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Cellular polyamines influence maturation and germination of somatic embryos from pro-embryonal masses of two grapevine cultivars

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Summary

Somatic embryos of grapevine multiply repeatedly and often fail to mature and germinate due to factors like dormancy and embryo teratology. The present investigation was carried out to improve the maturation and germination of somatic embryos from pro-embryonal masses (PEM) of 2A-Clone and 'Crimson Seedless', two seedless grapevine cultivars. Also, the aim was to study a correlation between cellular and residual polyamine (PA) levels in PEM and culture media. The efficiency of maturation and germination of embryos from PEM varied significantly between the two cultivars and depended on incubation period and type of PAs in the medium. HPLC analysis showed that higher levels of cellular putrescine in PEM had correlation with maturation and germination percentages in both cultivars. The levels of three PAs depleted in the media rapidly indicating its uptake by PEM. Of the three PAs, putrescine (PUT) was the most effective and resulted in 100.0 or 92.0 % maturation at 14 d or 30 d in 2A-Clone or 'Crimson Seedless', respectively. The maximum germination of somatic embryos was recorded with PUT at 14 d or 21 d in 2A-Clone or 'Crimson Seedless', respectively.

K e y w o r d s : '2A-Clone', 'Crimson Seedless', germination, grapevine, maturation, polyamines.

A b b r e v i a t i o n s : PAs - polyamines; PEM - pro-embryonal mass; PUT - putrescine; SPD - spermidine; SPM - spermine; µmol g^{-1} FW - micro moles per gram fresh weight.

Introduction

Growth and development of somatic cells of higher organisms is regulated by multiple controls. Somatic embryos of grapevine multiply repeatedly and often fail to mature and germinate due to factors like dormancy and embryo teratology. Apart from plant growth regulators, polyamines (PAs) have been implicated to play a positive role in maturation and germination of somatic embryos of eggplant (Fobert and Webb 1988) and carrot (Mengoli *et al.* 1989). Many plant processes regulated by different plant hormones have been correlated with PA metabolism (Kaur-Sawhrey *et al.* 2003). PAs, spermidine (SPD), spermine (SPM) and their diamine obligate precursor putrescine (PUT), are small aliphatic amines that are ubiquitous in all plant cells. Though the precise role of PAs is yet to be understood, extensive studies suggest their role in a variety of physiological processes ranging from cell growth and differentiation to stress responses.

Polyamines, like PUT behave like cations at their physiological pH and can interact with anionic macromolecules like DNA, RNA, acid phospholipids and proteins (Schuber 1989) and modify different plant processes. PAs have also been implicated in a wide range of biological processes, including growth, development and abiotic stress responses (Minocha *et al.* 1995). PAs have been reported to be key regulatory elements in morphogenesis during somatic and zygotic embryo development in grapevine (Faure *et al.* 1991). The cellular accumulation of PAs in relation to different plant morphogenic processes has not been studied extensively. Earlier reports have indicated correlation between cellular PA levels and maturation and germination of somatic embryos of several plant species (Faure *et al.* 1991; Yadav and Rajam 1997; Minocha *et al.* 1999). However, correlations between PAs and their biosynthetic enzymes and different plant growth processes are not universal and may be species dependant (Evans and Malmberg 1989). Though PAs were known to influence cell differentiation leading to somatic embryogenesis (Feirer *et al.* 1984), their time and duration dependant effects and the precise role of PAs in the regulation of somatic embryogenesis still remains unclear.

In our laboratory, we could establish pro-embryonal masses (PEM) of two grapevine cultivars; 2A-Clone and 'Crimson Seedless' from immature anthers, however, the frequencies of embryo maturation and germination were low resulting in poor embryo conversion to plantlets. The PEM consisted of embryogenic calli with small profuse globular pro-embryoids. Present investigation was carried out to study the correlation between cellular levels of PAs with maturation and germination of somatic embryos from PEM in the two economically important grapevine cultivars, 2A-Clone and 'Crimson Seedless'. The objective was to study a correlation between cellular and residual PA levels in PEM and culture media.

Materials and Methods

Pro-embryonal masses (PEM) of 2A-Clone and 'Crimson Seedless' obtained by anther culture could be proliferated continuously on half strength MS (Murashige and Skoog 1962) basal medium. However, to solve the prob-

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lem of lower percentage of maturation and germination, in the initial experiments, concentrations of putrescine (PUT), spermidine (SPD) or spermine (SPM) were optimized. PEM of both the cultivars were inoculated on half strength MS medium containing N^6-benzyladenine (BA) (0.89 µM) and supplemented with optimized concentrations of polyamines (PAs) - PUT (10 µM), SPD (50 µM) or SPM (40 µM). Sucrose (3 %), agar (0.7 %) and charcoal (0.2 %) were added to all media. Five PEM (500 mg each) were inoculated per petridish (55 mm) and each treatment consisted of five petridishes. Cultures were incubated under 16 h photoperiod at a light intensity of 12.2 µmhos·m^{-2}·s^{-1} at 25 ± 2 °C. Experiment was repeated thrice and observations on maturation (PEM showing well formed globular to torpedo shape embryos) and germination, levels of PAs in the PEM and in the culture media were analyzed by HPLC at weekly intervals. Data were subjected to analysis of variance (ANOVA).

Extraction of polyamines: For HPLC analysis, samples of PEM (200 mg) of both cultivars were drawn at weekly intervals. PEM of both the cultivars were ground separately in 2 ml of 4 % perchloric acid and homogenized. The mixture was kept at 4 °C for 1 h, and then the samples were mixed gently for 1 min and kept on ice for 4 min. Then these were centrifuged for 5 min at 5,000 g and filtered through glass wool.

Sample preparation for HPLC analysis: The test samples as well as PA standards were benzoylated according to the method described by FLORES and GALSTON (1982). The benzoylated PAs were dissolved in 100 µl of 64 % (v/v) methanol (HPLC grade; Merck, Germany) and the extract was analyzed by HPLC. Benzoylated PAs were analyzed with a Waters 2690-separation module HPLC equipped with 2487 Dual absorbance detector (Waters, USA). A delta pack C-18 column (4.6 X 250 mm, 15 µm particle size; Waters, USA) was used for the separation of PAs. The benzoylated PAs (50 µl) were injected manually and chromatographed at 28 °C. The solvent system consisted of methanol: water. Samples were run isocratically at 60 % methanol (v/v), with a flow rate of 0.5 ml·min^{-1} and were detected spectrophotometrically at 254 nm. The regression curves of each PA sample allowed the quantitative estimation of PAs in the sample. Amount of PAs in the samples were expressed as µmol·g^{-1} fresh weight (FW).

Results and Discussion

Percentage of maturation and germination of somatic embryos from PEM significantly varied between the two grapevine cultivars and depended on PAs in the medium and days of incubation. Among the three PAs tested, PUT affected the maximum maturation and germination in both grapevine cultivars. At 30 d of incubation, PUT resulted in 100.0, 92.2 % of maturation and 92.0, 84.6 % of germination in 2A-Clone and 'Crimson Seedless' (CS), respectively (Table). Between SPD and SPM, the latter affected

Table

Cellular PA content in PEM of 2A-Clone and Crimson Seedless cultured in the media supplemented with different PAs

Treatment	Days after inoculation	Maturation %		Germination %		PUT (µmol·g^{-1} FW)		SPD (µmol·g^{-1} FW)	
		2A-Clone	Crimson Seedless	2A-Clone	Crimson Seedless	2A-Clone	Crimson Seedless	2A-Clone	Crimson Seedless
Initial Explant	0	-	-	-	-	1.6	1.1	6.3	8.6
PUT (10 µM)	7	73.2	69.3	12.8	08.9	5.9	3.6	2.5	2.7
	14	100.0	86.6	92.0	74.7	13.8	6.8	0.2	1.7
	21	100.0	90.0	92.0	84.6	10.6	5.9	1.9	1.9
	30	100.0	92.2	92.0	84.6	7.4	4.9	1.8	1.9
SPD (50 µM)	7	00.0	00.0	00.0	00.0	2.4	3.3	8.8	8.0
	14	57.0	36.2	09.7	12.3	5.4	5.3	0.5	5.0
	21	59.1	54.7	21.3	24.5	1.9	4.0	3.0	3.9
	30	60.2	59.1	35.4	32.6	1.3	2.1	1.7	2.0
SPM (40 µM)	7	00.0	00.0	00.0	00.0	4.6	2.3	2.4	1.9
	14	52.6	47.2	23.7	26.3	10.4	3.9	1.9	1.7
	21	62.6	62.7	45.9	49.2	8.9	2.8	1.3	1.9
	30	66.1	65.8	54.3	53.2	7.0	1.8	1.0	1.8
Control	7	00.0	00.0	00.0	00.0	4.4	2.4	1.6	1.6
	14	25.4	14.7	00.0	00.0	4.7	3.1	15.3	10.3
	21	59.8	56.3	16.9	12.3	6.5	3.5	3.0	3.7
	30	83.0	82.1	35.4	31.6	9.7	4.3	1.7	1.9
SEM		1.7	1.6	1.5	2.9	0.5	0.3	0.6	0.5
± CD		6.5	6.1	5.9	11.3	1.5	0.9	1.8	1.4

*Basal medium used: ½MS + BA (0.89 µM) + Sucrose (3%), SEM = standard error of mean; CD = critical difference (p = 0.01).

higher percentage of maturation and germination in both cultivars. In all the treatments, except PUT, the maximum maturation and germination of somatic embryos from PEM of both cultivars was affected at 30 d of incubation. Among the three PAs, PUT was the most effective and resulted in the maximum maturation in 2A-Clone at 14 d, in CS at 30 d and maximum germination in 2A-Clone at 14 d and in CS at 21 d, respectively. PEM without PA treatment (Control) though resulted in high maturation percentage (83.0 and 82.1 at 30 d in 2A-Clone and CS, respectively), however, germination percentages in both cultivars were lower (35.4 and 31.6 for 2A-Clone and CS, respectively), hence was the need to carry out the present investigation. When comparing results of media supplemented with SPD or SPM with the control, it was observed that at 30 d, both PAs did not improve the maturation efficiency, though SPM resulted in higher germination percentages in both cultivars (Table).

In the present study, only free cellular PAs were estimated by HPLC, as they were considered to be abundant and the only active forms (Bagni *et al.* 1994). HPLC analysis showed that the accumulation of cellular PUT in PEM was maximum at 14 d in both cultivars. Further, at 21 and 30 d, cellular PUT levels decreased gradually (Table). A similar decreasing trend in cellular PUT levels from 14 d onwards in both cultivars was observed when medium was supplemented with SPD or SPM also. In control (medium without PA), PUT levels were lower compared to PA treatments in both cultivars and their levels were gradually increased towards 30 d after incubation, where the frequencies of maturation and germination of somatic embryos of both cultivars were the highest.

The cellular levels of PUT increased with progress of culture period and peaked at 14 d in both cultivars irrespective of type of PA supplemented in the media. In contrast to PUT, cellular levels of SPD were higher in the beginning and declined gradually showing an inverse correlation with maturation and germination. This trend was noticed in both grapevine cultivars (Table).

In addition to cellular PAs estimated from PEM during culture (at weekly interval), amount of residual PAs present in the media before (0 d) and during culture (at weekly interval) was estimated. Initial PUT level (0 d) in media supplemented with PUT (10 μM) was 8.8 μmol·g^{-1}. This level decreased differentially in culture media of two cultivars, though the decreasing trend was more or less identical (Figure, A). In case of medium supplemented with SPD, the decline in residual SPD level was sharp in 2A-Clone as compared to CS (Figure, B). The levels of residual SPM in media of both cultivars showed almost an identical trend (Figure, C).

Previous reports on PAs indicate its crucial role in somatic embryo development (Minocha *et al.* 1995). Changes in cellular PA metabolism during somatic embryogenesis have been reported earlier for several plant species (Minocha *et al.* 1995; Kumar *et al.* 1997). Higher levels of PAs have been associated with somatic embryogenesis in carrot (Fienberg *et al.* 1984). In the present study, higher cellular levels of PUT in the beginning might have stimulated maturation of somatic embryos from PEM, which

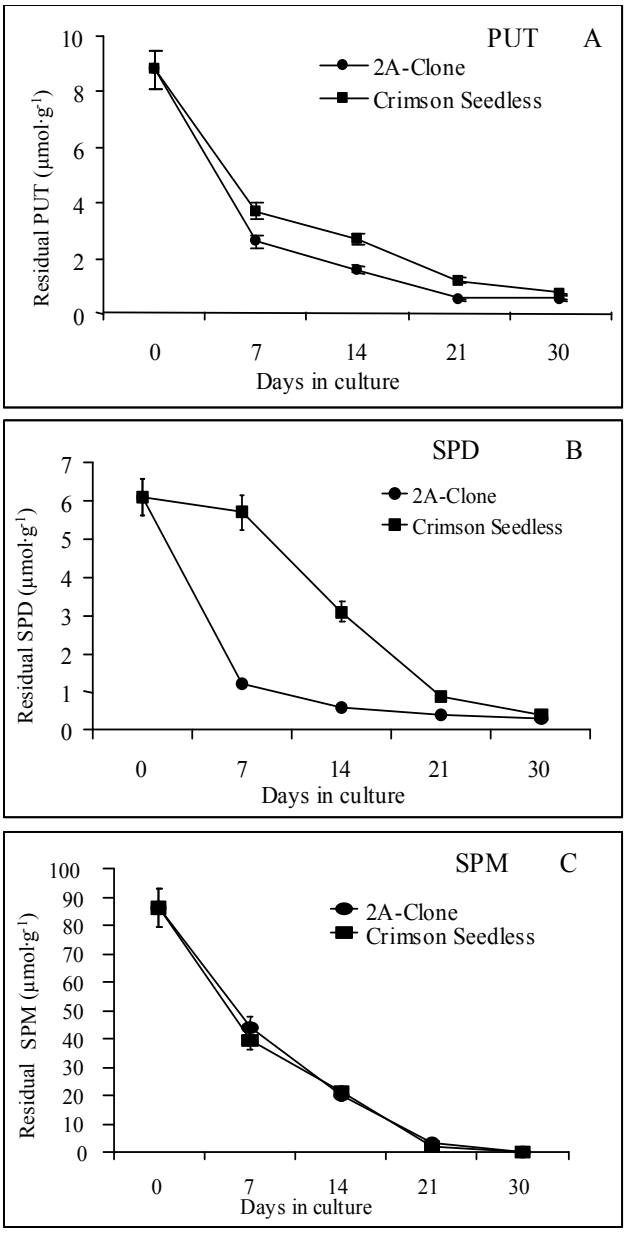

Figure: **A**: Residual PUT in the medium supplemented with PUT (10 μM). **B**: Residual SPD in the medium supplemented with SPD (50 μM). **C**: Residual SPM in the medium supplemented with SPM (40 μM). The values are the mean ± SE of three replications.

may be attributed to the rise in overall metabolic turn over of PAs and corresponding decrease in ethylene production as reported earlier (Andersen *et al.* 1998). Increased activities of arginine decarboxylase (ADC) and S-adenosylmethionine decarboxylase (SAMDC) have been reported as a result of higher PUT levels in tissues (Fienberg *et al.* 1984; Yadav and Rajam 1997). It was found that cellular SPD levels were least at 14 d in both cultivars. An inverse correlation was observed between cellular PUT and SPD levels in the PEM of both cultivars cultured either in PUT or SPD indicating an inter conversion of PUT and SPD by the action of enzymes in the PEM (Tassoni *et al.* 2000). A gradual decrease in PUT level in PEM with the advancement of culture period could be due to utilization of PAs during maturation and germination as reported earlier (Yadav and

Rajam, 1997). In another study on grapevine, increase in PUT content had correlation with the maturation response in Pinot noir (Helior *et al.* 1998). Decrease in the PA levels was observed during embryo transition from globular stage to developing plantlets (Bertoldi *et al.* 2004). In our study, PUT levels were higher during maturation stage while its level decreased during germination. These results are in conformity with earlier reports (Martinelli *et al.* 2001; Bertoldi *et al.* 2004), where a gradual decrease in PUT content was found to have correlation with high efficiencies of embryo germination and conversion to plantlets.

In general, cells undergoing expansion and elongation contain low levels of free PAs synthesized via arginine decarboxylase (ADC) (Galston and Kaur-Sawhney 1995). PUT levels increased substantially with addition of PAs to the media indicating a rapid uptake by PEM cells. PUT levels had a positive correlation with maturation. While, a reverse trend was observed with cellular SPD levels in PEM. A gradual increase in SPD content from bullet shape embryos stage to cotyledonary stage of somatic embryos of *Pinus radiata* has been reported (Minocha *et al.* 1999)

Medium supplemented with PUT showed lesser residual quantity of PUT in 2A-Clone as compared to CS indicating an early and rapid uptake of PUT by 2A-Clone than 'Crimson Seedless'. This could be a reason for higher efficiency of maturation and germination responses in 2A-clone. It was earlier reported in *Arabidopsis* that SPD supplied exogenously could largely be taken up from the culture medium and rapidly translocated to cotyledons (Tassoni *et al.* 2000). The presence of SPD and SPM in the media supplemented with PUT at later stages of culture could be attributed to the synthesis of SPD and SPM from their immediate precursor, PUT in the PEM tissues. The rate of uptake of PAs from the medium by PEM had a strong correlation with the frequency and earliness in maturation and germination of PEM of both grapevine cultivars.

Conclusion

Present study demonstrates the usefulness of exogenous supply of PUT in affecting maturation and germination of somatic embryos of two economically important grapevine cultivars. Cellular PA levels in PEM had correlation with morphogenic changes. The residual PAs measured in the media showed depletion with culture period indicating its ready uptake by PEM, and thereby affecting maturation and germination in two grapevine cultivars. The knowledge of endogenous changes in PA levels could be of help in formulating tissue culture media for development of an efficient somatic embryogenesis system in grapevine.

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