon using several explants (Vinoth and Ravindhran 2016; Ameri et al. 2015; Gnamien et al. 2013; Okumus et al. 2011; Suratman et al. 2009; Krug et al. 2005; Sultana et al. 2004; Chaturvedi and Bhatnagar 2001; Srivastava et al. 1989), they are not very efficient in producing a higher number of shoots thereby reducing the transformation frequency. Hence, an improved in vitro regeneration protocol with the potential of generating more shoots is a prerequisite to achieve a higher transformation efficiency. Amino acids are used in plant tissue culture media as an organic nitrogen source. It has been suggested that the presence of amino acids in the medium promoted shoot regeneration through cell division and differentiation of several crops (Satish et al. 2016; Pawar et al. 2015; Asad et al. 2009). Similarly, polyamines (PAs) have beneficial effects in plant cell culture and can act as

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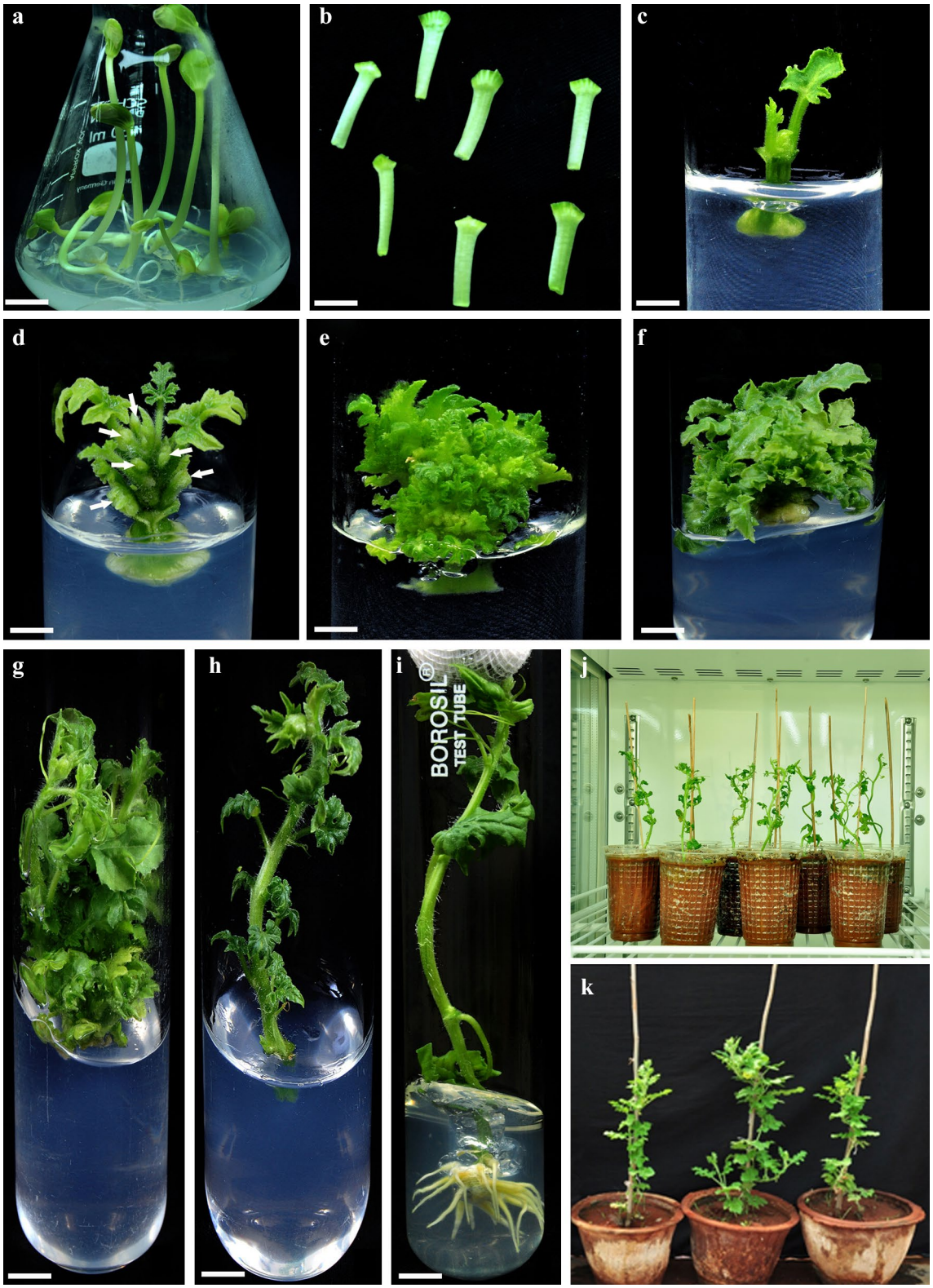

endogenous growth regulators or secondary hormonal messengers which trigger the emergence of the apical meristem (Sivanandhan et al. 2011). PAs have successfully been used in the regeneration of various crops like soybean (Arun et al. 2014), *Withania somnifera* (Sivanandhan et al. 2011), *Capsicum frutescens* (Kumar et al. 2007), and *Cucumis melo* (Tian et al. 1994), but were never tested in watermelon. The genetic stability of the regenerated plants is

◂**Fig. 1** The effect of polyamines on in vitro regeneration of watermelon cv. Arka manik. **a** 7-day-old in vitro grown seedlings; **b** cotyledonary node explants prepared from in vitro grown seedlings; **c** initiation of shoot buds from cotyledonary node on SIEM containing 1.0 mg l^{-1} BA, 5.0 mg l^{-1} leucine, and 10 mg l^{-1} spermidine after 7 days of culture; **d** multiple shoot induction from the cotyledonary node (*arrows* indicate the shoot buds) on SIEM containing 1.0 mg l^{-1} BA, 5.0 mg l^{-1} leucine, and 10 mg l^{-1} spermidine after 2 weeks of culture; **e** and **f** proliferation of shoots on SIEM containing 1.0 mg l^{-1} BA, 5.0 mg l^{-1} leucine, and 10 mg l^{-1} spermidine after 4 weeks and 6 weeks of culture, respectively; **g** elongated shoots on SIEM containing 1.0 mg l^{-1} BA, 5.0 mg l^{-1} leucine, and 10 mg l^{-1} spermidine; **h** elongated shoot in RM containing 1.0 mg l^{-1} IBA, 5.0 mg l^{-1} isoleucine, and 10 mg l^{-1} putrescine for rooting; **i** Shoot with profuse roots in RM containing 1.0 mg l^{-1} IBA, 5.0 mg l^{-1} isoleucine, and 10 mg l^{-1} putrescine; **j** hardened rooted plantlets in the growth chamber; **k** acclimatized plants in the greenhouse. *Arrows* indicates the initiation of adventitious shoots from cotyledonary node explant. *Bars* (**a**–1.0 cm; **b**, **c, d, e, f**–0.3 cm; **g, h**, **i**–0.5 cm)

an important parameter to be analyzed before adapting the regeneration protocol to obtain the transgenic plants. Several molecular markers play important roles in analyzing the clonal fidelity and among them, SCoT and RAPD markers have been widely used (Rathore et al. 2014). Based on the aforesaid facts, the present investigation aimed to investigate the synergistic effect of amino acids and PAs along with plant growth regulators (PGRs) in high frequency shoot regeneration and rooting using cotyledonary node as an explants. In addition, the clonal fidelity of the regenerated plants was evaluated using SCoT and RAPD markers.

The seeds of watermelon cv. Arka manik were procured from the Indian Institute of Horticultural Research (IIHR), Bengaluru, India. The seed germination was established according to the method of Li et al. (2011). Cotyledonary node explants were prepared from 7-day-old in vitro grown seedlings (Fig. 1a) and inoculated on shoot induction and elongation medium (SIEM) containing various concentrations (0.5–2.5 mg l^{-1}) of cytokinins (BA, TDZ, or KIN). After 6-weeks of culture on SIEM, the shoots measuring 4–5 cm were separated from the cotyledonary node and inoculated into the rooting medium (RM) supplemented with various concentrations (0.1–2.0 mg l^{-1}) of auxins (IBA, IAA, or NAA). Appropriate controls were maintained for shoot development and rooting on hormone free MS medium .

To determine the role of amino acids on plant regeneration, cotyledonary node explants (Fig. 1b) were inoculated in to SIEM containing an optimal concentration of BA (1.0 mg l^{-1}), and shoots were inoculated in to RM containing an optimal concentration of IBA (1.0 mg l^{-1}) in combination with various concentrations (5–20 mg l^{-1}) of amino acids such as leucine, isoleucine, methionine, proline, and glycine. Appropriate controls were maintained for shoot development and rooting in amino acids free SIEM and RM medium.

To evaluate the synergistic action of PAs on plant regeneration, cotyledonary node explants were inoculated in to SIEM containing an optimal concentration of BA (1.0 mg l^{-1}) and leucine (5.0 mg l^{-1}), and elongated shoots were inoculated in to RM containing an optimal concentration of IBA (1.0 mg l^{-1}) and isoleucine (5.0 mg l^{-1}) in combination with various concentrations (5–25 mg l^{-1}) of PAs such as spermidine, spermine, and putrescine. Appropriate controls were maintained for shoot development and rooting on PAs free SIEM and RM medium, respectively. In parallel, cotyledonary node explants were inoculated in to SIEM with 1.0 mg l^{-1} BA and elongated shoots were inoculated in to RM amended with 1.0 mg l^{-1} IBA in combination with various concentrations (5–25 mg l^{-1}) of PAs. All cultures were maintained at 25 ± 2 °C under a 16-h photoperiod at a light intensity of 50 µmol m^{-2} s^{-1}.

Well rooted plantlets were separated from the RM, washed thoroughly under running tap water and transplanted into plastic cups containing a mixture of autoclaved soil, sand, and vermiculite (3:1:1v/v/v). After hardening in the growth chamber for 3 weeks at 25 ± 2 °C with 80% relative humidity, the plantlets were transferred into earthen pots containing a sterile potting mixture for acclimatization in the greenhouse. The percentage of survival was recorded on 4th week after transfer into the greenhouse.

A completely randomized design was used for all treatments. All experiments were repeated thrice. The data were analyzed using one-way ANOVA, and the differences contrasted using Duncan's multiple range test (DMRT). All statistical analyses were performed at the level of *P* value less than 0.05 using SPSS 20 software (SPSS Inc, USA) for windows 7.0.

The photosynthetic pigments (chlorophyll a, b and carotenoids) were quantified from the leaves of in vitro regenerated plants following the method described by Aremu et al. (2012) and the concentrations were expressed as µg g^{-1} FW. Genomic DNA was extracted from eight randomly selected in vitro regenerated plants and in vivo grown plants (developed from the seed) using a genomic DNA isolation kit (Sigma-Aldrich, St. Louis, MO, USA). Genetic fidelity analysis was carried out by means of SCoT and RAPD markers. A total of 17 SCoT and 9 RAPD primers were employed to study the genetic fidelity of regenerated plants. The PCR reactions were carried out with specific primers in a PTC-100® thermal cycler (MJ research Inc, Waltham, Mass, USA) programmed with an initial denaturation at 94 °C for 4 min and 40 cycles at 94 °C for 1 min, 50 °C (37 °C for RAPD) for 1 min, 72 °C for 2 min which was followed by a final extension at 72 °C for 5 min. The amplified products were separated by electrophoresis on a 1.2% agarose gel. The amplified bands were scored and the genetic fidelity was analyzed as described by Agarwal et al. (2015).

Among the various cytokinins tested, SIEM supplemented with 1.0 mg l^{-1} BA showed the maximum response (82.33%) for shoot induction and elongation (Supplementary Table 1) from cotyledonary node explants. At this concentration, 13.23 shoots were induced from each cotyledonary node and the length of each shoot measuring 4.3 cm and each shoot comprises 4.26 nodes (Supplementary Table 1). SIEM devoid of cytokinins did not respond well for shoot induction and elongation (Supplementary Table 1). The addition of amino acids to the medium greatly influenced the regeneration potential of different crops (Asad et al. 2009; Vasudevan et al. 2008; Vasanth et al. 2006). In this report, different amino acids at various concentrations were amended in SIEM containing an optimum concentration of BA (1.0 mg l^{-1}). The results proved that the incorporation of leucine in SIEM significantly improved the response for shoot induction and shoot elongation compared to other amino acids tested. At an optimal concentration (5.0 mg l^{-1}) of leucine, 85% of cotyledonary node explants were responding for shoot induction and produced 29.4 shoots per explant. Each shoot measured 5.60 cm in length with 5.60 nodes (Supplementary Table 2). The results were in agreement with a previous report from cucumber where, the combination of BA and leucine significantly improved shoot induction and further development (Vasudevan et al. 2008). It was stated that the amino acids provide organic nitrogen, which requires less energy than inorganic nitrogen to be mobilized into the cells/tissue for differentiation and morphogenesis (Kim and Moon 2007).

The inclusion of PAs along with cytokinins showed a synergistic effect on shoot induction and elongation of numerous crops (Arun et al. 2014; Thiruvengadam et al. 2012; Sivanandhan et al. 2011). It has been reported that the exogenous supplementation of PAs triggers proliferation and growth of plant cells, and also leads to adventitious shoot formation (Thiruvengadam et al. 2012). In the present investigation, shoot induction and elongation from the cotyledonary node were critically influenced by different PAs in a dose dependent manner. The highest percentage (93%) of explants responded for shoot induction, shoot number (46.43 shoots per explant), shoot length (6.3 cm), and node number (7.3 nodes per shoot) was observed in SIEM supplemented with 1.0 mg l^{-1} BA, 5.0 mg l^{-1} leucine, and 10 mg l^{-1} spermidine (Table 1; Fig. 1c–h). However, when the spermidine concentration increased beyond 10 mg l^{-1}, the percentage of explants response for shoot induction, shoot number, and shoot length was significantly reduced (Table 1). SIEM amended with the optimum concentration of BA (1.0 mg l^{-1}) and various concentrations of PAs but lacking amino acids also showed a reduced regeneration potential of the cotyledonary node (Supplementary Table 3). It is assumed that leucine and spermidine provided nitrogen and amine sources, respectively, and along with BA exhibit a synergistic effect for shoot

Table 1 Effect of polyamines on multiple shoot induction from cotyledonary node explants derived from 7-day-old in vitro grown seedlings of *C. lanatus* cv.Arka manik on MS medium containing BA (1.0 mg l^{-1}) and leucine (5.0 mg l^{-1}) after 6 weeks of culture

PAs (mg l^{-1})	Percentage of explants responding (%)	Number of shoots per explant	Mean number of nodes per shoot	Mean shoot length (cm)
Control	85.00 ± 0.57bc	29.40 ± 0.65i	5.60 ± 0.95abc	5.60 ± 0.37abc
Spermidine				
5	86.33 ± 0.66b	37.40 ± 0.89c	6.00 ± 0.51abc	5.66 ± 0.63abc
10	93.00 ± 0.66a	46.43 ± 0.87a	7.30 ± 0.88a	6.30 ± 0.30a
15	83.00 ± 0.57cd	41.00 ± 0.56b	6.73 ± 0.63abc	5.36 ± 0.41abc
20	79.00 ± 0.57ef	37.10 ± 0.63c	6.36 ± 0.84abc	5.06 ± 0.08abc
25	72.66 ± 0.57hi	33.06 ± 0.52fg	5.70 ± 0.65abc	4.66 ± 0.66bc
Spermine				
5	78.00 ± 0.57fg	35.03 ± 0.60de	6.43 ± 0.23abc	5.70 ± 0.30abc
10	86.00 ± 1.15b	41.00 ± 0.60b	7.06 ± 0.52ab	6.00 ± 0.51ab
15	76.33 ± 0.57g	37.00 ± 0.63cd	6.40 ± 0.30abc	5.70 ± 0.30abc
20	72.00 ± 0.57hi	34.00 ± 0.49ef	6.00 ± 0.05abc	5.03 ± 0.52abc
25	69.33 ± 0.00j	32.33 ± 0.72fgh	5.70 ± 0.30abc	4.66 ± 0.33bc
Putrescine				
5	72.00 ± 0.57hi	30.73 ± 0.32hi	5.70 ± 0.35abc	5.06 ± 0.03abc
10	76.66 ± 0.57g	32.00 ± 0.63fgh	5.36 ± 0.37bc	5.33 ± 0.18abc
15	81.00 ± 1.00de	36.40 ± 0.87cd	6.00 ± 0.57abc	5.63 ± 0.41abc
20	73.00 ± 1.00h	33.06 ± 0.12fg	5.70 ± 0.60abc	5.06 ± 0.54abc
25	70.33 ± 0.57ij	31.36 ± 0.68gh	5.00 ± 0.05c	4.36 ± 0.41c

Values represent the mean ± standard error of three experiments. Mean values followed by the same letters within a column are not significantlydifferent according to Duncan's multiple range test at 5% level

induction and shoot differentiation from cotyledonary node explants. The results were in agreement with the report of Vasudevan et al. (2008), where the combination of leucine and spermidine significantly enhanced shoot regeneration in cucumber.

Among the various concentrations of auxins analyzed for the potential in root induction, 1.0 mg l^{-1} IBA in RM emerged as the optimal concentration. At this concentration of IBA, 83% of shoots responded to rooting and each shoot produced 5.03 roots measuring 2 cm in length (Supplementary Table 4). The rooting response was relatively lower in either auxin-free RM or RM supplemented with auxins other than IBA (Supplementary Table 4). The results were in accordance with Selvaraj (2002) who observed a maximum number of roots in *Cucumis sativus* measuring 3.2 cm in length in a medium containing IBA. The complementation of RM with amino acids further improved the potential of root induction. RM containing 1.0 mg l^{-1} IBA and 5.0 mg l^{-1} isoleucine showed a significantly higher percentage (80.33%) of rooting and each shoot produced 12 roots measuring 3.4 cm in length (Supplementary Table 5). The rooting response was relatively low in amino acid free RM or RM supplemented with amino acids other than isoleucine (Supplementary Table 5). Along with IBA and isoleucine, PAs also played a significant role in improving the rooting response. RM amended with 1.0 mg l^{-1} IBA, 5.0 mg l^{-1} isoleucine, and 10 mg l^{-1} putrescine showed a maximum rooting percentage of 95% with the production of 23.03 roots (Fig. 1i) per shoot measuring 4.32 cm in length (Table 2), which was significantly higher compared to RM containing 1.0 mg l^{-1} IBA and 5.0 mg l^{-1} isoleucine (Supplementary Table 5). RM supplemented with the optimal concentration of IBA (1.0 mg l^{-1}) and various concentrations of PAs but lacking amino acids showed a reduced rooting response (Supplementary Table 6). Similar to the present report, Vasudevan et al. (2008) observed a higher rooting response of *Cucumis sativus* in a medium containing the combination of auxin (IBA), amino acid (leucine), and PA (putrescine). Furthermore, the reports of Arun et al. (2014) in soybean, and Sivanandhan et al. (2011) in *W. somnifera* also proved the positive effect of putrescine in root induction and elongation. The well rooted plantlets were successfully hardened in the growth chamber (Fig. 1j) and acclimatized in the greenhouse (Fig. 1k) with success rate of 98%. This higher percentage of survival rate might be due to the putrescine in the RM which is believed to play an important role in the acclimatization of in vitro regenerated plants (Geneve and Hackett 1990).

PAs played a significant role in increasing the photosynthetic pigments of leaves collected from the regenerated watermelon plants. The plants that received 10 mg l^{-1} spermidine during their regeneration showed a fivefold increase in the level of photosynthetic pigments compared to their counterparts (Fig. 2a–d). PAs play a vital role in the

Table 2 Effect of polyamines on root induction from elongated shoots of *C. lanatus* cv. Arka manik on MS medium containing IBA (1.0 mg l^{-1}) and isoleucine (5.0 mg l^{-1}) after 4 weeks of culture

PAs (mg l^{-1})	Percentage of explants responding (%)	Number of roots per shoot	Mean root length (cm)
Control	80.00 ± 0.57fg	12.33 ± 0.86h	3.40 ± 0.20bcd
Spermidine			
5	81.33 ± 0.66ef	18.03 ± 0.54de	3.51 ± 0.23bcd
10	86.33 ± 0.33c	20.03 ± 0.03bc	3.81 ± 0.20abc
15	82.00 ± 0.00e	17.00 ± 0.57ef	3.62 ± 0.15bcd
20	80.00 ± 0.57fg	15.03 ± 0.60g	3.32 ± 0.20cde
25	77.33 ± 0.66j	12.66 ± 0.35h	3.12 ± 0.10de
Spermine			
5	78.00 ± 0.57ij	13.03 ± 0.54h	3.31 ± 0.25cde
10	83.66 ± 0.33d	16.03 ± 0.60fg	3.51 ± 0.20bcd
15	81.33 ± 0.66ef	14.66 ± 0.39g	3.21 ± 0.00cde
20	79.66 ± 0.33gh	12.03 ± 0.12h	3.01 ± 0.20de
25	78.33 ± 0.66hij	12.06 ± 0.49h	2.71 ± 0.17e
Putrescine			
5	84.00 ± 0.57d	20.36 ± 0.84b	4.01 ± 0.23ab
10	95.00 ± 0.00a	23.03 ± 0.57a	4.31 ± 0.26a
15	92.66 ± 0.66b	21.36 ± 0.63b	3.61 ± 0.26bcd
20	86.00 ± 0.57c	18.66 ± 0.33cd	3.21 ± 0.23cde
25	79.33 ± 0.33ghi	15.66 ± 0.29fg	3.01 ± 0.00de

Values represent the mean ± standard error of three experiments. Mean values followed by the same letters within a column are not significantlydifferent according to Duncan's multiple range test at 5% level

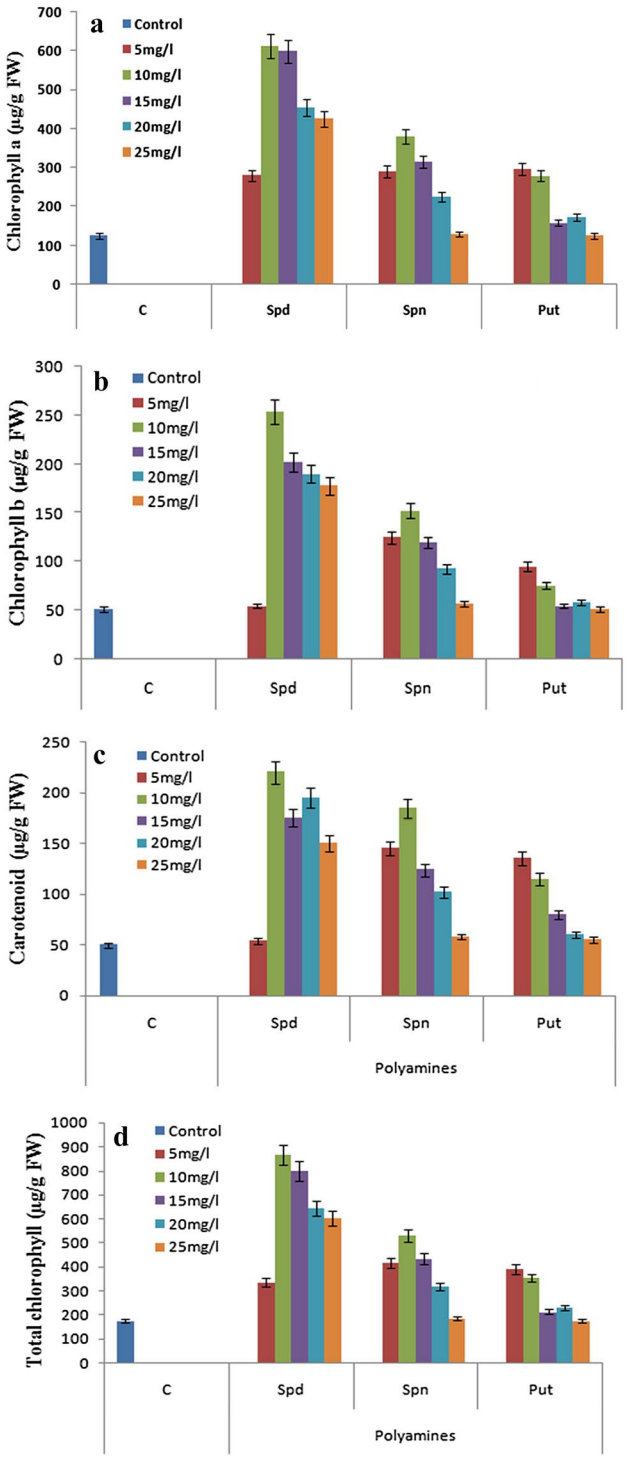

Fig. 2 Effect of polyamines treatment on the accumulation of photosynthetic pigments in in vitro regenerated plants of watermelon cv. Arka manik. **a** quantification of Chlorophyll a; **b** quantification of Chlorophyll b; **c** quantification of Carotenoid; **d** quantification of total Chlorophyll. *C* control, *Spd* spermidine, *Spn* spermine, *Put* putrescine

structure and photosynthetic subcomplexes including thylakoids, light-harvesting complexes and also the membranes of PSII system in photosynthetic pigments are enriched with putrescine, spermidine, and spermine (Navakoudis et al. 2003; Kotzabasis et al. 1993a, b). These PAs interact with phospholipids or other negatively charged groups of photosynthetic proteins and stabilize the membranes of the photosynthetic apparatus. Thus PAs improve the photosynthetic capacity of plants by increasing the level of the photochemical efficiency of PSII (Shu et al. 2012; Zhang et al. 2009). It was reported that the exogenous supplementation of spermidine in cucumber resulted in an increase of chlorophyll synthesis and net photosynthetic rate (Shu et al. 2012).

The genetic fidelity of the regenerated plants was assessed using SCoT and RAPD markers (Supplementary Tables 7, 8). Seventeen SCoT primers produced 47 homogenous and monomorphic fragments in the size range of 300 to 2000 bp (Supplementary Table 7; Supplementary Fig. 1), while 9 RAPD primers yielded 41 clear monomorphic fragments in the size range of 200–1800 bp (Supplementary Table 8; Supplementary Fig. 2). The results clearly showed that there was no genetic variation among the in vitro regenerated plants and in vivo grown plants. Similar to the present investigation, Agarwal et al. (2015) used RAPD and SCoT markers, and Kumar et al. (2015) employed RAPD markers to study the genetic uniformity of the regenerated plants of *Alhagi maurorum* and *Brassica oleracea*, respectively. Further, Agarwal et al. (2015) reported the advantage of using more than one marker in assessing the genetic stability of regenerated plants.

In conclusion, an efficient regeneration protocol was developed for watermelon by supplementing amino acids and polyamines in SIEM and RM. The amino acids acted as modulators of cellular growth and differentiation and improved the shooting and rooting response of watermelon. While, PAs behave as cations and interact with anionic macromolecules such as DNA, RNA, phospholipids, and proteins which influenced the explants to produce a higher number of shoots and roots. The SCoT and RAPD markers proved that there was no genetic variation among the regenerated watermelon plants. The regeneration protocol optimized in this communication could be useful in developing watermelons with better tolerance against several abiotic and biotic stresses by transferring desirable traits through genetic transformation.

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