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Genotype-dependent whole plant regeneration from protoplasts of red clover (*Trifolium pratense* L.)

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Abstract. Protoplasts are useful for subcellular studies, in vitro selection, somatic hybridization and transformation. Whole plant regeneration from protoplasts is a prerequisite to producing altered crop plants using these methods. Whole plant regeneration was achieved from leaf- and suspension culture-derived protoplasts of *T. pratense*. Regeneration was most dependent upon identifying genotypes with genetic capacity to regenerate. Additional factors that were used to select genotypes, but which proved to be less important, were a high rate of cell growth in culture and a high plating efficiency of protoplasts. One genotype was identified which had a regeneration response equivalent to that of *T. rubens* and which regenerated from both leaf- and suspension culture-derived protoplasts.

Introduction

Among the forage legumes, steady progress has been made in regeneration from protoplasts. Alfalfa (*Medicago sativa* L.) [3, 7, 13, 16, 18, 22, 42], *Trigonella corniculata* [8, 19], *T. foenumgraecum* [30], *Onobrychis viciifolia* [1], *Lotus corniculatus* [2, 37], and *Stylosanthes guianensis* [32] have been regenerated from protoplasts. In the genus *Trifolium* (L.), protoplast cultures of *T. arvense* [40], *T. hybridum* [6, 15, 38], *T. pratense* [15, 28], *T. repens* [1, 2, 4, 5, 6, 9, 14, 36, 37, 38, 39], *T. resupinatum* [23], and *T. rubens* [10] have been initiated. Whole plant regeneration has been achieved for *T. repens* [1, 4, 9, 37, 38, 39], *T. hybridum* [6, 38], and *T. rubens* [10]. Regeneration from

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callus and/or cell culture, but not from protoplasts, has been accomplished for *T. pratense* [4, 11, 12, 17, 25, 26, 31].

In the section *Trifolium* (Zoh.), *T. pratense* crosses with closely related annual species [33], but only with great difficulty to the perennial species. Hybrids of *T. pratense* with *T. sarosiense*, and *T. pratense* with *T. medium* were obtained [21, 27] via embryo rescue. Fertility of these hybrids was low, and attempts to introgress wild perennial germplasm into *T. pratense* had not met with success. An alternative approach to overcoming sterility and sexual incompatibility barriers is to use protoplasat fusion to produce somatic hybrids.

Only two reports exist of successful somatic hybridization in the forage legumes. Téoulé [34] regenerated hybrid plants from fusion of *M. sativa* and *M. falcata* protoplasts. *M. sativa* and *M. falcata* are sexually compatible but the production of this somatic hybrid does suggest that other somatic hybrids among foraged legumes are possible. Wright et al. [41] produced a somatic hybrid between sexually incompatible *Lotus corniculatus* and *L. coimbrensis*. Three important factors relating to success in obtaining somatic hybrid plants can be identified from these papers:
(1) at least one parent must be regenerable from protoplasts;
(2) both parents should be amenable to protoplast culture, and
(3) some means of selecting the heterokaryon or somatic hybrid must be devised. Preliminary screening work should concentrate on identifying parents with attributes (1) and (2).

Although often not emphasized, regeneration in the forage legumes is mostly (if not completely) genotype-dependent. Such is the case in alfalfa [16], white clover [1, 4, 9], red clover [17], and *T. rubens* [24]. Therefore, any scheme for crop improvement using in vitro techniques should require genotypes that regenerate at high frequency, show rapid and vigorous cell growth in culture, and have high protoplast plating efficiencies.

The objectives of this research were to identify *T. pratense* genotypes that would meet the above criteria. We report whole plant regeneration of *T. pratense* from protoplasts using a procedure modified from that of Grosser & Collins [10]. To our knowledge, this is the first report of regeneration from protoplasts of this species.

Materials and methods

Selection of genotypes

Preliminary experiments indicated that genotypic selection was necessary to

identify genotypes amenable to protoplast manipulations. Previously identified regenerator stocks [17, 25, 26] had been in culture for an extended period of time and had lost their capacity to regenerate.

Seeds of 150 genotypes from 'Arlington' were surface-sterilized, scarified and germinated on SGL medium [29] under continuous cool-white fluorescent light ($75\,\mu E\,m^{-2}\,s^{-1}$) at 26 °C. Seventy-five of these genotypes were progeny of specific crosses of known tissue culture regenerators; 40 were from polycrosses of known regenerators, and 35 were from open pollinations of known regenerators. One week after seed germination, hypocotyls were sectioned into 5-mm segments and placed on L2 medium [25] for callus production. After subculturing, 64 genotypes were selected for further study based on vigorous friable callus development (31 from specific crosses, 18 from polycrosses, and 15 from open pollinations). Growth in culture was evaluated by placing 0.5 g (FW) of friable callus of each genotype on L2 medium and measuring fresh weight gain after 30 days. This procedure was repeated over 3 successive 30-day cycles.

A second set of screenings for regeneration was conducted on a second batch of seedlings from the same set of materials as used previously. Those genotypes identified as having regeneration potential were subsequently evaluated for ability to produce vigorous suspension cultures and to produce suspension- and leaf-derived protoplasts. In addition, one highly regenerative genotype (designated R24T) from the cultivar Altaswede was provided by Marvin Schwartz, Agric. Canada Ltd., Saskatoon, Saskatchewan.

Recovery of plants

Friable callus of each genotype was placed on LSE and LSP115 media [25, 27] and subcultured over a 4-month period to evaluate regeneration capacity. Shoot cultures of genotypes that regenerated were maintained on either the ML8 [27] or LSP115 media. Shoots were rooted on either RL or CR2 media [10, 29], and rooted shoots were potted and transferred to the greenhouse.

Suspension culture initiation

Suspension cultures were initiated from all genotypes exhibiting regeneration capacity and/or vigorous callus growth. This was accomplished by placing approximately one gram of friable callus in 10 ml SL2 medium [26] in 125-ml Erlenmeyer flask maintained on a rotary shaker at 100 rpm under continuous cool-white fluorescent light ($9.5\,\mu E\,m^{-2}\,s^{-1}$) at 26 °C. After two

20-ml additions of SL2 medium over 1-week intervals, suspension cultures were subcultured weekly by combining approximately 20 ml of old cell suspension with 30–40 ml fresh SL2 medium. Once vigorous suspension cultures were obtained, their ability to yield large quantities of viable protoplasts was evaluated.

Protoplast isolation

Leaf-derived protoplasts were isolated from axenically grown shoot cultures maintained on LSP115 medium by monthly subcultures. Leaves were removed from one- to two-week old cultures and transferred to a 100 × 25 mm glass Petri dish containing 3 ml 8P medium [16]. Using a sharp scalpel, the leaflets were feathered in one-mm cuts perpendicular to the secondary veination of the leaflet. The leaflets were then transferred to a 125-ml side-arm Erlenmeyer flask containing 2.5–3.5 ml enzyme solution (cellulase, Onozuka RS, 1%; pectolyase Y-23, 0.2%; driselase, 0.25%; rhozyme HP 150, 0.25%; sorbitol, 0.35 M; mannitol, 0.35 M; $CaCl_2 \cdot 2H_2O$, 6 mM; 2-(N-morpholino)propanesulfonic acid (MES), 3 mM; NaH_2PO_4, 0.7 mM; pH 5.5; and filter-sterilized) and 7.5–6.5 ml 8P medium. A total of 10 ml enzyme solution/medium mixture was used for each isolation with the ratio of enzyme:8P medium being adjusted upward for less digestible genotypes. The flask was vacuum-infiltrated for 15 min followed by incubation for 6 h at 28 °C in the dark on a shaker rotating at 60 rpm.

Suspension culture-derived protoplasts were isolated from log-phase growth cultures at 3–4 days after subculture. Using a 5-ml disposable plastic pipet from which the tip had been removed, 2.5 ml of liquid cell suspension was pipetted into a sterile 60 × 20 mm plastic Petri dish. From 2.5–3.0 ml of enzyme solution was added followed by incubation of the sealed dish for 3 h under the conditions described above.

Protoplast purification

The crude protoplast mixtures were filtered through 45 μm mesh stainless steel filters packed with prefilters of glass wool. Mixtures were then centrifuged at 100 × *g* for 8 min. Supernatant was replacd with 5–10 ml 8P and the protoplasts resuspended by gently bubbling with a pasteur pipet. They were again centrifuged at 100 × *g* for 4 min, whereupon the supernatant was again removed and replaced with fresh 8P. The washing step was repeated an additional 2–3 times. After the final wash, protoplasts were counted with a hemacytometer and resuspended in 8P medium at a density of 5×10^4 and 10^5 protoplasts per ml. Protoplasts were plated in thin liquid

layers of 1.5 ml 8P in a 60 × 20 mm Petri dish (Falcon). For determination of plating efficiencies, protoplasts were plated in 1.5 ml 8P medium solidified with 0.2–0.45% low melting-point agarose (Bethesda Research Laboratories Inc. or International Biotechnologies Inc.). Plated protoplasts were sealed in an opaque box and incubated under 9.5 $\mu E\, m^{-2} s^{-1}$ light level at 26 °C.

Feeding and recovery of callus

One week after isolation, cultures were fed with 5–10 drops of C8P+ medium (2:1 C8P:SL2, [10]). A second feeding of 10 drops of SL2+ medium (1:3 C8P:SL2) was made during the second week. Weekly feedings thereafter were made with the addition of 10–15 drops SL2 medium. When colonies were 0.5 to 1.0 mm in diameter, they were transferred to solid L2 medium for callus growth. Plant regeneration and transfer to the greenhouse was achieved as described above, except for R24T, which exhibited somatic embryogenesis on L2 medium. In this case, embryogenic callus was transferred directly to LSP medium for production of shoots.

Results and discussion

Growth in culture

The screening criteria used to identify *T. pratense* genotypes amenable to protoplast manipulations included growth in culture, regeneration capacity, and ability to produce a vigorous suspension culture from which large quantities of viable protoplasts could be isolated.

Growth in culture was selected as a screening criteria because high rates of cell division should allow for the rapid recovery of callus from protoplasts. Growth was determined by measuring fresh-weight gain over 3 successive 30-day intervals (Table 1). Genotypes exhibiting high growth means demonstrated both stability and high performance in the 'L2' tissue culture system. Genotypes with high growth could also rapidly produce large amounts of callus tissue which could be utilized in regeneration experiments.

Statistical analysis of fresh-weight gain data indicated a significant rep × genotype interaction. The fitted model included reps and genotypes as main effects, and Tukey's [35] one degree of freedom for non-additivity. Preliminary analysis by methods described by Mandel [20] indicated that the one degree of freedom for 'concurrence' (equivalent to Tukey's one degree of

Table 1. Growth of callus from *T. pratense* genotypes.

Type of cross[1]	Genotype	Fresh-weight gain[2]	
		Mean (g)	Log transformed mean
OP	R578 C	10.98	2.359
Poly	B5C9 5	10.10	2.257
Poly	B5C9 B	7.94	1.895
OP	WR13 D	7.19	1.834
SC	B5C9 × R578 #C	6.05	1.737
SC	L × 2a #C	6.14	1.716
Poly	Arl 55 D	6.22	1.637
Poly	R578 C	6.04	1.634
OP	WR13 A	6.20	1.586
OP	WR13 O	5.31	1.545
SC	2m × C #A	4.57	1.476
SC	2m × C #E	6.57	1.422
SC	D × 2e #D	4.44	1.294
OP	WR13 C	5.04	1.291
SC	2R × E #B	3.67	1.133
Poly	R578 E	3.69	1.087
Poly	WR13 C	3.28	1.021
SC	R578 × B5C9 #B	5.07	1.011
SC	PCM6 × IM4 #D	4.04	1.007
SC	B5C9 × R578 #F	3.14	0.966
SC	PCM6 × IM4 #B	3.00	0.957
OP	WR13 B	2.69	0.943
Poly	Arl 55 B	3.59	0.934
Poly	R578 A	2.64	0.917
OP	Arl #C	2.43	0.845
SC	L × 2 #C	2.83	0.842
SC	2m × C #D	2.44	0.838
OP	WR13 N	2.33	0.800
SC	L × 2R #C	2.63	0.775
SC	L × 2e #C	2.31	0.676
SC	D × 3e #B	2.68	0.670
OP	WR13 L	2.24	0.667
Poly	WR13 B	1.94	0.639
SC	R578 × B5C9 #E	2.63	0.596
Poly	B5C9 C	1.89	0.587
SC	L × 2R #B	1.76	0.524
Poly	R578 H	1.67	0.495
SC	R578 × B5C9 #G	2.05	0.490
OP	WR13 J	1.77	0.488
SC	C × 2a #D	1.86	0.469
Poly	B5C9 E	1.66	0.440
Poly	R578 G	1.88	0.413
SC	C × 2a #A	1.40	0.311
OP	WR13 K	1.44	0.303
SC	2a × D #B	1.54	0.280

Table 1. Continued.

Type of cross[1]	Genotype	Fresh-weight gain[2]	
		Mean (g)	Log transformed mean
OP	R578 D	1.35	0.236
OP	WR13 E	1.33	0.232
SC	D × 2e #C	1.34	0.231
SC	L × 2e #B	1.25	0.211
SC	B5C9 × R578 #3	1.21	0.175
SC	C × 2a #C	1.17	0.142
OP	WR13 F	1.18	0.132
SC	L × 2a #B	1.14	0.129
SC	2F × L #C	1.15	0.123
OP	B5C9 E	1.14	0.119
Poly	B5C9 D	1.15	0.114
Poly	Arl 55 E	0.95	−0.052
SC	L × 2 #D	0.89	−0.118
Poly	B5C9 4	0.89	−0.119
SC	B5C9 × R578 #4	0.88	−0.174
SC	2m × C #F	0.81	−0.259
SC	L × 2e #A	0.78	−0.272
			LSD 0.05 = 0.624

[1] OP = open pollinated, poly = polycross, SC = single cross.
[2] Fresh-weight gain over three successive monthly repetitions starting with 0.5 g inoculum.

freedom for non-additivity) accounted for the majority of the interaction. This interaction was apparently due to genotypic adaptation, which was generally proportional to the vigour of each genotype at the time of initial plating. This indicates that genotypic selections for rapid growth should either be made over an extended period of time or else after the genotypes involved are sufficiently adapted to the tissue culture media. Early selections could lead to the elimination of genotypes which actually possess the desired growth capability. On the other hand, allowing time for cell lines to acclimate could result in the gain of deleterious variation and the loss of regeneration capacity.

Genotypic differences in growth were separated by performing an LSD test on log fresh-weight gain (Table 1), using the residual mean square from the fitted model as the estimate of error variance. The results demonstrated a large amount of genetic variation in growth rate, as measured by fresh-weight gain.

Division efficiencies

Overall protoplast division frequencies varied with tissue source and

Table 2. Division efficiencies of protoplast cultures 9–11 days after isolation from selected *T. pratense* genotypes.

Genotype	Source	Living[1]	Dividing[1]	Overall divisions[3]
L × 2e #C	leaf	22.1 ± 2.3	60.8 ± 3.6	18.3 ± 1.8
R24T	leaf	27.0 ± 3.9	46.6 ± 5.0	9.5 ± 0.6
R24T	suspension	23.2 ± 0.5	69.9 ± 0.9	14.7 ± 0.4
R578 OP C	suspension	50.4 ± 0.3	66.2 ± 0.4	34.5 ± 0.3
R578 OP 8	suspension	15.3 ± 0.7	57.3 ± 1.6	8.1 ± 0.3
R578 Poly 6	suspension	44.5 ± 2.0	62.2 ± 8.2	27.4 ± 2.3
R578 Poly 7	suspension	63.8 ± 1.6	58.5 ± 0.7	37.3 ± 1.4

[1] Number of living protoplasts/total number of living and dead protoplasts (mean ±SE, %)
[2] Number of dividing protoplasts/number of living protoplasts (mean ±SE, %)
[3] Number of dividing protoplasts/total number of protoplasts (mean ±SE, %)

genotype. Suspension culture-derived protoplasts had overall higher division frequencies than leaf-derived protoplasts, presumably because previous suspension culture of cells had allowed adaptation to culture conditions. For leaf-derived protoplasts, genotype L × 2e#C had approximately twice as high an overall division frequency as R24T, primarily because L × 2e#C had a higher proportion of living protoplasts that divided (Table 2). Protoplasts of R24T tended to be small and rapidly dividing compared to those of L × 2e#C. The small size and rapid division rate of R24T appeared to be related to the high regeneration capacity of this genotype.

For suspension culture-derived protoplasts, division frequencies ranged from 8 to 37% depending on the genotype (Table 2). Overall R578 OP C and R578 Poly 7 had the highest division frequencies. R578 OP C had a higher proportion of living protoplasts that divided, which might result in a higher regeneration frequency. Division frequency for suspension culture-derived protoplasts of R24T was relatively low (15%), but due to its high frequency of regeneration, would probably be quite useful in somatic hybridization experiments.

Regeneration potential of genotypes

The results of the genotypic screening involving the remaining criteria are presented in Table 3. Overall, regeneration capacity from callus was demonstrated in 14 out of 64 (22%) of the genotypes examined in the first screening. Of the 26 genotypes that produced vigorous suspension cultures, 15 (58%) were amenable to protoplast isolation. Of the regenerator genotypes,

8 of 14 (57%) were identified that produced vigorous suspension cultures from which large quantities of viable protoplasts could be isolated, therefore positively satisfying all of the original screening criteria. Among the additional 'Arlington' genotypes examined, three (R578 OP 8, R578 Poly 6, and R578 Poly 7) showed potential in satisfying the three requirements stated above. However, none were as satisfactory as R578 OP C. Many of the best genotypes used in this research had R578 parentage which passed on amenability to cell culture and regeneration ability to several of its open-pollinated and polycross progeny. These data suggest that R578 may possess one or more dominant factors for regeneration ability. Epistatic interactions of 'regeneration' genes with other genes affecting regeneration ability may have prevented regeneration in some R578 progeny.

Five genotypes demonstrated regeneration from direct plating of the suspension cultures onto LSE and LSP115 medium, and only 3 (L × 2e#C, R578 OP C, and R24T) were regenerated from protoplasts (Table 3, Figs. 1–4). More genotypes were able to regenerate from leaf-derived protoplasts than from suspension-derived protoplasts. Only R24T regenerated whole plants from suspension-derived protoplasts. The reduction in number of genotypes that regenerated from suspension and protoplast culture is probably related to the length of time in culture. Thus, only R24T, with very high regenerative potential, could be regenerated from all explant types. With the exception of R24T, protoclonal variation was observed among the regeneration (Fig. 4). This variation took the form of supernumerary leaflets, dwarfism, and sterility.

These data show that cell growth and protoplast division frequency in vitro are not associated with regeneration potential in the red clover genotypes examined in this study. A genotype must be amenable to cell or protoplast culture for regeneration to take place; however, some of the most culturable genotypes (i.e. Arl 55 Poly D) were non-regenerators, and the most highly regenerable genotype (R24T) showed only average division frequencies in protoplast culture. In identifying genotypes for use in somatic hybridization, primary emphasis should be placed on regeneration ability followed by amenability to protoplast culture. High rate of growth in culture could be ideal but not entirely necessary. Parental genotypes should also be sexually fertile, free of deleterious somaclonal variation, and of the 'normal' ploidy level. Thus, in the initial screen for regeneration ability, the original plant material should be maintained for donor tissue used in subsequent protoplast experiments.

It appears that R24T has the greatest potential for recovering whole plants from protoplasts and would be useful in somatic hybridization attempts. Visual comparisons to *T. rubens* genotypes with high regeneration

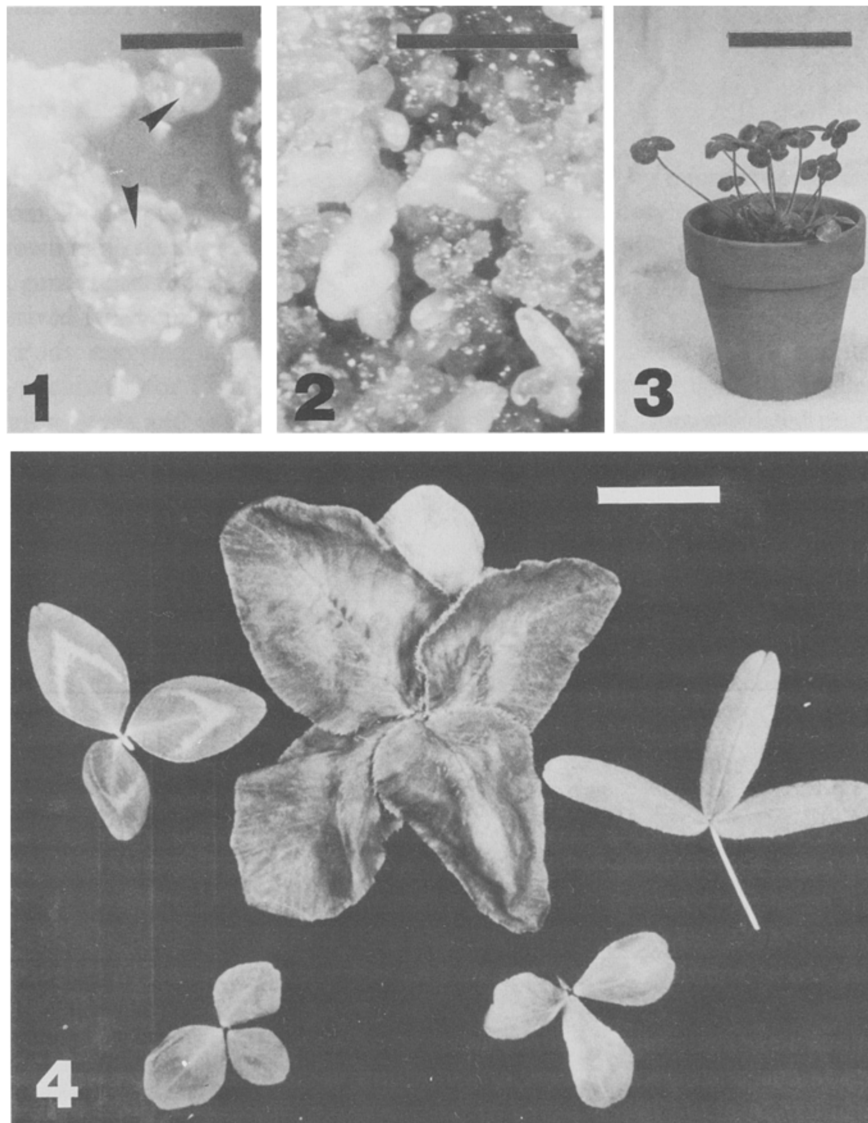
Table 3. Regeneration of *Trifolium pratense* genotypes for callus, suspension, and protoplast cultures.

Type[1] of cross	Genotype	Regeneration from callus	Vigorous suspension	Regenerates from suspension	Yields suspension-derived protoplasts	Yields leaf-derived protoplasts	Regenerates from suspension protoplasts	Regenerates from leaf protoplasts
SC	2a × D #B	–[2]						
SC	2F × L #C	+[2]	+	+	+			
SC	2m × C #A	+	+	–	–			
SC	2m × C #D	–	–					
SC	2m × C #E	–	–					
SC	2m × C #F	–						
SC	2R × E #B	–	–					
OP	Arl #C	(+)[3]	+	–	–			
Poly	Arl 55 I	+	+	–	–			
Poly	Arl 55 B	–	–					
Poly	Arl 55 D	–	+	–	+			
Poly	Arl 55 E	+	+	–	+			
OP	B5C9 E	–						
Poly	B5C9 4	–						
Poly	B5C9 5	–	+	–	+			
Poly	B5C9 B	–	+	–	+			
Poly	B5C9 C	+	+	–	+			
Poly	B5C9 E	–	–					
Poly	B5C9 D	–						
SC	B5C9 × R578 #3	–						
SC	B5C9 × R578 #4	–						
SC	B5C9 × R578 #C	–	+	–	–			
SC	B5C9 × R578 #F	–	–					
SC	C × 2a #A	–						
SC	C × 2a #C	–						
SC	C × 2a #D	–	–					
SC	D × 2e #C	–						
SC	D × 2e #D	–	–					
SC	D × 3e #B	+	+	–	–			
SC	D × 3e #B	–	–					
SC	L × 2 #C	–	–					
SC	L × 2 #D	–						
SC	L × 2a #C	–	+	–	+			
SC	L × 2e #A							
SC	L × 2a #B	–						

SC	L × 2e #B	−						
SC	L × 2e #C	+	+	−	−	+	−	+
SC	L × 2R #B	(+)	+	−	+			
SC	L × 2R #C	−	+	−	−			
SC	PCM6 × IM4 #B	−	−					
SC	PCM6 × IM4 #D	(+)	−					
OP	R578 8	(+)	+	−	+		−	
OP	R578 C	+	+	+	+	+	−	(+)
OP	R578 D	−						
Poly	R24T	+	+	+	+	+	+	+
Poly	R578 6	+	+	−	+		−	
Poly	R578 7	+	+	−	+		−	
Poly	R578 A	−	+	−	−			
Poly	R578 C	−	+	−	+			
Poly	R578 E	−	+	−	−			
Poly	R578 G	−	+	−	−			
Poly	R578 H	+	+	(+)	+	+	−	
SC	R578 × B5C9 #B	−	−					
SC	R578 × B5C9 #E	−	−					
SC	R578 × B5C9 #G	−	−					
OP	WR13 A	−	−					
OP	WR13 B	−	+	−	−			
OP	WR13 C	−		−	+			
OP	WR13 D	+	+	−	−			
OP	WR13 E	−						
OP	WR13 F	−						
OP	WR13 J	+	+	−	+	+	−	
OP	WR13 K	−						
OP	WR13 L	−	−					
OP	WR13 N	−	+	−	−			
OP	WR13 O	+	+	−	−			
Poly	WR13 B	+	+	+	+			
Poly	WR13 C	−	−					

[1] OP = open pollinated, poly = polycross, SC = single cross
[2] + indicates positive response, while − indicates a lack of response
[3] (+) indicates abnormal shoot formation

potential indicate that the response of R24T is quite similar. Its only drawback is that the original plants received from Schwartz (Agric. Canada Ltd.) were tetraploid ($2n = 4x = 28$, unpubl. data), presumably due to somatic doubling during the first cycle of tissue culture used to identify regenerating genotypes. This is a commonly observed occurrence in cell and protoplast cultures of *Medicago* and *Trifolium* (Myers, unpubl. observations [10, 14]). The tetraploid nature of R24T would make sexual transfer of



regeneration ability into diploid red clovers difficult, and might result in less chromosomally stable somatic hybrids.

With the genotypes identified in this study, the greatest chance for success in somatic hybridization would be to fuse leaf-derived protoplasts of both parents. The use of leaf-derived protoplasts is indicated since red clover appears to rapidly lose regeneration ability in culture. Either R578 OP C or R24T would be the red clover parent of choice. Success rate would be higher with R24T, but the use of R578 OP C would result in a symmetric allotetraploid since the parent plant was diploid ($2n = 2x = 14$).

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Fig. 1. Leaf protoplast-derived callus of R24T in liquid culture medium approximately one month after isolation. The compact globular structures (arrows) are proembryos. Bar = 0.5 mm.

Fig. 2. Somatic embryos and embryogenic callus from leaf protoplasts of R24T plated on solid LSP medium. Bar = 1.0 mm.

Fig. 3. Plant of R24T regenerated from leaf-derived protoplasts growing in the greenhouse. This plant has $2n = 4x = 28$ chromosomes. Bar = 5.0 cm.

Fig. 4. Leaves from *Trifolium* species regenerated from cell or protoplast culture. Clockwise from left: *T. pratense* P.I. 302576 (Turk 15) regenerated from callus culture, L × 2e#C regenerated from leaf-derived protoplasts, *T. rubens* regenerated from leaf-derived protoplasts, and R578 OP C regenerated from leaf-derived protoplasts (lower two leaves). L × 2e#C plants showed multifoliate leaves with a rugose texture. Plants were also tetraploid ($2n = 4x = 28$). R578 OP C plants were dwarfed and sterile with asymetrically shaped leaves. Bar = 2.0 cm.

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