Plant Cell, Tissue and Organ Culture **28**: 195–202, 1992.

Maturation and germination of walnut somatic embryos

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Received 3 January 1991; accepted in revised form 2 October 1991

Key words: cold storage, desiccation, gibberellic acid, liquid medium

Abstract

Walnut somatic embryos were multiplied by repetitive embryogenesis on a solid basal DKW medium at 25°C in the dark. When the embryos were isolated at early cotyledonary stage (1–2 mm long) from the primary embryos and cultured on the medium for 3 weeks, they developed into mature embryos showing white, enlarged cotyledons and shoot and root apex. After transfer to light on solid germination medium, however, few mature embryos (0–5%) germinated. Germination percentage increased to about 10% when the mature embryos were pretreated by a storage at 4°C in the dark for 2 months, or by desiccation at 25°C in the dark for 3 or 5 days under an air-humidity conditioned by saturated salt solutions ($Mg(NO_3)_2.6H_2O$, or $ZnSO_4.7H_2O$). Similar results were obtained by the addition of gibberellic acid (GA_3) to the germination medium. When mature embryos were desiccated and then placed on medical cotton compresses in liquid germination medium, 45% of the embryos germinated into complete plantlets. These plantlets continued their growth after transplanting to a mixture of peat and vermiculite in pots.

Abbreviations: GA_3 – gibberellic acid, DKW medium – Driver & Kuniyuki Walnut medium

Introduction

The Persian walnut (*Juglans regia* L.), the eastern black walnut (*J. nigra* L.) and their interspecific hybrid *J. nigra* × *J. regia* are commercially important species, producing wood of high quality. To meet the increasing need for plants, *in vitro* vegetative propagation has been intensively studied in several laboratories. In spite of recent progress, rooting of multiplied shoots has remained a difficult step in micropropagation (Rodriguez et al. 1989). Somatic embryogenesis is an alternative means of propagation and has the potential to produce complete plantlets directly (Tulecke & McGranahan 1985).

Somatic embryogenesis has been obtained from immature cotyledons of *J. regia*, *J. hindsii*, *Pterocarya*, *J. nigra* and *J. major* and interspecific hybrid *J. nigra* × *J. regia* (Tulecke & McGranahan 1985; Cornu 1988, 1989). The somatic embryos can be efficiently multiplied by repetitive or secondary embryogenesis on DKW medium (Driver & Kuniyuki 1984) without plant growth regulators. Complete plantlets have been regenerated in *J. regia* after a cold treatment of 8–10 weeks at 2–4°C (Tulecke & McGranahan 1985). However, Lee et al. (1988) reported a very low efficiency (0–15%) for this species. In fact, although somatic embryogenesis has been obtained for a great number of species including several forest plants, low efficiency of embryo germination and conversion to plantlets remains a major problem affecting most of the embryogenic systems described (Tulecke 1987; Am-

mirato 1989). In the present work, we studied the influence of development stage and quality of embryos on the germination of walnut somatic embryos. Cold storage and desiccation pretreatments, liquid germination medium and gibberellic acid (GA_3) were tested to promote embryo germination and to improve the recovery of transplantable plantlets.

Materials and methods

A repetitively embryogenic culture line was used in the present study. It was derived from the immature cotyledons of an hybrid nut collected on a *Juglans nigra* (NG23) pollinated by *Juglans regia* (Cornu 1988). This line has been maintained by subculture at 25°C in the dark for over 2 years. Proliferated embryos (both isolated embryos and clusters of small embryos) were subcultured every 2–3 weeks on a solid basal DKW medium without growth regulators and solidified with Gelrite at 0.3% (w/v).

Embryo maturation

Two to three weeks after the beginning of the subculture for repetitive embryogenesis, newly formed adventitious embryos were removed at early cotyledonary stage (1 × 1 mm, length × width) and cultured on solid medium (maturation medium) at 25°C in the dark for 3 weeks (87 × 15 mm Petri dish, 20 embryos per dish).

Pretreatments to promote germination

Embryos were stored at 4°C in the dark for 2 months before germination experiments. In the first series of cold storage experiments, 4 different types of embryos were stored on solid medium: white or translucent small embryos (2 × 1 mm, length × width, after 1 week in maturation medium), and white or translucent large embryos (5 × 10 mm, length × width, after 3 weeks in maturation medium). Each treatment consisted of 3 dishes with 10 embryos per dish. In the second series of cold storage experiments, white large embryos were treated in one of the following ways:

- embryos were placed on solid medium in small Petri dishes (50 × 12 mm);
- embryos were placed on solid medium in small Petri dishes that were individually closed in larger Petri dishes (87 × 15 mm) containing 10 ml water;
- embryos were placed in empty small Petri dishes that were individually closed in larger Petri dishes containing 10 ml distilled water.

Each treatment consisted of 5 dishes with 10 embryos per dish. Experiments were repeated twice.

For desiccation experiments, white large embryos were desiccated in empty Petri dishes (87 × 15 mm) at 25°C in the dark. Three dishes containing embryos, and three 100 ml beakers containing a supersaturated solution of either $ZnSO_4.7H_2O$, or $Mg(NO_3)_2.6H_2O$ were placed in an autoclaved plastic box (30 × 20 × 10 cm) sealed by 4 layers of Parafilm. At constant 25°C the $ZnSO_4.7H_2O$ created a relative humidity of about 90%, the $Mg(NO_3)_2.6H_2O$ about 60% (Merck Index 1983). Desiccation lasted 3 days with $Mg(NO_3)_2.6H_2O$, or 5 days with $ZnSO_4.7H_2O$. Each desiccation treatment consisted of 3 dishes with 40–50 embryos per dish. Each germination test of desiccated embryos consisted of 4 or 5 dishes with 10 embryos per dish. Experiments were repeated twice.

Embryo germination and plant development

For germination tests, embryos were placed on solid basal DKW medium for 3 weeks (87 × 15 mm Petri dish, 10 embryos per dish). In some experiments, desiccated embryos were placed for three weeks on medical cotton compresses (4 layers) in 10 ml liquid basal DKW medium, or 10 ml distilled water (87 × 15 mm Petri dish, 10 embryos per dish). In experiments involving gibberellic acid (90% GA_3, Sigma), a filter-sterilized GA_3 solution was added to the medium (or water) after autoclaving, to a final concentration of 0.1, 1 and 5 mg l^{-1}. In germination dishes, we firstly observed the elongation of the root apex and then the emergence of a green shoot. Only the embryos showing both an elongation of the root apex (1 cm or longer) and a development of the green shoot were considered as normally

germinated. Some embryos showed only an elongation of the root (1 cm or longer). They were recorded as 'root elongation'. When the roots attained 2 cm, complete plantlets and embryos showing only root were transferred to small jars (30 × 70 mm, three plants per jar) containing 20 ml solid basal DKW medium for a further 2–3 weeks. The complete plantlets showed good root and shoot growth. Some embryos that previously showed only root elongation in dishes became complete plantlets in the jars. The percentages of normal germination and of root elongation shown in the Results section were the final percentages recorded at this stage. For germination and further development in jars, embryos and plantlets were cultured in a growth room set at 25°C with a 16-h photoperiod under standard cool white fluorescent lamps that gave a photon flux density of about 72 μmol m^{-2} s^{-1}.

About 50 complete plantlets were transplanted to pots (1 plant per pot) containing a mixture of peat and vermiculite (4:1, v:v) in the growth room for 2 weeks. To avoid excess transpiration, the plantlets were covered by transparent plastic pots as described previously by Tulecke & McGranahan (1985). The plantlets were then moved to greenhouse for further development without transparent plastic pots.

Results

Embryo multiplication and maturation

Walnut somatic embryos were efficiently multiplied by repetitive embryogenesis on solid basal DKW medium without plant growth regulators, as previously described (Tulecke & McGranahan 1985). The site of secondary embryo differentiation was mainly the enlarged cotyledons. Especially on the down-side of cotyledons we often found embryo clusters. Secondary embryos were also formed at the root apex region. The adventitious embryos were generally well individualized, and slightly attached to their primary tissues.

However, most of the newly-formed embryos rapidly turned translucent or pale yellow and some died. When the newly formed embryos were isolated from the primary tissues at the early cotyledonary stage (Fig. 1A), most of them (80%) developed into mature embryos after 3 weeks of culture on the same solid medium, showing white, enlarged cotyledons, shoot and root apex (Fig. 1B, C). Some embryos became translucent or pale yellow or showed no obvious shoot apex. They could be used for secondary embryo production.

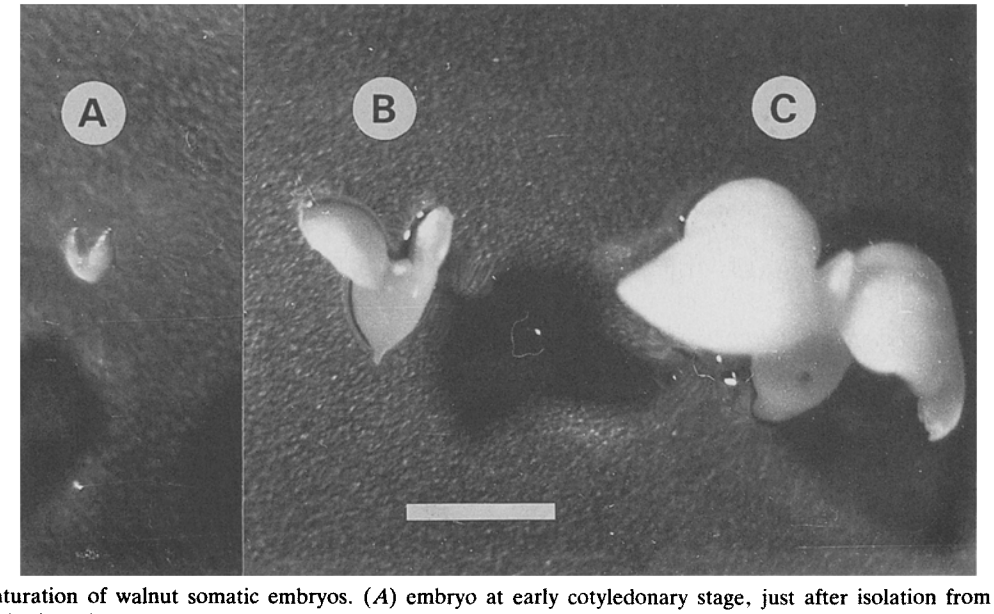

Fig. 1. Maturation of walnut somatic embryos. (*A*) embryo at early cotyledonary stage, just after isolation from the primary embryo; (*B*) after 10 days of culture in maturation medium; (*C*) mature embryo, after 20 days of culture in maturation medium.

Effect of cold pretreatment on embryo germination

When the well-developed mature embryos were kept on solid medium in the dark they did not germinate into plantlets. Although a few embryos (0–5%) showed an elongation of root apex, most of the embryos continued to produce secondary embryos and then became brown. After transfer from dark to light, the germination percentage of mature embryos was also very poor (0–5%). Most of the embryos showed swelling green and red parts without clear organization. In some embryos, the cotyledons turned green and continued to form secondary embryos (Fig. 2A), while their root and shoot apex failed to develop. Some embryos had well-elongated roots but their apical buds remained inactive (Fig. 2B). Only the correctly germinated embryos showing root and green shoot (Fig. 2C) developed into transplantable plantlets (Fig. 2D) and survived after transfer to pots. However, no acclimatization experiments have been done in the present work.

To promote embryo germination, different types of embryos were transferred to solid medium and stored at 4°C for 2 months before germination experiments (Table 1). The well-developed embryos (large, white) showed 10% germination after the cold pretreatment. On the other hand, translucent embryos and the small embryos did not germinate even after a cold treatment. Thus, only large white embryos were used in the experiments described below.

Effects of different cold-storage conditions on embryo germination were further studied (Table 1). No significant difference was observed between the different treatments.

Effect of desiccation pretreatment and germination in liquid medium

When embryos were placed at 25°C in the dark in a closed box under an air-humidity determined by a saturated $Mg(NO_3)_2.6H_2O$ solution for 3 days, or a saturated $ZnSO_4.7H_2O$ solution for 5 days, they lost 50–70% of their fresh weight (data not shown). On solid medium, the desiccated embryos showed higher percentages of root elongation and of normal germination than non-desiccated ones (Table 2). A higher germination percentage (45%) was observed when desiccated embryos were placed on medical cotton compresses in liquid germination medium compared to a similar treatment in distilled water.

A poor germination percentage (10%) was also found for desiccated embryos placed on solid medium compared to the highest germination percentage (45%) in liquid germination medium, even if root elongation percentages are comparable. Moreover we found that roots appeared much earlier in liquid medium than on solid medium. Practically all germinated embryos and embryos showing root elongation were obtained in one week in liquid medium instead of 3 weeks on solid medium.

Effect of GA_3 on embryo germination

Mature embryos were cultured in the light on solid germination medium containing GA_3 at three concentrations (0.1, 1 and 5 mg l^{-1}). In the presence of GA_3 (5 mg l^{-1}), germination percentage increased to 10% (Table 2), a result similar to a cold pretreatment. When embryos were stored at 4°C for 2 months and then transferred on solid germination medium containing 5 mg l^{-1} GA_3, the germination percentage was not significantly different from that of cold pretreatment or GA_3 addition alone (Table 2). However, GA_3 addition reduced the germination percentage of desiccated embryos in liquid medium.

Discussion

We showed here that isolation of embryos at early cotyledonary stage allows a normal maturation of walnut somatic embryos on solid basal DKW medium without plant growth regulators. Moreover, maturity and white appearance of embryos seem to be an important precondition for germination. Translucency of the embryos may reflect their lack of starch and/or protein storage (Tulecke & McGranahan 1985). This may explain why translucent embryos are unable to germinate. Biochemical analysis of the embryos are required to verify this hypothesis.

As shown by Tulecke & McGranahan (1985)

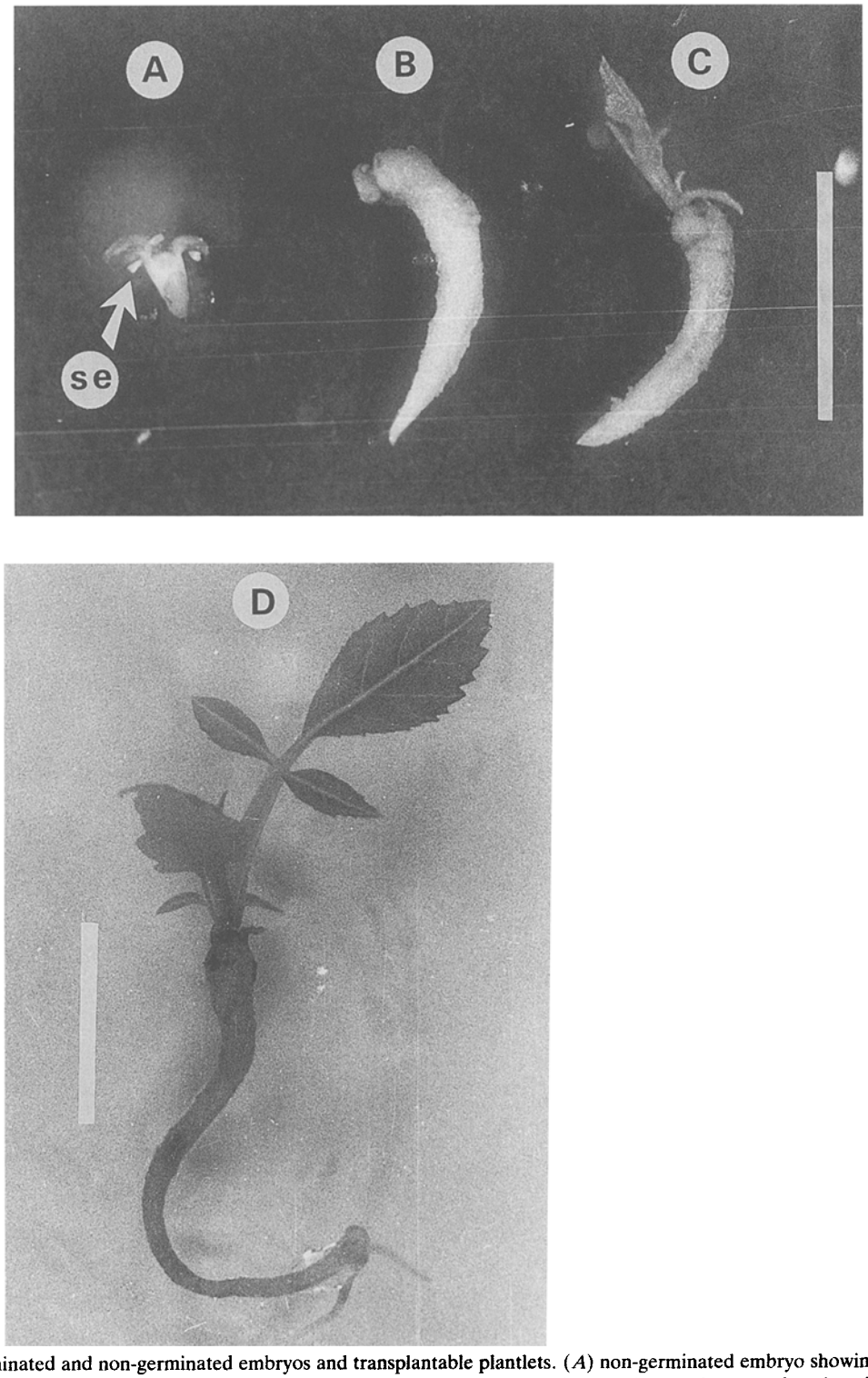

Fig. 2. Germinated and non-germinated embryos and transplantable plantlets. (*A*) non-germinated embryo showing formation of secondary embryos (se) on the cotyledons; (*B*) non-germinated embryo showing an elongated root and an inactive apical bud; (*C*) germinated embryo showing elongated root and shoot. (*D*) transplantable plantlet showing well-developed root and shoot, 2 weeks after the transfer from a germination dish to a jar. Scale bar = 10 mm.

Table 1. Effect of pretreatment in cold on embryo germination. White and translucent small embryos (2 × 1 mm, length × width) or large embryos (5 × 10 mm, length × width) were compared. 'Medium', Medium, H_2O' and 'H_2O' refer to different cold storage conditions described in Materials and methods. Embryo germination and root elongation were tested on solid medium.

Conditions of pretreatment	Embryos		Embryos tested	Root elongation (%) *	Germination (%) *
	Size	Quality			
Medium	Large	White	30	30 a	10 a
		Translucent	30	0 b	0 b
	Small	White	30	3 b	0 b
		Translucent	30	0 b	0 b
Medium	Large	White	52	29 a	10 a
Medium, H_2O	Large	White	52	27 a	10 a
H_2O	Large	White	50	40 a	14 a

* Percentages followed by a same letter are not significantly different (X^2 test of independence, $p > 0.05$).

Table 2. Effects of cold storage, desiccation and germination medium on embryo germination. Cold storage or desiccation-pretreated mature embryos were allowed to germinate on solid medium (S), or on medical cotton compress in liquid medium (L), or on compress in distilled H_2O containing or not GA_3 (5 mg l^{-1}). Control: embryos without pretreatment. The percentage of embryos showing only root elongation was also given.

Pretreatment	Germination medium	Embryos tested	Root elongation (%)	Germination* (%)
Control	S	80	6	2 a
	S + GA_3	60	20	10 b
Cold storage	S	181	21	9 b
	S + GA_3	40	22	18 b
Desiccation with $ZnSO_4.7H_2O$	S	52	48	10 b
	S + GA_3	40	52	23 b
	L	49	41	45 c
	L + GA_3	52	62	21 b
	H_2O	40	16	12 b
	H_2O + GA_3	48	57	6 ab
Desiccation with $Mg(NO_3)_2.6H_2O$	S	50	36	12 b
	L	50	38	46 c

* Germination percentages followed by a same letter are not significantly different (X^2 test of independence, $p > 0.05$).

and Tulecke et al. (1988), a cold treatment of 2 months at 4°C promotes the germination of walnut somatic embryos. The effect of cold treatment may be due to the breaking of embryo dormancy as suggested by Tulecke & McGranahan (1985). It has been reported that abscisic acid accumulates during the maturation of walnut zygotic embryos and thought to be involved in their dormancy (Marx et al. 1988). Cold treatment has been shown to reduce the level of endogenous abscisic acid and to induce the germination of Vitis somatic embryos (Rajasekaran et al. 1982). In addition, GA_3 or GA_3-like substances have been detected in developing pecan fruits (Wood 1982) and grape somatic embryos (Pearce et al. 1987). Although the involvement of GA_3 in germination control has been well documented for zygotic embryos

(Oishi & Bewley 1990; Le Page-Degivry et al. 1990), the effect of GA_3 on the germination of somatic embryos has seldom been reported (Ghazi et al. 1986; Radojevic 1988; Preece et al. 1989). With walnut somatic embryos, we showed that the addition of GA_3 in the germination medium slightly stimulates embryo germination.

Desiccation pretreatment promotes the germination of walnut somatic embryos as previously reported in other species including soybean (Buchheim et al. 1989), pecan (Wetzstein et al. 1989), grape (Gray, 1987) and red oak (Gingas & Lineberger 1989). According to Kermode et al. (1986), desiccation may redirect metabolism from a maturation mode to a germination one. However, the mechanism by which desiccation produces such a transition is unknown. Desiccation may cause a decrease in the level of endogenous abscisic acid as shown in developing maize kernels (Oishi & Bewley 1990), or it may initiate a cascade of other biochemical and physiological changes such as membrane permeability and embryo sensitivity to endogenous plant growth regulators. It has been proposed that germination requires both a decrease of endogenous abscisic acid level and an increased sensitivity to GA_3 (Le Page-Degivry et al. 1990; Oishi & Bewley 1990). Seed drying and soaking are two steps commonly used in the germination of some crop plants. To our best knowledge, however, no previous studies reported the association of somatic embryos desiccation and liquid germination medium. In walnut, the germination of desiccated embryos was further strongly promoted by the use of liquid germination medium. After placing the desiccated embryos on compresses in liquid medium, we observed a rapidly developing yellow coloration zone on the compresses, suggesting the leaching of substances from the desiccated embryos to the medium. However, we do not understand why the presence of GA_3 reduced the percentage of embryo germination in the liquid germination medium.

As compared to a cold storage of 2 months (Tulecke & McGranahan 1985), desiccation pretreatment (3–5 days) combined with the use of a liquid germination medium is less time-consuming and more efficient in promoting embryo germination. Desiccation or dehydration is a normal part of the seed development and it appears to play a role in preparing the embryos for germination (Ammirato 1989). In this respect, somatic embryos behave similarly to zygotic embryos, as concluded by Buchheim et al. (1989). We are currently investigating the changes of the levels of plant growth regulators, especially abscisic acid, during somatic embryo maturation, desiccation and germination in liquid medium in order to better understand the endogenous control.

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