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Genetic control of in Vitro shoot regeneration from leaf explants in *Solanum chacoense* Bitt.

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Abstract Although the heritable nature of plant tissue culture responses is now well documented in many species, only a few studies have been conducted to elucidate complete inheritance patterns. Genetic control of in vitro shoot regeneration from leaf explants was investigated in *Solanum chacoense* using parental, F_1 and F_2 generations. Broad-sense heritability estimates were high for frequency (percentage) of responsive leaf explants 61–83%) and number of shoots regenerated per responsive explant (53–75%). Consistent with high heritability estimates, a hypothesis involving three genes could be formulated to explain the variability in the response observed in this study. This model implies that homozygous recessive alleles at any two (out of three) loci are required for the highest response, i.e., more than two shoots per explant in more than 40% of the explants. The presence of homozygous recessive alleles at any one of the three loci produces an intermediate response, i.e., fewer than 40% of the explants regenerating fewer than two shoots per explant, and a dominant allele at all the three loci results in non-responsiveness. Additional minor modifier genes, each with a small effect, would also be required to account for the variable intensity of regeneration within groups. Such a relatively simple genetic control of in vitro regenerability suggests that incorporation of this trait should be easy in potato improvement programmes.

Key words Genes · Heritability · Inheritance · Potato · Regenerability

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Introduction

In vitro responsiveness is a heritable character and the existence of wide genetic variation for this trait is now well documented in many plant species (Cappadocia 1990; Armstrong et al. 1992; Cowen et al. 1992). The type and extent of the response can be modulated through media modifications. However, in spite of our efforts for optimizing culture conditions for efficient regeneration, many agronomically elite genotypes remain unresponsive (Ball et al. 1992; Hulme et al. 1992; Calleberg and Johnson 1993). Thus, although it would be most desirable to remove the genotype limitation altogether, a more realistic approach to overcome this problem is to transfer in vitro regenerability through breeding (Wenzel and Uhrig 1981; Foroughi-Wehr et al. 1982; Uhrig 1985; Armstrong et al. 1992). An availability of genetically well-characterized genotypes and a knowledge of the genetic system controlling in vitro regenerability will greatly help in this endeavor, and the question is now being addressed in an increasing number of studies.

Solanum chacoense, a highly polymorphic tuber-bearing diploid ($2n = 2x = 24$) potato species, holds great promise for use in the genetic improvement of cultivated potato (Hawkes and Hjerting 1989). Whereas tetrasomic segregation due to the autotetraploid nature of cultivated potato makes it difficult to follow inheritance patterns, *S. chacoense* can be profitably used in genetic studies. A number of genotypes of this species have been well characterized for their responses to in vitro culture (Cappadocia 1990; Veronneau et al. 1992). Data on the segregation of many molecular [restriction fragment length polymorphism (RFLP)] markers is now available, and a RFLP genetic map of this species is about to be completed in our laboratory (Rivard et al. 1989; SR Rivard unpublished). All this offers exiting possibilities for understanding the genetic mechanisms involved in in vitro regenerability by integrating classical genetics and molecular biology. In the investigation

described here, the inheritance of shoot regeneration from leaf explants was studied in *S. chacoense*.

Materials and methods

Plant material

All material originated from two diploid (2n = 2x = 24) self-incompatible clones (PI 230582 and PI 458314) of *Solanum chacoense* Bitt. obtained from the Potato Introduction Station, Sturgeon Bay, Wisconsin, USA. Their RFLP profiles and their response to anther and leaf disc culture have been extensively studied (Rivard et al. 1989; Cappadocia 1990; Veronneau et al. 1992). These two parental clones, three of their reciprocal F_1 hybrids (namely A2, B1 and B2) and three F_2 progenies produced by sib-mating the three F_1 hybrids (see footnotes on Table 1) were used in this investigation. Each of the F_2 populations was represented by 20 genotypes.

The three F_1 hybrids were selected for their in vitro behaviour with B1 being unresponsive to leaf disc culture, and A2 and B2 being capable of regeneration (Veronneau et al. 1992). Parental clones PI 230582 and PI 458314 were both unresponsive to leaf disc culture.

Establishment of axenic cultures

Parents and F_1s

Nodal cuttings 2 cm long were obtained from greenhouse-grown plants immersed for 20 min in calcium hypochlorite 5% (w/v), rinsed three times in sterile water and then cultured in 7 × 7 × 10 cm Magenta GA7 vessels (four nodes per box) containing 50 ml of culture medium. The medium contained Nitsh's (1969) basal constituents: 20 g l^{-1} sucrose, 5 g l^{-1} activated charcoal (Sigma) and 6 g l^{-1} agar (Difco-Bacto). The pH was adjusted to 5.8 with 0.5 *N* NaOH before adding agar and activated charcoal, and the medium was autoclaved for 30 min at a pressure of 0.5 kg cm^{-2} at 121°C. The cultures were maintained in vitro through periodic (about 6 weeks) shoot subculture.

F_2 progenies

True seeds were immersed for 10 min in calcium hypochlorite 10% (w/v), rinsed three times in sterile water and transferred into 15 × 100-mm sterile petri dishes containing Murashige and Skoog (MS) (1962) basal salts and 7 g l^{-1} agar (Difco-Bacto) for germination. The medium was autoclaved for 30 min at a pressured of 0.5 kg cm^{-2} at 121°C. The petri dishes were kept in the dark until the radicals started coming out of the seeds, at which time the petri dishes were transferred to light conditions. After 10–15 days, the seedlings were transferred into test tubes (1 seedling per test tube) containing the same medium as described for the parents and F_1s. These in vitro plants were then propagated through nodal cuttings and finally cultured in 7 × 7 × 10-cm Magenta GA7 vessels as described for the parents and F_1s.

Leaf explant culture

Leaf explant culture was essentially performed as described by Veronneau et al. (1992) using leaf explants of about 0.5 cm^2 removed from in vitro plant material that had last been subcultured approximately 6 weeks before. For each genotype, 100 explants removed from 16 plants were cultured on induction medium composed of MS basal medium supplemented with 1 mg l^{-1} naphthalene acetic acid (NAA), 9 mg l^{-1} benzylaminopurine (BA), 30 g l^{-1} sucrose and 7 g l^{-1} agar (Difco Bacto). The pH was adjusted to 5.6 with 0.5 *N* NaOH before adding agar, and the medium was autoclaved for 30 min at a pressure of 0.5 kg cm^{-2} at 121 °C. Twenty-five explants were inculated in each 15 × 100-mm petri dish, and the dish was then sealed with parafilm. The experiment was replicated four times.

Four weeks later, the number of leaf explants still green and those producing macrocallus (callus visible to naked eye) microcallus (callus visible only under microscope) were recorded for each petri dish, and the leaf explants were transferred to the regeneration medium. This medium was identical to the induction medium except that NAA was omitted, a reduced concentration of BA (3 mg l^{-1}) was used, and 5 mg l^{-1} filter-sterilized gibberellic acid (GA_3) was added after autoclaving. After 6 weeks, the number of leaf explants exhibiting shoot formation and the number of shoots produced per responsive explant were recorded for each petri dish.

All in vitro donor plants and cultures were incubated in a culture room at 22° ± 2 °C under 75 μ mol $m^{-2}s^{-1}$ light intensity provided by fluorescent tubes (Valencia, cool-white 40W) at a 16-h photoperiod.

Statistical analysis

The percentage of leaf explants remaining green at the end of the induction phase, of leaf explants producing micro and/or macrocallus and of responsive leaf explants in which shoot regeneration had occured were transformed to arcsin square root before statistical analyses. The number of shoots per responsive leaf explant was also transformed to square root. Analyses of variance of percentage of responsive leaf explants and number of shoots per responsive leaf explant were done according to Snedecor (1968) for a completely randomized design using replication means. Each F_2 population was analyzed separately. The phenotypic variance was taken as the sum of the error variance and genetic variance, the latter being calculated by subtracting the error mean square from the genotypic mean square and dividing the remainder by the number of replications. Broad-sense heritability was calculated according to Johnson et al. (1955). Estimates of the number of effective factors segregating were computed by the following formula (Mather and Jinks 1982):

$$\hat{K} = \frac{(L_1 - L_2)^2}{4\sigma_g^2}$$

where $\hat{K}$ = number of effective factors; L_1 and L_2 are extreme progenies; and σ_g^2 = genetic variance

In order to analyse the frequency of responsive leaf explants and number of shoots per responsive explant as qualitative characters, F_2 genotypes were classified into phenotypic classes (groups). For establishing the boundaries of phenotypic classes, the performance of F_1 genotypes and frequency distribution of F_2 genotypes was used as reference (Table 1). Chi-square values were calculated following Snedecor (1968). Spearman's rank correlation coefficients were computed among various in vitro characteristics (Snedecor 1968) using genotypic means.

Results

None of the leaf explants of the two parents and none of those of one of the F_1 hybrids, B1, regenerated shoots. On a mean basis, 18% of the leaf explants of F_1 hybrid A2 regenerated 1.57 shoots per responsive explant and 34% of the explants of B2 formed 1.82 shoots per responsive explant. The three F_2 progenies segregated for these characteristics, and the genotypes differed significantly amongst themselves (Tables 1 and 2). Ten (17%) F_2 genotypes performed better than their respective parents.

Estimates of broad-sense heritability were high for both frequency of responding leaf explants and number of shoots per responding explant (Table 3). Three effective factors (genes) were usually involved in the genetic

Table 1 Frequency (percentage) of responsive leaf explants and number of shoots per responsive explant in parental, F_1 and F_2 genotypes

Genotype/ progeny	Frequency of responsive explants (%)			Number of shoots per responsive explant		
	Range	Mean	Phenotypic classes	Range	Mean	Phenotypic classes
Parents						
PI 230582	–	0.0		–	0.0	
PI 458314	–	0.0	I = 0.0	–	0.0	I = 0.0
F_1 genotypes[a]						
A2	–	18.0		–	1.57	
B1	–	0.0	II > 0.0 and ≤ 40.0	–	0.0	II > 0.0 and ≤ 2.0
B2	–	34.0		–	1.82	
F_2 progenies[b]						
K	0.0 – 99.7	15.0	III > 40.0	0.0 – 10.5	1.7	III > 2.0
L	0.0 – 73.5	15.8		0.0 – 3.7	1.5	
M	0.0 – 50.5	9.1		0.0 – 2.6	1.1	

[a] Parentages: A2, PI 458314 × 230582; B1 and B2, PI 230582 × 458314
[b] Parentages: K, B1 × A2; L, B1 × B2; M, A2 × B2

Table 2 Analysis of variance for the frequency[a] (in percentage) of responsive leaf explants and number of shoots per responsive explant[b] in three F_2 progenies

Progeny	Source	*df*	Mean sum of squares for:	
			Frequency of responsive explants	Number of shoots per responsive explant
K	Genotype	19	0.185**	2.939**
	Error	60	0.011	0.224
L	Genotype	19	0.145**	1.356**
	Error	60	0.007	0.175
M	Genotype	19	0.073**	1.076**
	Error	60	0.010	0.196

** $P < 0.01$
[a] Percentage data transformed using arcsin of the square root prior to analysis
[b] Data transformed using square root prior to analysis

Table 3 Estimates of genetic (σ_g^2) and phenotypic (σ_p^2) variances, broad-sense heritability (h^2) and number of effective factors ($\hat{K}$) for frequency of responsive leaf explants and number of shoots per responsive explant in three F_2 progenies (Variances and mean genotypical values (L_1 and L_2) were obtained after data transformation; $h^2 = \sigma_g^2/\sigma_p^2$; $\hat{K} = (L_1 - L_2)^2/4\,\sigma_g^2$)

Response	Progeny	σ_g^2	σ_p^2	h^2	L_1	L_2	$\hat{K}$
Frequency of responsive explants	K	0.0435	0.0545	0.80	0.0	0.871	4
	L	0.0345	0.0415	0.83	0.0	0.590	3
	M	0.0158	0.0258	0.61	0.0	0.453	3
No. of shoots per responsive explant	K	0.6788	0.9028	0.75	0.0	3.243	4
	L	0.2953	0.4703	0.63	0.0	1.918	3
	M	0.2200	0.4160	0.53	0.0	1.607	3

control of these two tissue culture responses (Table 3). The four effective factors obtained in one of the F_2 progeny was probably due to the performance of 1 outstanding genotype, K23.

The production of responsive F_1 genotypes by hybridizing two non-responsive parents and the isolation of transgressive segregants in F_2 progenies indicate that these characters are controlled by either recessive alleles or by epistatic interaction of genes present singly in the parents. To elucidate the inheritance of these two responses, F_2 genotypes were classified into three phenotypic classes: class I- non-responsive, class II- intermediate and class III- high (Tables 1, 4, 5). On the basis of this classification, both parents and one of the F_1 hybrids B1, belong to class I, while the remaining two F_1s belong to class II. The data could not be fitted to a single- or two-genes model, hence a three-gene model was tested. This genetic model assumes that frequency of response and number of shoots regenerated per responsive explant are controlled by three genes and that homozygous recessive alleles at any two of the three loci are required for the highest response, whereas a dominant allele at any two of the three loci would result in an intermediate response. Finally, a dominant allele at all three loci would give non-responsiveness. Additional minor modifier genes, each with a small effect, would then influence the intensity of regeneration within classes. The results obtained indicated that this model is acceptable. It is therefore possible to propose that the genetic constitution of the parents and F_1 hybrids is as follows: PI 230582 – AaBbCc; PI 458314 – AaBbCc; A2 – AabbCc; B1 – AaBbCc; and B2 – AaBbcc. On the basis of the above presented genetic make-up of parents and F_1s, the F_2 genotypes may be classified into the three following classes:

Class I: A—B—C—
Class II: aaB—C—; A—bbC—; A—B—cc
Class III: aabbC—; aaB—cc; A—bbcc

The two parents, heterozygous at all the three loci, would produce F_1 populations segregating for the two responses. Results obtained by Veronneau et al. (1992) are also in agreement with this assumption. Further-

Table 4 Observed and expected segregation ratios for frequency of responsive explants in three F_2 progenies

Progeny	Number of genotypes in phenotypes classes							
	Observed			Expected			χ^2	P
	I	II	III	I	II	III		
K	7	11	2	7.5	10	2.5	0.2333	0.70 – 0.90
	7		(13)[b]	7.5		(12.5)	(0.0533)	(0.70 – 0.90)
L	4	12	4	5.625	9.375	5	1.4044	0.30 – 0.50
	4		(16)[b]	5.625		(14.375)	(0.6531)	(0.30 – 0.50)
	(16)[a]		4	(15)		5	(0.2667)	(0.50 – 0.70)
M	6	13	1	7.5	10	2.5	2.1	0.30 – 0.50
	6		(14)[b]	7.5		(12.5)	(0.48)	(0.30 – 0.50)

[a] After summing up of I and II classes
[b] After summing up of II and III classes

Table 5 Observed and expected segregation ratios for number of shoots per responsive explants in three F_2 progenies

Progeny	Number of genotypes in phenotypes classes							
	Observed			Expected			χ^2	P
	I	II	III	I	II	III		
K	7	10	3	7.5	10	2.5	0.1333	0.90 – 0.95
	7		(13)[b]	7.5		(12.5)	(0.0533)	(0.70 – 0.90)
L	4	12	4	5.625	9.375	5	1.4044	0.30 – 0.50
	4		(16)[b]	5.625		(14.375)	(0.6531)	(0.30 – 0.50)
	(16)[a]		4	(15)		5	(0.2666)	(0.50 – 0.70)
M	6	11	3	7.5	10	2.5	0.50	0.70 – 0.90
	6		(14)[b]	7.5		(12.5)	(0.48)	(0.30 – 0.50)

[a] After summing up of I and II classes
[b] After summing up of II and III classes

more, if the three F_1s do have the above proposed genetic make-up, then the F_2 progenies should segregate accordingly. The number of plants in different phenotypic classes is shown in Tables 4 and 5. The deviation of the observed ratio from the expected one is not significant; therefore the proposed hypothesis cannot be rejected (Tables 4 and 5).

Finally, correlation studies among various in vitro characteristics, revealed four significant associations (Table 6). In particular, a strong positive correlation ($r = 0.79^{**}$) was found between frequency of leaf explants capable of shoot regeneration and the number of shoots carried by each explant.

Table 6 Spearman's rank correlation coefficients among various in vitro culture responses

Characteristics	X1	X2	X3	X4	X5
Frequency of green explants (X1)	1.00	0.78**	0.59**	0.15	0.15
Frequency of microcallus producing explants (X2)	0.78**	1.00	0.74**	0.11	0.19
Frequency of macrocallus producing explants (X3)	0.59**	0.74**	1.00	0.14	0.19
Frequency of responsive explants (X4)	0.15	0.11	0.14	1.00	0.79**
Number of shoots per responsive explant (X5)	0.15	0.19	0.19	0.79**	1.00

** $P < 0.01$

Discussion

In this study response of the parental clones as well as that of their F_1s was similar to that reported previously, except that a relatively lower proportion of leaf explants of A2 and B2 produced fewer shoots. This could be due to the fact that in the present investigation leaf explants were kept for 4 weeks in the induction medium instead of the 2 weeks reported by Veronneau et al. (1992). In addition, the use of leaf pieces instead of discs could also have influenced the response through a reduced wound surface.

The heritable nature and the existence of genetic variation in tissue culture responsiveness have been shown in many plant species, including alfalfa (Reisch and Bingham 1980), barley (Foroughi-Wehr et al. 1982; Dunwell et al. 1987), *Cucumis* (Nadolska-Orczyk and Malepszy 1989), maize (Cowen et al. 1992), potato (Wenzel and Uhrig 1981; Uhrig 1985; Veronneau et al. 1992; Meyer et al. 1993), tomato (Koornneef et al. 1987) and wheat (Lazar et al. 1984; Ekiz and Konzak 1991). Segregation for the ability to regenerate shoots as observed by us in the F_1 and F_2 populations confirms the overriding genotypic effect on in vitro culture

response and thus is in full agreement with earlier findings.

Partitioning of this variation into heritable and non-heritable components through analysis of variance showed that the magnitude of genetic variation is much higher than that of environmental variance. This was reflected in our high estimates of broad-sense heritability. Heritability, in association with genetic variance estimates, can be used to calculate the number of effective factors (genes) involved in the control of given characters (Mather and Jinks 1982). High heritability and a very reduced number of effective factors, as observed here, can be interpreted as the result of additive gene action or simple genetic control (Falconer 1987). However, it should be borne in mind that estimates of heritability and effective factors, like estimates of any genetic parameter, are properties not only of a specific trait but also of the population and of the environmental conditions to which the individuals are subjected (Falconer 1987). Thus, the ultimate value of the estimates of genetic parameters depends upon the magnitude of all the components of variance. Therefore, a change in any of these components will alter the values of the estimates. Moreover, all of the genetic components are influenced by gene frequencies, which differ from population to population and vary with the past selection history, the breeding methods employed to develop a given population and the accompanying cultural practices (Birhman and Kaul 1989). In addition, heritability values depend not only on the heritable proportion of variation in the population but also on the amount of non-heritable variance (Mather and Jinks 1982; Falconer 1987). For valid estimates it is therefore essential to evaluate a large number of genotypes under uniform environmental conditions. This was done in the investigation reported here. Hence, the computed estimates provided here should be quite representative.

High estimates of heritability of in vitro plant regeneration that are comparable to those found in the present study have been previously reported in *Cucumis* (Nadolska-Orczyk and Malepszy 1989), potato (Coleman et al. 1990; Veronneau et al. 1992), tomato (Koornneef et al. 1987) and wheat (Lazar et al. 1984). The conclusion drawn from most of these studies was similar to the one arrived at in the present case, that relatively few genes may be involved in the control of tissue responses.

As mentioned earlier in this paper, many studies have been conducted on the partitioning of genetic variation for tissue culture responsiveness into heritable and non-heritable components, but there are actually only a few papers presenting complete models of inheritance. Reisch and Bingham (1980) presented the type of inheritance for bud formation from callus cultures of *Medicago sativa* L. Segregation ratios suggested that bud differentiation from callus was controlled by two dominant genes. One dominant gene was necessary to obtain regeneration. Dominant alleles at both loci had to be present in order to obtain the highest regeneration. Additional genetic factors were also found to be necessary to obtain regeneration from suspension cultures. In *Trifolium pratense*, on the other hand, plant regeneration from somatic tissue was found to be determined, as in the present study, by three recessive genes (Broda 1984). Koornneef et al. (1987) suggested that two dominant genes control regeneration ability from leaf explants in interspecific hybrids of *Lycopersicon peruvianum* and *L. esculentum*. In addition to these two major genes, minor genes were also involved in modifying regenerability in the population studied. The ability to regenerate plants from leaf explants of *Cucumis sativus* L. was evaluated by Nadolska-Orczyk and Malepszy (1989). Two dominant genes, characterized by complementary and probably with additive interaction, controlled plant regeneration from leaf explants. Working with *Solanum phureja*, Cheng and Veilleux (1990) concluded that protoplast culturability was controlled by two independent loci with complete dominance. Additional minor loci also influenced the character. More recently Taylor and Veilleux (1992) reported on the inheritance of competence for leaf disc regeneration and protoplast culturability in *Solanum phureja* and suggested a two-gene model for both cultures. In particular, for plant regeneration from leaf discs, recessive alleles at each of the two loci were required for the highest response, a dominant allele at either of the loci giving only a marginal response and dominant alleles at both loci resulting in no response. The difference between this two-gene model versus a three-gene model proposed by us might be due to the fact that these authors investigated only one single F_2 progeny, which led to the identification of a lower number of genes than were actually involved.

In conclusion, the results reported here reinforce the view that genes responsible for competence in in vitro plant regeneration can indeed be identified (nowadays by the use of molecular markers), integrated in breeding programmes and accumulated into agronomically elite genotypes.

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