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Plant regeneration from hypocotyl and petiole callus of *Trifolium pratense* L.

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ABSTRACT

Red clover (Trifolium pratense L.) seedlings were screened for the ability to regenerate plantlets from hypocotyl-derived callus tissue. Media sequences described by Beach and Smith (1979) and Collins and Phillips (1982) and a variation using media from both sequences were tested. Plantlets were regenerated from three out of 642 genotypes. In all three cases, callus was initiated on B5C medium and regeneration was accomplished on SPL medium. Attempts to regenerate plants from petiole-derived callus tissue have so far been successful only with regenerants of clone F49. Petiole callus from epicotyl-derived F49 plants proved to be non-regenerative. Pollen viability varied significantly among individuals regenerated from callus cultures of clone F49. Root tip squashes from F49 regenerants revealed the normal diploid chromosome number (2n=14). The frequency of regeneration within progeny from reciprocal crosses between F49 regenerants and several non-regenerative genotypes was 29%.

Key words: red clover, Trifolium pratense, plant regeneration

Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid, BAP - benzylaminopurine, KN - kinetin, NAA - α-naphthaleneacetic acid

INTRODUCTION

Beach and Smith (1979) and Phillips and Collins (1979) determined that plant regeneration from red clover callus tissue could not be accomplished using protocols developed for other species. They responded by developing protocols specific for callus induction, somatic embryo induction and plantlet development of red clover. Wang and Holl (1988) studied red clover regeneration and were unsuccessful on the Beach and Smith (1979) regeneration medium but they observed regeneration and evaluated somaclonal variation using a Collins and Phillips (1982) medium. Phillips and Collins (1979) stated that the frequency of genotypes capable of regeneration from non-meristematic tissue-derived callus was about one percent.

The ability of red clover callus cultures to undergo somatic embryogenesis is reported to be due to additive genetic effects (Keyes et al., 1980). There have been suggestions to develop germplasm lines of red clover with high regeneration capacities (Beach and Smith, 1979; Keyes et al., 1980), but to our knowledge none have been produced.

In this paper we describe the selection of red clover genotypes capable of plant regeneration from hypocotyl and petiole callus. Heritability of the regeneration trait was also investigated. The program is aimed at genetic improvement of red clover through in vitro techniques.

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MATERIALS AND METHODS

Plant Material and Culture Conditions

Cultures were initiated from seedlings of red clover (Trifolium pratense L.) cultivars Arlington, Florex, Marino, Renova and Tepa grown in vitro. All media were adjusted to pH 5·8 prior to autoclaving (1·1 kg cm^{-2}; 120°C; 15 min) and were solidified with 7·0 gl^{-1} purified grade agar (Fisher Scientific, Fair Lawn, New Jersey). Cultures were grown at day/night temperatures of 24°C/18°C with a 16 h photoperiod under cool white fluorescent light. Intensity of illumination was 14-15 $\mu Em^{-2}s^{-1}$ for callus cultures and 160-250 $\mu Em^{-2}s^{-1}$ for in vitro plantlets.

Culture Initiation

Seeds were scarified with sandpaper, washed in 2% Liqui-Nox (Alconox, Inc., New York, N.Y.) detergent solution, rinsed in deionized water, then surface-sterilized for 20 min in 33% Javex solution (2% w/v sodium hypochlorite) and rinsed three times with sterile distilled water. Disinfected seeds were germinated on basal B5 medium (Gamborg et al., 1968) without sucrose in GA7 culture vessels (Magenta Corp., Chicago). Hypocotyl segments from 7 to 9-day-old seedlings were placed on callus induction media in sterile disposable 24 cell well plates (Corning) with 2 ml medium per well. The epicotyls were placed on 10 ml hormone-free L2 agar medium (Phillips and Collins, 1979) in 150 mm x 25 mm culture tubes. The number of seedlings evaluated necessitated dividing the study into 2 separate experiments. Media are described in Table 1.

Hypocotyl Culture

Evaluation of the regenerative capacity of hypocotyl callus was performed using media sequences consisting of three steps (Stages I, II and III) of 4 weeks duration each. Hypocotyls were cut in two segments, approximately 8 mm each. The two hypocotyl segments from each seedling were plated on different media in experiment 1 whereas the two sections were plated in separate culture wells on the same medium in experiment 2 (Table 2). After a total of 12 weeks in culture (Stages I, II and III), vigorous calli were subcultured on SPL medium in culture tubes (Stage IV).

Table 1. Media and their use for red clover as proposed in the literature

Medium	Salts and Vitamins	Growth Regulators (mg l^{-1}) Auxin	Cytokinin	Proposed Use
B5C[a]	B5[b]	NAA 2·0 2,4-D 2·0	KN 2·0	Callus and induction of regeneration
B5E[a]	B5	NAA 2·0	adenine 2·0	Regeneration
L2[c]	L2	picloram 0·06	BAP 0·1	Callus
SEL[c]	L2	2,4-D 0·01	adenine 2·0	Induction of somatic embryogenesis
SPL[c]	L2	picloram 0·002	BAP 0·2	Promotion of shoot development

[a]Media after Beach and Smith (1979)

[b]Gamborg, Miller and Ojima (1968)

[c]Designations as reported by Collins and Phillips (1982)

Table 2. Callus production and shoot regeneration from hypocotyl segment explants on three media sequences

Expt	No. Genotypes	Media Sequences[a] (Stage) I	II	III	IV	No. Callus Cultures	No. Callus Cultures with Shoots
1	354	B5C—— >	B5E—>	B5E—>	SPL	257	1
		L2 —— >	SEL—>	SPL—>	SPL		0
2	288	B5C—— >	B5E—>	SPL—>	SPL	263	2
	642					520	3

a Media as described in Table 1.

Plant material for experiment 1 consisted of 48 cv. Arlington, 96 cv. Florex, 96 cv. Marino, 90 cv. Renova and 24 cv. Tepa seedlings for a total of 354. Experiment 2 involved hypocotyl explants of 288 cv. Florex seedlings. Red clover is a cross-pollinated crop. Therefore each seedling is considered an individual genotype.

Plantlets regenerated from callus were grown and multiplied on SPL medium in culture tubes. Well-developed plantlets were subsequently maintained on hormone-free L2 medium with 2·5% sucrose in culture tubes.

Petiole Culture

Petiole sections were excised from 36 *in vitro* regenerated plantlets of clone F49 (authors' designation for the regenerative culture selected in experiment 1), from 6 epicotyl-derived F49 plantlets and from 1 epicotyl-derived plantlet each of 16 non-regenerative cv. Florex control genotypes. Ten petiole segments approximately 8 mm long, were cut from young leaves of each of the 58 plantlets described above. The 10 petiole segments were placed on B5C medium in 60 x 20 mm sterile disposable petri dishes for 4 weeks (Stage I). Five callussed sections from each plate were transferred onto B5E medium while the other 5 sections were transferred to SPL medium for 4 weeks (Stage II). All sections were then plated onto SPL medium for 4 weeks (Stage III). Regeneration was evaluated at the end of Stages II and III. Media sequences are summarized below (Table 3).

Progeny Evaluation

Eleven F49 regenerants, three F49 epicotyl-derived plants and 10 epicotyl-derived plants from individual seedlings which had produced non-regenerative hypocotyl callus cultures were acclimated in a mist frame, then potted in a soil: perlite:peat moss mixture (1:1:1). Regenerants and non-regenerative plants were crossed by hand without emasculation. Regenerability of each progeny was evaluated in three replications using the three-step protocol of Collins and Phillips (1982). Three petiole sections, 7-10 mm long, were plated in each replication. Progeny from which plantlet regeneration was observed in at least two replications were scored as regenerative genotypes.

Chromosome Number Determination

Root tips were collected from F49 regenerants, pretreated, fixed and stained with modified carbol fuchsin solution (Kao, 1982) according to the procedure described by Beach and Smith (1979).

Pollen Viability

Pollen viability was characterized by examining pollen grains which had been stained with a solution of 2% acetocarmine:glycerol (1:1). Pollen grains which were plump and took up the stain were scored as viable while those which were shrivelled and did not stain were regarded as nonviable. Each slide was prepared using anthers from three flowers of an influorescence and 200 pollen grains were scored per slide. Three slides were prepared for each plant. Percentage viable pollen data was analyzed by the F-test using a nested classification (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Hypocotyl-derived callus cultures were obtained and maintained for the 12 week evaluation period (Stages I, II and III) from 520 genotypes out of an initial number of 642 seedlings (Table 2). Callus was readily obtained from most genotypes on B5C and L2 media. This supports findings by Keyes et al. (1980) who concluded that several in vitro traits, including callus growth, are genotype specific in red clover. Callus of only three of 520 genotypes (designated as F49, F329, and F464) regenerated plantlets under the described conditions. In all three cases, regeneration occurred on SPL medium with callus which had been induced on B5C medium. Two of the three regenerative cultures (F49 and F329) required an additional step (Stage IV) on SPL medium in culture tubes before regeneration became evident. In later petiole callus studies, however, plants regenerated from F49R callus during Stage II on SPL medium.

Propagation and maintenance of the three regenerative cultures was accomplished by multiplication of somatic embryos on SPL medium. The first embryos to be visible arose from callus tissue but subsequent embryos appeared to result from proliferation of existing embryogenic tissue. All embryos were easily detached from surrounding tissue. Plantlet development and embryo proliferation occurred simultaneously on SPL medium in culture tubes.

Table 3. Effect of genotype and media sequence on frequency of regeneration from petiole calli at Stage II and Stage III

Genotype	Media Sequence	Frequency of Regeneration[b] Stage II	Stage III
F49-M[c]	A	0/6	0/6
	B	0/6	0/6
F49-R[d]	A	4/36	27/33
	B	17/35	26/32
cv. Florex	A	0/16	0/15
Control plants	B	0/16	0/13

[a]Media sequence A: B5C——>B5E——>SPL
Media sequence B: B5C——>SPL——>SPL

[b]No. of regenerative cultures/no. of cultures evaluated

[c]F49-M refers to clone F49 mother plants (epicotyl-derived)

[d]F49-R refers to clone F49 callus regenerants

It appears from the petiole callus results (Table 3) that efficient regeneration can be achieved employing a two-step protocol (Stage I on B5C medium and Stage II on SPL medium) if regenerative genotypes (such as F49) are used. A follow-up study, evaluating the media sequences after Beach and Smith (1979) and Collins and Phillips (1982), as well as four combinations of the two revealed that petiole callus from F49 regenerants will undergo embryogenesis on SPL medium regardless of whether L2 or B5C medium is used to induce callus (Stage I) and whether SEL or SPL is employed for Stage II culture (data not presented). Regeneration was also observed when F49R suspension aliquots [SL-2 liquid medium (Phillips and Collins, 1980)] were plated on SPL medium.

The results obtained with the petiole callus as well as with the hypocotyl callus demonstrate that the critical stage for embryogenesis and/or plantlet conversion is culture on SPL medium. Beach and Smith (1979) stated that red clover plantlets were regenerated from hypocotyl and ovary-derived callus on B5E medium. Despite several attempts, we achieved limited regeneration with F49 callus tissue on B5E medium. Wang and Holl (1988) were unsuccessful in attempts to regenerate red clover plantlets from callus tissue on this medium.

While petiole callus from F49 regenerants was highly embryogenic, petiole callus from F49 mother plantlets failed to regenerate (Table 3). It appears then that some difference exists between the F49 mother plantlets and those which were regenerated from hypocotyl callus, even though both originated from the same seedling. It is possible that a genetic change occurred in the callus thereby permitting regeneration. Handcrosses between F49 regenerants and F49 mother plants did not produce seeds. It appears, then, that the S-allele gametophytic incompatibility system which inhibits selfing in red clover is operating between these two groups of plants.

All F49 regenerants contained the normal diploid chromosome complement (2n=14). Pollen viability data give a measure of the somaclonal variation of clone F49. An analysis of variance confirmed that there were no significant differences among pollen viability means of the three groups (namely, F49 mother plants, F49 regenerants and ten control cv. Florex plants). A very high degree of variability was confirmed, however, among plants within groups. When the data were sorted by plant group it was apparent that the F49 mother plants had very consistent pollen viability estimates while the F49 regenerants varied in the degree of pollen viability to a similar extent as the group of cv. Florex control plants (Table 4). The variability among percent pollen viability estimates within plants was very low regardless of group (std. dev. = 4·78%). The phenomenon of reduced (although statistically insignificant) as well as variable pollen viability estimates provides additional evidence of genetic variation.

Table 4. Percentage stained pollen of F49-M[a], F49-R[b] and cv. Florex control plants

Genotype	No. Plants	% Stained Pollen[c]
F49-M	3	91·23 ± 0·78
F49-R	11	65·09 ± 30·25
cv. Florex control plants	10	74·34 ± 25·93

[a]F49-M refers to clone F49 mother plants (epicotyl-derived)

[b]F49-R refers to clone F49 callus regenerants

[c]Percentages and standard deviations calculated from counting 200 pollen grains replicated three times for each plant.

We are presently studying heritability of the regeneration trait. Progeny having an F49 regenerant as either the male or female parent have shown a regeneration frequency of approximately 29 percent (29/101). This is the first report to prove that regenerability of a red clover clone is a highly heritable trait. Regeneration from callus tissue has been shown to be highly heritable in alfalfa (Bingham et al., 1975; Wan et al., 1988).

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