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Heritability estimates for callus growth and regeneration in desmodium

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Abstract The F_2 and F_3 generations of two crosses (6123×13083 and 6123×144, with 6123 the regenerating parent) were evaluated for callus growth and regeneration capacity. Based on joint scaling tests and variance partitioning, neither callus growth nor regeneration fitted a simple additive-dominant genetic model. Heritability estimates obtained from parent-offspring regression analyses ranged from 0.65 to 0.77 for callus growth and from 0.19 to 0.46 for regeneration, with the range in both influenced by the cross and numerical scale employed. Members of two F_3 families exhibited much more vigorous and prolific regeneration than the regenerating parental genotype. Because many individuals in the segregating generations showed no evidence of regeneration, population distributions for this trait were severely truncated, or censored. Regression-order analysis was used to estimate means and variances of these censored populations. The association between poor callus growth and high regeneration capacity observed in the parental lines was absent from the F_2 and F_3 generations, indicating that no association between callus growth and regeneration was present.

Key words In vitro regeneration · Heritability · Forage legumes · Censored data sets · *Desmodium*

Introduction

Desmodium (*Desmodium* sp.) is a forage legume widely grown in pastures of the tropics and subtropical regions of Australia and the United States. In order to apply emerging crop-improvement technologies to this crop, reliable and consistent in vitro regeneration must be achieved. A callus-based regeneration protocol has been developed for desmodium, but regeneration has been highly genotype-specific (Wofford et al. 1992). Regeneration has been inconsistent even in the best regenerating genotypes so far identified (Krottje 1995). In addition, the improvement of regeneration in non-regenerating genotypes by modifying various media components has been relatively unsuccessful.

Genetic selection has been successful in improving regeneration capacity in other forage legumes, including alfalfa (Reisch and Bingham 1980) and red clover (Quesenberry and Smith 1993). The objective of the present study was to evaluate genetic aspects of callus growth and regeneration in desmodium to determine if selection could be effective.

Materials and methods

Two regenerating and two non-regenerating lines were selected for hand crossing in this naturally self-pollinating genus. The regenerators, IRFL 6123 and IRFL 6128, are classified as *D. heterocarpon* ssp. *angustifolium* (Craig) Ohashi. These regenerating lines are known to produce less callus growth than the non-regenerating parents used in this study (Wofford et al. 1992). In the non-regenerators, UF 144 is classified as *D. ovalifolium* Wall., and CIAT 13083 possesses morphological plant characteristics intermediate between *D. heterocarpon* and *D. ovalifolium*.

Flowering of parental lines was induced by simulating a 10-h day-length in the greenhouse. Donor pollen was obtained by tripping flowers using a toothpick with a small piece of fine sandpaper glued to one end in such a way that the dehiscing anthers would strike the sandpaper and pollen would adhere. Pollen was then transferred to flowers of the maternal parent by tripping in a similar manner. Flowers of the maternal parent were not emasculated because they were extremely sensitive to manipulation, and emasculation resulted in flower abscission. Pollinations were carried out at a time of day when the flowers had fully opened, but self-tripping had not yet occurred. At least 100 flowers for each possible combination of regenerator and non-regenerator parent, including reciprocals, were pollinated in this manner.

The seeds resulting from these crosses were germinated and planted in the greenhouse. Hybrids possessed a leaf morphology intermediate between parents, and were readily identified when the plants were 3–4 weeks old. Since F_1 plants could not be identified as hybrids until this advanced seedling stage, in vitro data could not

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be collected for the F_1 generation. The F_1 plants were allowed to self-pollinate, but the flowers were hand tripped to increase seed set.

The F_2 seed, harvested from the F_1 hybrids, was scarified by a 12-min immersion in concentrated sulfuric acid (H_2SO_4), followed with several rinses in sterile deionized water, and germinated in Petri dishes on seed germination medium (SGL) (Collins and Phillips 1982). Hypocotyls were excised and placed onto the callus-induction medium described below, and epicotyls were returned to SGL medium for rooting. Rooted epicotyls were transferred to potting soil after 10 days and grown to maturity in the greenhouse where they were allowed to self-pollinate to produce the F_3 generation.

Hypocotyls from the parental lines, F_2, and F_3 populations were cultured under a three-step regeneration protocol. Initial callus production was on L2 medium (Phillips and Collins 1979) supplemented with 0.06 mg l^{-1} picloram and 0.2 mg l^{-1} BA for 28 days. Shoot buds were induced on L2 with 1.0 mg l^{-1} 2,4-D and 2.0 mg l^{-1} adenine for 14 days, and shoot elongation was induced on L2 with 0.012 mg l^{-1} picloram and 0.4 mg l^{-1} BA for 28 days. Shoots were rooted on L2 medium lacking plant growth regulators.

Callus fresh weights were measured at the time of transfer from callus medium to bud-induction medium. For statistical analysis, callus weights were transformed to $\log_{10}$ of callus weight in mg.

A visual rating scale was devised for the regeneration trait, as this trait presented major obstacles to genetic analysis. In studies of this type, it is highly desirable to be able to evaluate each plant in terms of a metric variable, such as percent of responding explants or number of shoots per explant. Because hypocotyl explants were used in the present study, only one explant could be examined in each plant, so the data could not be expressed as the percent of responding explants. Expression of regeneration as the number of shoots per explant was impossible because a high frequency of explants produced no buds or shoots. The trait was expressed on a visual rating scale of 1 to 7 with the following characteristics associated with each value: 1=no evidence of regeneration; 2=slight indication of regeneration in the form of localized deep-green coloration; 3=formation of a single, well-defined but non-elongated bud; 4=multiple bud formation, usually with some elongation; 5=one or two well-elongated shoots; 6=between three and five elongated shoots; and 7=more than five elongated shoots. This type of rating scale can be treated as a quasi-continuous, metric variable if the ratings yield a reasonably normally distributed error variance. This can be tested by examining the distribution of a population with no genetic variance component. The regenerating parental population (genotype 6123) is such a population. The distribution of regeneration scores for this population is not seriously skewed, nor is it bimodal (see Fig. 1), suggesting that there was little or no scale-induced non-normality.

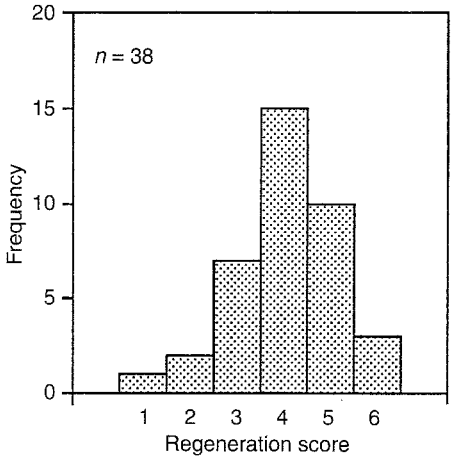

Fig. 1 Distribution of regeneration scores in the regenerating parent, genotype 6123

Results and discussion

Of the 590 seeds obtained from approximately 2200 pollinations, two F_1 plants from cross 6123×13083 and three F_1 plants from cross 6123×144 were identified and used to produce the F_2 and F_3 generations analyzed in this study.

Since genotypes 6123 and 6128 are nearly identical, both in gross morphology and in vitro performance, crosses 6123×13083 and 13083×6128 were essentially reciprocals. However, severe morphological abnormalities appeared in the F_1 of cross 13083×6128, and persisted through the F_3 generation. The three F_1 seedlings from this cross appeared normal through approximately 6 weeks of age, after which new growth showed severely stunted leaves and internodes. Stunting was evident from the early seedling stage in many F_2 and F_3 individuals, and appeared at late seedling stages in all individuals. Stunting was accompanied by extremely poor flower production and seed-set. The F_2 of cross 13083×6128 also exhibited a depressed callus growth rate relative to the two other crosses and to the parental lines , with a high incidence of callus necrosis and death. Since cross 13083×6128 is essentially the reciprocal of cross 6123×13083, the abnormalities may have been the result of interactions between the cytoplasm of parent 13083 and nuclear genes of parent 6128. The observed abnormalities could potentially mask the expression of the in vitro characters of interest, as well as exerting confounding selective pressures in the F_2 and F_3 generations. For these reasons, cross 13083×6128 was excluded from genetic analysis.

Joint scaling tests (Cavalli 1951) were applied to callus weight for crosses 6123×13083 and 6123×144 (Table 1). Due to the limited number of generations available, only simple additive-dominant models were tested. Significant test results were not obtained for either cross. Examination of the generation means suggests a factor that may have confounded the joint scaling tests. In both crosses, F_2 and F_3 means are significantly lower than the mid-parent mean, and are fairly close to one another. In the case of cross 6123×13083, the F_3 mean is higher than the F_2 mean, while in cross 6123×144 the F_3 mean is lower. If a simple additive-dominant model is assumed, the deviations of the F_2 and F_3 from the mid-parent mean suggest a large, negative dominance effect for reduced callus growth. In contrast,

Table 1 Callus weight means ($\log_{10}$ mg) and standard errors for parental lines, F_2, and F_3 populations

Cross	Generation	Mean	SE	*n*
6123 × 144	P_1 (6123)	2.413	0.037	35
	P_2 (144)	2.844	0.032	26
	F_2	2.445	0.037	92
	F_3	2.231	0.024	291
6123 × 13083	P_1 (6123)	2.413	0.037	35
	P_2 (13083)	2.833	0.036	20
	F_2	2.319	0.050	65
	F_3	2.412	0.030	181

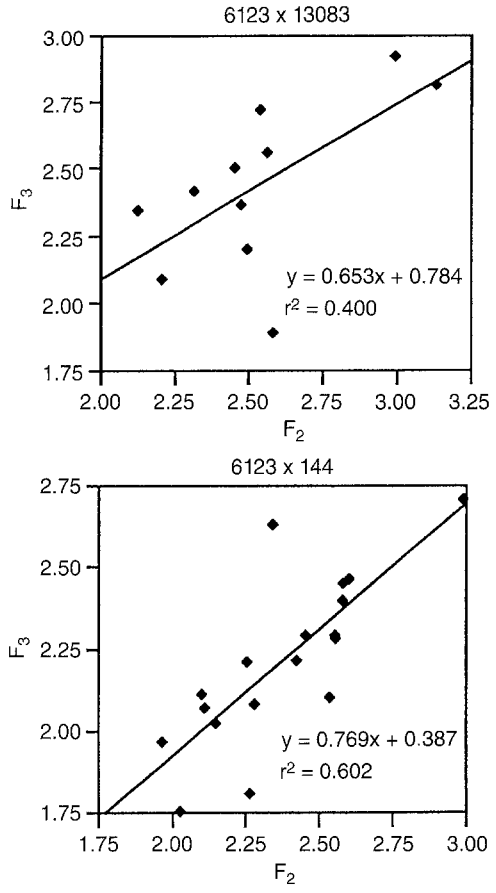

Fig. 2 Regression of F_3 family mean callus weight on F_2 parental callus weight for crosses 6123 × 13083 and 6123 × 144. Weights are expressed as $\log_{10}$ of callus weight in mg

the relationship between F_2 and F_3 means suggests a small dominance effect, negative for cross 6123×13083, and positive for cross 6123×144. The discrepancy between the dominance effect predicted by the relationship of the progeny means to the mid-parent mean and that predicted by the relationship between F_2 and F_3 means makes it impossible to obtain a significant result in an additive-dominant joint scaling test. A cytoplasmic effect acting to depress callus growth in the F_2 and F_3 generations may account for this discrepancy. Each cross was also analyzed by the weighted, iterative variance partitioning method described by Mather and Jinks (1971). As with the joint scaling test, only a simple additive-dominant model could be tested and, once again, no meaningful pattern could be determined.

Regression of F_3 family mean callus weight on F_2 parent callus weight yielded highly significant results (Fig. 2). Regression coefficients, which by definition represent realized heritabilities, were 0.653 and 0.769, for crosses 6123×13083 and 6123×144, respectively. In the case of selfing, parent-offspring regression is in part a function of dominant and epistatic genetic effects, but these effects play a much smaller role in the regression than does additive genetic variance (Mather and Jinks 1971).

A large number of individuals in all populations showed no sign of regeneration (score=1). All individuals in the non-regenerating parent populations (genotypes 144 and 13083) were rated as 1. Distributions of this type cannot be rendered normal by any mathematical transformation, since we lack any information about differences among those individuals that fall below the observable threshold, and no transformation can restore this missing information. For example, regardless of transformation, the two non-regenerating parental populations will always have equal means, and variances of zero. This type of data structure can be treated as "threshold" (Falconer 1981) or "censored" (Newman et al. 1989) data. Both approaches assume that there exists an underlying, normally distributed, continuous scale of proclivity toward a certain condition – in the case of the present work, tendency to regenerate – that can only be detected when the level of proclivity rises above a given threshold. Falconer's treatment dealt with only one or two discrete response classes above the threshold, while the conceptually similar treatment of Newman et al. (1989) involved a continuous, or semicontinuous, response variable.

Several techniques have been described for "uncensoring" censored data sets. The most intuitively appealing method is regression-order analysis. This method involves "replacement of censored observations with their predicted value from a linear regression of observed values on regression-order scores" (Newman et al. 1989). That is, a normal quantile regression is performed on a truncated population in order to estimate the mean and variance of the intact population. The accuracy of population parameters estimated by this method is limited by the number of observations lying above the censoring threshold. However, reasonably accurate results can be obtained even when the majority of observations are below the threshold, as long as several observations lie above the threshold. Although this is a less than ideal approach, in cases of severe censoring it may be the most acceptable alternative. The option of simply analyzing the raw regeneration scores – either transformed or otherwise – is undesirable because this entails accepting the clearly incorrect assumption that all individuals receiving regeneration scores of one have the same proclivity for regeneration. The contrasting options, then, are to ignore the left-hand tails of many of the population distributions, or to use an acknowledgedly problematic method of reconstructing these tails.

The public-domain computer program UNCENSOR (Newman et al. 1989) was used to perform regression-order analysis on regeneration scores. Estimates of means and standard errors obtained in this way, as well as means and standard errors of the raw data, are presented in Tables 2 and 3. In general, means derived from regression-order analysis are lower than those obtained from the raw data, and variances are higher. This is not unexpected, since truncation of the low end of a data set – as occurs in the raw data – will artificially raise a population's mean and lower its variance. For the raw data, a significant (α=0.05), positive relationship exists between F_3 family variance and family mean, due at least in part to the fact that the lower

Table 2 Parental, F_2, and F_3 means and standard errors for regeneration score[a] based on raw and uncensored data from the cross 6123 × 13083

Generation	Raw data		Uncensored data		n	$n > 1$
	Mean	SE	Mean	SE		
6123 (regenerating parent)	4.053	0.181	4.075	0.169	38	37
F_2 (overall)	1.868	0.137	1.679	0.176	57	29
F_2 (F_3 parents)	1.636	0.279	2.078	0.211	11	4
F_3 (overall)	1.594	0.070	1.185	0.107	165	67
F_3 Families						
1	2.000	0.299	2.116	0.297	13	7
2	1.462	0.183	1.520	0.202	13	6
3	1.333	0.144	1.391	0.187	21	5
4	2.083	0.336	1.982	0.407	12	7
5	2.417	0.468	2.045	0.506	12	8
6	1.440	0.142	1.391	0.182	25	8
7	1.600	0.267	1.612	0.314	10	5
8	1.214	0.114	–	–	14	3
9	1.923	0.178	2.032	0.156	13	11
10	1.214	0.114	–	–	14	3
11	1.389	0.200	–0.067	0.523	18	4

[a] The rating scale used was as follows: 1 = no evidence of regeneration; 2 = slight indication of regeneration in the form of localized deep-green coloration; 3 = formation of a single, well-defined but non-elongated bud; 4 = multiple bud formation, usually with some elongation; 5 = one or two well-elongated shoots; 6 = between three and five elongated shoots; and 7 = more than five elongated shoots

Table 3 Parental, F_2, and F_3 means and standard errors for regeneration score[a] based on raw and uncensored data from the cross 6123 × 144

Generation	Raw data		Uncensored data		n	$n > 1$
	Mean	SE	Mean	SE		
6123 (regenerating parent)	4.053	0.327	4.075	0.169	38	37
F_2 (overall)	1.994	0.158	1.278	0.233	80	43
F_2 (F_3 parents)	2.267	0.365	1.553	0.620	17	8
F_3 (overall)	2.058	0.089	1.554	0.120	260	136
F_3 Families						
1	1.571	0.251	0.900	0.461	14	6
2	1.769	0.410	–	–	13	3
3	1.500	0.289	–	–	4	2
4	3.850	0.386	3.700	0.319	20	20
5	3.706	0.361	3.667	0.314	17	16
6	1.667	0.494	–	–	6	2
7	1.545	0.171	1.635	0.186	22	8
8	2.000	0.392	1.319	0.632	13	6
9	1.500	0.203	1.438	0.261	14	5
10	1.273	0.195	–	–	11	2
11	2.733	0.371	2.641	0.414	15	12
12	1.650	0.167	1.753	0.160	20	10
13	2.625	0.324	2.625	0.294	8	7
14	1.714	0.240	1.220	0.141	21	8
15	2.000	0.275	2.356	0.315	12	7
16	1.611	0.183	1.680	0.193	18	8
17	1.789	0.271	1.056	0.469	19	8
18	1.615	0.241	1.386	0.324	13	6

[a] The rating scale used was as follows: 1 = no evidence of regeneration; 2 = slight indication of regeneration in the form of localized deep-green coloration; 3 = formation of a single, well-defined but non-elongated bud; 4 = multiple bud formation, usually with some elongation; 5 = one or two well-elongated shoots; 6 = between three and five elongated shoots; and 7 = more than five elongated shoots

the mean, the more severely truncated the distribution. This relationship is absent from the uncensored data because the truncated lower ends of the distributions have been reconstructed. The unusually high variances observed for some F_3 families may be due to the segregation of major genes within these families, resulting in a somewhat bimodal distribution and an exaggerated variance. This effect is most pronounced in the uncensored data. Population sizes for the F_3 families are too small to allow distinguishing between spurious bimodality and bimodality resulting from genetic causes. Bimodality was not observed in the F_2 populations, nor in the complete F_3 populations, and

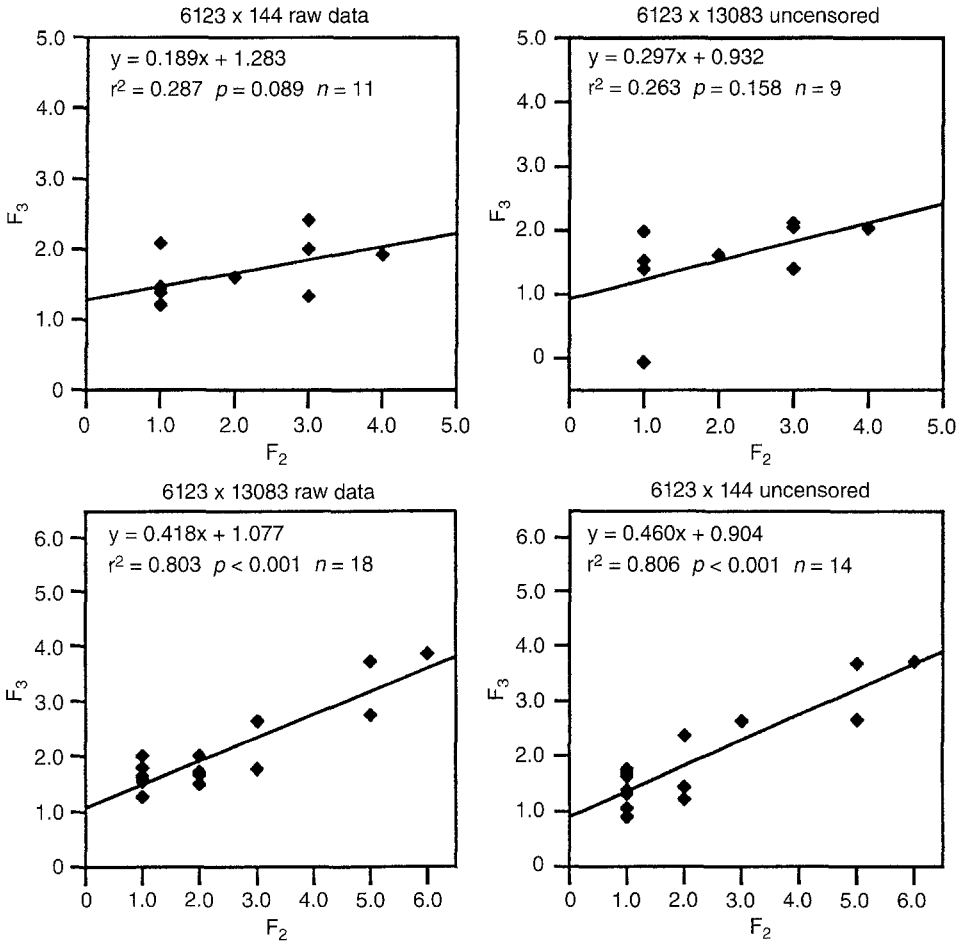

Fig. 3 Regression of F_3 family mean regeneration score on F_2 parental regeneration score for crosses 6123 × 13083 and 6123 × 144 utilizing raw and uncensored data

these distributions appeared normally distributed except for the truncated left-hand tails.

Due to the difficulties discussed above, analysis of the regeneration trait was restricted to parent-offspring regression. Regressions of F_3 family mean on the F_2 parent for the two crosses, utilizing both uncensored and raw data, are presented in Fig. 3. Cross 6123×144 shows a strong parent-offspring relationship, with a moderately high regression coefficient. The coefficients of determination (r^2) are very similar for the raw and uncensored data (0.803 and 0.806, respectively), but the slope is greater for the uncensored data (0.460 versus 0.418 for the raw data). In the case of cross 6123×13083, no significant regression is observed. One cause for this may be the small sample size, particularly the low number of parents with regeneration scores higher than one. Another cause is the broad range of mean offspring regeneration scores for parents with regeneration scores of one. It is likely that the "true" regeneration scores for some of the parents (particularly the parent at the far lower left of the plot) lie somewhat below the censoring threshold. If it were possible to measure parental regeneration scores of less than one, the observations at the lower left corner of the plot might be distributed farther to the left, resulting in a stronger regression. It is noteworthy that for both callus growth and regeneration score, several F_3 individuals greatly exceeded any individual from the inbred parental populations. Members of two F_3 families from cross 6123×144 showed particularly profuse regeneration.

While regeneration was associated with poor callus production in the parental lines, this association was not observed in the F_2 and F_3 generations. For the combined F_3 population, a weak, but significant (α=0.01), positive correlation between the two traits was observed. The geometric mean callus weight for F_3 individuals exhibiting multiple shoot production (regeneration score of 6 or 7) was 348 mg, in contrast to 200 mg for the entire F_3 population and 258 mg for the regenerating parent. Thus, it appears that the relationship between regeneration ability and poor callus production observed in the parental lines has no genetic basis, but rather is the result of a chance distribution of alleles. It should therefore be possible to select for both traits simultaneously.

Although neither the callus growth nor regeneration trait could be described by an additive-dominant genetic model, parent-offspring regression has shown that both traits are heritable. Compared to analyzing the raw data, application of an uncensoring technique to deal with the highly censored regeneration data sets did not have a great effect on the results of parent-offspring regression. However, it is felt that uncensoring may under some circumstances be a useful tool for handling this type of data.

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