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Auxin-stimulated somatic embryogenesis from immature cotyledons of white clover

W. A. Parrott

Department of Agronomy, University of Georgia, Athens, GA 30602, USA

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Abstract. Cotyledons from immature embryos of white clover (*Trifolium repens* L.) cv. Osceola were exposed to 2,4-D or NAA to induce somatic embryogenesis. NAA at 10 or 20 mg l^{-1} was very inefficient at stimulating embryogenesis, while concentrations of 30 or 40 mg l^{-1} resulted in death of the explant tissue. Continuous exposure of cotyledons to 40 mg l^{-1} 2,4-D resulted in somatic embryos which were arrested at the globular stage, or which underwent cycles of secondary embryogenesis, never proceeding beyond the globular stage. A 10 day exposure time to 2,4-D at the same concentration led to formation of somatic embryos, most of which had poorly developed cotyledons. Almost 10% of the somatic embryos converted into plants following transfer to medium devoid of growth regulators. Attempts to improve morphology of somatic embryos by using shorter exposure times to 2,4-D at 40 mg l^{-1}, or by maintaining the 10 day exposure time while varying the concentration of 2,4-D, were not successful. Plants were obtained from all parents evaluated, although at different frequencies.

Key words: Somatic embryogenesis - *Trifolium repens* - White clover

Introduction

White clover (*Trifolium repens* L.) is a forage legume grown in various parts of the world, including the southeastern United States, northwestern Europe, and New Zealand (Carlson et al. 1985). Sporadic recovery of shoots from callus cultures of white clover has occurred following the use of protocols based on the use of various combinations of auxins and cytokinins (Pelletier and Pelletier 1971; Oswald et al. 1977; Gresshoff 1980; Mohapatra and Gresshoff 1982; Ahuja et al. 1983; Bond and Webb 1989; Webb et al. 1987a).

While the mode of regeneration in white clover has been reported to be somatic embryogenesis from callus tissues (Bhojwani et al. 1984), the mode of regeneration is not always clear in other cases. A recent report of regeneration in white clover is organogenesis (Webb et al. 1987a). Pederson (1986) was able to obtain somatic embryos in culture, but these failed to develop. Nevertheless, somatic embryogenesis from callus exposed to auxin-cytokinin combinations does occur in several species of the genus *Trifolium*, including red clover, *T. pratense* L., (Phillips and Collins 1980; Bhojwani et al. 1984; McGee et al. 1989), *T. rubens* L. (Parrott and Collins 1983; Cui et al. 1988; McGee et al. 1989), *T. arvense* L. (Bhojwani et al. 1984), *T. incarnatum* L., and *T. vesiculosum* Savi (Pederson 1986).

White clover has also been regenerated from somatic embryos induced on immature zygotic embryos exposed to BA at 0.2-2.0 mg l^{-1} without the presence of an auxin in the medium (Maheswaran and Williams 1984, 1985, 1986). In contrast, auxins alone, and not cytokinins, have been used to induce somatic embryogenesis from immature zygotic embryos of several legumes (e.g., Lazzeri et al. 1985; Ozias-Akins 1989; Merkle and Wiecko 1989; Trigiano et al. 1988). The research presented here was conducted to determine if auxins alone could induce somatic embryo formation from zygotic embryos of white clover.

Materials and Methods

Seeds of the cultivar Osceola were obtained from the Regional Plant Introduction Station in Experiment, GA, and established in the greenhouse at the University of Georgia in Athens. Four plants were selected at random to serve as the source of zygotic embryos. These plants were crossed by hand in all possible combinations, and the seed heads collected at 7-8 days after pollination, when developing embryos had reached the cotyledonary stage and the endosperm had become firm. Florets with developing pods were enclosed in a cheesecloth pouch and

surface-sterilized in 70% 2-propanol for 30 seconds, followed by a soak in 1.05% NaOCl (20% Clorox) for 12 minutes, and by three rinses in sterile water. Pods were removed from the corolla and individual seeds excised. The end of the seed containing the embryonic axis was cut off, and the cotyledons removed from the seed coat and endosperm with the aid of a dissecting microscope. The two cotyledons were placed, adaxial side up, in 100 x 20 mm disposable petri plates containing EC6 medium (Maheswaran and Williams 1984) with 3 g l^{-1} of Gelrite as the solidifying agent and either 2,4-D or NAA as the sole growth regulator. All culture plates were sealed with Nescofilm and maintained at 25°C under a 23 hour photoperiod provided by cool white fluorescent bulbs. Light intensity averaged 75-100 μM $m^{-2}s^{-1}$.

The initial experiment compared the effect of continuous exposure to 2,4-D (Finer 1988; Lazzeri et al. 1985) versus a 10 day auxin pulse (Parrott et al. 1988). One hundred cotyledons were explanted on to EC6D40 (EC6 basal medium with 40 mg l^{-1} of 2,4-D). After 10 days, half of the cotyledons were transferred to MS0 medium (growth regulator-free medium containing MS salts (Murashige and Skoog 1962), B_5 vitamins (Gamborg et al. 1968), 3% sucrose, and solidified with 3 g l^{-1} Gelrite) for 20 days, at the end of which both treatments were examined for the presence of somatic embryos.

The second experiment directly compared the effect of 40 mg l^{-1} of 2,4-D with that of NAA at 10 mg l^{-1}. In all, 1058 cotyledons were placed on EC6D40 medium, and 1986 were placed on EC6N10 medium (EC6 supplemented with 10 mg l^{-1} NAA). All cotyledons were transferred to MS0 after 10 days. The resulting somatic embryos were counted after 20 days and transferred to fresh MS0 medium. Upon germination of the somatic embryos, the resulting plants were transferred to soil and maintained at 100% relative humidity in covered seed trays for establishment. The cover was increasingly opened over a period of a week, and finally removed entirely. The plants were transferred to the greenhouse and grown to maturity.

A third experiment compared the effectiveness of 0, 3, 6, or 9 day pulse times with 2,4-D at 40 mg l^{-1}. A minimum of 160 cotyledons was used for each exposure time. Following exposure to the auxin, the cotyledons were transferred to MS0, such that the number of days on 2,4-D plus the number of days on MS0 totaled 30.

Alternatively, the possibility of varying the level of 2,4-D while maintaining a 10 day pulse time was explored. A minimum of 150 cotyledons was exposed to 10, 20, 30, or 40 mg l^{-1} of 2,4-D for 10 days, then transferred to MS0 medium. The effectiveness of NAA levels greater than 10 mg l^{-1} was also evaluated. At least 80 cotyledons were exposed to NAA at 10, 20, 30, or 40 mg l^{-1} for 10 days, at which time the cotyledons were transferred to MS0. Finally, the effectiveness of an MS basal medium, instead of an EC6 medium, was evaluated by placing 1000 cotyledons on a medium containing MS salts, B5 vitamins, and 40 mg l^{-1} 2,4-D.

Results

Continuous vs pulsed exposure to 2,4-D

Globular stage somatic embryos became visible after 14 days on the immature cotyledons, regardless of whether they had been transferred onto MS0 medium or had remained on EC6D40. However, development of the embryos differed greatly, depending on the medium. The somatic embryos which formed on cotyledons exposed continuously to 2,4-D usually did not develop past the globular stage. In two cases, the globular stage somatic embryos underwent secondary embryogenesis. When the secondary embryos reached the globular stage, the cycle was repeated, forming branched chains of globular stage somatic embryos, suggesting that these somatic embryos were locked into a particular developmental stage from which they could not proceed in the presence of the auxin (Figure 1).

Somatic embryos developing on cotyledons transferred to growth regulator-free medium after a 10 day exposure to 2,4-D continued their development past the globular stage, and reached full size by 21 days. These embryos tended to have a very well developed axis, but poorly developed cotyledons (Figure 2). Nevertheless, somatic embryos with well-developed cotyledons developed occasionally (extreme left of Figure 2, and Figure 3).

Table 1. Percent regeneration and average number of somatic embryos obtained per cotyledon plated from immature embryos of different parents

Cross[a]	# cot.[b]	% Resp.[c]	# emb./ cot.[d]
1x2	33	6.06	0.15±0.10
1x3	357	12.61	0.31±0.06
2x1	33	15.15	0.39±0.17
2x4	115	20.86	0.65±0.17
3x1	311	18.97	0.62±0.11
3x4	9	11.11	0.33±0.31
4x2	184	15.22	0.42±0.10
4x3	16	43.75	1.25±0.51

[a]Four white clover plants were selected as parents and crossed pairwise. The first represents the female parent, the second represents the the pollen parent
[b]Total number of cotyledons placed on EC6D40 medium
[c]Percent of plated cotyledons that gave rise to somatic embryos
[d]Average number of somatic embryos per cotyledon placed in culture ± standard error

2,4-D vs NAA

The numbers of somatic embryos formed from immature cotyledons exposed to 2,4-D at 40 mg l^{-1} for 10 days are given in Table 1. In all, 542 somatic embryos were obtained from 1058 cotyledons, representing an overall efficiency of 0.51 somatic embryos per cotyledon. Of the total cotyledons explanted, 16.2% formed somatic

embryos, and the average number of somatic embryos per responding cotyledon was 2.9, with a range from 1 to 17. At the end of 30 days from the time of explanting (10 days on EC6D40 + 20 days on MS0), all embryos were transferred to MS0 medium. Some of the embryos began to germinate immediately, while others turned brown and died. Those embryos alive after 14 days were transferred to fresh MS0 until plants were obtained or death occurred. In all, 9.8% of the somatic embryos converted into plants.

Callus production and profuse rooting ocurred in the presence of NAA (Figure 4). Out of 928 cotyledons placed on NAA, only 3 somatic embryos were obtained. These three somatic embryos began to germinate while still on the original cotyledon explant.

Pulse treatment with 2,4-D

Exposure to 2,4-D at 40 mg l^{-1} was varied in an effort to improve morphology of the somatic embryos. Cotyledons were exposed to 2,4-D for 0, 3, 6, or 9 days, and then transferred to MS0 medium. The resulting data in terms of embryo numbers are presented in Table 2. No obvious morphological differences were evident between those embryos induced on 6 days 2,4-D and those on 9 days, nor were there any differences between conversion rates.

Table 2. Effect of 2,4-D concentration on the number of somatic embryos recovered and the subsequent conversion of these embryos into plants

mg l^{-1} 2,4-D	Somatic embryos/ cotyledon	% Conversion
10	0.24	3
20	0.19	3
30	0.06	9
40	0.16	50

Level of 2,4-D

As varying the exposure time to 2,4-D was not an effective means towards improving embryo quality, an attempt was made to maintain the 10 day exposure time, but vary the concentration of 2,4-D. Concentrations of 2,4-D of 10, 20, 30, and 40 mg l^{-1} were compared for their effectiveness in the induction of somatic embryogenesis. These data are in Table 3.

Level of NAA

The possibility that a level of NAA higher than 10 mg l^{-1} would be effective in the induction of somatic embryos was investigated by exposing cotyledons for 10 days to levels of 10, 20, 30, or 40 mg l^{-1} NAA. As with the earlier NAA test, the frequency of embryo production was very low. The 10 and 20 mg l^{-1} treatments gave 0.01 and 0.001 somatic embryos per cotyledon, respectively. No embryos were obtained with 30 or 40 mg l^{-1} NAA, concentrations which resulted in death of almost all the explanted cotyledons.

Use of MS basal medium

Finally, the use of an MS basal medium instead of the EC6 basal medium during the induction phase was investigated, as the use of one single basal medium during all the phases of the regeneration procedure would simplify the number of stock solutions that must be maintained in the laboratory. All cotyledons initially placed on MS medium instead of EC6 failed to survive.

Table 3. Effect of 2,4-D concentration on the number of somatic embryos recovered and the subsequent conversion of these embryos into plants

mg l^{-1} 2,4-D	Somatic embryos/ cotyledon	% Conversion
10	0.24	3
20	0.19	3
30	0.06	9
40	0.16	50

Discussion

Immature zygotic embryos of white clover had previously been shown to undergo somatic embryogenesis upon exposure to low levels of a cytokinin (Maheswaran and Williams 1984, 1985). This study shows that immature zygotic embryos of white clover are also capable of undergoing auxin-stimulated somatic embryogenesis, as has been the case with several other species. Cytokinin-stimulated somatic embryos formed along the axis of intact immature zygotic embryos (Maheswaran and Williams 1984, 1985), whereas auxin-stimulated somatic embryos from this study formed on cotyledons detached from immature zygotic embryos. The arrangement of the somatic embryos around the periphery of the explanted zygotic cotyledon (Figure 2) is reminiscent of that described for soybean by Hartweck et al. (1988).

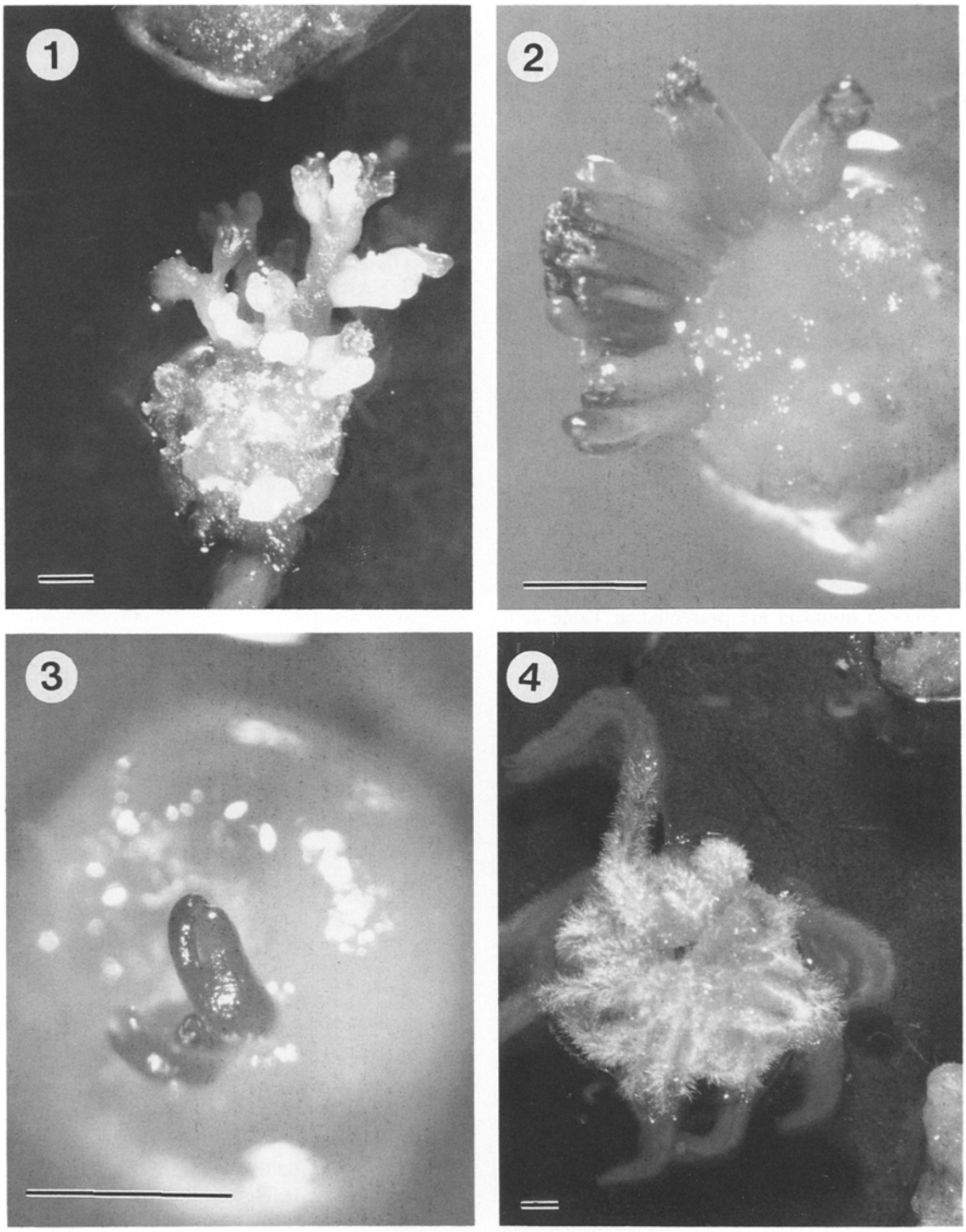

Fig. 1. Immature cotyledon of white clover after 30 days on EC6 medium supplemented with 40 mg l^{-1} of 2,4-D, with somatic embryos which have undergone secondary embryogenesis (Bar =1 mm). **Fig. 2.** Somatic embryos forming on an immature cotyledon of white clover. The cotyledon was exposed to EC6 medium supplemented with 40 mg l^{-1} of 2,4-D for 10 days, then transferred to growth regulator-free medium for 15 days (Bar = 1 mm). **Fig. 3.** A somatic embryo of white clover showing normal development of the cotyledons, forming 5 days after the explanted cotyledon was transferred to growth regulator-free medium. The explanted cotyledon had been previously exposed to EC6 medium with 40 mg l^{-1} of 2,4-D (Bar = 1 mm). **Fig. 4.** An immature cotyledon of white clover showing extensive root formation. The cotyledon was exposed to EC6 medium supplemented with 10 mg l^{-1} of NAA for 10 days, then transferred to growth regulator-free medium for 10 days (Bar = 1 mm).

It appears that different legumes respond differently to NAA and 2,4-D. In soybean, NAA is very effective, and gives somatic embryos more normal than those induced with 2,4-D (Lazzeri et al. 1987; Hartweck et al. 1988). In contrast, peanut does not respond to NAA (Ozias-Akins 1989), and white clover responds very poorly. In this study, best results were obtained by exposing the immature cotyledons to 40 mg l^{-1} of 2,4-D for 10 days, followed by transfer of the cotyledons to growth regulator-free medium. Approximately 10% of the somatic embryos thus formed converted into plants after separation from the explant tissue and transfer to growth regulator-free medium. Exposures to 2,4-D for less than 10 days resulted in low numbers of embryos, and conversion into plants of embryos induced with less than 40 mg l^{-1} of 2,4-D was poor. The latter was surprising, as excessive exposure to an inducing auxin has long been associated with poor development of somatic embryo apical meristems (Halperin and Wetherell 1964).

One of the features of white clover regeneration from callus has been the great genotype specificity. Efficient regeneration has been limited to a few genotypes with high regeneration capacity from cell or protoplast culture (Bhojwani et al. 1984; White 1984; Webb et al. 1987b; Yamada 1989). Because white clover is a highly heterozygous cross pollinated species, every immature embryo sampled represents a different genotype. Given that it was possible to obtain somatic embryos from every parental combination attempted, auxin-stimulated somatic embryogenesis is apparently not as sensitive to the strong genotype specificity as other white clover regeneration protocols, or alleles conferring embryogenic capacity were present in a relatively high frequency in the individuals tested. The information in Table 1 does suggest that different parental combinations resulted in progeny which varied in their ability to regenerate, as evidenced by the frequencies of somatic embryos per cotyledon that ranged from 0.15 to 1.25 for the full sib families.

Given that auxin-stimulated somatic embryogenesis of white clover is apparently applicable to several genotypes and can be used to obtain cycles of secondary embryogenesis, this method has the potential to be used to obtain transgenic plants. Secondary embryogenesis has proven useful to obtain transgenic walnut plants (McGranahan et al. 1988, 1990). In addition, the globular stage repetitive somatic embryogenesis observed for clover is similar to that observed in soybean explants continuously exposed to a high level of 2,4-D (Finer 1988). These have been used to establish embryogenic suspensions (Finer and Nagasawa 1988) amenable to microprojectile transformation (McMullen and Finer 1990).

As a species, white clover is amenable to a wide range of regeneration protocols. In addition to direct and indirect organogenesis, it is amenable to both auxin-stimulated and cytokinin-stimulated embryogenesis. Such versatility can make white clover a model species in which to study regeneration.

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