

Mannose: a potential selection system for genetic transformation of annatto

V.B. PAIVA NETO^{*1}, C.R. CARVALHO^{**} and W.C. OTONI^{*}

Departamento de Biologia Vegetal/BIOAGRO* and Departamento de Biologia Geral**, Universidade Federal de Viçosa, Campus Universitário, 36570-000, Viçosa, MG, Brasil

Abstract

The aim of the present work was to evaluate the feasibility of mannose as a selection system for the future genetic transformation of annatto (*Bixa orellana* L.). Hypocotyl segments, inverted hypocotyls and immature zygotic embryos were inoculated onto a Murashige and Skoog's medium supplemented with B5 vitamins, 87.6 mM sucrose and mannose in different combinations, 2.8 g dm⁻³ *Phytigel*[®], and 4.56 µM zeatin (organogenesis) or 2.26 µM 2,4-dichlorophenoxyacetic acid and 4.52 µM kinetin (somatic embryogenesis). Annatto explants did not regenerate on medium with mannose as the only carbon source when inverted hypocotyls and immature zygotic embryos were used. However, organogenesis (5 % frequency) occurred exclusively in hypocotyl-derived explants nearest to the crown (collar) of the seedlings. No further shoot development was achieved. Therefore the substitution of sucrose by mannose inhibited both organogenesis and embryogenesis, and thus the employment of mannose could constitute an alternative selective agent in protocols for genetic transformation of this species.

Additional key words: *Bixa orellana*, organogenesis, phosphomannose isomerase, somatic embryogenesis.

The species *Bixa orellana* L. (Bixaceae), known as annatto, is native to tropical America, and has become of increasing importance because of natural dyes, mainly bixin and norbixin carotenoids.

The use of antibiotics and herbicides as selective agents has caused a widespread public concern due to the limited knowledge on the possible impacts of these compounds upon environment and human health (Wang *et al.* 2000). In addition, antibiotic resistance markers including kanamycin, hygromycin, geneticin or phosphinothricin have limited use in monocots (Wilmink and Dons 1993), and antibiotic resistance in a final product is often undesirable (Hansen and Wright 1999). To avoid the use of these agents, alternative selection systems have been proposed and developed (Haldrup *et al.* 1998, Joersbo 2001). Recently a system selection based on the toxicity of the monosaccharide mannose to plants has been proposed. The toxic action of mannose makes it a useful selection agent for the generation of

transgenic plants in which the *Escherichia coli manA* gene (PMI, EC 5.3.1.8) encoding phosphomannose isomerase enzyme is used as a resistance marker (Joersbo *et al.* 1998). PMI is common in nature and found across kingdoms, being however less present in plants (Reed *et al.* 2001). According to Wang *et al.* (2000) and (Reed *et al.* 2001), the mannose selection system does not cause any risk to animal, human or environment safety. The *manA* gene expressed in maize cells conferred the ability to utilize mannose as a sugar source, providing an alternative selectable marker for transformation of maize and other plants (Wang *et al.* 2000).

The main purpose of this research is to examine the usefulness of mannose as selection system for future genetic transformation of annatto, providing a foundation for subsequent genetic manipulation and control of specific secondary metabolite pathway related to compounds as bixin.

Received 14 August 2002, accepted 28 April 2003.

Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; MS - Murashige and Skoog's medium; PMI - phosphomannose isomerase.

Acknowledgements: V.B. Paiva Neto is a recipient of a graduate fellowship from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior, Brasil).

¹Present address: FEAD Minas, Rua Cláudio Manoel, 1162, Funcionários, 30140-100, Belo Horizonte, MG, Brasil.

Corresponding author (W.C. Otoni); fax: (+55) 31 38992580, e-mail: wotoni@ufv.br

Seeds of annatto (*Bixa orellana* L) were collected from field-grown plant in an experimental area of the Federal University of Viçosa, Brazil. Seeds were manually scarified with a scalpel, by wounding the seed coat extremity opposite to embryo root axis, in order to overcome physical dormancy. Following, seeds were dipped into a solution of 70 % (v/v) ethanol for 1 min, and afterwards in a solution of 10 % (v/v) calcium hypochlorite for 5 min, and then rinsed 5 times with sterile distilled water. After surface-sterilization process, seeds were germinated in test tubes (150 × 25 mm) containing 10 cm³ of MS-based salts (Murashige and Skoog 1962), supplemented with B5 vitamins (Gamborg *et al.* 1968), 58.4 mM sucrose, 100 mg dm⁻³ myo-inositol, pH 5.7 ± 0.1, and solidified with 2.8 g dm⁻³ *Phytigel*[®] (Sigma Chemical Co., St. Louis, USA). Cultures were maintained under a 16-h photoperiod, irradiance of 36 µmol m⁻² s⁻¹ (two fluorescent tubes; *Luz do Dia Especial*, 20 W, Osram, São Paulo, Brazil) and temperature 27 ± 2 °C. *In vitro* grown seedlings (15 - 20 d after germination) were used as the source of hypocotyls explants.

Hypocotyl segments (9 - 10 mm length) and inverted hypocotyls (40 - 50 mm length) were aseptically removed and inoculated in sterile disposable Petri dishes (90 × 15 mm) and test tubes (150 × 25 mm) containing shoot induction medium consisting of MS-based salts, B5 vitamins, 87.6 mM of different combinations of sucrose and mannose (as specified in Fig. 1), 4.56 µM zeatin, 100 mg dm⁻³ myo-inositol, pH 5.7 ± 0.1, and solidified with 2.8 g dm⁻³ *Phytigel*[®]. Cultures were maintained under same conditions as previously described.

For somatic embryogenesis induction, green, immature capsules were collected from field-grown annatto plants (approximately 10 years old). At harvest (approximately 70 d after anthesis), seed coats were bright and reddish in colour, and the embryos were green. Seeds were removed from capsules, and surface-sterilized as described before, the seeds coat were dissected out and the immature embryos were inoculated onto embryogenic medium containing MS-based salts, supplemented with B5 vitamins, 87.6 mM of different combinations of sucrose and mannose, 1.0 g dm⁻³ activated charcoal, 2.26 µM 2,4-D, 4.52 µM kinetin, 100 mg dm⁻³ myo-inositol, pH 5.7 ± 0.1, and solidified with 2.8 g dm⁻³ *Phytigel*[®]. Cultures conditions were as previously described.

The experiment was performed twice, in a completely randomized design, with five replicates per treatment (10 explants per Petri dish) for both hypocotyl segments and immature embryos, and four replicates per treatment (three test tubes per replicate, containing each one explant) for inverted hypocotyls. Data were collected 40 and 60 d after hypocotyls and immature embryos inoculation, respectively, and both organogenesis and embryogenesis frequencies were recorded.

In order to evaluate the sensitivity of the explants to mannose, dose-response experiments were performed and the effects of mannose and sucrose under different combinations on *in vitro* organogenesis and embryogenesis of annatto were determined. Regardless the explant type no regeneration response was observed with 43.8 mM (data not shown) or 87.6 mM mannose as the only carbon source in the culture medium, mainly when inverted hypocotyls (Figs. 1A, 2F) or immature zygotic embryos (Figs. 1C, 2B) were used as explants. Interestingly, when hypocotyl segments were used, 5 % of organogenesis frequency was obtained (Fig. 1B), but restricted to hypocotyl explants derived from the most proximal segment of seedlings crown (Fig. 2D), resulting in an intriguing response. When mannose under higher concentrations was partially or totally replaced by sucrose, increased organogenesis frequencies were observed, reaching maximum values of 91 % (inverted hypocotyls) and 86 % (hypocotyl segments) with 21.9 mM mannose + 65.7 mM sucrose, and 87.6 mM sucrose, respectively (Figs. 1A, B). A similar response was observed for embryogenesis frequency, reaching 65 % with 87.6 mM sucrose as the only carbon source

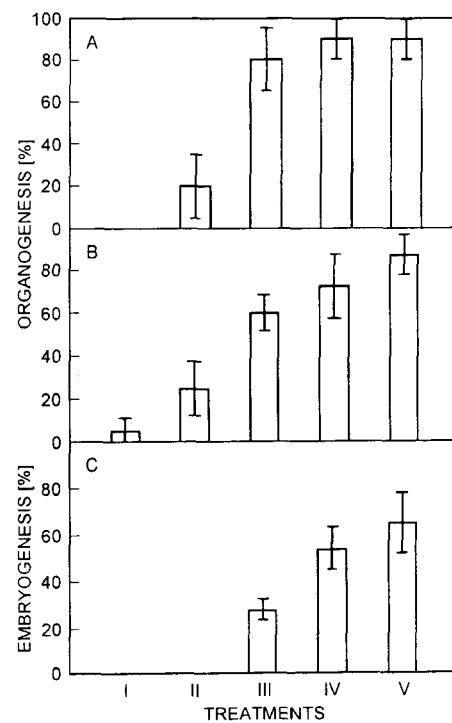


Fig. 1. Organogenesis frequency in inverted hypocotyls (A), hypocotyl segments (B), and embryogenesis frequency in immature zygotic embryos (C) of annatto 40 (A, B) and 60 (C) d after inoculation onto MS medium supplemented with mannose and sucrose under different combinations: I - 87.6 mM mannose; II - 65.7 mM mannose + 21.9 mM sucrose; III - 43.8 mM mannose + 43.8 mM sucrose; IV - 21.9 mM mannose + 65.7 mM sucrose; V - 87.6 mM sucrose. Bars indicate the standard deviations.

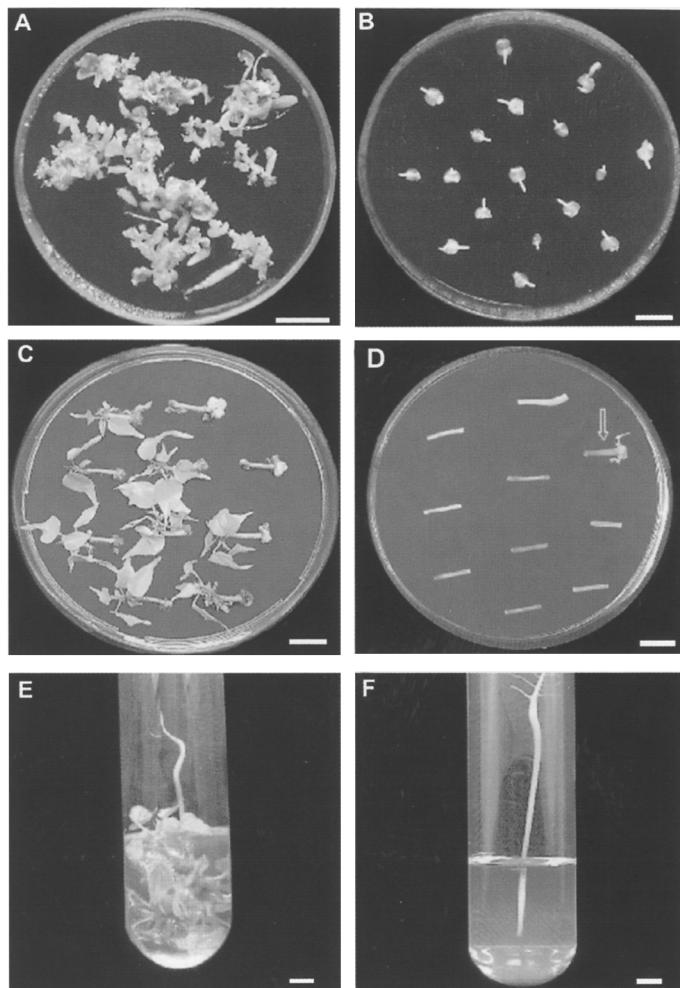


Fig. 2. Immature zygotic embryos (A, B), hypocotyl segments (C, D), and inverted hypocotyl segments (E, F) of annatto 40 d after inoculation onto MS medium supplemented with 87.6 mM sucrose (A, C, E) or 87.6 mM mannose (B, D, F). Bars: 5 mm.

(Fig. 1C). This behavior suggested that sucrose supply was essential to inducing morphogenesis in annatto. Analogous results were found on the influence of sucrose and mannose in the induction and growth of maize callus (Wang *et al.* 2000). The importance of the carbon source for *in vitro* morphogenic process has also been pointed out elsewhere (Petersen *et al.* 1999, Fuentes *et al.* 2000, Ramarosandratana *et al.* 2001).

With traditional transformation protocols, plant cells are placed on culture media containing salts, hormones and a carbon source, usually sucrose. In phosphomannose isomerase (PMI)/mannose selection system, plant tissues are cultured on a similar medium supplemented with either mannose as the unique carbon source or with media containing both sucrose and mannose (Reed *et al.* 2001). Indeed, the enzyme PMI was reported to be found in soybeans and several other legumes, but not in many plant species (Goldsworthy and Street 1965, Lee and Matheson 1984).

Annatto explants that remained for long period on MS medium supplemented with mannose as the only carbon

source grew pale losing the initial green coloration. So, apparently they maintained alive since tissue browning or necrosis did not occur (Figs. 2B,D). One line of evidence to confirm the explant viability was the callogenetic response of the hypocotyl segments after transference to a sucrose-supplemented medium, even after remaining for 90 d in original mannose-supplemented medium. Mannose-regenerated shoots appeared yellowish in color, and also exhibited lower rates of shoot elongation and leaf expansion (Fig. 2D), as compared to those ones differentiated in sucrose-supplemented medium (Figs. 2C,E). When sucrose was present in the medium inoculated with hypocotyls (21.9 to 87.6 mM sucrose) or immature zygotic embryos (43.8 and 87.6 mM sucrose), the explants that did not show shoot formation or embryogenesis conserved the initial green coloration. Therefore, sucrose was able to counteract the inhibitory effects of mannose, even in minor concentration, avoiding that explants grow pale. Likewise, Joersbo *et al.* (1999) found that four frequently employed saccharides (sucrose, glucose, fructose, and maltose) counteract

strongly with the phytotoxic effects of mannose in *Beta vulgaris*, and that the transformation frequencies were also dependent on the nature and concentration of the added sugars, but in this respect sucrose resulted in the highest transformation frequencies. So, the employment of mannose in combination with sucrose, as a selective agent and non-toxic carbon source, respectively, may allow a gradual selective process, like the one used with success by Joersbo *et al.* (1998, 1999) in the transformation of *B. vulgaris*.

The fact that the explants failed to develop when cultured with mannose partially occurs because roots readily take up the sugar and convert it to mannose-6-phosphate by the action of hexokinase. However, mannose-6-P is not further used due to a deficiency of phosphomannose isomerase in cultured cells, which is necessary for its conversion to fructose-6-P. The irreversible formation of mannose-6-P also inhibits respiration by depleting cells of orthophosphate required for ATP production (Goldsworthy and Street 1965, Loughman 1966). In addition, mannose and other hexoses repress transcription of genes required for

photosynthesis and glyoxylate cycle (Jang and Sheen 1994). In *Arabidopsis* the mannose phosphorylation discharge a signal cascade resulting in the repression of genes and energetic depletion during seed germination (Pego *et al.* 1999).

Taking together the data led to conclude that replacement of sucrose by mannose inhibited the annatto morphogenesis, suggesting the usefulness of mannose as a selective agent in protocols for genetic transformation of this species. However, considering the observed organogenic response, it is not recommended the use of the basal portion of hypocotyls nearest the crown for transformation purposes. It can be supposed that shoot differentiation as affected by mannose may be related to a remaining sucrose gradient decreasing acropetally along the seedling hypocotyl, bearing in mind that the seedlings were obtained from a sucrose-supplemented medium.

In short, these results have already been used in our laboratory for establishing a genetic transformation protocol of this species, enabling future prospects on the manipulation of genes involved in pigment biosynthesis and accumulation.

References

Fuentes, S.R.L., Calheiros, M.B.P., Manetti-Filho, J., Vieira, L.G.E.: The effects of silver nitrate and different carbohydrate sources on somatic embryogenesis in *Coffea canephora*. - Plant Cell Tissue Organ Cult. **60**: 5-13, 2000.

Gamborg, O.L., Miller, R.A., Ojima, K.: Nutrient requirements of suspension cultures of soybean root cells. - Exp. Cell Res. **50**: 151-158, 1968.

Goldsworthy, A., Street, H.E.: The carbohydrate nutrition of tomato roots VIII: mechanism of the inhibition by D-mannose of the respiration of excised roots. - Ann. Bot. **29**: 45-58, 1965.

Haldrup, A., Petersen, S.G., Okkels, F.T.: The xylose isomerase gene from *Thermoanaerobacterium thermosulfurogenes* allows effective selection of transgenic plant cells using D-xylose as the selection agent. - Plant mol. Biol. **37**: 287-296, 1998.

Hansen, G., Wright, M.S.: Recent advances in the transformation of plants. - Trends Plant Sci. **4**: 226-231, 1999.

Jang, J.C., Sheen, J.: Sugar sensing in higher plants. - Plant Cell **6**: 1665-1679, 1994.

Joersbo, M.: Advances in the selection of transgenic plants using non-antibiotic marker genes. - Physiol. Plant. **111**: 269-272, 2001.

Joersbo, M., Donaldson, I., Kreiberg, J., Petersen, S.G., Brunstedt, J., Okkels, F.T.: Analysis of mannose selection used for transformation of sugar beet. - Mol. Breed. **4**: 111-117, 1998.

Joersbo, M., Petersen, S.G., Okkels, F.T.: Parameters interacting with mannose selection employed for the production of transgenic sugar beet. - Physiol. Plant. **105**: 109-115, 1999.

Lee, B.T., Matheson, N.K.: Phosphomannoisomerase and phosphoglucoisomerase in seeds of *Cassia coluteoides* and some other legumes that synthesize galactomannan. - Phytochemistry **23**: 983-987, 1984.

Loughman, B.C.: The mechanism of absorption and utilization of phosphate by barley plants in relation to subsequent transport to the shoot. - New Phytol. **65**: 388-397, 1966.

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

Pego, J.V., Weisbeek, P.J., Smeekens, S.C.M.: Mannose inhibits *Arabidopsis* germination via a hexokinase-mediated step. - Plant Physiol. **119**: 1017-1023, 1999.

Petersen, K.K., Hansen, J., Krogstrup, P.: Significance of different carbon sources and sterilization methods on callus induction and plant regeneration of *Miscanthus × ogiformis* Honda 'Giganteus'. - Plant Cell Tissue Organ Cult. **58**: 189-197, 1999.

Ramarosandratana, A., Harvengt, L., Bouvet, A., Galvayrac, R., Pâques, M.: Effects of carbohydrate source, polyethylene glycol and gellan gum concentration on embryonal-suspensor mass (ESM) proliferation and maturation of maritime pine somatic embryos. - *In Vitro* cell. dev. Biol. Plant **37**: 29-34, 2001.

Reed, J., Privalle, L., Powell, M.L., Meghji, M., Dawson, J., Dunder, E., Suttie, J., Wenck, A., Launis, K., Kramer, C., Chang, Y., Hansen, G., Wright, M.: Phosphomannose isomerase: an efficient selectable marker for plant transformation. - *In Vitro* cell. dev. Biol. Plant **37**: 127-132, 2001.

Wang, A.S., Evans, R.A., Altendorf, P.R., Hanten, J.A., Doyle, M.C., Rosichan, J.L.: A mannose selection system for production of fertile transgenic maize plants from protoplasts. - Plant Cell Rep. **19**: 654-660, 2000.

Wilmink, A., Dons, J.J.M.: Selective agents and marker genes for use in transformation of monocotyledonous plants. - Plant mol. Biol. Rep. **11**: 165-185, 1993.