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Somatic embryogenesis and plant regeneration from stem explants of *Moricandia arvensis*

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Abstract Efficient and reproducible plant regeneration has been established from stem internode explants of *Moricandia arvensis*, a crucifer of special interest due to its C_3-C_4 intermediate photosynthetic activity. Somatic embryogenesis was induced in one-third of explants cultured on Murashige and Skoog based medium containing 9 mM 2,4-dichlorophenoxyacetic acid. High frequencies of plant regeneration (>90%) resulted when somatic embryos were germinated on medium lacking growth regulators. Regenerated plants were diploid, fertile and morphologically similar to seed-derived plants of *M. arvensis*. This is the first report of somatic embryogenesis in *M. arvensis*. This plant regeneration system should facilitate gene identification and localisation studies of C_3-C_4 physiology by insertional mutagenesis, a prerequisite for the isolation and transfer of genes involved in C_3-C_4 metabolism from *Moricandia* to cultivated brassicas.

Key words *Moricandia arvensis* · *Brassicaceae* · C_3-C_4 intermediate · Stem explants · Somatic embryogenesis

Abbreviations *2,4-D* 2,4-Dichlorophenoxyacetic acid · *GA_3*, Gibberellic acid · *MS* Murashige and Skoog

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Introduction

Moricandia arvensis (2n=28), a member of the subtribe *Moricandiinae* in the family *Cruciferae*, is a wild relative of the crop brassicas and has a photosynthetic/photorespiratory system intermediate between C_3 and C_4 plants (Holaday et al. 1981). Differential expression of active glycine decarboxylase in leaf tissues, combined with a Kranz-like leaf anatomy, results in efficient recapture of carbon dioxide from photosynthesis and a low carbon dioxide compensation point in this plant. Apel (1994) suggested that improved water efficiency, from C_4 photosynthesis, was of primary importance in the distribution and dominance of C_4 species in semi-arid habitats and that this improvement resulted from a stepwise process via C_3-C_4 intermediate species. Genetic analysis of the C_3-C_4 character of *M. arvensis* would be of interest in studying the evolution of photosynthetic systems, while transfer of genes for C_3-C_4 physiology in crop improvement programmes could increase the adaptability of cultivated plants to drought.

Somatic embryos have been studied extensively as models for understanding the genetic and chemical regulation of early plant devlopment (for reviews see Carman 1990; West and Harada 1993; Zimmerman 1993). Somatic embryogenesis has been reported, in some *Cruciferae*, to improve plant regeneration from explants and protoplast-derived tissues compared with regeneration via organogenesis (O'Neill and Mathias 1993; Hadfi and Batschauer 1994; Pua 1994). Additionally, in other systems, somatic-embryo-derived plants exhibit reduced somaclonal variation since the plants are, in general, of single-cell origin (Pavingerová et al. 1994).

Insertional mutagenesis by transformation of a recipient cell with a DNA fragment is one of the possible routes to identify new genes on the basis of their loss of function. Genes involved in physiological processes can be identified by this approach, provided that recipient cells are totipotent and that a large collection of independent insertions can be generated and screened. The development of an efficient plant regeneration system will allow mutagenic

studies to be undertaken to elucidate the major gene(s) involved in *M. arvensis* C_3-C_4 metabolism. Plant regeneration from *M. arvensis*, via organogenesis, has been reported by Khehra and Mathias (1992). This first report of *M. arvensis* somatic embryogenesis provides an alternative regeneration system for physiological and genetic manipulation studies.

Materials and methods

Plant material

Seeds of *M. arvensis* were surface sterilised with a 30 s rinse in 70% (vol/vol) ethanol, followed by 20 min in 20% (vol/vol) Domestos bleach (Lever, Runcorn, UK). Seeds were washed three times in sterile water. After sterilisation, the seeds were placed, 30 per jar, in 175-ml glass, screw-capped Powder Round glass jars (Beatson Clark, Rotherham, UK), each jar containing 50 ml of Murashige and Skoog (1962) (MS)-based medium supplemented with 3% (wt/vol) sucrose, 50 μM indole-3-acetic acid, 14 mM kinetin, 2.3 μM folic acid, and 0.8% (wt/vol) agar (Sigma, Poole, UK), at pH 5.6 (designated BGS). All cultures were maintained under a 16-h photoperiod (21.45 μE m^{-2} s^{-1} Cool White fluorescent tubes) at 22 ± 2°C. Shoot tips (three nodes in length) of 45-day-old plants were transferred to fresh BGS medium with subculture every 56 days.

Preparation and culture of explants

Internodal sections (0.5 cm in length) were excised from stock plants and randomly plated, ten per dish, onto 25-ml aliquots of MSD2 medium [MS-based medium containing 9 mM 2,4-dichlorophenoxyacetic acid (2,4-D)] in 9-cm-diameter Petri dishes (Bibby-Sterilin, Stone, UK). After 56 days, explants bearing somatic embryos were subcultured to 9-cm Petri dishes each containing 25 ml of either agar-solidified MS-based medium lacking growth regulators (designated MS0), or R1 medium [half-strength MS salts, 3% (wt/vol) sucrose, 0.8% Sigma agar, 0.5 mM 2,4-D, 2.9 mM gibberellic acid (GA_3), pH 5.6; Matsumoto et al. 1991].

Plant regeneration from somatic embryos

After 14–21 days of explant growth on MS0 or R1 media, germinated embryos were excised from the explants and transferred, five per 9-cm Petri dish, onto a 7-cm-diameter Whatman no. 1 filter paper overlaying 25 ml of either MS0 agar medium or GA medium (MS-based medium with 2.9 mM GA_3; Matsumoto et al. 1991). A filter paper was included in each dish, since such a support was reported to promote dehydration and to increase plant regeneration in *Oryza sativa* (Tsukahara and Hirosawa 1992). Embryo-derived plants were transferred, two per jar, to 175-ml glass jars each containing 50-ml aliquots of either agar-solidified MS0 or BGS medium to promote rooting.

Transfer of plants to the glasshouse

Plants were transferred to the glasshouse when 4–5 cm in height. Excess agar medium was removed from their roots by washing in distilled water and individual plants were placed in 8 cm plastic pots each containing a 1:1 (vol:vol) mixture of Levington M3 (Fisons, Ipswich, UK) and John Innes no. 3 (J. Bentley, Barrow-on-Humber, UK) composts. The plants were grown at minimum night and maximum day temperatures of 22°C and 24°C, respectively, under natural daylight supplemented with a 16 h photoperiod of 180 μE m^{-2} s^{-1} (Philips TLD fluorescent tubes). High humidity was maintained, for the first 7–10 days after transfer, by covering the plants with polythene bags, which were perforated on day 10 and removed 20 days after transfer. At least ten seed-derived plants were grown alongside their tissue-culture-derived counterparts. Once flower buds appeared, each inflorescence was covered with a 8½″×10½″ Kenro photographic negative bag (KJP Ltd, Nottingham, UK) to prevent outcrossing. Mature dry siliques were harvested and the seed number per silique recorded.

Pollen viability

Pollen was examined from ten randomly selected somatic-embryo-derived plants and ten randomly selected control, seed-derived plants. Pollen was removed from the anthers of flowers 1 day after anthesis and collected in 1.5-ml Eppendorf tubes, each containing 1 ml of CPW salts solution (Frearson et al. 1973) with 13% mannitol (CPW13 M solution) and 0.005% fluorescein diacetate (FDA; Sigma, UK.). After incubation at 22°C for 5 min, a drop of pollen suspension was placed on a glass microscope slide and examined under UV illumination using a Nikon Diaphot-TMD inverted microscope. Viable pollen fluoresced yellow-green. The percent viability was calculated by scoring at least 600 pollen grains per sample from two samples per plant.

Histological analysis

Samples were fixed overnight in 2.5% (vol/vol) glutaraldehyde (Agar Scientific, Stansted, UK) in 100 mM phosphate buffer, pH 7.0, at 4°C. Fixed tissues were dehydrated through a graded ethanol series and embedded in LR White medium grade resin (London Resin Co., Reading, UK). Semi-thin (2 μm) sections were cut on glass knives, collected on glass slides and stained in 0.5% (wt/vol) toluidine blue in 0.1% (wt/vol) sodium tetraborate (2 min, 60°C) prior to light microscopy.

Cytological analysis

Chromosome spreads were prepared using an enzyme digestion/air-drying procedure. Root tips were collected from 14-day-old glasshouse-grown plants and placed in 1.5-ml Eppendorf tubes, each containing 1 ml of 0.05% (wt/vol) aqueous colchicine (Sigma, UK) solution and stored at 22°C for 2.5 h. The colchicine solution was replaced with a freshly prepared solution of 3:1 (vol:vol) absolute ethanol:glacial acetic acid. Root tips were stored at –20°C. Samples were removed from the fixative and washed (15 min) in distilled water. Individual root tips were transferred to microscope slides, blotted with filter paper and treated with 100 μl of a prewarmed (37°C), filter-sterilised enzyme solution consisting of 2% (wt/vol) Cellulase RS (Yakult Honsha, Nishinomiya Hyogo, Japan), 1.5% (wt/vol) Macerozyme R200 (Yakult Honsha), 0.3% (wt/vol) Pectolyase Y23 (Seishin, Tokyo, Japan) and 1 mM NaEDTA at pH 4.2. Root tips were incubated at 37°C in a moist chamber for 40 min, blotted dry and washed with 100 μl distilled water. The water was replaced with freshly prepared fixative solution and cells spread by tapping with a pair of fine forceps. Preparations were allowed to air dry before staining in 2% (wt/vol) Giemsa RS (BDH, Poole, UK) in 0.05 M phosphate buffer, pH 6.8, at 22°C for 1 h. After washing in distilled water and air drying, preparations were mounted in Styromount (R. A. Lamb, London, UK) and chromosomes counted. Ten spreads per slide and five slides were assessed for each plant.

Results and discussion

Induction of somatic embryogenesis from cultured explants

After 28–30 days of culture on MSD2 medium, 66.9±1.7% of explants had swollen and produced non-chlorophyllous

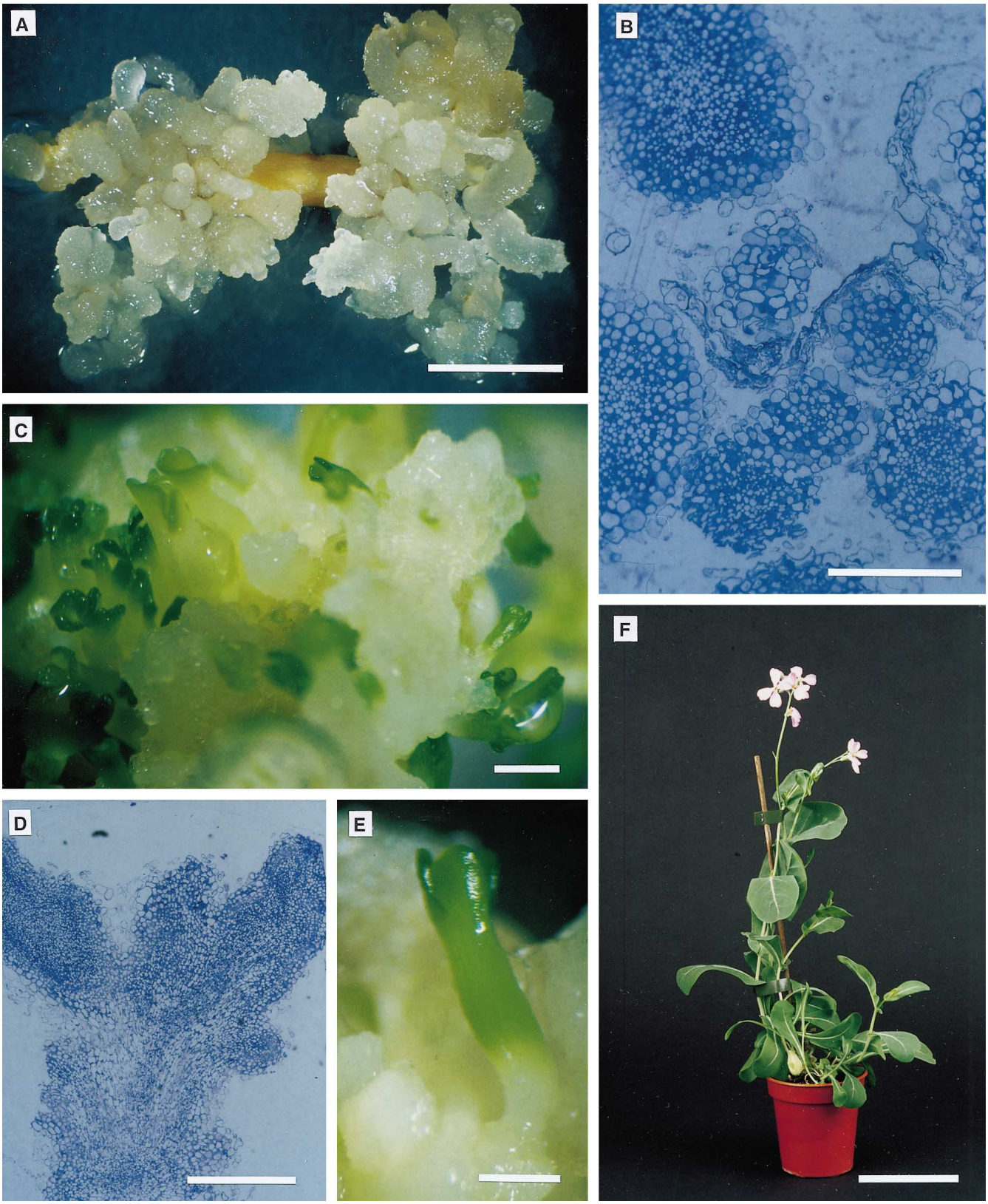

Fig. 1A–F Plant regeneration, by somatic embryogenesis, from *Moricandia arvensis* stem explants. **A** Stem explant exhibiting somatic embryogenesis after 58 days of culture on MSD2 medium (*bar* 0.5 cm). **B** Light micrograph of a transverse section through embryogenic callus showing the production of globular somatic embryos (*bar* 1 mm). **C–E** Germinating somatic embryos, including a longitudinal section of an embryo (**D**), after transfer of stem explants to MS0 agar medium (72 days of culture) (*bar*: **C**, **E** 0.5 cm, **D** 1 mm). **F** A mature, somatic embryo-derived plant in the glasshouse (*bar* 6 cm)

Table 1 Plant regeneration from stem explants of *Moricandia arvensis* by somatic embryogenesis. *Arrows* indicate media transfers during plant regeneration (*M* MS0, *B* BGS, *G* GA medium). Values in parentheses are actual populations of embryos/plants assessed

Experiment	Number of stem explants	Embryogenic explants (%±SD)	Percentage of germinating embryos		Percentage survival of plants ex vitro			
			MS0	GA	M→M	M→B	G→M	G→B
1	20	35.0±49.5	88.6 (31/35)	94.3 (33/35)	72.2 (13/18)	61.1 (11/18)	0 (0/0)	0 (0/0)
2	120	32.0±46.9	96.1 (173/180)	99.4 (179/180)	100 (48/48)	97.9 (47/48)	97.9 (47/48)	100 (48/48)
3	120	32.5±48.1	98.3 (177/180)	97.8 (176/180)	97.9 (47/48)	95.9 (46/48)	100 (48/48)	97.9 (47/48)
Mean±SD	–	33.1±1.7	94.3±5.1	97.2±2.6	90.1±15.5	85.0±20.7	99.0±1.5	99.0±1.5

callus which developed into friable tissue during the next 28 days of culture. By this time, the remaining explants were necrotic, but had produced callus at their cut ends. This callus exhibited a nodular appearance and underwent somatic embryogenesis after 56–60 days of culture (Fig. 1A). Embryogenesis occurred either from 90–100% of explants in each culture dish, or all explants within a dish failed to produce somatic embryos. This response was reflected in the large standard deviations for the three replicate experiments at this stage of regeneration (Table 1). Extracellular proteins are reported to be secreted into the culture medium by tissues undergoing somatic embryogenesis (Zimmermann 1993), which may account for the non-random distribution of embryogenesis, with signals from responding explants triggering cells of nearby explants along the same developmental route. Light microscopy of sections through embryogenic calli revealed highly differentiated structures interspersed with undifferentiated cells (Fig. 1B). Such structures lacked vascular connections with the original explants and were identified as globular somatic embryos. Somatic embryogenesis was the only regeneration response observed. Shoot regeneration, via organogenesis, did not occur in any of the cultured explants.

Germination of somatic embryos and transfer of regenerated plants to the glasshouse

Germinating heart- and torpedo-stage embryos were seen 14 days after explants producing globular somatic embryos were transferred from MSD2 medium to MS0 medium (Fig. 1C–E). Embryos also germinated on explants that were transferred from MSD2 medium to R1 medium. Interestingly, the transfer to growth-regulator-free medium at this stage of somatic embryo development has been beneficial in stimulating embryo germination in other plants, including cassava (Szabados et al. 1987), tobacco (Gill and Saxena 1993) and white clover (Wessinger and Parrott 1993). In carrot cells, a reduction or removal of exogenous auxins is required to alter the activity of genes which modulate embryogenic development (Zimmerman 1993).

Somatic embryos of *Moricandia* grew rapidly following excision from the explants, and developed into plantlets on MS0 or GA media (Table 1). In experiment 1, some somatic embryos exhibited abnormal growth including distorted, fused or multiple cotyledons and non-extended hypocotyls. However, normal growth and root production occurred in subsequent experiments.

Equal numbers of plantlets (Table 1) from MS0 and GA media were transferred to MS0 or BGS media to establish plants *in vitro*. Overall, plant survival rates (72.2±11.0%), on transfer to compost in the glasshouse, were similar for regenerants from all media combinations. The morphology of regenerated plants was identical to that of seed-derived plants (Fig. 1F).

Analysis of regenerated plants

At anthesis, the mean percentage viability of pollen from the somatic-embryo-derived plants (79.9±4.0%) was not significantly different ($P<0.05$) to the viability of pollen from seed-derived plants (70.7±2.4%), according to the Student's *t*-test (Table 2). In addition, the number of seeds per silique from self-pollinated embryo-derived plants was not significantly different ($P<0.05$; Student's *t*-test) from that of seed-derived plants (Table 2). Cytological assessments revealed a normal diploid number of 2n=2x=28 for every root preparation examined. Cells with aberrant chromosome numbers were not observed in any preparations.

Overall, the simple culture procedure, involving MSD2 medium containing 2,4-D followed by MS0 medium lacking growth regulators, is effective for inducing somatic embryogenesis in stem explants of *M. arvensis*.This regeneration system is an efficient alternative to shoot regeneration by organogenesis reported previously (Khehra and Mathias 1992) for the production of fertile, diploid plants.

Insertional mutagenesis or gene tagging is a well-established method for mutant induction in *Arabidopsis thali-*

Table 2 Fertility of somatic-embryo-derived plants compared with seed-derived plants of *M. arvensis*. Data shown are the mean ±SE of ten plants, two samples per plant

Plant origin	Pollen viability	Number of seeds per silique	Chromosome number
Seed derived	70.72±2.39	41.30±2.03	2n=2x=28
Somatic embryo	79.86±4.04	43.35±1.81	2n=2x=28

ana, a close relative of *M. arvensis* and cultivated brassicas. *Agrobacterium*-mediated T-DNA insertional mutagenesis has been exploited to further the understanding of flower development (Van Lijsebettens et al. 1991) and plant embryo development (Errampalli et al. 1996). Additional advances are possible with the refinement of the vector-selection system developed by Bouchez et al. (1993), which utilises a promotorless β-glucuronidase reporter gene, together with two selectable marker genes in the T-DNA insert conferring resistance to kanamycin and the herbicide Basta, and the modified *Dissociation* (*Ds*) transposon-tagging strategy of Wilson et al. (1996) designed to recover dominant gain-of-function alleles. It is envisaged that a combination of the somatic embryogenesis regeneration system reported here with these, or similar, T-DNA or transposon-tagging procedures, could facilitate the identification and isolation of C_3-C_4 gene(s) from *M. arvensis*, prior to their transfer to cultivated brassicas.

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References

Apel P (1994) Water use efficiency in *Flaveria* and *Moricandia* species. Biol Plant 36:243–246

Bouchez D, Camilleri C, Caboche M (1993) A binary vector based on Basta resistance for *in planta* transformation of *Arabidopsis thaliana*. C R Acad Sci III de la Vie 316:1188–1193

Carman JG (1990) Embryogenic cells in plant tissue cultures: occurrence and behaviour. In Vitro Cell Dev Biol 26:746–753

Errampalli D, Patton D, Castle L, Mickelson L, Hansen K, Schnall J, Feldmann K, Meinke D (1991) Embryogenic lethals and T-DNA insertional mutagenesis in *Arabidopsis*. Plant Cell 3:149–157

Frearson EM, Power JB, Cocking EC (1973) The isolation, culture and regeneration of *Petunia* leaf protoplasts. Develop Biol 33:130–137

Gill R, Saxena PK (1993) Somatic embryogenesis in *Nicotiana tabacum* L.: induction by thidiazuron of direct embryo differentiation from cultured leaf discs. Plant Cell Rep. 12:154–159

Hadfi K, Batschauer A (1994) *Agrobacterium*-mediated transformation of white mustard (*Sinapis alba* L.) and regeneration of transgenic plants. Plant Cell Rep 13:130–134

Holaday AS, Shieth YJ, Lee KW, Chollet R (1981) Anatomical, ultrastuctural and enzymatic studies of leaves of *Moricandia arvensis*, a C3-4 intermediate species. Biochem Biophys Acta 637:334–341

Khehra GS, Mathias RJ (1992) Plant regeneration from hypocotyls, cotyledons and stem segments of the C_3–C_4 intermediate species *Moricandia arvensis*. Plant Cell Rep 11:412–415

Matsumoto K, Cabral GB, Teixeira JB, Rech EL (1991) *In vitro* somatic embryogenesis from immature leaves of cassava. R Bras Fisiol Veg 3:107–110

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

O'Neill CM, Mathias RJ (1993) Regeneration of *Arabidopsis thaliana* L. cv. Columbia (C24), via direct embryogenesis. J Exp Bot 44:1579–1585

Pavingerová D, Dostá l J, Bísková R, Benetka V (1994) Somatic embryogenesis and *Agrobacterium*-mediated transformation of chrysanthemum. Plant Sci 97:95–101

Pua E-C (1994) Expression variability of marker genes during plant regeneration from protoplasts of R1 transgenic mustard (*Brassica juncea*) via somatic embryogenesis. J Plant Physiol 143: 363–371

Szabados L, Hoyos R, Roca W (1987) *In vitro* somatic embryogenesis and plant regeneration of cassava. Plant Cell Rep 6:248–251

Tsukahara M, Hirosawa T (1992) Simple dehydration treatment promotes plantlet regeneration of rice (*Oryza sativa* L.) callus. Plant Cell Rep 11:550–553

Van Lijsebettens M, Boer B den, Hernalsteens J-P, Van Montagu M (1991) Insertional mutagenesis in *Arabidopsis thaliana*. Plant Sci 80:27–37

Weissinger AK II, Parrott WA (1993) Repetitive somatic embryogenesis and plant recovery in white clover. Plant Cell Rep 12:125–128

West MAL, Harada JJ (1993) Embryogenesis in higher plants: an overview. Plant Cell 5:1361–1369

Wilson K, Long D, Swinburne J, Coupland G (1996) A *Dissociation* insertion causes a semidominant mutation that increases expression of *TINY*, an *Arabidopsis* gene related to *APETALA2*. Plant Cell 8:659–671

Zimmerman JL (1993) Somatic embryogenesis: a model for early development in higher plants. Plant Cell 5:1411–1423