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Heritability of regeneration in tissue cultures of sweet potato (*Ipomoea batatas* L.)*

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Summary. A population of open-pollinated progeny from 12 parents, and the 12 parents, was surveyed for *in vitro* growth and regeneration characteristics. Four different tissue culture procedures involving different media and the use of different explants to initiate the cultures were used. Petiole explants from young leaves were used as explants for initiation of callus cultures. These were evaluated for callus growth rate, friability, and callus color and texture, before transferring to each of three different regeneration media for evaluation of morphogenetic potential. Small shoot tips also were used to initiate callus cultures, which were evaluated for the same growth characteristics and transferred to growth-regulator free regeneration media. Regeneration occurred through root or shoot regeneration or through embryogenesis. Tissue culture treatment effects, as well as genotypic effects, were highly significant in determining: the types of callus produced, callus growth rates, color and texture on the two types of media used for the second and third subcultures. The family × treatment interaction was generally not statistically significant, affecting only callus color. Estimates of narrow sense heritability for callus growth rate in both the second and third subcultures were high enough (0.35 and 0.63, respectively) for the evaluation of parental lines for selection procedures. These characteristics were also the only early culture callus traits that were consistently correlated with later morphogenesis of the cultures. They were negatively correlated with root or shoot regeneration. The occurence of somatic embryogenesis was not correlated with early callus growth characteristics. Genetic and treatment effects were highly significant in the evaluation of morphogenetic potential, through root or shoot regeneration, or through embryogenesis. Regeneration of all types was of low frequency for all procedures, expressed in ≦ 11% of the cultures of the total population.

Key words: *Ipomoea batatas* L. – Sweet potato – Embryogenesis – Morphogenesis

Introduction

The application of tissue culture technology to the improvement of sweet potato, a clonally propagated crop, depends upon the ability to successfully initiate callus cultures from explants of mature plants, maintain them as callus or suspension cultures, and finally, to regenerate plants from long term *in vitro* cultures.

Earlier investigations of sweet potato tissue culture have been concerned with finding the optimum explant source, cultural conditions and media compositions for the accomplishment of these goals (Gunckel et al. 1972; Yamaguchi and Nakajima 1973; Tsay and Tseng 1979; Hwang et al. 1983; Liu and Cantliffe 1984; Jarrett et al. 1984).

Several *in vitro* studies with sweet potato indicate that the cultivar used can influence the results of the experiment profoundly. This has been demonstrated in regeneration from anther cultures (Tsay and Tseng 1979), lateral bud cultures (Litz and Conover 1978), and regeneration from callus derived from explants of storage root tissue (Gunckel et al. 1972; Yamaguchi and Nakajima 1973). Jarrett et al. (1984) tested 9 genotypes from the CATIE/GTZ germplasm collection and found that both the optimal medium for embryogenesis and the frequency of cultures that were embryogenic differed with genotype. Previous observations in our laboratory also indicated that genotypic differences were an important source of

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variability in sweet potato tissue cultures, and that the wide variation in success in attempts to proliferate cultured cells and regenerate whole plants reported in the literature could be due to this significant genetic effect. Genotypic differences in tissue culture characteristics and regeneration have been reported for a variety of economic crops, including wheat (Larkin et al. 1984), tomato (Greshof and Doy 1972) red clover (Keyes et al. 1980) and potato (Simon and Peloquin 1977).

The explant used to initiate callus culture can also influence the regeneration potential. A number of explant sources have been used in the initiation of sweet potato tissue cultures, including storage root tissue (Gunckel et al. 1972; Hwang et al. 1983), cured and stored storage root tissue (Yamaguchi and Nakajima 1973), leaf tissue (Sehgal 1978; Carswell and Locy 1984) and leaf and stem tissue (Carswell and Locy 1984), with varying degrees of success. Hwang et al. (1983) found that results using explants of storage root tissue may include preformed tertiary shoot meristems which could confound the results of regeneration studies. In all the above examples, regeneration was found only in primary cultures and not in long-term callus cultures. Recently, somatic embryogenesis from secondary cultures has been achieved using very small shoot tips or shoot meristems as the initial explants (Liu and Cantliffe 1984; Jarret et al. 1984).

The objectives of this study were: 1) to determine the optimal explant source and tissue culture protocols and 2) to investigate the genetic variability for growth, development and regeneration capacity in secondary callus cultures of a large sweet potato population.

Materials and methods

Plant material

Twelve parents were randomly selected from the 1982 polycross nursery maintained at Clinton, NC. The twelve parents and 24 seedlings from each half-sib family were used in this study. Parental lines for this study were from a population of sweet potato breeding lines developed by Dr. Alfred Jones of the U.S. Vegetable Laboratory, U.S. Department of Agriculture, Charleston, South Carolina, USA. They included W 103, W 109, W 125, W 128, W 138, W 147, W 149, W 157, W 159, W 167, W 174 and 79 BM 17.

Seeds were acid-scarified and planted individually in cell trays of a sterilized medium containing equal parts of soil, sand and peat. They were maintained in a growth chamber at 27 °C day/22 °C night under a 16 h photoperiod. Plants were watered as necessary and fertilized with a balanced fertilizer on a biweekly basis. Parental lines were maintained by cuttings under these same environmental conditions.

In vitro procedures

Two separate tissue culture protocols were used to examine the propensity for regeneration of secondary cultures, involving different explant sources and different media. For both procedures explant material was surface-sterilized in 0.525% Na-hypochlorite for 12 min, rinsed three times with sterile double-distilled water and blotted dry on sterile filter paper. Each different medium for this study was prepared in a 25 l lot, as a 10× stock prepared from dry components. Stocks were divided into 100 ml aliquots and frozen in plastic bags at −20 °C. Frozen stocks were thawed and reconstituted as needed and the pH adjusted to 5.8. This procedure effectively eliminated any significant differences due to media preparation or batch. All media were solidified with 0.8% w/v Bactoagar and sterilized by autoclaving for 18 min at 15 psi, in 0.5 l lots, before being pipetted (10 ml per plate) into 20×60 mm disposable Petri plates.

Procedure A

This procedure was developed from earlier studies by our laboratory as an adaptation of that used by Sehgal (1975) and used small pieces of petiole from young leaves (approximately 1 cm in length) as the explant material. Young leaves were excised at the basal end of the petiole, surface-sterilized as described above and the petiole sectioned into four 2 mm sections. Four sections from a single petiole were placed onto a 20×60 mm disposable Petri plate containing 10 ml of callus initiation medium (MS-AI), consisting of the basal salts of Murashige and Skoog (1962) medium, with myo-inositol (100 mg/l), niacin (0.5 mg/l), pyridoxine HCl (0.5 mg/l), thiamine HCl (4 mg/l), sucrose (30 g/l), and supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin at 2 mg/l each. After 5 weeks on this initiation medium, each explant and its callus was transferred to callus proliferation media (MS-AP), consisting of the same salts, carbohydrates and vitamins as the MS-AI initiation medium, but with decreased levels of 2,4-D and kinetin (0.2 mg/l each). Five weeks on this medium generally produced a large amount of callus which was then subcultured onto three separate media. The three regeneration media used in Procedure A include:

A1 – consisting of the same Murashige-Skoog salts, carbohydrates and vitamins as in the initiation medium MS-AI, but with no growth regulators.

A2 – equivalent to Proliferation Medium MS-AP.

A3 – White's salts (1934), niacin (1 mg/l), pyridoxine HCl (1 mg/l), thiamine HCl (10 mg/l), 2% sucrose and the growth regulators naphthalene acetic acid (NAA) at 2 mg/l and 6-benzyladenine (6-BA) at 0.2 mg/l.

After 10 weeks on MS-AP (with a subculture onto the same media at 5 weeks), the callus cultures were transferred onto Regeneration Media, and then transferred to fresh, identical Regeneration Media after five weeks. Only callus cells were used for subculturing unless the cultures were too small to successfully excise the callus.

Procedure B

This procedure was adapted from that used by Liu and Cantliffe (1984a) and used small, actively growing shoot tips as the explant material. Shoot tips were surface-sterilized as above, and dissected to obtain the explant, about 2 mm long, consisting of the meristem and a few, very small leaf primordia. Two apical shoot tip cultures were initiated for each genotype, and placed on Initiation Medium (MS-BI), a Murashige-Skoog salt medium with thiamine (4 mg/l), inositol (100 mg/l), 3% sucrose and 2,4-D (2 mg/l). After five weeks, the resulting calli were transferred to Proliferation Medium (MS-BP), which was identical to the Initiation Medium MS-BI except for the addition of kinetin at 0.25 mg/l. Cultures went through two five-week subcultures on Proliferation Media. A single regeneration medium was used for Procedure B, and consisted of the same salts, thiamine and carbohydrates as the Initiation Medium MS-BI, but with no growth regulators. As in Procedure B, callus cultures were transferred to fresh Regeneration Medium after five weeks.

Data collection and analysis

At 5 and 10 weeks after subculture on callus Proliferation Media, the cultures were evaluated individually for growth rate, presence of orange and green pigments, darkening and the presence of white, crystalline-appearing cells termed "snow" in this study. These cells appear as elongated filamentous cells on the surface of the callus when viewed with a dissecting microscope. The rating scales ranged from a low rating of 0 (no expression of the characteristic) to a highest level of 5 (except for growth rate, for which the highest rating was 10). Friability ratings were also taken at the end of the second subculture.

Regeneration ratings were taken after 10 weeks on Regeneration Media (with the subculture onto the same media occurring midway in the 10 weeks), and are presented as a percentage of the cultures exhibiting the development of roots, shoot or somatic embryos.

Values for each genotype × treatment were averaged and the means used for the analysis of variance. The analysis of variance was performed on a reduced data set, consisting of 18 offspring for each of the 12 parents. This reduction, based on using only those genotypes for which complete data were available, produced a balanced data set, facilitating the computer calculations. Also to conserve the size of the X'X matrix, the interactions of individual genotypes with treatments were not partitioned and are included in the residual term.

Treatments (tissue culture protocols) are considered as fixed effects in this model, as they were representative of the media cited in the literature or used in our preliminary studies. The families and within-family sources of variation were random. For these models, the treatment mean squares were compared to the mean square of the family × treatment interaction for the calculation of the F statistics. The forms of the analysis of variance on two callus growth media and for the four regeneration protocols are shown in Tables 1 and 2, respectively. The narrow-sense heritability estimates were calculated from the mean squares in these models as follows:

$$h^2 = \frac{4\,(\sigma^2_{\text{family}})}{\sigma^2_{\text{phenotype}}} = \frac{\sigma^2_{\text{additive}}}{\sigma^2_{\text{phenotype}}}.$$

Heritability was also calculated using parent: offspring regression. For that calculation, the same reduced data set was used, but with the unaveraged ratings of the individual cultures, and so included four replications for each genotype. Parental values used in the regression were the mean of three separate cultures, each including four replicates.

Table 1a. Form of the analysis of variance for data on two different callus growth media – a mixed model

Source	d.f.	Expected mean square
Families	11	$\sigma^2 + 2\,\sigma^2_{P(F)} + 36\,\sigma^2_F$
Progeny (families)	204	$\sigma^2 + 2\,\sigma^2_{P(F)}$
Treatments – FIXED	1	$\sigma^2 + 18\,\sigma^2_{FT} + 216\,\theta^2_T$
Family × treatment	33	$\sigma^2 + 18\,\sigma^2_{FT}$
Error	204	σ^2

Table 1b. Form of the analysis of variance for data on four separate generation media – a mixed model

Source	d.f.	Expected mean square
Families	11	$\sigma^2 + 4\,\sigma^2_{P(F)} + 72\,\sigma^2_F$
Progeny (families)	204	$\sigma^2 + 4\,\sigma^2_{P(F)}$
Treatments – FIXED	3	$\sigma^2 + 18\,\sigma^2_{FT} + 216\,\theta^2_T$
Family × treatment	33	$\sigma^2 + 18\,\sigma^2_{FT}$
Error	612	σ^2

Table 2. Analysis of variance for callus growth characteristics in two different *in vitro* protocols after the second and third subcultures

Source	Second subculture						
	d.f.	Growth	Friability	Orange	Green	Dark	Snow
Family	11	**	**	**	**	**	ns
Progeny (family)	204	**	*	**	*	**	ns
Treatment	1	**	**	**	**	**	**
Family × treatment	11	ns	ns	*	**	ns	ns
Error	204						
	Third subculture						
	d.f.	Growth		Orange	Green	Dark	Snow
Family	11	**		**	**	**	*
Progeny (family)	204	**		**	**	**	**
Treatment	1	**		*	ns	**	**
Family × treatment	11	ns		ns	**	ns	ns
Error	204						

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

Results and discussion

Genotypic variation in the adaptability to *in vitro* procedures has been reported for several agronomic crops. Quantitative genetic studies on this variation have been completed for wheat anther cultures (Lazar et al. 1984) and red clover callus regeneration (Keyes et al. 1980) which showed that additive genetic variance was the primary source of variability in these systems, allowing for potential rapid gain from selection through a breeding program for improved *in vitro* aptitude. However, Lu et al. (1984), working with eleven different hybrid corn cultivars, found that environmental factors, such as the sucrose concentration in the culture medium, the developmental stage of the immature embryos used as explants and the field conditions during embryo formation were of greater consequence in determining the efficiency of embryogenic callus formation than were the genotypes of the plant material.

In this study, genotypic effects (both among families and among progeny within families) were highly significant in determining the type of callus produced, affecting the growth rate, texture (friability) and color of the callus produced on the two media in both the second and third subculture (Table 2). The only exception to this case was the presence of the white, crystalline-appearing cells (snow), which was independent of genotype in the second subculture, although genotype was a significant factor in the third subculture. Treatment effects, which compound both media effects (Media A vs. Media B) and explant differences (petiole vs meristem explant), were also significant for most characteristics. The family × treatment interaction was significant only for callus color characteristics, the presence of orange pigments in the callus after the second subculture and the presence of green pigmentation after both the second and third subcultures. The greening of callus after the third subculture, in fact, showed an insignificant treatment effect, but a highly significant family × treatment interaction.

Relative values of the callus traits studied on the different callus maintenance media (Table 3) showed more clearly the extremely high significance of the differences in the treatment effects. In general, the cultures in protocol A (petiole segments on Medium A) grew more slowly, were less friable and were more likely to contain either orange or green pigment than the cultures initiated with shoot tip explants on Medium B. These differences between treatments were less extreme after the third subculture. Overall, the cultures were more highly pigmented (orange and green pigments) and darker after the third subculture.

Narrow sense heritability estimates (h^2) for callus characteristics (Table 4) were calculated separately for the two treatments (A and B) by regression of the offspring values on those of the single parent, and for the combined treatments by the variance-covariance method using the nested design shown in Table 1. Overall, the heritability estimates were too low to use successfully in a selection procedure based on parental phenotypes. However, the heritability estimates for callus growth characteristics were high enough to allow

Table 3. Mean values of callus characteristics on two different growth media after the second and third subcultures[a]

Medium	Second subculture					
	Growth	Friability	Orange	Green	Dark	Snow
A	1.02	2.81	0.43	0.72	1.87	0.50
B	2.67	3.54	0.27	0.10	3.22	0.07
lsd	0.11	0.16	0.11	0.13	0.15	0.14
$P>F$	0.0001	0.0001	0.003	0.0001	0.0001	0.0001
	Third subculture					
	Growth		Orange	Green	Dark	Snow
A	5.60		0.52	1.12	2.22	0.25
B	4.51		0.40	1.00	2.69	0.69
lsd	0.15		0.10	0.15	0.17	0.15
$P>F$	0.003		0.016	0.11	0.0001	0.0001

[a] The rating scale for each characteristic ranged from a low rating of 0 to a high rating of 5, with the exception of the growth measurement for the third subculture, for which the upper limit was a rating of 10

Table 4. Heritability estimates for tissue culture characteristics in two procedures (A & B)

Response variable	Subculture	Heritability		
		Parent: offspring regression		Nested design[a] combined
		Medium A	Medium B	
Growth	2	0.29**	0.37*	0.35
Friability	2	0.37**	0.31*	0.17
Orange	2	0.09	0.38*	0.09
Green	2	0.64**	0	0.17
Dark	2	0.39	0.34	0.05
Snow	2	0.08	0	0
Growth	3	0.27**	0.90**	0.63
Orange	3	0.09	0	0.15
Green	3	0.77**	0.26	0.18
Dark	3	0.40*	0.13	0.35
Snow	3	0.39*	0.40	0.15

*,** Narrow sense heritability estimate significant at the 5% or 1% level of probability, respectively

[a] Significance levels were not determined for h^2 estimates calculated by use of the nested design variance component method

successful evaluation of the parental lines for selection procedures. In addition, growth rate in the second and third subcultures were the only callus characteristics consistently correlated with the later morphogenesis of that culture (Table 5). The growth rate of the callus was negatively correlated (at the 1% significance level) with later shoot or root regeneration. Several other characteristics (orange pigment and "snow" phenotype in the second subculture and the darkness of the callus in the third subculture) showed a significant correlation at the 5% level with later regeneration. None of the early growth characteristics were significantly correlated with later regeneration in procedure B, or with embryogenesis in any procedure. This would severely limit the feasibility of using early culture growth characteristics to screen for cultures with high regeneration frequencies, as embryogenesis is generally the preferred morphogenetic response. The analysis of variance for data with the four separate regeneration protocols takes the form of a nested design similar to that used for the callus characteristics (Table 6). This analysis applies only to the data for root and shoot regeneration, since embryogenesis was found only in two of the four regeneration protocols. The analysis of variance for embryogenesis was similar to that used for callus characteristics. The use of four treatment protocols allows a further breakdown of the treatment effects into two contrasts – the combined A protocols vs the B protocol (petiole vs small shoot tip explants) and among the three A (petiole) protocols. The genetic effects were again significant, for both the among families and the among progeny with families components. Treatment effects were also significant, and the breakdown into the two contrasts showed that most of the treatment variation was due to the differences between protocols A and B (petiole vs meristem), which were significant at the 1% level. The differences among the three A protocols were significant at the 5% level for the trait of root regeneration, and not significant at the 5% level for the trait of shoot regeneration.

The analysis of variance for the occurence of embryogenesis in the cultures showed that, for this trait, the only significant variation was that among families. The variation among progeny within the families was insignificant, as were treatment differences and the family × treatment interaction. It should be reinforced, however, that only the two treatments which produced at least some somatic embryos were included in the analysis. Two other treatments did not produce somatic embryos and so, if included in this analysis, would definitely result in a highly significant treatment effect.

Regeneration of all three types, root and shoot morphogenesis and somatic embryogenesis, was of low frequency by these measurements (the percentage of cultures expressing the trait). The significant treatment effects (Table 7) were apparent for root and shoot morphogenesis, as are the differences between the A treatments and treatment B, and the nonsignificance of the slight differences among the A treatments for shoot regeneration. The two growth regulator-free regeneration media (A1 and B) induced the highest frequency of callus that developed roots, significantly more than the other two media which included 2,4-D (A2) or NAA (A3). Shoot regeneration was consistently low, with significant differences only between procedures A3 and B. Only two of the procedures (A2 and B) produced any cultures capable of somatic embryogenesis. These procedures differed both in growth regulators (A2 included both 2,4-D and kinetin, while B was growth regulator-free) and in the explant source. Sweet potato, a clonally propagated crop, does not allow the option of using immature embryos of established cultivars as

Table 5. Correlation of callus growth characteristics with regeneration in secondary cultures

Response variable	Sub	Root regeneration Procedure		Shoot regeneration Procedure		Embryogenesis Procedure	
		A	B	A	B	A	B
Growth	2	** (–)	ns	** (–)	ns	ns	ns
Friability	2	ns	ns	ns	ns	ns	ns
Orange	2	ns	ns	ns	ns	* (–)	ns
Green	2	ns	ns	ns	ns	ns	ns
Dark	2	ns	ns	ns	ns	ns	ns
Snow	2	ns	ns	* (+)	ns	ns	ns
Growth	3	** (–)	ns	* (–)	ns	ns	ns
Orange	3	ns	ns	ns	ns	ns	ns
Green	3	ns	ns	ns	ns	ns	ns
Dark	3	* (–)	ns	ns	ns	* (–)	ns
Snow	3	ns	ns	ns	ns	ns	ns

*,** The correlation is significant at the 5% or 1% level of probability, respectively
(–, +) = The two traits are negatively or positively correlated, respectively

Table 6. Analysis of variance for shoot and root regeneration in four different *in vitro* protocols

Source	d.f.	Roots	Shoots
Family	11	**	*
Progeny (family)	204	*	**
Treatment	3	**	*
Petiole vs meristem	1	**	**
Among petiole	2	*	ns
Family × treatment	33	ns	ns
Error	612		

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

Table 7. Mean values of the frequency of regeneration in the four *in vitro* protocols[a]

Protocol	Roots	Shoots	Embryogenesis
A1	0.11 a	0.02 ab	–
A2	0.03 b	0.02 ab	0.04
A3	0.06 b	0.04 a	–
B	0.11 a	0.002 b	0.06
lsd	0.04	0.02	0.03
$P>F$	0.0001	0.011	0.35

[a] Within columns, values followed by different letters are significantly different at $P=0.05$

Table 8. Heritability estimates for regeneration in secondary callus culture for four *in vitro* protocols

Response variable	Protocols				Overall[a]
	A1	A2	A3	B	
Root development	0.12	0	0.28*	0.12	0.19
Shoot development	0.19	0.18	0	0	0.05
Embryogenesis	–	0.17	–	0.26*	0.25

* Narrow sense heritability estimate significant at the 5% level of probability

[a] Significance levels were not determined for h^2 estimates calculated by use of the nested design variance-covariance method

the explant material for the initiation of callus. Shoot tips and apical meristems provide the best explant material, and the smallest explant (i.e. the meristematic dome alone) is the most efficient source of embryogenic callus (Templeton-Somers and Collins, unpublished data; Liu and Cantliffe 1984 b), although the explant mortality rate is much higher with such small and delicate explants.

The heritability estimates for the regeneration traits in this study were low (Table 8), partially as a reflection of the means of data collection. At the initiation of this experiment, it was decided that any regeneration of root, shoot or embryo in a culture would be considered significant, and so each culture was rated as only positive or negative for that regeneration trait. Analysis was then performed on the frequency of the cultures of each genotype expressing that trait. A more precise rating system, perhaps counting the number of roots, shoots, or embryos produced by each culture over time, could increase the accuracy of the heritability estimates. Greater precision in rating the regeneration capacity of cultures could increase the magnitude of the heritability estimates to a level high enough to rely on the parental phenotype in the selection techniques and with less dependence on extensive progeny testing.

Conclusion

Significant, heritable genetic differences were found for all morphogenetic responses and for most callus growth characteristics. In general, there was significant variation among families, progeny within families and among treatments, which represented the different tissue culture protocols followed.

There were significant differences due to the four different tissue culture protocols followed. Only two of the four regeneration protocols resulted in regeneration through somatic embryogenesis. All four treatments resulted in a low frequency of shoot regeneration. There were no significant differences among treatments for root regeneration. Family × treatment interactions were not significant for any morphogenetic response or most callus growth characteristics with the exception of callus pigmentation.

Standard sweet potato breeding lines could provide a potential base for a breeding program for improved regeneration from secondary tissue cultures. The resulting population, improved for adaptability to *in vitro* techniques, could provide breeders with the potential for incorporating novel genotypes, obtained through tissue culture procedures such as *in vitro* selection, into the genetic base of the breeding program.

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