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ARTIFICIAL SEEDS OF ALFALFA (*MEDICAGO SATIVA* L.). INDUCTION OF DESICCATION TOLERANCE IN SOMATIC EMBRYOS

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SUMMARY

The use of somatic embryos from cell culture systems in the clonal propagation of plants would be greatly facilitated if the somatic embryos could be dried and stored in a dormant state similar to true seeds. A cell culture system was developed for alfalfa (*Medicago sativa* L.) line RL34 which gave high yields of somatic embryos in an approximately synchronized pattern. These somatic embryos were treated with abscisic acid (ABA) at the cotyledonary stage of development to induce desiccation tolerance. With no visual preselection, approximately 60% of the dried embryos converted into plants upon reimbibition. When high quality embryos were selected prior to drying, 90 to 100% conversion rates were observed. The timing of the application of ABA in terms of embryo development was critical with an optimum being at cotyledonary stage spanning approximately 4 days; thus, synchronized embryo development is required for optimal expression in bulk samples. The vigor of the seedlings from dried somatic embryos was greater than those from embryos which had not been dried, but remained substantially lower than those from true seeds.

Key words: artificial seeds; alfalfa; somatic embryogenesis; desiccation tolerance.

INTRODUCTION

The potential use of somatic embryos for the clonal propagation of plants has been recognized for some time, but the technology necessary to use these embryos as a propagation system analogous to seeds has only been recently developed (Redenbaugh et al., 1987). In these first artificial seed systems, the somatic embryo is encapsulated in a protective alginate matrix which provides mechanical support and protection and is coated with a wax film to prevent desiccation. However, the storage life and vigor of these artificial seeds is limited, requiring cold storage to maintain viability for 3 months. Zygotic embryos undergo desiccation in vivo during their development into mature seeds. In the quiescent state of a seed the dry zygotic embryo remains viable for years (Priestley, 1986). Somatic embryos developing *in vitro* follow morphological stages similar to zygotic embryos *in vivo*. If these somatic embryos could be induced to follow the same genetic program as true seeds and acquire desiccation tolerance, then the potential use of somatic embryos as artificial seeds, in terms of long term storage and handling, would be greatly facilitated.

Some studies have shown limited success in inducing desiccation tolerance in cell cultures. The earliest report was on the successful drying of carrot callus in the air stream of laminar flow chamber over night but embryos were not tested (Nitzsche, 1980). Kitto and Janick (1985) dried carrot somatic embryos encapsulated in a "polyex" wafer and reported 4% survival after 36 h, but no survival was obtained with unprotected embryos, suggesting that the wafer simply impeded the loss of water from the somatic embryos. Gray (1987) and Gray et al. (1987) reported somewhat better success with 20% survival in grape somatic embryos following desiccation.

The objective of this study was to induce desiccation tolerance in alfalfa somatic embryos. The plant growth regulator ABA was a natural choice to use as an inductive agent. ABA has been shown to induce stress tolerance including chilling (Ackerson, 1982; Rikin et al., 1979), freezing (Mohapatra et al., 1987), and drought (Zeevaart, 1980). Other reports also suggest that ABA induced freezing tolerance in cell cultures (Chen and Gusta, 1983; Keith and McKersie, 1986) and ABA has also been used to mature zygotic embryos of Brassica *in vitro* (Finkelstein

and Crouch, 1987). In the developing seed, ABA accumulates and has been implicated in imposing dormancy in zygotic embryos (Bewley and Black, 1982). In this paper we describe a process which can induce complete tolerance of desiccation in somatic embryos of alfalfa (*Medicago sativa* L.) using ABA, enabling storage for prolonged periods of 1 year or more with high rates of viability.

Materials and Methods

Plants of alfalfa (*Medicago sativa* L.) cv. Rangelander line RL-34 were obtained from Dr. D. C. W. Brown, Agriculture Canada, Ottawa and were grown in growth rooms with 300 μmoles/m²/sec light intensity, 16 h photoperiod, at temperatures of 23 / 17° C (day/night). The petioles of young fully expanded leaves of plants in the vegetative stage were harvested periodically. Petioles were surface sterilized sequentially in 75% ethanol for 20 sec, 5% calcium hypochlorite for 4 min, and rinsed 3 times with sterile water. Petioles were then cut into usually 2 sections of 1.0 to 1.5 cm.

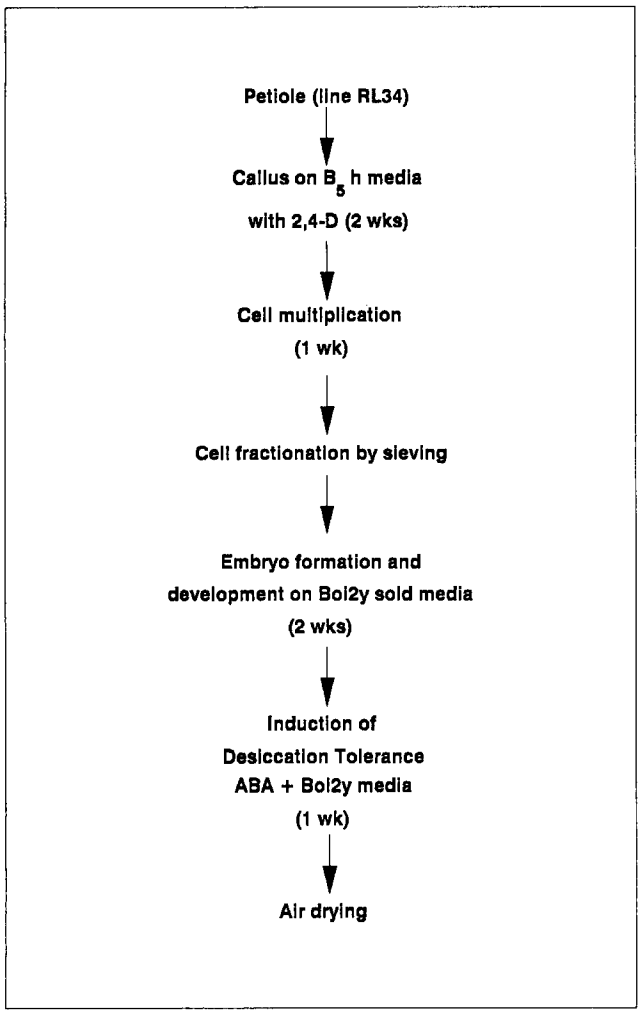

FIG. 1. Schematic diagram showing the cell culture system used to produce dry somatic embryos of alfalfa (*Medicago sativa* L.) line RL34.

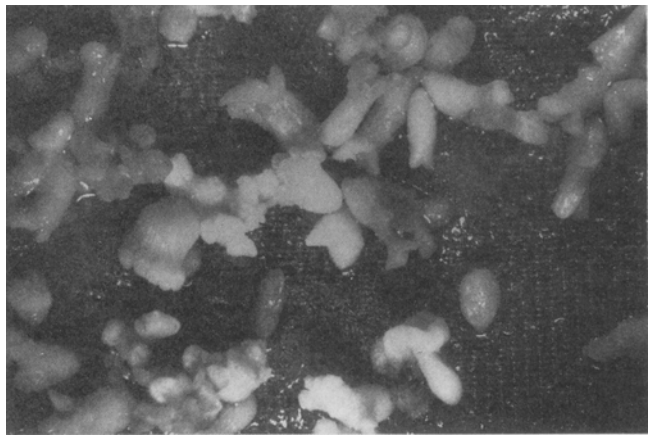

FIG. 2. Somatic embryos of alfalfa (*Medicago sativa* L.) line RL34 growing on a 200 μm nylon mesh on an agar plate containing BOi2Y medium.

The petioles were cultured on B_5h medium (Atanosov and Brown, 1984), which consists of B_5 medium (Gamborg et al., 1968) modified to contain 1 mg/L 2,4-D, 0.2 mg/L kinetin, 30 g/L sucrose, 3 g/L KNO_3, 895 mg/L $CaCl_2$, 800 mg/L L-glutamine, 500 mg/L $MgSO_4 \cdot 7H_2O$, 100 mg/L serine, 10 mg/L L-glutathione, 1.0 mg/L adenine, 9 g/L Bacto agar. Callus formed after 2 weeks on B_5h medium, and was transferred into 25 ml of liquid B_5 medium containing 1.0 mg/L 2,4 D and 0.1 mg/L NAA in 250 ml Erlenmeyer flasks and the flasks were gently shaken. After 1 week in suspension the cell material was sieved sequentially through 500 μm and 200 μm nylon meshes (Nitex). The material collected on the 500 μm mesh was discarded, and the cell fraction collected on the 200 μm mesh was collected and spread into a thin layer on pieces of 200 μm mesh (7 cm² each). There was usually sufficient callus in one flask to coat 10 screens. The screens were placed on hormone-free BOi2Y (Bingham et al., 1975) solid agar medium in 9 cm petri plates to allow embryo development.

ABA (Sigma Chemical Co.) was dissolved in 0.22% $NaHCO_3$ and incorporated into the BOi2Y medium before autoclaving. The cell fraction growing on the BOi2Y media (without ABA) as described above was transferred along with the mesh onto the BOi2Y media containing ABA, and incubated at 25° C at a light intensity of 80 μmoles/m²/sec for 7 days.

ABA-treated embryos were placed along with the mesh into sterile water, rinsed to remove other cell material with water, and collected by decanting the rinse water. Embryos were spread in the petri dish and kept in a laminar flow hood (with air flow) for 24 h for air drying. A sample of embryos equilibrated in the air was taken and

TABLE 1

RATE OF CONVERSION INTO SEEDLINGS OF SOMATIC EMBRYOS OF ALFALFA (*MEDICAGO SATIVA* L.) LINE RL 34 FOLLOWING DRYING TO 15% MOISTURE AND STORAGE FOR 3 WEEKS IN THE DRY STATE

ABA Concentration ($\times 10^{-6}$ Molar)	Embryo Age (Days)		
	11	14	18
0	8 ± 4	14 ± 6	15 ± 6
5	51 ± 9	53 ± 5	65 ± 7
10	58 ± 7	68 ± 6	69 ± 9
No ABA and No drying	60 ± 6	66 ± 3	67 ± 4

Embryo age is the number of days on hormone free BOi2Y media.
Values are the mean of 5 separate experiments ± standard error of the mean. Over 100 embryos without preselection were used in each experiment.

heated at 90° C over night to estimate the moisture content and dry weight. The dried embryos were stored in a petri dish in a laboratory drawer at ambient temperature with no humidity control (% relative humidity was between 20 to 40%) for at least 3 weeks.

For routine viability assessments, embryos were germinated on a germination paper saturated with sterile water in a magenta box (Magenta Corp., Chicago). The embryos with both roots and trifoliolate leaves were scored as converted into plants. In some experiments, dry embryos were planted directly into moistened peat pellets or into sterile soil at 3 mm depths in a magenta box. In these latter cases, the number of seedlings which established plants were counted to determine survival.

Results and Discussion

The alfalfa line RL-34 which was used in these studies produced substantial quantities of callus and was highly embryogenic when the procedures outlined in Fig. 1 were followed. Five petiole sections of 1 cm length weighed approximately 25 mg and produced about 1 g callus in 2 weeks on B_5h media. When calli were placed into liquid medium, they were dispersed into a fine suspension which became quite dense at the end of the 1 week incubation. The suspension culture contained predominantly 3 types of cells: embryoids or abnormal embryo-like structures which settled at the bottom of the flask, small cell clusters of undifferentiated, dividing meristematic cells and large, elongated single cells. Preliminary studies indicated that only the clusters or meristematic cells would develop into good quality embryos with a high conversion rate into plants. Sieving the suspension through a series of 500 and 200 μm nylon mesh screens separated these 3 cell types. The large cell clusters and abnormal embryos were collected on the 500 μm mesh and were discarded. The single cells passed through the 200 μm mesh. Thus, the cell material collected on the 200 μm mesh was predominantly the desired embryogenic fraction, consisting of the small meristematic cell clusters. When this cell fraction was plated as a thin layer on a 200 μm nylon mesh placed on hormone free BOi2Y medium, the somatic embryos began to develop (Fig. 2). The nylon mesh served as a cushion between the cells and the media surface. The embryos were not embedded in the media and this reduced the browning of the cells which were in contact with the agar surface, probably as a result of better aeration between the cells and the media surface. The mesh also provided a convenient way of handling the cultures since the embryos could be moved easily along with the mesh from one media to another without disturbing their microenvironment.

Four days after plating on hormone-free media the somatic embryos were apparent as green dots. The embryos appeared in two groups, approximately 5 to 6 days apart, with about 60% of the embryos forming in the first group. From 25 mg of petiole tissue, 1500-2200 embryos could be obtained in the first group (300-440 embryos per petiole). This represents a rate of embryo formation of 36 000-52 800 embryos per 1 g of petiole tissue. Without any desiccation treatment, over 60% of these embryos routinely converted into normal plants; thus 1 g of petiole tissue from RL34 could produce approximately 20 000 plants.

In the first experiments, at 11 to 18 days following transfer to BOi2Y media, somatic embryos were transferred to media containing 5 to 10 μmolar ABA. The embryos were green in color and most had rudimentary cotyledons. When placed on ABA containing media the embryos gradually lost their green color and appeared yellowish-white within 5 to 7 days. Embryos treated with ABA were larger and had a higher dry weight than the

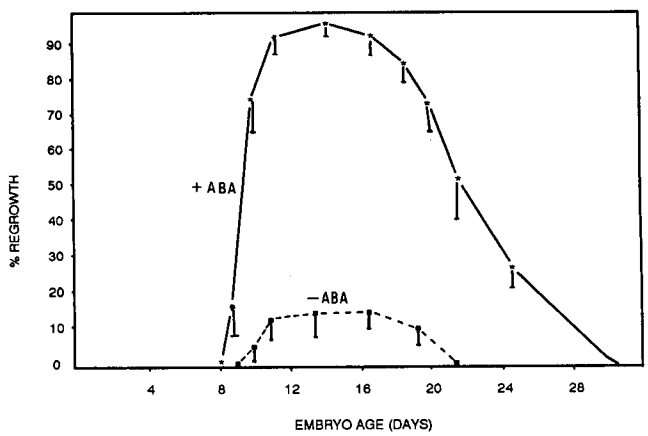

FIG. 3. Regrowth of dried somatic embryos of alfalfa (*Medicago sativa* L.) line RL34 following transfer to BOi2Y medium containing 10^{-5} M ABA at different stages of development. Vertical bar represents the standard error of the mean.

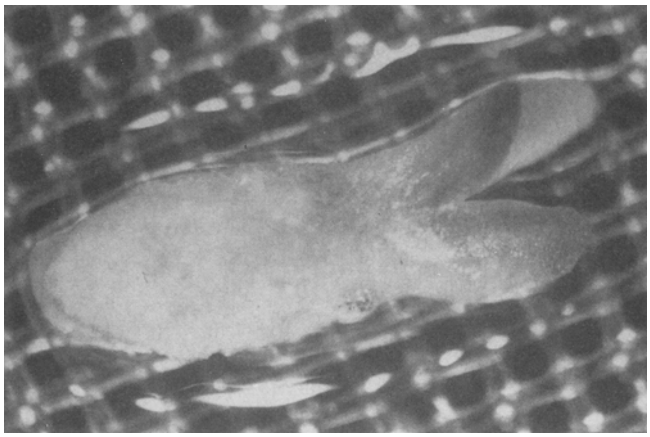

FIG. 4. Somatic embryo of alfalfa (*Medicago sativa* L.) line RL34 at the optimum stage for transfer to ABA containing medium. Note elongate torpedo shape and rudimentary cotyledons.

untreated embryos. For example, a 10 day old embryo which was treated with ABA weighed on average 1.18 mg (dry weight) compared to 0.98 mg for an untreated embryo. After 7 days on ABA containing media, the embryos were air dried to between 8 and 15% moisture. When the dry embryos were rehydrated on moist filter paper at 25° C, up to 69% of the embryos survived the desiccation process and converted into plants (Table 1). This is a similar rate of conversion to that observed for non-dried somatic embryos and indicated no significant loss of viability. Some of the dried embryos were planted in peat pellets or sown directly into sterile soil. The viability estimated from the number of seedlings established was approximately 60%, which is similar to that observed when the embryos were rehydrated on filter paper. After a one year storage period at room temperature without humidity control, samples from these same embryos germinated at a rate of 60% into seedlings indicating that the dried somatic embryos can be stored for prolonged periods without losing viability.

When all the embryos were removed from the media without preselection and dried, viability after desiccation was 60% when averaged over many experiments. If the embryos at cotyledonary stage were selected visually prior to drying, the viability after desiccation was 90 to 100%. This observation confirmed that the desiccation *per se* did not reduce viability. The relatively lower (60%) viability of the non selected embryos was a result of the developmental stage and quality of the embryo.

To investigate the interaction between ABA and developmental stage of the embryo in more detail, embryos were collected only from the first group which developed. On the ABA containing BOi2Y media, the somatic embryos lost their green color regardless of their stage of development. The induction of desiccation tolerance by ABA was clearly age dependent (Fig. 3). If the ABA treatment was imposed on the embryos before 8 days of development, it did not induce desiccation tolerance. Following day 8, the ability of the somatic embryos to respond to ABA increased such that by days 12 to 16 over 90% of the embryos were able to successfully convert into plants following drying and storage for 3 weeks in the dried state. Following day 16, the ability to respond was lost gradually, such that by 31 days of age the somatic embryos, which had begun to convert into plants, were no longer able to respond to the ABA. The precise timing of the optimum stage for ABA treatment may vary depending on the growth conditions and species. Generally, alfalfa somatic embryos were most responsive to ABA for a short period of time with an optimum at the torpedo to cotyledonary stage of development (Fig. 4).

ABA has also been used to "harden" carrot somatic embryos prior to desiccation (Kitto and Janick, 1985). In these experiments, ABA was incorporated into the suspension culture at the embryo induction phase when other growth regulators were present, which is the phase equivalent to our suspension culture phase (Fig. 1). Subsequently, as in the alfalfa system used here, the cells from suspension culture were transferred to a growth regulator free medium to allow embryo development. Then the embryos were coated with a "polyex" coating and dried. In that procedure, the embryos did not survive desiccation at all without the coating, and coated embryos survived only 1 to 2 days after desiccation. However, the ABA treatment improved the survival of encapsulated embryos after desiccation. The treatment procedure of Kitto and Janick (1985) was conducted in our alfalfa cell culture system and compared to the procedure described above. ABA at a final concentration of 10^{-5} M was incorporated into the liquid medium during the suspension culture phase along with 2,4-D and NAA as described in the normal B_5 liquid medium. The ABA treatment was also administered after the embryos had

TABLE 2

SURVIVAL OF DESICCATED SOMATIC EMBRYOS OF ALFALFA (*MEDICAGO SATIVA* L.) LINE RL34 AFTER A 10^{-5} M ABA TREATMENT AT THE EMBRYO INDUCTION STAGE AND AT THE EMBRYO DEVELOPMENT STAGE

Time of ABA Treatment		% Survival After Desiccation (regrowth)
Embryo Induction Stage	Embryo Development Stage	
Yes	Yes	64 ± 8
Yes	No	4 ± 3
No	Yes	78 ± 9
No	No	0

ABA treatment was imposed either in the cell suspension culture (embryo induction stage) or in the hormone-free BOi2Y medium (embryo development stage) as outlined in figure 1.

Values are the mean of 3 separate experiments ± standard error of the mean.

TABLE 3

VIGOR OF ALFALFA (*MEDICAGO SATIVA* L.) LINE RL34 SEEDLINGS PRODUCED FROM SOMATIC EMBRYOS AND MEASURED 14 DAYS FOLLOWING EMERGENCE OF THE RADICLE

Seedling Growth	Control	ABA Treated and Dried	True Seed
Fresh weight (mg)	5.8 ± 0.6	8.4 ± 0.9	147 ± 15
Shoot length (mm)	7.2 ± 0.8	14.5 ± 1.6	37 ± 4

The somatic embryos were either treated with 10^{-5} M ABA to induce desiccation tolerance and dried to 15% moisture or not ABA treated and allowed to germinate as a control. Values are the mean of 50 seedlings ± standard error.

developed on the hormone-free medium for 10 days. A difference as a result of the timing of ABA application was apparent (Table 2). The ABA treatment applied only in the suspension culture phase did not induce desiccation tolerance. In contrast, if the ABA treatment was applied to the embryos at 10 days after transfer to the BOi2Y medium, desiccation tolerance was induced regardless of the treatment imposed during the suspension culture phase. Furthermore, when ABA was incorporated into the suspension culture medium, cell mass in the suspension was 50% lower. Consequently, incorporation of ABA into the suspension culture may have reduced cell division and consequently embryo yield.

The vigor or growth rate of the seedling from a somatic embryo was lower than the vigor of a seedling from a true seed (Table 3). For example, the vigor of a seedling from a non-dried somatic embryo of alfalfa at about 14 days following germination is similar to that of a 4 day old seedling from a true seed. The vigor of seedlings from dried somatic embryos was greater than those from somatic embryos which had not been dried (Table 3), although still considerably less than those from a true seed. In true seeds the desiccation step is assumed to facilitate a switch from a "development mode" to a "germination mode" by destroying certain mRNA and synthesizing new mRNA (Kermode and Bewley, 1985). It is likely the somatic embryo responded in a similar fashion to desiccation, thus enhancing its vigor.

The exact mechanism by which ABA induces desiccation tolerance is unknown. However, there are suggestions that the desiccation injury in plant tissues may be mediated by a free radical mechanism (Senaratna et al., 1985a) and that the presence of free radical scavenging molecules (antioxidants) provide tolerance of desiccation in seeds (Senaratna et al., 1985b). Another hypothesis is that the accumulation of sugars maintain the stability of the membranes in dry state by replacing the water molecules at the charged exterior surface of membranes (Crowe et al., 1984). Whether any of these tolerance mechanisms are induced by ABA treatment in somatic embryos needs further investigation.

The data reported here clearly demonstrate that desiccation tolerance can be induced in alfalfa somatic embryos by treatment with ABA only if applied at a specific stage in their development. Since timing of ABA treatment is critical for positive response to ABA, a high degree of synchrony in somatic embryo development or selection of embryos is required to achieve high germination rates. The dessicated embryo germinates in a manner analogous to the true zygotic seed and seedlings are more vigorous than those from nondried somatic embryos. Although these experiments were conducted using alfalfa somatic embryos, it is anticipated that somatic embryos from other species with an equally refined cell culture system will respond similarly.

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