BIOLOGIA PLANTARUM 55 (2): 375-378, 2011

BRIEF COMMUNICATION

Role of auxins, polyamines and ethylene in root formation and growth in sweet orange

A.F.S. MENDES[1], L.C. CIDADE[1], W.C. OTONI[2], W.S. SOARES-FILHO[3] and M.G.C. COSTA[1]*

Center for Biotechnology and Genetics, Biological Sciences Department, State University of Santa Cruz, Ilhéus-45662-000, Brazil[1]
Plant Biology Department, Federal University of Viçosa, Viçosa-36570-000, Brazil[2]
Embrapa Cassava and Tropical Fruit, Cruz das Almas-44380-000, Brazil[3]

Abstract

The primary objective of this work was to investigate the role of polyamines (PAs) on root formation and growth in two sweet orange (*Citrus sinensis* L. Osb.) cultivars Pineapple and Pêra. Adventitious shoots (30-d-old) derived from epicotyl explants were transferred to root induction medium containing Murashige and Skoog salts at different strengths and supplemented with different concentrations and combinations of auxins. Root formation and development decreased in both sweet orange cultivars concomitant with the reduction of medium strength. The α-naphtaleneacetic acid was important during the root differentiation phase, but its combination with indole-3-butyric acid was essential for root elongation. The addition of PAs significantly improved root formation and/or growth, depending on their concentration, whereas the presence of inhibitor of PAs biosynthesis α-difluoromethylornithine (DFMO) inhibited these processes. The rooting impairment caused by DFMO was partially reversed by the supplementation of putrescine. Aminoethoxyvinylglycine AVG and $AgNO_3$ also inhibited *in vitro* rooting in both sweet orange cultivars, indicating that ethylene was likewise important for rhizogenesis in sweet orange.

Additional key words: *Citrus sinensis*, rooting, putrescine, spermidine, spermine.

Significant achievements in the genetic transformation of citrus have been made in the past decades (Moore *et al.* 1992, Gutiérrez *et al.* 1997, Cervera *et al.* 1998, Costa *et al.* 2002, Almeida *et al.* 2003, Li *et al.* 2009, Oliveira *et al.* 2009). However, rooting of the regenerated transgenic shoots remains as a major bottleneck in the currently available protocols. This limitation has been imposed by the poor rooting ability of citrus, especially sweet orange (*Citrus sinensis* L. Osb.), becoming the system inefficient and more complicated due to the need of micrografting. To our knowledge, only a few systematic studies evaluated the possible factors affecting *in vitro* rhizogenesis in citrus, which included auxins (Moreira-Dias *et al.* 2000, Mendes *et al.* 2008), temperature and photosynthetic radiation (Duran-Vila *et al.* 1992). The role of polyamines (PAs) in this process has not been investigated, despite their relevance acquired during the last decade in relation to rhizogenesis (Couée *et al.* 2004). PAs, primarily spermidine (Spd), spermine (Spm) and their precursor putrescine (Put), are considered essential for cellular proliferation and normal cellular function (Bais and Ravishankar 2002). Therefore, the objective of this study was to investigate the relationship of PAs and related metabolic pathways

Received 4 November 2009, *accepted* 11 May 2010.
Abbreviations: AVG - aminoethoxyvinylglycine; BAP - 6-benzylaminopurine; DFMO - α-difluoromethylornithine; IBA - indole-3-butyric acid; MS - Murashige and Skoog; NAA - α-naphtaleneacetic acid; put - putrescine; RIM - rooting induction medium; SIM - shoot induction medium; Spd - spermidine; Spm - spermine.
Acknowledgements to International Foundation for Science (IFS) for financial support; to The State of Bahia Research Foundation (FAPESB) for financial and fellowship supports; to National Council for Scientific and Technological Development (CNPq) and Coordination of Higher Education and Graduate Training (CAPES) for fellowships support.
* Corresponding author; fax: (+55) 73 36805226, e-mail: mcosta@labbi.uesc.br

(ethylene biosynthesis) with root formation and growth in sweet orange, an economically important citrus species highly recalcitrant to *in vitro* rooting. The effects of the strength of Murashige and Skoog (1962; MS) medium and auxins in the rhizogenesis of sweet orange were also investigated.

Seeds of *Citrus sinensis* (L.) Osb. (cv. Pineapple and Pêra) were collected at Citrus Germplasm Collection of Embrapa Cassava and Tropical Fruit (Cruz das Almas, Bahia, Brazil). Preparation of epicotyl explants from etiolated seedlings and regeneration of the adventitious shoots were as described in Mendes *et al.* (2008). The elongated shoots (≥ 0.5 cm) were individually transferred to root induction medium (RIM). The RIM was composed by inorganic salts of MS medium (full-strength, ½-strength and ¼-strength MS salts), in combination with α-naphtaleneacetic acid (NAA) alone (1.0 mg dm^{-3}) or NAA (0.5 mg dm^{-3}) + indole-3-butyric acid (IBA) (0.5 mg dm^{-3}), 25 g dm^{-3} sucrose and 8 g dm^{-3} agar (*Sigma*, St. Louis, USA), pH 5.7 ± 0.1. The auxins NAA and IBA were added to RIM before autoclaving. The media were dispensed as 40 cm^3 aliquots in Magenta boxes (*Sigma*).

Rooting of the shoots was also carried out in RIM containing different concentrations (1 - 1000 μM) of the PAs (Put, Spd, and Spm). Further, inhibitors of polyamine [1 μM α-difluoromethylornithine (DFMO) in combination or not with 1 μM Put] and ethylene [50 μM aminoethoxyvinylglycine (AVG)] synthesis and ethylene action [20 - 50 μM silver nitrate ($AgNO_3$)] were added. For this set of experiments, the RIM was composed by full-strength MS salts, 25 g dm^{-3} sucrose, 0.5 mg dm^{-3} NAA, 0.5 mg dm^{-3} IBA, 8 g dm^{-3} agar, pH 5.7 ± 0.1. PAs, DFMO, AVG, and $AgNO_3$ were filtered through 0.22 μm filters (*Fisher Scientific*, Springfield, USA) and separately added to culture medium after autoclaving.

Experiments were performed with ten shoots per replicate, five replicates per treatment, and repeated at least once. Data of root formation (average number of roots per shoot) and growth (average root length) were evaluated after 45 d of incubation. Statistical analysis was performed with the software *BIOESTAT* (Universidade Federal do Pará, Brazil), which tested the experiments as a completely randomized design. Analysis of variance (*ANOVA*) was applied and for means comparison Bonferroni's test was used, with a critical value of $P \leq 0.05$.

Root formation and growth in sweet orange were significantly affected by the strength of the medium and auxins (Table 1). The average root number and length decreased in both sweet orange cultivars concomitant with the reduction of the ionic strength in the RIM. Whereas higher root numbers were obtained on RIM supplemented with NAA alone, the roots were longer on RIM containing a combination of NAA and IBA. Root initiation is a process that requires a lot of energy, and the demand for salts and sugars in the medium is high (Mohammed and Vivalder 1988). We can conclude that NAA was important in the root differentiation, but its combination with IBA is essential in the root elongation.

The supplementation of exogenous PAs in RIM had a significant effect on the average number of roots per shoot and average root length (Table 2). In cv. Pineapple, more roots per shoot were produced on RIM containing 1000 μM Put or 1 - 100 μM Spd as compared to RIM devoid of PAs (control). However, its roots were shorter in the presence than in the absence of PAs. In cv. Pêra, a significant improvement in the average number of roots per shoot was obtained in RIM supplemented with 100 μM Put, 1 μM Spm, or 1 - 100 μM Spd. Put at 1000 μM and Spd at 1 or 1000 μM also significantly increased the root length in comparison to the control. Spm at 1000 μM completely inhibited root formation and growth in both cultivars, similarly as reported in Virginia pine (Tang and Newton 2005).

The role of PAs on root formation and growth in sweet orange was further investigated using the irreversible inhibitor of PA biosynthesis DFMO in RIM. The results revealed that the inhibition of PA biosynthesis significantly decreased the average root number per shoot and average root length in Pineapple (Table 2). The addition of the 1 μM Put to medium containing DFMO

Table 1. Effects of the strength of MS medium and auxins on *in vitro* root formation and growth in two sweet orange cultivars, Pineapple and Pêra. Means ± SE, $n = 50$. Data are from two independent experiments. Means followed by the same letter in a column are not statistically significant by the Bonferroni's test ($P = 0.05$).

Strength	Auxins [mg dm^{-3}]	Pineapple root number	root length [mm]	Pêra root number	root length [mm]
Full-strength	NAA (1.0)	4.3 ± 0.5 a	21.0 ± 2.0 b	4.4 ± 0.5 a	8.8 ± 0.3 b
	NAA (0.5) + IBA (0.5)	2.2 ± 0.1 b	28.3 ± 1.2 a	3.0 ± 0.3 b	17.5 ± 1.0 a
½-strength	NAA (1.0)	2.4 ± 0.3 b	10.0 ± 0.3 e	2.2 ± 0.4 c	5.0 ± 0.3 b,c
	NAA (0.5) + IBA (0.5)	1.9 ± 0.1 b	16.6 ± 1.2 c	1.4 ± 0.4 c	7.4 ± 0.5 b
¼-strength	NAA (1.0)	1.3 ± 0.2 c	5.9 ± 0.4 f	0.3 ± 0.2 d	4.4 ± 0.2 c
	NAA (0.5) + IBA (0.5)	1.2 ± 0.1 c	13.5 ± 0.9 d	1.0 ± 0.2 c,d	6.7 ± 1.0 b

Table 2. Effects of PAs, DFMO, AVG, and $AgNO_3$ on *in vitro* root formation and growth in sweet orange. Means ± SE, $n = 50$. Data are from two independent experiments. Means followed by the same letter in a column are not statistically significant by the Bonferroni's test ($P = 0.05$ level).

Polyamine [μM]	Inhibitor [μM]	Pineapple root number	Pineapple root length [mm]	Pêra root number	Pêra root length [mm]
0	0	1.8 ± 0.2 b	28 ± 2 b	2.2 ± 0.3 b	16 ± 2 b
Put (1)	0	1.9 ± 0.1 b	19 ± 3 c	2.4 ± 0.3 b	18 ± 1 b
Put (100)	0	1.9 ± 0.2 b	18 ± 3 c	2.8 ± 0.4 a	15 ± 1 b
Put (1000)	0	2.3 ± 0.2 a	21 ± 2 c	2.3 ± 0.3 b	24 ± 3 a
Spm (1)	0	1.7 ± 0.1 b	18 ± 1 c	2.8 ± 0.2 a	18 ± 1 b
Spm (100)	0	1.8 ± 0.2 b	17 ± 2 c	2.4 ± 0.3 b	18 ± 2 b
Spm (1000)	0	0.0 ± 0.0 e	0 ± 0 f	0.0 ± 0.0 d	0 ± 0 e
Spd (1)	0	2.1 ± 0.2 a	15 ± 2 c	3.0 ± 0.1 a	20 ± 1 a
Spd (100)	0	2.1 ± 0.2 a	10 ± 1 d	3.4 ± 0.2 a	19 ± 2 b
Spd (1000)	0	1.9 ± 0.1 b	10 ± 1 d	2.1 ± 0.2 b	21 ± 1 a
0	DFMO (1)	1.0 ± 0.1 c	13 ± 1 d	-	-
Put (1)	DFMO (1)	1.0 ± 0.1 c	49 ± 9 a	-	-
0	AVG (50)	0.0 ± 0.0 e	0 ± 0 f	1.0 ± 0.1 c	6 ± 1 d
0	$AgNO_3$ (20)	1.2 ± 0.4 c	20 ± 2 c	1.0 ± 0.1 c	15 ± 2 b
0	$AgNO_3$ (50)	0.4 ± 0.2 d	5 ± 1 e	1.0 ± 0.1 c	11 ± 1 c

was able to restore root elongation, but not root differentiation. Altogether, these data demonstrate for the first time the critical role that PAs play on citrus rhizogenesis, as was reported in other plant species (Biondi *et al.* 1990, Hausman *et al.* 1994, 1995, Faivre-Rampant *et al.* 2000, Tonon *et al.* 2001, Tang and Newton 2005, Naija *et al.* 2009).

The effects of the inhibition of ethylene biosynthesis (AVG) and action ($AgNO_3$) on *in vitro* rhizogenesis in sweet orange were also investigated, since PAs and ethylene are both derived from related metabolic pathways and probably compete for SAM, a common precursor. The presence of AVG in the RIM significantly reduced the average root numbers and lengths in both cultivars (Table 2). Pineapple showed a higher sensitivity to AVG than Pêra, with no root formation observed in the presence of AVG. Thus, the inhibition of ethylene synthesis, which tends to redirect greater amount of SAM to the PA biosynthesis, cannot improve rhizogenesis in citrus. $AgNO_3$ in the RIM also decreased both root numbers and lengths, irrespective of the sweet orange cultivar. The root growth was severely affected at the higher concentration of $AgNO_3$. Together, these results revealed that ethylene also play an important role in citrus rhizogenesis.

As conclusion, the present work provides new insights into the hormonal and metabolic control of root development in sweet orange. PAs and ethylene, as well as auxins and inorganic salts, play important roles in this process. These findings have important implications from both fundamental and biotechnological perspectives.

References

Almeida, W.A.B., Mourão-Filho, F.A.A., Pino, L.E., Boscariol, R.L., Rodriguez, A.P.M., Mendes, B.M.J.: Genetic transformation and plant recovery from mature tissues of *Citrus sinensis* L. Osbeck. - Plant Sci. **164**: 203-211, 2003.

Bais, H.P., Ravishankar, G.A.: Role of polyamines in the ontogeny of plants and their biotechnological applications. - Plant Cell Tissue Organ Cult. **69**: 1-34, 2002.

Biondi, S., Diaz, T., Iglesias, I., Gamberini, G., Bagni, N.: Polyamines and ethylene in relation to adventitious root formation in *Prunus avium* shoot cultures. - Physiol. Plant. **78**: 474-483, 1990.

Cervera, M., Juarez, J., Navarro, A., Pina, J.A., Durán-Vila, N., Navarro, L., Peña, L.: Genetic transformation and regeneration of mature tissues of woody fruit plants bypassing the juvenile stage. - Transgenic Res. **7**: 51-59, 1998.

Costa, M.G.C., Otoni, W.C., Moore, G.A.: An evaluation of factors affecting the efficiency of *Agrobacterium*-mediated transformation of *Citrus paradisi* (Macf.) and production of transgenic plants containing carotenoid biosynthetic genes. - Plant Cell Rep. **21**: 365-373, 2002.

Couée, I., Hummel, I., Sulmon, C., Gouesbet, G., Amrani, A.E.: Involvement of polyamines in root development. - Plant Cell Tissue Organ Cult. **76**: 1-10, 2004.

Duran-Vila, N., Gogorcena, Y., Ortega, V., Ortiz, J., Navarro, L.: Morphogenesis and tissue culture of sweet orange [*Citrus sinensis* (L.) Osb.]: effect of temperature and photosynthetic radiation. - Plant Cell Tissue Organ Cult. **29**:

11-18, 1992.

Faivre-Rampant, O., Kevers, C., Dommes, J., Gaspar, T.: The recalcitrance to rooting of micropropagated shoots of the *rac* tobacco mutant: implication of polyamines and the polyamine metabolism. - Plant Physiol. Biochem. **38**: 441-448, 2000.

Gutiérrez, E.M.A., Luth, D., Moore, G.A.: Factors affecting *Agrobacterium*-mediated transformation in citrus and production of sour orange (*Citrus aurantium* L.) plants expressing the coat protein gene of *Citrus tristeza* virus. - Plant Cell Rep. **16**: 745-753, 1997.

Hausman, J.F., Kevers, C., Gaspar, T.: Involvement of putrescine in the inductive rooting phase of poplar shoots raised *in vitro*. - Physiol. Plant. **92**: 201-206, 1994.

Hausman, J.F., Kevers, C., Gaspar, T.: Auxin-polyamine interaction in the control of the rooting inductive phase of poplar shoot *in vitro*. - Plant Sci. **110**: 63-71, 1995.

Li, D.L., Tan, B., Duan, Y.X, Guo, W.W.: Regeneration of transgenic citrus plants from the trimmed shoot/root region of etiolated seedlings. - Biol. Plant. **53**: 578-582, 2009.

Mendes, A.F.S., Cidade, L.C., Manzoli, G.N., Otoni, W.C., Soares-Filho, W.S., Costa, M.G.C.: Tissue culture parameters in sweet orange cultivars. - Pesq. agropec. bras. **43**: 1093-1096, 2008.

Mohammed, D.G., Vivalder, W.E.: Root production and plantlet development in tissue cultured conifers. - Plant Cell Tissue Organ Cult. **14**:137-160, 1988.

Moore, G.A., Jacono, C.C., Neidigh, J.L., Lawrence, S.D., Cline, K.: *Agrobacterium*-mediated transformation of *Citrus* stem segments and regeneration of transgenic plants. - Plant Cell Rep. **11**: 238-242, 1992.

Moreira-Dias, J.M., Molina, R.V., Bordón, Y., Guardiola, J.L., Garcia-Luis, A.: Direct and indirect shoot organogenic pathways in epicotyl cuttings of Troyer citrange differ in hormone requirements and their response to light. - Ann. Bot. **85**:103-110, 2000.

Murashige, T., Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Naija, S., Elloumi, N., Ammar, S., Kevers, C., Dommes, J.: Involvement of polyamines in the adventitious rooting of micropropagated shoots of the apple rootstock MM106. - *In Vitro* cell. dev. Biol. Plant **45**: 83-91, 2009.

Oliveira, M.L.P., Febres, V.J., Costa, M.G.C., Moore, G.A., Otoni, W.C.: High-efficiency *Agrobacterium*-mediated transformation of citrus *via* sonication and vacuum infiltration. - Plant Cell Rep. **28**: 387-395, 2009.

Tang, W., Newton, R.J.: Polyamines promote root elongation and growth by increasing root cell division in regenerated Virginia pine (*Pinus virginiana* Mill.) plantlets. - Plant Cell Rep. **24**: 581-589, 2005.

Tonon, G., Kevers, C., Gaspar, T.: Changes in polyamines, auxins and peroxidase activity during *in vitro* rooting of *Fraxinus angustifolia* shoots: an auxin-independent rooting model. - Tree Physiol. **21**: 655-663, 2001.