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Repetitive somatic embryogenesis and plant recovery in white clover

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Summary. Breeding and selection was used to generate a population of white clover (*Trifolium repens* L.) from cultivar Osceola with a high embryogenic capacity. Somatic embryos were obtained from immature cotyledons of white clover placed onto EC6 basal medium containing 40 mg L^{-1} of 2,4-D and 6% sucrose. The effects of 2,4-D at 20 and 40 mg L^{-1} and of the carbohydrates, sucrose and maltose, were evaluated for their influence in the establishment of repetitive somatic embryogenesis. To determine the optimal protocol for plant recovery from somatic embryos, the effects of MS vs. EC6 basal salts, sucrose vs. maltose, B5 vitamins vs. yeast extract, and inclusion or exclusion of activated charcoal were evaluated. Repeated subculture of white clover somatic embryos on EC6 basal medium containing 6% sucrose with 2,4-D at 20 or 40 mg L^{-1} effectively maintains repetitive embryogenesis. Medium containing MS salts with 6% maltose as the carbohydrate source was the most efficient for plant recovery.

Key words: Maltose - *Trifolium repens*

Introduction

Auxin-stimulated induction of somatic embryogenesis is an effective mode of regeneration for several crop plants. Among the attributes of regeneration systems based on somatic embryogenesis are the wide range of species that are able to form somatic embryos, the production of complete propagules, and the ability of auxin-stimulated somatic embryogenesis systems to become repetitive. Not only can such a repetitive somatic embryogenesis system be used for mass propagation, it has become a valuable tool in genetic transformation protocols, especially for those species which do not regenerate readily from callus. The value of repetitive embryogenesis to obtain stable transformants was originally demonstrated by McGranahan et al. (1988; 1990) in walnut. Subsequently, repetitive embryogenesis in a liquid culture system has been used for stable transformation of both soybean and cotton (Finer and McMullen 1990; 1991).

White clover is a pasture legume widely utilized in New Zealand and the southeastern United States (Carlson et al. 1985). Induction of auxin-stimulated somatic embryogenesis in white clover (Parrott, 1991) has made it feasible to develop a repetitive embryogenesis culture system for this crop. This type of regeneration system relies on the constant presence of an auxin in the growth medium which, after inducing the formation of somatic embryos from the explant tissue, locks those embryos at an early stage of development from which they cannot proceed. Instead of maturing into cotyledonary-stage embryos, the embryos give rise to new, secondary, embryos in a cycle that may be repeated indefinitely (Terzi and LoSchiavo 1990).

The specific goals of this study were to initiate and maintain repetitive somatic embryogenesis cultures for white clover, define parameters for the optimal maintenance of these repetitively embryogenic cultures, and define parameters important to the efficient conversion of embryos to plants.

Materials and Methods

Selection for regeneration capacity (plant materials sources and culture conditions). Four plants from the cultivar Osceola were crossed in all possible combinations. Resulting pods were collected from seed heads 8-

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10 days after pollination as described previously (Parrott 1991), placed in a tea infuser, and surface-sterilized by immersion in 70% 2-propanol for 30 seconds, followed by immersion in 1% NaOCl (20% commercial bleach, v/v) for 12 minutes, and three rinses in sterile water. The resulting zygotic embryos were excised from the immature seed, and cotyledons were placed, adaxial side up, on EC6D40 medium consisting of EC6 salts (Maheswaran and Williams 1984) supplemented with 1 g L^{-1} yeast extract, 6% sucrose, and 40 mg L^{-1} of 2,4-D, pH adjusted to 5.8 and solidified using 3 g L^{-1} of Gelrite (Chemical Dynamics Corporation, South Plainfield, NJ) in 100 × 20 mm disposable plastic petri plates. Each plate contained 20 cotyledons. Cultures were sealed with Nescofilm (Karlan Research Products Corp., Santa Rosa, CA) and maintained at 25°C under a 23 hr photoperiod provided by cool white flourescent bulbs. Light intensity averaged 75-100 μM photons m^{-2} s^{-1}.

After 10 days, the cotyledons were transferred to growth-regulator-free MS medium with B5 vitamins (Gamborg et al. 1968) and 3% sucrose (MSO medium). After three weeks, individual somatic embryos were separated from the explant tissue and transferred to fresh MSO until plantlets were recovered. Regenerated plants were paired, intercrossed, and the process repeated two additional times. To help lessen inbreeding depression, only non-sib regenerated plants were crossed with each other during the selection process.

To determine the extent to which the cycles of regeneration and intercrossing were effective in increasing the frequency of somatic embryos recovered per explanted cotyledon, the original parental plants were once again intercrossed, as were the plants from the first and second cycles of selection, and the resulting immature embryos evaluated for their embryogenic capacity on EC6D40 medium.

Repetitive Embryogenesis. To assess repetitive embryogenesis, plants obtained from the second cycle of selection were intercrossed, and 180 immature cotyledons placed on EC6D40 as described. Approximately five weeks after explanting, 180 somatic embryos at the globular-stage of development were removed from the cotyledons and placed on fresh EC6D40 with twenty embryos per plate. This was repeated every five weeks for a total of four rounds of embryogenesis, with each successive round using 180 somatic embryos formed during the previous round. At the conclusion of each round, the number of somatic embryos formed per embryo was assessed.

The second experiment compared the relative ability of four medium formulations to promote and maintain repetitive embryogenesis. These were as follows: EC6D40 (described above), EC6D20 (basal EC6 medium with 20 mg L^{-1} of 2,4-D), EC6D40M (EC6D40 with 6% maltose substituted for 6% sucrose), EC6D20M (EC6D20 with 6% maltose substituted for 6% sucrose). One hundred twenty somatic embryos excised at the end of the second round were subcultured onto each of these media. After five weeks, the number of new somatic embryos formed per explant was assessed.

Data collected at the conclusion of the second experiment suggested that a more thorough comparison between EC6D40 and EC6D20 was merited. In this third experiment, 960 somatic embryos, removed from EC6D40 medium at the end of the third round, were divided between EC6D40 and EC6D20 plates. At the end of six weeks, the newly formed somatic embryos were counted and the number of new embryos per embryo recorded.

Plant recovery. In the final experiment, thirteen growth-regulator-free media were compared for their ability to permit the further development, maturation, and conversion of globular-stage somatic embryos to plantlets. One hundred globular-stage embryos, ten embryos per plate, were placed on each of the media listed in Table 4. After six weeks on each medium or media sequence, the number of embryos that produced plantlets was recorded.

Data Analysis. For all experiments, raw data were transformed using a square root transformation after adding 0.5 to each data point (Steel and Torrie 1980) and evaluated using SAS PROC ANOVA. Depending on the experiment, rounds or media were used as main effects, plates within medium or rounds as repetitions, and embryos within plates as subsamples. All treatment results consisting exclusively of zero values were not included in the analysis of variance. Treatment means were separated using Fisher's Protected Least Significant Difference. Finally, the means obtained through the use of SAS PROC ANOVA are reported in the tables as the square of the transformed mean plus the mean square error (Steel and Torrie 1980).

Results and Discussion

Selection for regeneration capability

A substantial increase in regeneration capacity, as determined by the number of somatic embryos recovered per explanted cotyledon, was achieved in each cycle of intercrossing and regeneration (Table 1). The rapid rate of gain from selection suggests that this embryogenic capacity is under relatively simple genetic control. Alfalfa is another legume in which breeding and selection also resulted in increased frequency of somatic embryogenesis (Bingham et al. 1975; Ray and Bingham 1989). In both of these cases, 2 cycles of selection increased regeneration capacity to about 66% of individuals in the population. By contrast, selection only increased the white clover regeneration frequency to just under 40%. However, the original regeneration frequencies of the unselected populations of alfalfa were higher than that of the unselected white clover population used in this study.

Table 1. Regeneration capacity of immature cotyledons derived from recurrent selection using EC6D40 medium.

Cycle	N	% Cots with SE*	# SE* Formed	Avg. SE* per cot.
0	165	4	13	0.08
1	272	12	71	0.26
2	54	39	93	1.72

* = somatic embryos

Evaluation of repetitive embryogenesis

New embryos would form on somatic embryos transferred to fresh medium, but these would not proceed past a

globular-stage of development. When the average number of embryos that formed per somatic embryo was evaluated for four rounds of repetitive embryogenesis on EC6D40 medium, the number of embryos formed varied significantly among rounds (Table 2). Although large numbers of embryos were produced in each round, the number of embryos was subject to fluctuations from round to round, without any long-term trend towards an increase or decrease in embryo number. Morphologically, there was no difference for any of the four rounds evaluated.

Table 2. Frequency of embryo production over rounds of repetitive embryogenesis using EC6D40 medium.

Round	N	Mean # embryos/explant
1	180	4.797 c[a]
2	180	5.313 c
3	180	8.510 a
4	180	6.776 b

[a] Means followed by the same letter are not significantly different at the 0.05 level.

Effect of 2,4-D level and carbohydrate source

More embryos were produced per explant when the medium contained 40 mg L^{-1} 2,4-D than when it contained 20 mg L^{-1}, regardless of the carbohydrate source (Table 3). However, by the end of the fourth round, there was a significantly higher average number of embryos formed per explant for the embryos on medium containing 20 mg L^{-1} 2,4-D versus 40 mg L^{-1}. This suggests an inherent variability in the frequency of somatic embryogenesis that is larger than the effect of auxin level. However, there was an observable increase in size and green color of somatic embryos formed on all media containing 20 instead of 40 mg L^{-1} 2,4-D (Figure 1a & b).

Table 3. Effect of carbohydrate source and 2,4-D level on frequency of embryo formation.

Medium	N	Round	Mean # embryos/explant
EC6D40	120	3	10.255 a[a]
EC6D20	120	3	7.841 b
EC6D40M	120	3	5.067 c
EC6D20M	120	3	4.934 c
EC6D40	440	4	7.684 d
EC6D20	440	4	9.951 e

[a] Means followed by the same number are not significantly different at the 0.05 level.

The use of 6% sucrose in the medium gave higher numbers of embryos at both 2,4-D levels than did the use of 6% maltose. There was no significant difference in average number of embryos formed per explant between the two maltose-containing media. This suggests that sucrose, used as the sole carbohydrate source, is more conducive to the formation of globular-stage white clover somatic embryos than is maltose. This finding is in contrast to results from similar experiments in both alfalfa and orange, in which maltose was more effective than sucrose for embryo formation (Strickland et al. 1987; Button 1978).

Plant recovery

Once repetitive embryogenesis is induced and maintained, it is neccesary to stop embryogenesis from recurring and allow the newly formed embryos to complete development into plants. Conventionally, this is accomplished by first placing globular-stage somatic embryos on a medium devoid of growth regulators, permitting the embryo to develop to a cotyledon-stage, followed by a maturation period to permit the accumulation of storage reserves (Parrott et al. 1991). Sometimes, it becomes necessary to include activated charcoal to increase auxin removal from the developing embryo. The inclusion of activated charcoal can be introduced during maturation of somatic embryos (Buchheim et al. 1989) or prior to the histodifferentiation of embryos (Ebert and Taylor 1990). For white clover, all but one of the media containing activated charcoal resulted in death of the embryos. The only treatment that did not cause embryo death was seven days on MS0 medium containing 0.5% activated charcoal, after which the embryos were transferred to MS0 medium (Table 4). It may be that for clover, a week's exposure to activated charcoal is too long to achieve the normal embryo development and maturation required prior to efficient plant conversion.

In addition to the removal of growth regulators, the carbohydrate source has also been reported to be an important parameter in the conversion of embryos to plantlets. Specifically, when substituted for sucrose, maltose has been reported to increase efficiency of embryo to plant conversion (Button 1978; Strickland et al. 1987). Maltose is also used for the conversion of soybean embryos (Finer and McMullen 1991). In this work, media with maltose instead of sucrose gave higher conversion frequencies. Use of maltose with MS salts resulted in the highest conversion percentages, with 6% being the best concentration of maltose tested (Table 4).

The use of EC6 salts was reported to be more effective than MS salts for embryogenesis of white clover (Parrott

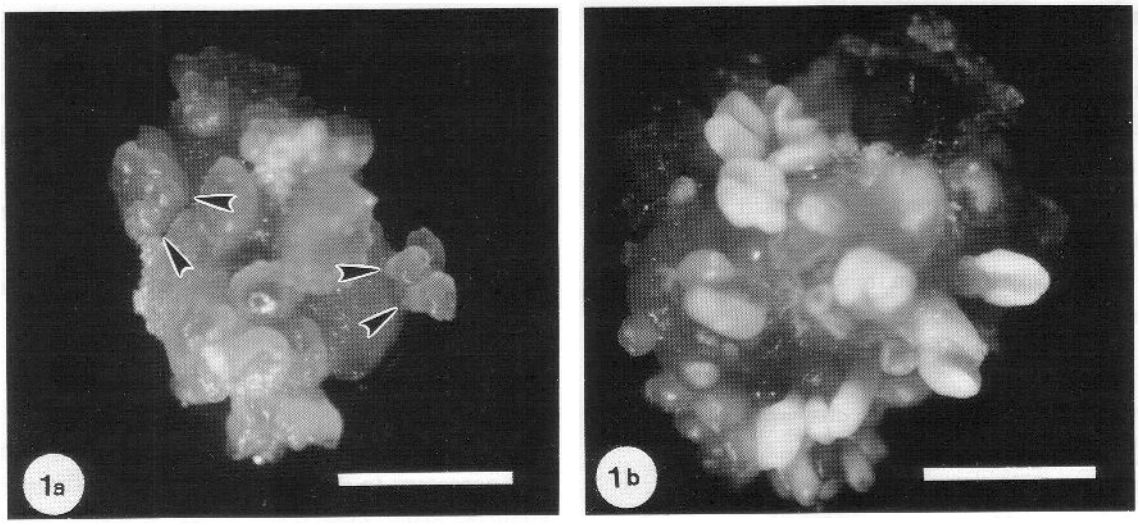

Fig. 1 Embryos formed after 35 days on EC6D40 medium (arrows in 1a) of after 40 days on ECD20 medium (1b). Bar = 1 mm.

1991), but information was not available on the relative effectiveness of these two salt formulations for the conversion of embryos into plants. For conversion, the results show that media using MS salts instead of EC6 salts had the highest success frequencies (Table 4). Thus, it may be possible that different stages of embryogenesis have optimal salt formulations.

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Table 4. Composition and effectiveness of different media for plant recovery.

Basal Salts	Carbohydrate	Vitamin Source	Days on Activated Charcoal	Mean % Plant Recoverya
MS	maltose 6%	B5	0	14.4 a[d]
MS	maltose 1.5%	B5	0	12.4 ab
MS	maltose 3%	B5	0	11.2 ab
EC6	maltose 3%	YE	0	8.3 abc
MS	sucrose 3%	B5	0	6.1 bcd
EC6	sucrose 6%	YE[b]	0	5.7 bcd
EC6	sucrose 6%	B5[c]	0	4.8 cd
EC6	maltose 6%	YE	0	3.8 cd
MS	sucrose 3%	B5	7	3.2 d
EC6	maltose 12%	YE	0	0[e]
EC6	sucrose 6%	YE	7	0
EC6	sucrose 6%	YE	14	0
MS	sucrose 3%	B5	14	0

[a] N = 10 plates, 10 embryos per plate.
[b] Yeast extract.
[c] B5 vitamins (Gamborg et al. 1968).
[d] Means followed by the same letter are not significantly different at the 0.05 level.
[e] Media that resulted in zero percent conversion were excluded from data analysis.

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