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Regeneration by somatic embryogenesis of triploid plants from endosperm of walnut, *Juglans regia* L. cv Manregian

Walt Tulecke[1], **Gale McGranahan**[2], **and Hamid Ahmadi**[3]

[1] Antioch College, Yellow Springs, OH 45387, USA
[2] USDA/ARS, Department of Pomology, University of California, Davis, CA 95616, USA
[3] Department of Pomology, University of California, Davis, CA 95616, USA

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ABSTRACT

Plants were regenerated by somatic embryogenesis from endosperm tissue of open-pollinated seeds of Juglans regia L. cv Manregian. These plants were obtained by growing endosperm tissue on media similar to those used for plant regeneration from walnut cotyledons (Tulecke and McGranahan 1985). The plants appear morphologically uniform and have a triploid chromosome number of 3n = 48. Nine plants have been grown to a young sapling stage in soil. This embryogenic line from endosperm has been maintained in culture for two years by the process of repetitive somatic embryogenesis.

INTRODUCTION

Endosperm tissues in culture provide the material for regenerating plants with triploid chromosome number, as shown by the reports of the successful regeneration by organogenesis and somatic embryogenesis. Reviews on endosperm (Bhatnagar and Sawhney 1981), endosperm culture (Johri and Bhojawani 1977), and triploids in woody perennials (Lakshmi Sita 1987) have appeared recently. Research on walnut tissue culture has been reviewed (McGranahan et al. 1987) and includes reports on endosperm (Cheema and Mehra 1982), micropropagation (Driver and Kuniyuki 1984), somatic embryogenesis (Tulecke and McGranahan 1985) and embryo rescue (McGranahan et al. 1986).

This work was undertaken to regenerate triploid walnuts for use in rootstock improvement. The induction of somatic embryos from a tissue culture of endosperm was the method used. It is but one of several techniques which may be useful in obtaining new germplasm for the walnut improvement program, especially if screening techniques can be developed and applied to early stages of propagation systems.

METHODS

The media and techniques used in this study are similar to those previously described (Tulecke and McGranahan 1985). Endosperm tissues of open-pollinated walnut Juglans regia L. cultivars were placed on Medium-1 (Table 1) at approximately weekly intervals from 4-12 weeks post-pollination. The developing nuts used for these cultures were stored for several days at 2-4°C prior to surface sterilization and use. After sterilization the nuts were opened, the white embryo tissue was removed and a portion of the gelatinous endosperm tissue was cultured on Medium-1. Particular care was taken to avoid culturing embryonic tissue and to avoid getting the exudate from the cut fruit on the endosperm tissue. After 2-3 weeks exposure to Medium-1, which is based on the walnut medium of Driver and Kuniyuki (1984) with the addition of 6-benzylaminopurine (1.0 mg/l), kinetin (1.0 mg/l) and indole-3-butyric acid (0.01 mg/l), the endosperm tissue was transferred to the basal medium without added growth substances. Many other media were used in experiments designed to induce a tissue culture from endosperm tissue or to induce somatic embryogene-

Table 1. Media used for the induction of somatic embryogenesis in endosperm tissue of Juglans regia L. cv. Manregian.

	Medium-1 mg/l	Medium-2 mg/l	Basal Medium mg/l
NH_4NO_3	1416	825	1416
$Ca(NO_3)_2{+}4H_2O$	1968	–	1968
K_2SO_4	1559	–	1559
$MgSO_4{+}7H_2O$	740	740	740
$CaCl_2{+}2H_2O$	149	149	149
KH_2PO_4	265	170	265
m-Inositol	100	20	100
Sucrose	30,000	10,000	30,000
1-Glutamine	250	250	–
$ZnSO_4{+}7H_2O$	–	1.7	–
$Zn(NO_3)_2$	17.0	–	17.0
KI	–	1.6	–
$MnSO_4{+}H_2O$	33.4	4.5	33.4
$CuSO_4{+}5H_2O$	0.25	0.005	0.25
H_3BO_3	4.8	1.2	4.8
Na_2MoO_4	0.39	0.05	0.39
$CoCl_2{+}6H_2O$	–	0.005	–
$FeSO_4{+}7H_2O$	33.8	33.8	33.8
NaEDTA	45.4	45.4	45.4
Thiamin HCl	2.0	0.02	2.0
Nicotinic acid	1.0	0.1	1.0
Pyridoxine	–	0.1	–
Glycine	2.0	0.4	2.0
6-Benzylaminopurine	1.0	0.5	–
Kinetin	2.0	–	–
Indole-3-butyric acid	0.01	0.1	–

Offprint requests to: W. Tulecke

sis. Most of these tests will not be reported here, since the results were negative. These included the use of gibberellic acid with casein hydrolysate, additions of abscisic acid, naphthaleneacetic acid, indole-3-butyric acid and kinetin in interactive grids at various concentrations. The protocol of media used to grow the endosperm tissues as sustained tissue cultures and to obtain somatic embryogenesis are given in the results. In addition to basal medium and Medium-1 with added growth substances, another medium with less nitrogen, calcium, potassium and other constituents but increased indole-3-butyric acid was used to obtain somatic embryos from the endosperm tissue cultures (Medium-2, Table 1).

Chromosome counts were made using the procedure of Bradley (1982). This involved the pre-treatment of root tips with p-dichlorobenzene at 4-5°C for 3-4 h, fixation in ethanol: acetic acid (3:1) at 4-5°C for 12-18 h, two water washes and storage in 70% ethanol in the cold. Staining was done with Feulgen's and the tissue was hydrolyzed in a pectinase/cellulase mixture 1:1, prior to applying the squash technique. The roots of two plants derived from somatic embryos from endosperm tissue cultures were used to determine the chromosome number. The roots were taken from potted plants growing in soil.

RESULTS

Tissue cultures of walnut endosperm were established from several cultivars of Juglans regia L. by serial culture on Medium-1, Table 1. These tissue cultures of endosperm were obtained from immature open-pollinated seeds of the cultivars, Payne, Early Ehrhardt, and Manregian. The optimal time for obtaining explants was approximately 8 weeks after pollination. The endosperm tissue of cultivars Hartley, Tehama, Scharsch-Franquette, and Juglans hindsii (Jeps) Jeps. were also cultured, but they did not produce a tissue culture under the same culture procedures.

Excellent growth from the endosperm of Manregian was obtained by culturing the tissue on Medium-1 at 8 weeks after pollination. Three weeks later, the tissue was transferred to the basal medium with the following additions: benzylaminopurine (0.1 mg/l), indole-3-butyric acid (1.0 mg/l), kinetin (0.2 mg/l) and l-glutamine (125 mg/l). After two weeks the tissue was transferred and maintained on Medium-1. Sixteen weeks after initiation of the tissue culture from endosperm a cluster of globular and cotyledonary somatic embryos was observed (Fig. 1). This was the beginning of the embryogenic line from the endosperm of cultivar Manregian. The embryogenic tissue and the somatic embryos were immediately transferred to the basal medium without hormones or glutamine. Improvement in the growth of this embryogenic line was observed after transfer to basal plus 6% sucrose for two weeks and then Medium-2 for three weeks, followed by serial transfers on basal medium.

Five months after the initiation of a tissue culture from the endosperm of Manregian a repetitively embryogenic line developed. A total of 27 globular and 5 cotyledonary somatic embryos were obtained from 9 culture vials. This embryogenic line was maintained by continuous subculture on basal medium using repetitive somatic embryogenesis from the roots and hypocotyl of somatic embryos as the source of additional somatic embryos. Brown hypertrophied callus masses from aged somatic embryos were also used as a source of somatic embryos when repeatedly transferred on basal medium.

An interesting observation was made on the culture of a single root cap from a somatic embryo from this embryogenic line. An ageotropic root grew vertically upward in a culture tube after pretreatment with basal medium containing 10 mg/l abscisic acid (filter sterilized). The loose root cap was lifted off the root and placed on the "6 b" Sequoia rooting medium of Ball, Morris and Rydelius (1978). Nine days later a somatic embryo was observed (Fig. 2). After thirty days in culture five other somatic embryos were observed from the same root cap and the original somatic embryo was well developed (Fig. 3).

Selected somatic embryos which were opaque with starch were placed in the cold at 2-6°C for 2 months to overcome dormancy and prevent rosetting. They were then transferred to peat plugs soaked in dilute White's medium for three weeks until leaf development and greening was sufficient for transfer to potting soil in covered plastic cups (Tulecke and McGranahan 1985). Later, they were planted in 8 inch pots and enclosed in plastic bags, finally being acclimatized and transferred to the greenhouse and then to the field. Only those somatic embryos which showed normal morphology, a white starch storing appearance in the root and hypocotyl region and some vigor were selected for growth into plants. Other abnormal forms were observed in the embryogenic line but these were not selected for further growth.

Chromosome counts on 6 roots from 2 plants were made. The number was verified as triploid, 48, as shown in Fig. 4.

A total of 9 plants have grown to a young sapling stage in soil in the field. They are several years old and appear healthy.

DISCUSSION

Earlier work by Cheema and Mehra (1982) demonstrated the possibility of obtaining tissue cultures from the endosperm of walnut. Their study confirmed the triploid character (3n = 48) of endosperm derived tissue and roots. They also obtained shoot and bud structures but no somatic embryos or plants. Other workers have reported success with apple endosperm (Mu et al. 1977), sandalwood (Lakshmi Sita, Raghaven, Ram and Vaidyanathan 1980) and other plants (Muniyamma 1977; Huang, Huangfu and Xu 1983; Srivastava 1973).

One of the potential uses of triploid plants from walnut is as rootstock. Seeds from the open-pollination of Manregian trees are sometimes used as a source of rootstock, hence the endosperm-derived plants reported here from Manregian endosperm will be evaluated for this use. The plants from Manregian endosperm represent novel germplasm which is composed of two genetic components from the maternal side (Manregian) and one from the paternal side (unknown). The history of the Manregian cultivars has been summarized by Gibson (1964), Wepster (1970) and McGranahan and Catlin (1987).

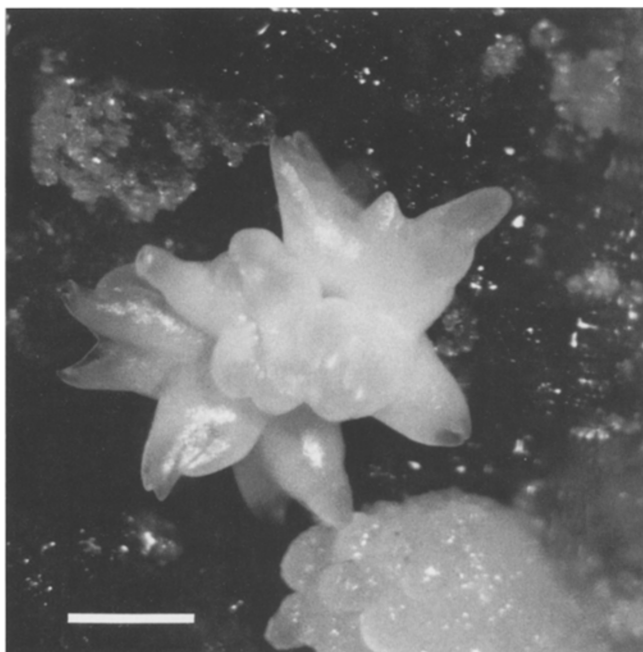

Fig. 1 Callus tissue, globular and cotyledonary somatic embryos obtained from walnut endosperm, Juglans regia L. cv Manregian. The embryogenic line was derived from this tissue. (Scale bar = 1mm).

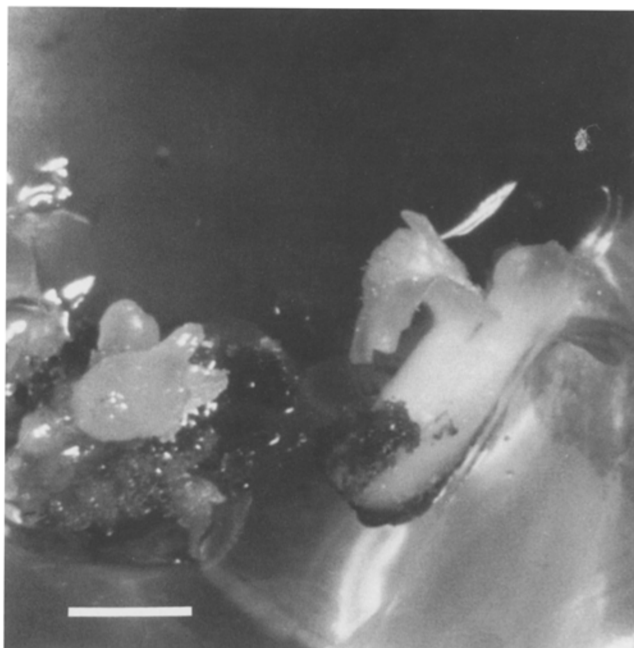

Fig. 3 This is the same root cap culture as shown in Figure 2, but 30 days after culture. The original somatic embryo was removed to the right; five additional somatic embryos in different stages of development may be seen on the original root cap tissue. (Scale bar = 1mm).

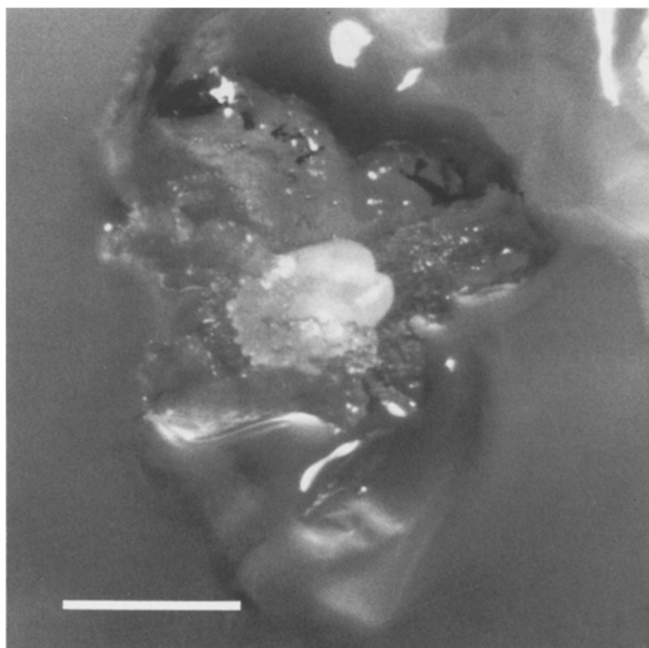

Fig. 2 A somatic embryo developing from root cap tissue. The root cap was loose and easily removed from an ageotropic root of a somatic embryo derived from the endosperm of open-pollinated seeds of Juglans regia L. cv Manregian. The root cap is shown after nine days in culture. (Scale bar = 1mm).

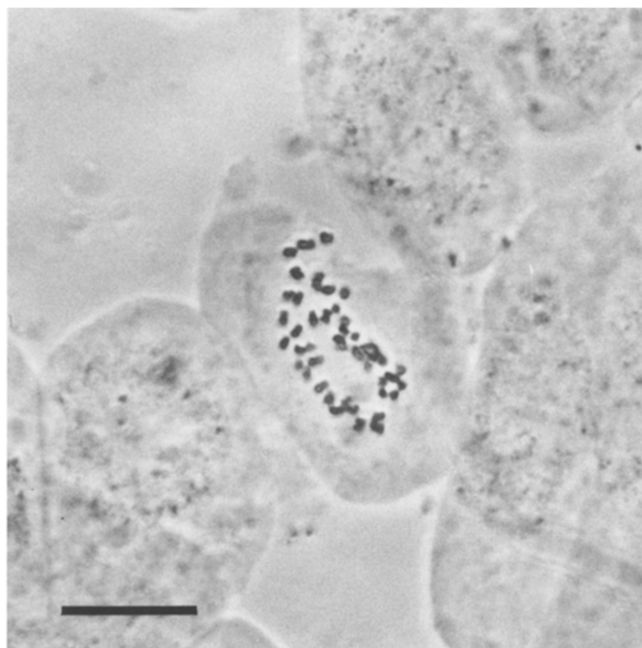

Fig. 4 Feulgen stained preparation of a root tip showing that the chromosome number is triploid (3n = 48). The root tip was taken from a plant growing in soil; the plant was grown from a somatic embryo derived from the endosperm tissue of open-pollinated seeds of Juglans regia L. cv Manregian. (Scale bar = 50μ).

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