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Genetic Variation in Tissue Cultures of Red Clover*

G.J. Keyes, G.B. Collins and N.L. Taylor
Department of Agronomy, University of Kentucky (USA)

Summary. Design II matings were made among randomly selected clones of 'Arlington' red clover (*Trifolium pratense* L.). Progeny were evaluated in vitro on two regeneration media for callus growth and differentiation. Additive genetic variance was a significant source of variability for nearly all traits evaluated, including somatic embryogenesis. In vitro traits, such as rapid callus growth, colony vascularization, root initiation, chlorophyll production and embryogenesis were highly heritable and should respond to breeding and selection. Dominance genetic variance was significant for only a few in vitro characters. Maternal and cytoplasmic factors were significant primarily in the early subcultures. Highly significant additive genetic correlation of performance on two regeneration media was found. A population selected on one of the regeneration media for such characteristics as improved plantlet regeneration, rapid callus growth, long term colony viability or the frequency of root initiation should show correlated improvement on the other medium. No significant differences for embryogenesis were attributable to differences in the regeneration media used. Furthermore, no interaction of additive genetic effects with regeneration media were observed. These data indicate that improvement in the frequency of plantlet regeneration from callus of red clover could effectively be achieved by breeding and selection for embryogenic types.

Key words: Tissue culture – Somatic embryogenesis – Genetic variance – Plant breeding – *Trifolium pratense* L.

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Introduction

Application of tissue culture technology to the improvement of red clover depends upon the ability to initiate, maintain, manipulate and finally regenerate plants from in vitro cultures. Previous investigations have been concerned with determining the cultural practices and media compositions which favored growth and differentiation of red clover cultured in vitro (Phillips and Collins 1979; Phillips and Collins 1980; Beach and Smith 1979).

Investigators working with a variety of economic plant species have observed that successful initiation, growth and redifferentiation of tissue cultures is often dependent on the genotype selected for study. Genetic effects have been reported to contribute to differences observed in tissue cultures of common bean, *Phaseolus vulgaris* L. (Mok and Mok 1977); rice, *Oryza sativa* L. (Guha-Mukherjee 1973); corn, *Zea mays* L. (Green and Phillips 1975; Shannon and Batey 1973); tobacco, *Nicotiana tabacum* L. (Phillips and Collins 1977; Ogura and Tsuji 1977; Cheng and Smith 1973; Hlasnikova 1977); tomato, *Lycopersicum esculentum* Mill. (Gresshof and Doy 1972); birdsfoot trefoil, *Lotus corniculatus* L. (Nizeki and Grant 1971); sweet clover, *Melilotus alba* Desr. (Taira et al. 1977); alfalfa, *Medicago sativa* (Saunders and Bingham 1972; Saunders and Bingham 1975; Walker et al. 1978; Keyes and Bingham 1979); potato, *Solanum* spp. L. (Simon and Peloquin 1977); petunia, *Petunia* spp. L. (Izhar and Power 1977); and wheat, *Triticum aestivum* L. (Shimada and Makino 1975).

Observations made in this laboratory indicated that genotypic differences were an important source of variability in the success of proliferating and redifferentiating callus of red clover, *Trifolium pratense* L. (unpublished data).

This research was designed to investigate: (1) genetic variability for growth, development, and somatic embryo-

genesis in callus cultures of red clover; and (2) the genetic correlation of growth and differentiation on two regeneration media.

Materials and Methods

Mating Design

Random samples of Arlington red clover genotypes were germinated and grown to maturity in a greenhouse. Twenty-four genotypes were randomly selected, divided into three sets of eight each, and a 4 × 4 Design II mating with reciprocals was constructed from each set (Comstock and Robinson 1948). Crosses were made by hand without emasculation. Plants were maintained in a greenhouse until seed had been hand harvested.

In Vitro Method

Seeds from reciprocal full-sib crosses were disinfected with 95% ethyl alcohol for 5 min and with 2% w:v calcium hypochlorite for 20 min, followed by three washes in sterile de-ionized water. Seeds were germinated under aseptic conditions in sterile disposable petri plates. Twenty-four seedlings from each full sib family, 12 from each seed parent, were drawn at random and hypocotyls of each were sectioned into four 2 mm sections. The four sections of a single seedling were inoculated onto a 20 × 60 mm sterile disposable petri plate containing 15 ml of KB2 medium, consisting of the basal components of the PCL medium (Phillips and Collins 1979), supplemented with 2 mg/liter each of 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalenacetic acid (NAA) and 6-furfurylaminopurine (kinetin). The medium was solidified with 0.8% w:v agar. KB2 was steam autoclaved for 18 min, at 15 psi, in one-half-liter lots, prior to being poured into culture plates under aseptic conditions. The culture medium was replicated in four batches. The 12 seedlings of a reciprocal full-sib family were divided into four groups of three seedlings each, and one group was assigned at random to each of the four replicate batches of KB2. Initiation cultures were maintained under continuous artificial light at 23 C for 35 days.

At the conclusion of the initiation phase of culture, callus colonies were transferred to two different regeneration media. The regeneration media were designated 'D', a PCL basal medium supplemented with 0.01 mg/liter 2,4-D and 5.0 mg/liter adenine (Phillips and Collins 1980); and 'B', a B5 basal medium (Gamborg et al. 1968) supplemented with 20 mg/liter thiamine, 1 mg/liter NAA, and 5.0 mg/liter adenine (Beach and Smith 1979). Regeneration media were replicated in two batches. Batches were prepared as 10x stocks from dry components. Stocks were packaged in 100-ml aliquots, in plastic bags, and stored in a freezer at −20° C. Tissue assigned to a batch of regeneration media was transferred to the same batch in subsequent subcultures.

The regeneration media selected for this study had been shown in previous studies to be effective in promoting continued growth and differentiation of red clover callus (Phillips and Collins 1980; Beach and Smith 1979). These media differed with respect to growth regulator, vitamin, and basal composition. Sub_1 cultures were initiated by transfer of callus colonies of a single genotype to two replications of each regeneration media, one colony to each rep of both media. Thus, every genotype was assigned to both regeneration media. Furthermore, rep 1 and rep 2 of each media contained the same genotypes. In this way, differences between replications of a single regeneration medium, as well as differences between culture media, could not result from drawing different samples of genotypes for assignment to media treatments or to replications of media treatments. Cultures were maintained as described previously for a period of 35 days. At the conclusion of Sub_1 culture, callus fresh weight ('fresh-weight') percentage of colonies rooting ('rooting'), percentage of colonies producing a dense cover of elongated filaments massed at the callus surface ('snow'), percentage of non-nodulated, smooth colonies ('smooth'), and percentage of hard, dark green colonies ('dark green') were recorded.

To initiate Sub_2 cultures, tissue was transferred to sterile disposable petri plates containing 50 ml of fresh regeneration media. Transfers were made on a plate for plate basis. After 28 days in Sub_2 culture, plates were visually rated for callus vigor ('vigor'), the percentage of colonies initiating roots ('rooting') and the total number of roots initiated ('root count').

Sub_3 cultures were initiated in the above fashion. After 35 days plates were visually rated for colony viability or the absence of browning and necrosis ('viability') and continued callus growth ('vigor'). The total number of roots initiated ('root count'), the percentage of colonies which had proliferated white or creamy friable callus ('white callus'), and the percentage of hard, dark green colonies ('dark green') were also recorded.

No data was collected on Sub_4 cultures. Sub_5 cultures were initiated in the standard fashion and at the conclusion of 35 days the percentage of embryogenesis was noted.

Data Analysis

Percentage data were transformed on an angular scale measured in radians. Previous experience indicated that root count data should be log-normally distributed. The natural log transformation was applied to root count data to satisfy assumptions of the analysis of variance.

Analysis was based on the average performance of 12 genotypes sampled from each reciprocal full sib family. Data collected on 'B' medium was analyzed separately from data collected on 'D' medium. The form of the analysis of variance along with the expectation of mean squares is shown in Table 1. The underlying assumptions of the analysis are disomic inheritance, linkage equilibrium, no epistasis, that relatives are not inbred and that relatives are a random sample of a non-inbred base population. Additive and dominance genetic variances, as well as narrow sense heritability, were estimated for each response variable after the method of Comstock and Robinson (1948). The genetic interpretations of mean squares follows the discussion of Cockerham (1963). The variance of rows within sets and the variance of columns within sets are equivalent to the covariance of row half-sibs and column half sibs, respectively. Each provides an estimate of 1/4 the additive genetic variance. The variance of the interaction of rows and columns in sets provides an estimate of 1/4 the dominance genetic variance. Phenotypic and additive genetic correlation coefficients were calculated for each response variable measured on the two regeneration media.

Following the analysis of data separated by media, data were pooled and a single analysis of variance was carried out. The form of the analysis of variance is shown in Table 2. To conserve the size of the X'X matrix the interactions of replications with all effects as well as the interaction of regeneration media with reciprocals, mating sets and female parents in sets were not partitioned and comprise the residual term. In this analysis media 'B' and media 'D' are assumed to be a random sample of regeneration media which support growth and differentiation of red clover callus. For this reason, inferences are to regeneration media of this type.

Table 1. Form of the analysis of variance for data on separate regeneration media, a random effects model

Source	d.f.	EMS
Replications	1	$\sigma^2 + 96\sigma^2_{Reps}$
Mating sets	2	$\sigma^2 + 2\sigma^2_{Recip} + 4\sigma^2_{RxC(S)} + 16\sigma^2_{C(S)} + 16\sigma^2_{R(S)} + 32\sigma^2_{FP(S)} + 64\sigma^2_{S}$
Female parents in sets	3	$\sigma^2 + 2\sigma^2_{Recip} + 32\sigma^2_{FP(S)}$
Rows in sets[a]	9	$\sigma^2 + 2\sigma^2_{Recip} + 4\sigma^2_{RxC(S)} + 16\sigma^2_{R(S)}$
Columns in sets	9	$\sigma^2 + 2\sigma^2_{Recip} + 4\sigma^2_{RxC(S)} + 16\sigma^2_{C(S)}$
Rows x columns in sets	27	$\sigma^2 + 2\sigma^2_{Recip} + 4\sigma^2_{RxC(S)}$
Reciprocals	45	$\sigma^2 + 2\sigma^2_{Recip}$
Experimental error[b]	95	σ^2

[a] Estimates of additive genetic variance and narrow sense heritability are based upon the pooled SS for rows in sets and columns in sets with 18 d.f.
[b] Experimental error consists of the pooled interactions of replications with all other effects

Table 2. Analysis of variance for percentage of embryogenesis in Sub_5 culture with data pooled from media 'B' and media 'D', a random effects model

Source of variability	d.f.	Mean square	EMS
Replications	1	0.01435	$\sigma^2 + k_9\sigma^2_{Reps}$
Regeneration media = RM	1	0.00071	$\sigma^2 + k_8\sigma^2_{RM}$
Mating sets	2	1.5780	$\sigma^2 + k_3\sigma^2_{Recip} + k_4\sigma^2_{RxC(S)} + k_5\sigma^2_{C(S)} + k_5\sigma^2_{R(S)} + k_6\sigma^2_{FP(S)} + k_7\sigma^2_{MS}$
Female parents in sets	3	0.0558	$\sigma^2 + k_3\sigma^2_{Recip} + k_6\sigma^2_{FP(S)}$
Rows in sets	9	0.41277**	$\sigma^2 + k_3\sigma^2_{Recip} + k_4\sigma^2_{RxC(S)} + k_5\sigma^2_{R(S)}$
Columns in sets	9	0.34875*	$\sigma^2 + k_3\sigma^2_{Recip} + k_4\sigma^2_{RxC(S)} + k_5\sigma^2_{C(S)}$
Rows x columns in sets	27	0.11625	$\sigma^2 + k_3\sigma^2_{Recip} + k_4\sigma^2_{RxC(S)}$
Reciprocals	45	0.07165	$\sigma^2 + k_3\sigma^2_{Recip}$
RM x rows in sets[a]	9	0.18526*	$\sigma^2 + k_1\sigma^2_{RMxRxC(S)} + k_2\sigma^2_{RMxR(S)}$
RM x columns in sets	9	0.11697	$\sigma^2 + k_1\sigma^2_{RMxRxC(S)} + k_2\sigma^2_{RMxC(S)}$
RM x rows x columns in sets	27	0.07826	$\sigma^2 + k_1\sigma^2_{RMxRxC(S)}$
Residual	241	0.07274	σ^2

[a] The pooled estimate for the interaction of regeneration media with rows in sets and columns in sets is not significant
* F-value significant at the 5% level of probability
** F-value significant at the 1% level of probability

Results

Analyzing data separated by regeneration media, significant or highly significant estimates of additive genetic variance (Table 3) were reported for 13 of 14 in vitro traits measured on Medium 'B' and for 12 or 14 in vitro traits measured on Medium 'D'. The estimates of narrow sense heritability showed no trend toward either increasing or decreasing magnitude through successive subcultures (Table 4). Heritability in the narrow sense was 0.54 on medium 'B' and 0.25 on medium 'D' for the percentage of embryogenesis recorded in the fifth subculture.

Significant or highly significant estimates for dominance genetic variance were obtained for only 3 of 14 characters measured on Medium 'B' and for only 4 of 14 characters measured on Medium 'D' (Table 3). Both callus 'snow' morphology and callus 'smooth' morphology were significant for genetic dominance in the first subculture on

Table 3. Significance estimates for genetic variances of 14 tissue culture characters measured on two regeneration media

Response variable	Sub_X	Additive variance		Dominance variance		Reciprocal variance	
Regeneration Media		'B'	'D'	'B'	'D'	'B'	'D'
Freshweight	1	**	**	NS	NS	**	**
Rooting	1	**	**	NS	NS	**	**
Snow	1	NS	NS	*	**	**	**
Smooth	1	**	**	**	*	**	**
Dark green	1	*	**	NS	NS	**	**
Vigor	2	**	**	NS	NS	**	**
Rooting	2	**	**	**	NS	*	**
Root count	2	**	**	NS	NS	**	**
Viability	3	**	**	NS	NS	*	**
Vigor	3	**	**	NS	NS	**	**
Root count	3	**	NS	NS	**	**	NS
White callus	3	**	**	NS	NS	NS	**
Dark green	3	*	*	NS	**	*	NS
Embryogenesis	5	**	*	NS	NS	NS	NS

* F-value significant at the 5% level of probability
** F-value significant at the 1% level of probability
NS F-value not significant

Table 4. Narrow sense heritability estimates for 14 tissue culture characters measured on two regeneration media

Response variable	Sub_X	h^2	
		Medium 'B'	Medium 'D'
Freshweight	1	0.54**	0.69**
Rooting	1	0.80**	>1**[a]
Snow	1	0.29	0.18
Smooth	1	0.99**	>1**[a]
Dark green	1	0.36*	0.58**
Vigor	2	0.72**	0.60**
Rooting	2	0.30**	0.77**
Root count	2	0.40**	0.61**
Viability	3	0.94**	0.79**
Vigor	3	0.62**	0.62**
Root count	3	0.41**	0.11
White callus	3	0.68**	0.77**
Dark green	3	0.32*	0.33*
Embryogenesis	5	0.54**	0.25*

* Narrow sense heritability estimate significant at the 5% level of probability
** Narrow sense heritability estimate significant at the 1% level of probability
[a] Parameter estimates which exceed the theoretical maximum $h^2 = 1$ are assumed to be the result of sampling error

both regeneration media. The percentage of colonies initiating roots in the second subculture on Medium 'B' was highly significant for dominance, as were the total number of roots initiated and the frequency of colonies showing a 'dark green' callus morphology in the third subculture on Medium 'D'. Thus, in 3 of 7 cases, where dominance was significant, the choice of regeneration media significantly affected its expression.

Significant or highly significant reciprocal effects were estimated for 12 or 14 characters measured on Medium 'B' and for 11 of 14 characters measured on Medium 'D' (Table 3). Significant reciprocal effects were not estimated for the percentage of friable 'white' colonies or the percentage of embryogenesis measured on Medium 'B' in the third and fifth subcultures, respectively. Significant reciprocal effects were not estimated for total 'root count' or the percentage of 'dark green' colonies measured on Medium 'D' in the third subculture, nor were reciprocal effects significant for the percentage of embryogenesis measured in the fifth subculture.

In three of five cases in which reciprocaleffects were not significant, differences among regeneration media contributed to the differential expression of reciprocal effects.

The analysis of variance for the percentage of embryogenic colonies in Sub_5 culture with data pooled from Media 'B' and Media 'D' is shown in Table 2. Significant or highly significant estimates for additive genetic effects were obtained for the frequency of embryogenesis across two media in the fifth subculture (Table 2). However, neither dominance genetic effects nor reciprocal effects contributed significantly to variability for that character (Table 2). Differences between the regeneration media did not account for significant variability in embryogenesis. The interaction of regeneration media with rows in sets tested significant, but the interaction of regeneration media with additive genetic effects pooled from the rows in sets and columns in sets sums of squares was not significant. Thus, only additive genetic effects could be said with confidence to have accounted for the observed differences in the percentage of embryogenesis.

The performances of reciprocal full sibs on two different regeneration media were significantly correlated for each in vitro trait (Table 5). In general, phenotypic correlations became smaller in successive subcultures. Correlation of additive genetic effects for response on two regeneration media was significant or highly significant for 12 in vitro traits (Table 5). Genetic correlation coefficients were calculated only when the additive genetic variance component estimate was significant on both regeneration media. No trend was apparent over successive subcultures for the magnitude of genetic correlations.

Table 5. Phenotypic and additive genetic correlation for in vitro growth and differentiation on two regeneration media

Callus morphology	Sub_x	r_p	r_A
Freshweight	1	0.78**	>1**[a]
Rooting	1	0.66**	0.97**
Snow	1	0.58**	y
Smooth	1	0.91**	0.96**
Dark green	1	0.84**	0.97**
Vigor	2	0.45**	0.63**
Rooting	2	0.44**	1.0 **
Root count	2	0.52**	0.61**
Viability	3	0.35**	0.67**
Vigor	3	0.52**	0.68**
Root count	3	0.37**	y
White callus	3	0.33**	0.87**
Dark green	3	0.34**	0.48*
Embryogenesis	5	0.20**	0.90**

y = additive effects not significant on both media
* Estimate of correlation coefficient significant at the 5% level of probability
** Estimate of correlation coefficient significant at the 1% level of probability
[a] Parameter estimates which exceed the theoretical maximum $r_A = 1$ are assumed to be the result of sampling error

Discussion

The results of this experiment suggest that the successful establishment of rapidly growing cell and tissue cultures may depend as much upon the choice of genetic materials as it does upon in vitro cultural factors. Adequate sampling of genetic variability is necessary to ensure that in vitro cultural practices are not based upon the performance of only a few unique genotypes. Highly significant additive genetic effects for callus morphology and differentiation indicate that heritable genetic factors are involved in the growth and redifferentiation of in vitro callus cultures of red clover. Significant additive genetic effects have previously been noted in in vitro callus cultures of corn (Tabata and Motoyoshi 1965), wild cabbage (Buiatti et al. 1974), and alfalfa at the diploid level (Keyes and Bingham 1979).

Since in vitro characters, such as continued undifferentiated growth, vascularization, root initiation, chlorophyll production, etc. are highly heritable, genetic lines which are uniform and predictable in their organizational patterns could be developed by breeding and selection. Such materials could be useful in studies of the anatomy or biochemistry of differentiation in culture.

Highly significant heritable variability for the percentage of somatic embryogenesis on both regeneration media indicates that the frequency of regenerable genotypes in the base population can also be raised by genetic selection. Heritable genetic factors have not yet been exploited to improve regeneration in red clover. Since heritable variability accounts for more than half of the variability for embryogenesis on one medium and for one quarter of the variability on another medium, continued improvements in the regeneration response are likely to come from breeding and selection for embryogenic types.

Breeding techniques applied to another forage legume, alfalfa, *Medicago sativa* L., resulted in improved plantlet regeneration from callus cultures at both the diploid and tetraploid levels (McCoy and Bingham 1977; Bingham et al. 1975). The improved tetraploid population has been used by Walker and colleagues (1978, 1979) to examine hormonal control of organ formation in alfalfa callus and to study the temporal separation of induction processes from differentiation processes. A diploid line selected for high frequency regeneration following suspension culture has been used by Reisch and Bingham (1979)[1] to select and regenerate lines which are resistant to in vitro growth inhibition by ethionine, an analogue of methionine. Regenerated lines are currently being evaluated for overproduction of free methionine. Thus, breeding and selection for regeneration in alfalfa has resulted in materials which have aided both the study of basic developmental processes of in vitro callus cultures and the recovery of plants from variant selection at the cellular level.

The development of regenerable lines of red clover would significantly enhance the usefulness of in vitro methods for clover improvement in two important respects. First, a large number of highly culturable genotypes could be drawn upon for in vitro variant selection efforts, thus increasing the probability of successfully regenerating variant types. Second, by regenerating variant types in genetically different backgrounds the effect of the variant character could be evaluated in a broad sample of the cultivar germplasm. Further, a poly-cross of genetically different variant types could lead to a directly useful synthetic population which is stable for the variant character.

Differential expression of dominance genetic effects across two media was reported in this study. These results indicated that intra-allelic interactions which acted to produce an effect on one regeneration media did not produce the same effect on another medium. Similarly, examining heterosis for the growth of alfalfa callus, Keyes and Bingham (1979) reported that heterosis was much greater on one of the two media used in that experiment. However, in the present study, genetic dominance correlation coefficients for the percentage of colonies showing 'smooth' or 'snow' callus morphologies in the first subculture on media 'B' and 'D' were positive and significant, indicating that at least in these two cases specific gene combinations produced similar effects on the different regeneration media.

1 Reisch and Bingham (1979): In vitro selection of ethionine-resistant variants of diploid alfalfa. Agronomy Abstracts, p. 74

Differential expression of reciprocal effects was also reported. Since the same genotypes with the same reciprocal effects appear on both media it would seem that differences between the two media are responsible for this differential expression.

The magnitude of reciprocal effects tended to decrease in successive subcultures, indicating that such effects are more important in the earlier subcultures.

Correlation analysis for in vitro performance on two regeneration media was carried out. It is possible that different genes are responsible for a particular in vitro response among genetically identical cultures grown on different regeneration media. The genetic correlation of response to different regeneration media provides an indication of the extent to which common genes are active on both media. Pleiotropy and/or linkage are assumed as the underlying mechanism which account for a correlation (Falconer 1960). Significant positive genetic correlations indicated that the same heritable genetic factors produced similar effects on the different media, even though basal constituents, vitamin supplementation, and phytohormonal composition of the two regeneration media differed in most respects. Thus, while dominance genetic effects and reciprocal effects were differentially expressed on the two regeneration media in a number of cases, heritable genetic factors were stable across media for their contribution to growth and differentiation. Highly significant correlation of additive genetic effects for embryogenesis suggests that breeding and selection practiced in one regeneration system should produce a correlated improvement in the other.

Conclusions

Significant, heritable, genetic differences are present for in vitro callus growth and plantlet regeneration in cultivated red clover. Standard cultivars could serve as the base population in a breeding program for improved regeneration of plants from callus tissue cultures. Agronomically adapted populations, which have been improved for in vitro culturability, should provide the red clover breeder with a useful tool for incorporating variant characters, which arise spontaneously or through direct selection in culture, into existing cultivars.

Dominance genetic variance was significant for very few in vitro traits. In three of seven cases the choice of regeneration media determined the expression of dominance genetic variance.

Reciprocal effects were significant for in vitro traits in the early subcultures but did not account for differences in somatic embryogenesis.

The performance of reciprocal full-sibs measured on two regeneration media was highly correlated. Significant positive correlation of additive genetic effects was found for 12 in vitro traits. It was concluded that common genes account for performance on both regeneration media.

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Mr. G.J. Keyes
Cell Biology Group
Monsanto Ag. Products. Co.
800 N. Lindbergh Blvd.
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Dr. G.B. Collins
Prof. Dr. N.L. Tailor
Department of Agronomy
University of Kentucky
Lexington Ky. 40546 (USA)