

Characterization of recurrent somatic embryogenesis of alfalfa on auxin-free medium

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Abstract

Callus cultures from 300 genotypes of alfalfa (*Medicago sativa* L.) were initiated from leaf, petiole, and internode explants placed on Blaydes medium containing 10.74 μM α -naphthaleneacetic acid, 11.42 μM indole-3-acetic acid, and 9.29 μM kinetin. Five genotypes produced somatic embryos. Upon transfer of these embryos to growth regulator-free Murashige and Skoog medium with B₅ vitamins, new somatic embryos repeatedly formed directly on older somatic embryos without an intervening callus phase in a cycle lasting about 30 days. These cultures have been maintained for two years, during which time their embryogenic capacity has remained stable. New embryogenic cultures could be started repeatedly from these genotypes. The elimination of sugars from the medium could stop recurrent embryogenesis. Glucose, maltose, and fructose stimulated recurrent embryogenesis more effectively than sucrose. Sucrose was superior to lactose, while sorbitol and mannitol did not stimulate recurrent somatic embryogenesis. The absence of nicotinic acid in the medium, as long as sucrose was present, was lethal to embryos of three of the five tested genotypes. The ability of this system to propagate embryos exponentially offers potential for development of new gene transfer systems and application to artificial seed technology.

Abbreviations: NAA – α -naphthaleneacetic acid, RSE – recurrent somatic embryogenesis

Introduction

Recurrent somatic embryogenesis (RSE) of alfalfa (*Medicago sativa* L.) was originally described by dos Santos et al. (1983) and by Lupotto (1983, 1986). This phenomenon occurred on either Blaydes (Lupotto 1983) or Murashige & Skoog (dos Santos et al. 1983; Lupotto 1986) media that were devoid of growth regulators, but supplemented with yeast extract. In Lupotto's system, somatic embryos of alfalfa continuously gave rise to new embryos, without an intervening callus phase, in a 20 day cycle. The somatic embryos would germinate, producing roots and primary leaves, but would cease growth as soon

as somatic embryos appeared in the crown region. Although it was difficult to convert the embryos into plants, plants could be recovered by excising the shoots from germinating embryos and allowing them to root on growth regulator-free medium. Alternatively, some plants could be obtained by placing the somatic embryos directly into soil. This RSE remained stable for the two years during which it was monitored (Lupotto 1983, 1986).

RSE has been shown to be a useful system for the transformation of plants (McGranahan et al. 1988; McGranahan et al. 1990). Furthermore, RSE could be integrated into artificial seed production technology (Fujii et al. 1989; McKersie et

al. 1989; Slade et al. 1989; Redenbaugh & Walker 1990) as a system capable of providing an unlimited number of somatic embryos. Consequently, cultures of alfalfa undergoing RSE were initiated and characterized in an effort to obtain a better understanding of this phenomenon and achieve a greater control over the process.

Materials and methods

Plant material and initiation of cultures

Plants from 'Georgia-TE', an unreleased alfalfa germplasm (derived from the intermating of equal numbers of selected plants from 'Saranac AR', 'Apollo', and 'Florida 77', and provided by Dr. J.H. Bouton, Dept. of Agronomy, University of Georgia) were initiated from seed in a greenhouse. When plants were 10 cm tall, the top 5 cm were cut off and surface-disinfested in 70% 2-propanol for 30 sec, followed by immersion in 1.05% NaOCl (20% Clorox) for 12 min, and three rinses in sterile, deionized water. Petiole and leaf sections were placed on BIINK medium, consisting of Blaydes (1966) salts and vitamins, 3% sucrose, 3 g l⁻¹ of Gelrite as a solidifying agent, pH adjusted to 5.8, and supplemented with 11.42 µM indole-3-acetic acid, 10.74 µM NAA, and 9.29 µM kinetin. This combination of growth regulators previously had been found to be particularly conducive to callus growth of alfalfa (Bingham et al. 1975). The medium was dispensed into 100 × 20 mm plastic petri dishes. A total of 300 genotypes was placed in culture. Calluses were allowed to grow for one month, then subcultured onto fresh medium at biweekly intervals for an additional 2 months. All cultures were maintained at 25–27 °C, with a 23-h photoperiod and a photosynthetic photon flux of 75–125 µmol m⁻² s⁻¹ provided by cool white fluorescent tubes.

Proliferation of somatic embryos

Embryos developing on the calluses appeared within the first 6 weeks, and were allowed to develop to the cotyledon stage, then transferred to MS0 medium, consisting of Murashige and

Skoog (1962) salts, B₅ vitamins (Gamborg et al. 1968), 3% sucrose, 2 g l⁻¹ Gelrite as a solidifying agent, and pH adjusted to 5.8. Plates and culture conditions were as described above. After 30 days, the recurrent embryos that had developed on the primary embryos were picked off and transferred to fresh medium. This cycle was repeated every 30 days. Five embryogenic genotypes were isolated and used in subsequent experiments. These were identified as TE26, TE66, TE108, TE110, and TE200.

Beginning on the fifth month, the stability of RSE was monitored in genotypes TE26 and TE66. Ten somatic embryos from each genotype were selected at random, and the number of repetitive embryos forming on each embryo was counted. The process was repeated monthly for 18 months. The data for both genotypes were analysed by regressing the number of embryos formed on cycle of embryogenesis. Analysis of variance was also performed on the data using the SAS PROC ANOVA procedure (SAS Institute 1982). Cycle of embryogenesis, genotype, and replication within genotype were the main effects. All sources of variation were tested against the error term.

Reinitiation of cultures undergoing RSE

Leaves, petioles, and internodes were collected from TE26 and TE66 plants and surface-disinfested as described. Tissue pieces were placed on BIINK medium for callus induction. Callus cultures were initiated from 81 leaf pieces, 82 petioles, and 40 internodes of TE26, and from 43 leaf pieces, 66 petioles, and 26 internodes of TE66. The calluses were evaluated after 5 weeks, and any somatic embryos present were transferred to MS0 medium to ensure that they would undergo RSE. Tissues were collected from both the parental plants and plants previously regenerated from the parental plants.

Attempts to stop RSE

Almost all somatic embryos underwent RSE instead of converting into plants. The actual spontaneous rate of conversion into plants was determined by placing 200 3-week old embryos each from TE66, TE108, TE110 and TE200 into

soil, and counting the resulting number of plants one month later.

In an attempt to determine the factors involved in RSE, 25 embryos each from genotypes TE108 and TE110 were evaluated on MS0 medium, MS0 medium without B₅ vitamins or sucrose, MS0 medium with vitamins but no sucrose, and MS0 medium with sucrose but no vitamins. Embryos were evaluated after 30 days for the presence of RSE. This experiment was repeated a second time, using 25 embryos each of TE66, TE108, and TE110.

In another series of treatments, 25 embryos each of TE108 and TE110 were placed on MS0 medium lacking one of the B₅ vitamins. Media were made from which nicotinic acid, thiamine, pyridoxine, or *myo*-inositol had been omitted. This experiment was repeated a second time using 25 embryos each of TE66, TE108, TE110, and TE200. Since embryos on media without nicotinic acid died, the apparent requirement for nicotinic acid was verified by placing 25 embryos from all five genotypes on media with nicotinic acid as the only vitamin with and without sucrose, and on medium lacking sucrose and all vitamins.

The effect of carbohydrates on RSE was further evaluated by placing 25 embryos each of genotypes TE66, TE108, TE110, and TE200 on equimolar amounts of various sugars equivalent to the molarity of 3% sucrose. The selected sugars were fructose and glucose (monosaccharides), lactose and maltose (disaccharides), and sorbitol and mannitol (sugar alcohols). Medium with sucrose and medium with no sugar were used as controls for each genotype. The number of cotyledon-stage somatic embryos forming on each embryo was counted after 30 days. Data were subjected to analysis of variance, and the means separated by F-LSD (SAS Institute 1982). The sugarless, sorbitol, and mannitol treatments were not included in the analysis, since none of these treatments underwent RSE. Prior to analyses, data were transformed using the square root transformation. Since the data consisted mostly of small numbers and zero values, 0.5 was added to each embryo count prior to transformation (Steel & Torrie 1980).

Finally, 25 embryos of genotype TE66 and 50

1, 2, or 3% sucrose. Analysis of variance was performed on embryo number data using SAS PROC GLM as described previously. Since genotypes were non-significant, the data were reanalyzed with PROC ANOVA, using genotypes as replications and embryos within replications as subsamples. The effect of sucrose level was evaluated in more detail by repeating the experiment using TE26, TE66, TE108, and TE200 at both 2 and 3% sucrose.

Results and discussion

Proliferation of somatic embryos

Out of 300 genotypes placed into culture, five genotypes formed somatic embryos within 6 weeks, even though the callus had never been removed from the auxin. This is unusual in alfalfa, because the development of normal somatic embryos is normally associated with the removal of auxins from the medium (Bingham et al. 1988). Embryos from all five genotypes began to undergo RSE upon transfer to MS0 medium (Fig. 1), exactly as described by Lupotto (1983, 1986). Embryos of TE66 transferred to fresh medium enlarged and began to germinate, producing one or two trifoliate leaves before ceasing further development. This was accompanied by the appearance of somatic embryos along the entire length of the germinating embryo, from the root tip to just below the apical meristem, with the greatest number of somatic embryos forming on the hypocotyl. Embryos from the remaining genotypes underwent RSE before germinating, or before germination proceeded very far. Regardless of the genotype, one entire cycle could be obtained every month. Somatic embryos were very loosely attached to the parental embryo, and frequently became disassociated from it, a phenomenon that suggests these embryos originate from a single-cell or at most a very small group of cells (dos Santos et al. 1983; Williams & Maheswaran 1986).

The establishment of RSE on growth regulator-free medium is not unique to alfalfa, having also been described in sweet orange, *Citrus sinensis* (Kochba & Button 1974; Kochba & Seigal 1977), winter oilseed rape, *Brassica*

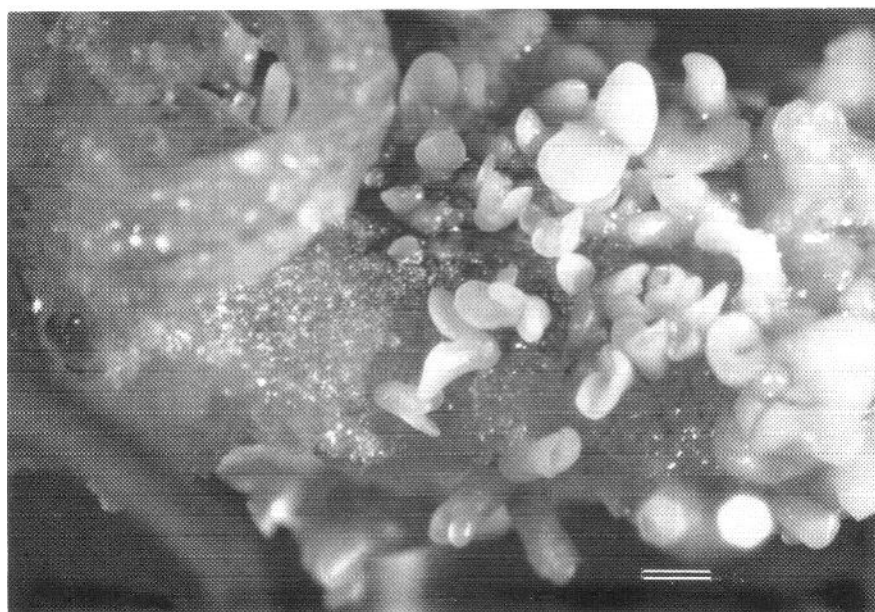


Fig. 1. Somatic embryos forming directly on an older somatic embryo of alfalfa (Bar = 1 mm).

napus spp. *oleracea* (Loh & Ingram 1982, 1983; Loh et al. 1983;); walnut, *Juglans regia* (Tulecke & McGranahan 1985); carrot, *Daucus carota* (Smith & Krikorian 1989); and pecan, *Carya illinoensis* (Wetzstein et al. 1989). Alfalfa does appear to be unique in the respect that somatic embryos may have actually germinated prior to the initiation of RSE. RSE in these systems may be a reflection of endogenous growth regulator levels (Smith & Krikorian 1989). In turn, the growth regulator level probably is a direct reflection of a developmental gene expression program that cannot be discontinued at the appropriate time, or alternatively, of a program that cannot be initiated at the appropriate time.

Of the two genotypes monitored for the stability of RSE, TE66 consistently produced significantly more somatic embryos than TE26 ($p < 0.01$) (Fig. 2). The slopes of the regression lines for both genotypes were not significantly different from zero, indicating that RSE had remained stable in both genotypes. During the evaluation period, TE26 produced an average of 20 somatic embryos per embryo per cycle, while TE66 produced 30. At this rate, a single embryo of TE66 could produce 5.31×10^{17} somatic embryos through RSE in a year's time, underscoring the tremendous potential of this system for proliferation of somatic embryos.

Reinitiation of cultures undergoing RSE

The occurrence of cultures undergoing RSE raised several questions concerning the initiation of somatic embryogenesis. Therefore, attempts were made to determine:

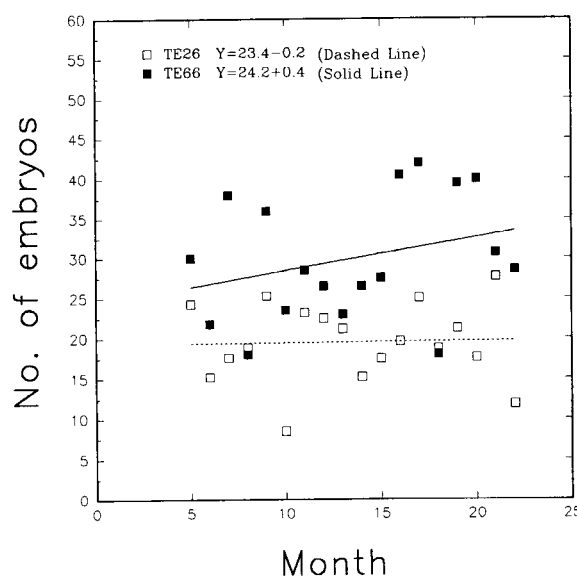


Fig. 2. Average production of somatic embryos by two genotypes of alfalfa over a two-year period.

- if the source of explant tissue affected RSE potential, or
- if the ability to undergo RSE was due to a mutation acquired in culture.

Because only a limited number of callus lines from a given genotype initiate RSE, several callus lines per genotype must be screened to detect RSE. In the case of TE26, an average of 15% of all callus lines initiated from it had produced somatic embryos within 5 weeks of initiation. Nineteen percent of callus lines derived from leaf tissue, 17% of lines derived from petiole tissue, and 8% of lines derived from stem tissue gave rise to somatic embryos, all of which underwent RSE. Callus of TE66 did not form any somatic embryos in this particular experiment, but has done so in subsequent attempts. The parent plants and plants obtained from the somatic embryos did not differ in their ability to initiate rounds of RSE from new callus cultures, implying that the ability to undergo RSE is a property present in the original plant, and not one acquired in culture.

The capacity of alfalfa to form somatic embryos is generally considered to be under genetic control, and as such is the property of individual genotypes (Bingham et al. 1988). The results from this study suggest that only a limited number of genotypes from the germplasm used have the ability to undergo RSE. While the capacity for regeneration of alfalfa has been attributed to the presence of two dominant genes (Reisch & Bingham 1980; Wan et al. 1988; Hernández-Fernández & Christie 1989), it is currently impossible to determine if the known gene(s) that condition for somatic embryogenesis in alfalfa are allelic to ones that condition for RSE.

Attempts to stop RSE

As noted by Loh & Ingram (1983), the maintenance of embryogenic cultures on media devoid of growth regulators most likely selects for further embryogenic capacity rather than the ability to convert into plants. Accordingly, in these experiments, only 1% of somatic embryos of TE108, TE110 and TE200 placed in soil converted to plants. The percent conversion for TE66 was 6%.

Previous results showed RSE would stop if the embryos were placed directly into soil (Lupotto 1986). An inference that can be drawn from this is that something absent in the soil but not the medium is responsible for RSE. Alternatively, something present in the soil but not the medium has the ability to stop RSE. The first possibility is the easier to test, and the most obvious ingredients present in the medium but absent in soil were sucrose and B₅ vitamins.

Placing somatic embryos on medium lacking sucrose, vitamins, or both had striking results, and the results are summarized in Table 1. When sucrose and vitamins were both present in the medium (MS0 medium), embryo survival was high, and embryos underwent RSE within 2 weeks. When the medium lacked both sucrose and vitamins, no RSE occurred. All these embryos survived for 6 weeks, after which they bleached and died. Similar results were observed when the medium lacked sucrose but contained vitamins. However, when the medium contained sucrose but no vitamins, most embryos of TE110, and TE200 died within two weeks. About half of the embryos of TE66 and TE108 survived and began to undergo RSE, but at an

Table 1. Effect of organic supplements on RSE. Numbers are mean percentages of surviving somatic embryos and mean percentage of somatic embryos undergoing RSE from two experiments. Data were collected at 2 weeks.

Genotype	Vitamins + sucrose		Vitamins – sucrose		Sucrose – vitamins		No organics	
	Alive	RSE	Alive	RSE	Alive	RSE	Alive	RSE
TE26	– ^a	–	–	–	–	–	100%	0%
TE66	100%	100%	88%	0%	48%	0%	100	0
TE108	100	100	32	0	52	0	100	0
TE110	100	100	100	0	4	4	100	0
TE200	88	88	100	0	2	0	100	0

^aData not available

Table 2. Effect of B₅ vitamin supplements on RSE. Numbers are mean percentages of surviving somatic embryos and mean percentage of total somatic embryos undergoing RSE from two experiments. Data were collected at 4 weeks.

Genotype	Thiamine		Nicotinic acid		Pyridine		myo-Inositol	
	Alive	RSE	Alive	RSE	Alive	RSE	Alive	RSE
TE26	90%	90%	14%	14%	100%	100%	100%	100%
TE66	100	100	68	60	96	96	100	100
TE108	100	100	82	82	100	98	88	84
TE110	100	100	10	10	96	96	88	84
TE200	100	100	10	6	96	96	96	96

initial rate two weeks slower than that of their counterparts on MS0.

The requirement for vitamins in the presence of sucrose was evaluated further by placing somatic embryos on media lacking only one of the B₅ vitamins (Table 2). RSE occurred normally on media lacking thiamine, pyridoxine, or myo-inositol. In contrast, when nicotinic acid alone was omitted from the medium, 84–90% of embryos of TE26, TE110, and TE200 died within 2 weeks. In contrast, only 18 and 32% of embryos of TE66 and TE108 did not survive on medium lacking nicotinic acid. When the requirement for nicotinic acid was verified by using nicotinic acid as the sole vitamin, embryos survived and underwent RSE normally if sucrose was present. Otherwise, embryo death occurred within 6 weeks.

The use of vitamins in plant tissue culture has been called into question (Murray 1990), and these results support the contention that thiamine, pyridoxine, and myo-inositol are unnecessary for this particular system of alfalfa regeneration. However, three of the genotypes evaluated have a requirement for nicotinic acid for short-term survival in the presence, but not the absence, of sucrose. Even the tolerant genotypes, TE66 and TE108, performed better in the presence of nicotinic acid (Table 2). The previously observed requirement for yeast extract (Lupotto 1983) can probably be explained by the presence of nicotinic acid at concentrations between 250 and 700 µg g⁻¹ in yeast extract (Bridson 1978).

Previously, carbohydrate source had been found to affect somatic embryogenesis in alfalfa, with maltose, but not glucose, having the ability to promote the formation of large numbers of normal somatic embryos (Strickland et al. 1987).

The effect of various carbohydrates on RSE is presented in Table 3. As determined by analysis of variance, genotype and carbohydrate were highly significant ($p < 0.01$) sources of variation for RSE. No single carbohydrate was best for all genotypes, a fact reflected in the highly significant carbohydrate × genotype interaction. Overall, the highest number of RSE was obtained with maltose. Glucose and fructose were superior to sucrose, but not as efficient as maltose. Lactose, which is not a plant sugar, was very inefficient at stimulating RSE. There was no incidence of RSE on medium lacking a carbohydrate source, or on media with sorbitol or mannitol. In addition to the quantitative differences in RSE in the presence of various carbohydrates, there were qualitative differences. The embryos with the most normal morphology were produced in the presence of sucrose. Those produced on lactose, while very normal in morphology, were minute in size.

Sorbitol and mannitol are not metabolized by plant cells to a great extent, and did not lead to RSE, even though they were present in the media in equimolar amounts. This suggests that the role of sugars in RSE is not merely that of an

Table 3. Effectiveness of various sugars on the formation of cotyledon-stage somatic embryos via RSE.

Carbohydrate	Embryo no ^a
Maltose	11.5 a ^b
Glucose	8.1 b
Fructose	7.2 bc
Sucrose	5.6 c
Lactose	3.5 d
LSD _(0.05)	2.1

^aAverage number of embryos per embryo for four genotypes.

^bMeans with the same letter are not significantly different, as determined by F-LSD.

osmoticum, and that the energy status within the embryo may be an important factor for RSE. While one possibility is that carbohydrates in the medium directly stimulate the occurrence of RSE, an alternative possibility is that some other factor is responsible for RSE, and that the presence of a carbohydrate in the medium is only necessary to provide the energy required for RSE. The effect of carbohydrates in the medium was further evaluated by comparing RSE across sucrose levels ranging from 1% to 3%. Sucrose level was highly significant, with the highest frequency of RSE occurring at 2% sucrose. When the experiment was repeated a second time with a greater number of genotypes, the reduction in embryos produced was due almost exclusively to TE108, which was more productive at 2 than 3% sucrose. Genotypes TE26 and TE66 produced identical numbers of embryos at both sucrose concentrations, while TE200, a genotype not used in the first trial, produced more embryos at 3 than 2% sucrose. These results suggest that genotypes differ in their sensitivity to sucrose levels, and these may need to be optimized for some genotypes.

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