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IN VITRO GROWTH AND REGENERATION CHARACTERISTICS OF DIVERSE POPULATIONS OF SWEET POTATO (IPOMEA BATATAS (L.) Lam)

(Croissance et caractéristiques de régénération in vitro de diverses populations de Patate douce)

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SUMMARY

Populations of open-pollinated seedlings from three distinc gene pools were surveyed for in vitro growth and developmental characteristics, using four different tissue culture procedures. Petiole explants from young leaves were used to initiate callus cultures (Murashige-Skoog agar medium with vitamins, 3 per cent sucrose, 1 mg/l 2,4-D and 1 mg/l kinetin) which were maintained on the same medium with a reduced level of 2,4-D (0.2 mg/L) and evaluated for callus growth rate, friability and callus color. Callus from these cultures were transferred to three separate regeneration media for the evaluation of morphogenetic potential. Small shoot tips were also used to initiate callus cultures on MS medium supplemented with thiamine, 3 per cent sucrose and 2 mg/l 2,4-D, which were transferred to proliferation media including 0.25 mg/L kinetin before transfer to growth regulator-free regeneration medium. Early callus characteristics were significantly correlated with later regeneration. Significant differences in shoot and root regeneration and in somatic embryogenesis were found among and within populations and among culture protocols.

RESUME

Des populations de plantules obtenues par pollinisation libre à partir de 3 pools géniques distincts ont été analysées pour la croissance et les caractéristiques de développement in vitro, en utilisant quatre méthodes différentes de culture de tissu.

Des pétioles de jeunes feuilles sont utilisées pour initier des cultures des cals (milieu gélosé de Murashige et Skoog avec vitamines, 3 pour cent de saccharose, 1 mg/l de 2,4D et 1m/l/l de kinétine.

On évalue le taux de croissance des cals, leur friabilité et leur couleur. A partir de ces cultures, les cals sont transférés sur trois différents milieux de régénération pour l'évaluation du potentiel morphogénétique.

Des petites tiges sont aussi utilisées pour initier des cultures de cals sur le milieu MD supplémenté avec de la thiamine, 3 pour cent de saccharose et mg/l de 2,4D. Ces cals sont transférés sur un milieu de prolifération contenant 0,25 mg/l de kinétine avant leur passage sur un milieu de régénération dépourvu de régulateur de croissance. Les caractéristiques de précocité des cals sont significativement corrélées avec leur régénération tardive.

Des différences significatives dans la régénération des tiges et des racines ainsi que dans l'embryogenèse somatique sont trouvées entre et à l'intérieur des populations tout aussi bien qu'entre les protocoles de culture.

Tissue culture of sweet potatoes (*Ipomea batatas* (L.) Lam.) has received much attention from researchers in recent years. Potential applications of tissue culture techniques to a vegetatively propagated food crop such as sweet potatoes are numerous. For example, tissue culture could eliminate much of the expense, time and labor now involved in plant production in temperate climates and could insure a reliable source of disease and insect-free plants in tropical climates.

Aplication of such techniques in the improvement of this important world food crop depends on successfully initiating callus, maintaining callus cultures and regenerating plants from those cultures. Many *in vitro* studies, therefore, have been concerned mainly with optimum explant source, cultural conditions and media composition.

Previous reports indicate a significant effect of cultivar on results of *in vitro* techniques. In regenerating plants from another cultures, TSAY and TSENG (1979) found cultivar differences in callus production from another cultures of sweet potato and reported plantlet regeneration from embryoids in only one clone. LITS and CONOVER (1978) saw similar differences in lateral bud cultures and other researchers found differences in regeneration using callus derived from explants of storage root tissue (YAMAGUCHI and NAKAJAMA, 1973). JARRET et al (1984) found that the optimal medium for embryogenesis and the frequency of embryogenic cultures differed with genotype. This phenomen has previously been reported for other crops in tissue culture such as corn (LU et al, 1983), wheat (LARKIN et al, 1984), tomato (GRESHOFF and Doy, 1972), red clover (KEYES et al, 1980) and potato (SIMON and PELOQUIN, 1977).

Recent observations in our laboratory indicated that genotypic differences are important in the variability or reports from sweet potato tissue culture studies. The varying degrees of success reported in the literature in proliferating cells and regenerating whole plants could thus

be due in large part to this significant genetic effect. In addition a survey of large numbers of diverse genotypes might identify clones or related groups of clones that are significantly more adapted to *in vitro* culture than others.

The purposes of this study were 1) to survey a large group of divers genotypes of sweet potatoes to determine genetic variability for growth, development and regeneration capacity in secondary callus cultures ; 2) to determine the effects of various tissue culture protocols and explant sources on growth, development and regeneration, and 3) to determine if early callus characteristics can be correlated with potential for secondary regeneration.

MATERIALS AND METHODS

Open-pollinated seeds were obtained from plants of four distinct gene pools designated Puerto Rico, Philippines, Taiwan and North Carolina. Seeds were acid-scarified (H_2SO_4, 20 min.), planted individually in cell trays of soil mix (1 soil:1 sand:1 peat by vol.) and maintained in a growth chamber at 27°C day/22°C night under a 16 hour photoperiod. Plants were watered as necessary and fertilized with a balanced fertilizer every other week.

Two separate tissue culture protocols were used to examine the propensity for regeneration of secondary cultures utilizing different tissues as explants. These are identified as Protocol A and Protocol B. To test different media, Protocol A was further divided into three protocols by using three different regeneration media. These protocols are identified as A1, A2 and A3. For both procedures the explant material was surface-sterilized in 0.525 per cent sodium-hypochlorite for 12 minutes, rinsed three times with sterile double-distilled water and blotted dry on sterile filter paper. Each medium for this study was prepared in a 25 l lot, as a 10 X 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 variation due to media preparation or batch. All media were solidified with 0.8 per cent w/v Bacto-agar and sterilized by autoclaving for 18 min. at 10.4 x 10 Pa in 0.5 L lots, before being pipetted (10 ml per plate) into 20 x 60 mm disposable petri plates.

Protocol A. This procedure was developed from earlier studies in our laboratory as an adaptation of that used by SEHGAL (1975) and utilized small pieces of petiole from young leaves (about 1 cm in length) as the explant material. Young leaves were excised at the stem, surface-sterilized as described above and the petiole were placed into a 20 x 60 mm disposable petri plate containing 10 ml of callus initiation medium, 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 were transferred to callus proliferation 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 subcultured onto three separate regeneration media. The three regeneration media used in Protocol A included :

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

A2- equivalent to proliferation medium.

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

After ten weeks on the proliferation medium (with a subculture into the same medium at five weeks), the callus cultures were transferred onto the regeneration media for ten weeks (with a subculture onto fresh media at five weeks). Only callus cells were used for subculturing unless the cultures were too small to excise the callus successfully.

Protocol B. This procedure was adapted from that used by LIU and CANTLIFFE (1984) and utilized small, actively growing shoot tips as the explant material. Shoot tips were surface-sterilized as described previously, 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 an initiation medium : a Murashige-Skoog salt medium with thiamine (4 mg/l), inositol (100 mg/l), 3 per cent sucrose and 2,4-D (2 mg/l). After five weeks, the resulting calli were transferred to proliferation medium, which was identical to the initiation medium with addition of kinetin at 0.25 mg/l. The cultures went through two five-week subcultures on proliferation medium. A single regeneration medium was used for Procedure B, and consisted of the same salts, thiamine and carbohydrates as the initiation medium, but with no growth regulators. As in Procedure A, callus cultures were transferred to fresh regeneration medium after five weeks.

For all protocols at five and ten weeks after subculture on callus proliferation media, cultures were individually evaluated for growth rate, the presence of orange and green pigments, darkening and the presence of white, crystalline-appearing cells termed "snow" in this study. These cells appeared as elongated filamentous cells on the surface of the callus when viewed with a dissecting microscope. The

rating scale ranged from a low rating of 0 (no response) to a high rating of 5 (greatest response). Friability ratings were also taken at the end of the second subculture.

Regeneration ratings were taken after ten weeks on regeneration medium (with the subculture onto the same media occuring at five weeks), and are presented as a percentage of the cultures exhibiting root, shoot or somatic embryo development.

Values for each genotype-treatment combination were average and the means used for analysis of variance. Analysis of variance was performed on a reduced data set, consisting of 36 clones for each of the Taiwan and Philippine populations, 72 clones for the population from Puerto-Rico, and 216 clones from the North Carolina population. This reduction produced a balanced data set facilitating computer calculations. This reduction produced a balanced data set facilitating computer calculations. Also, to conserve the size of the X'X matrix , 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 are representative of the media used by other researchers and in our preliminary studies. Populations and genotypes within population sources of variation were considered random. For these models, treatment mean squares were compared to mean square of population x treatment interaction for calculation of F. statistics.

RESULTS AND DISCUSSION

Significant differences in callus characteristics were apparent early in the establishment of cell cultures (Table 1). Treatment (protocol) differences were detected in the analysis of variance in all characteristics by the second subculture when all populations were combined. By the end of the third subculture some of those differences between treatments appear to dissipate. Population and genotypes within populations were significant sources of variation in most characters at the second subculture and remained significant by the third subculture. Population x treatment interactions were generally nonsignificant after the third subculture with the exception of growth.

Protocol A, which utilized small pieces of petiole from young leaves as the explant source, clearly gave an advantage in early growth (Table 2). However, by the third subculture, Protocol B, utilizing actively growing shoot tips as the explant source, was as effective in supporting growth as Protocol A. Differences between populations which were

Table 1. - Analysis of variance for sweet potato callus growth characteristics of four populations using two *in vitro* protocols after the second and third subcultures.

		Second Subculture					
Source	d.f	friability	growth	orange	green	dark	snow
Population	3	**	**	**	**	ns	ns
Genotype (Pop)	168	ns	**	ns	**	**	ns
Treatment	1	**	**	**	**	**	**
Population x Trt	23	**	*	ns	**	**	ns
Error	579						

		Third Subculture				
Source	d.f	growth	orange	green	dark	snow
Population	3	**	**	**	ns	**
Genotype (Pop.)	168	**	ns	**	**	ns
Treatment	1	ns	*	**	**	**
Ppulation x Trt	3	**	ns	ns	ns	ns
Error	579					

*,** - Estimate significant at the 1% or 5% level of probability, respectively.

ns - Estimate not significant at the 5% level of probability.

Table 2. Mean values of callus characteristics on two different sweet potato *in vitro* protocols after the second and third subcultures (all populations combined)[z].

	Second subculture					
Protocol	friability	growth	orange	green	dark	snow
A	0.91	6.29	0.33	0.89	1.92	0.46
B	3.36	2.73	0.18	0.09	3.22	0.04
lsd	0.13	0.39	0.08	0.11	0.12	0.09
p F	0.0001	0.0001	0.0004	0.0001	0.0001	0.0001

	Third subculture				
Protocol	growth	orange	green	dark	snow
A	4.77	0.40	1.33	2.25	0.29
B	4.67	0.30	1.09	2.76	0.67
Lsd	0.26	0.09	0.15	0.14	0.15
p F	0.42	0.034	0.0003	0.0001	0.0001

[z] - The rating scale for each characteristics ranged from a low rating of 0 to a high rating of 5, with the exception of the growth measurements, for which the upper limit was a rating of 10.

apparent at the second subculture remained about the same apparent at the second subculture remained about the same at the third subculture (Table 3). The Taiwan population was clearly superior in growth.

Analysis of variance for shoot and root regeneration and somatic embryogenesis using four *in vitro* protocols is shown in Table 4. While genotype (either population or genotypes within population) was an important source of variation in root and shoot regeneration, only *in vitro* col (treatment) was a significant factor in somatic embryogenesis and suggests that frequency of embryogenesis may not be increased with appropriate selection of genotype. Population x treatment interactions were not significant for any type of regeneration.

Overall, frequency of cultures undergoing regeneration in secondary culture was low. When examined by tissue culture protocol (Table 5), the highest percentage of cultures undergoing regeneration in secondary culture was approximately 8.4 per cent in Protocol B utilizing shoot tips. These findings are similar to those of LIU and CANTLIFFE (1984). They also showed that the frequency of somatic embryogenesis could be improved greatly by using even smaller pieces of shoot tips for the initial explant. By using only the meristem itself as an explant, they were able to increase the frequency of embryogenesis to over 90 per cent.

Treatment effects were significant for root and shoot regeneration and somatic embrogenesis in secondary cultures. Procedures A1 and B, the two procedures with a regeneration medium free of growth regulators, were the most successful in inducing root formation. Shoot regeneration frequency was consistently low for all protocols, but Protocol B was unique in supporting virtually no shoot morphogenesis. This procedure, however, was the most successful in obtaining somatic embryogenesis, with 5.0 per cent of the cultures producing embryos. Procedures A1 and A3 were completely unsuccessful in inducing embryogenesis.

Frequency of root regeneration was significantly affected by germplasm origin (population), with less than 1 per cent of the cultures from the Taiwan population producing roots (Table 6). The Taiwan population had a significantly higher growth rate (Table 3) than the other populations. Perhaps the emphasis on callus growth reduced the potential for root morphogenesis in that population. Frequencies of shoot regeneration and somatic embryogenesis were not significantly affected by population.

Correlations between early callus growth characteristics and regeneration in secondary cultures over all populations are shown in Table 7. While the reasons for these correlations may be of theoretical importance, the actual values appear to be too low to be of significance in the choice of highly embryogenic or regenerative genotypes.

Table 3. Mean values of callus characteristics in four distinct populations of sweet potatoes after the second and third subcultures (protocols combined)[z].

Population origin	Second subculture					
	friability	growth	orange	green	dark	snow
NC	3.17 b	4.01 c	0.34 a	0.38 b	2.64	0.26
Philippines	3.34 ab	4.83 b	0.08 b	0.45 b	2.68	0.30
Taiwan	3.45 a	6.05 a	0.04 b	0.48 b	2.44	0.14
Puerto Rico	2.83 c	4.77 b	0.17 b	0.75 a	2.54	0.18
lsd	0.24	0.72	0.15	0.21	0.23	0.18
p>F	0.0001	0.0001	0.0001	0.0001	0.209	0.274

Population origin	Third subculture				
	growth	orange	green	dark	snow
NC	4.21 c	0.46 a	1.03 c	2.50	0.46 b
Philippines	5.17 b	0.16 bc	1.31 bc	2.54	0.55 b
Taiwan	7.03 a	0.01 c	1.64 a	2.38	0.90 a
Puerto Rico	4.98 b	0.25 b	1.51 ab	2.62	0.39 b
lsd	0.495	0.167	0.286	0.255	0.214
p>F	0.0001	0.0001	0.0001	0.347	0.0001

[z] - 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 measurements, for which the upper limit was a rating of 10.

Table 4. Analysis of variance for sweet potato shoot and root regeneration and somatic embryogenesis for four *in vitro* protocols.[z]

Pop'n	d.f.	Roots	Shoots	Embryogenesis
Population	3	**	ns	ns
Genotypes (population)	165	ns	**	ns
Treatment	3	**	**	**
Population x Trt.	9	ns	ns	ns
Error	1257			

*,** - Estimate significant at the 5% or 1% of probability, respectively.

ns - Estimate not significant at the 5% level of probability.

Table 5. Mean values of the percentage of sweet potato cultures undergoing regeneration for four *in vitro* protocols.

Protocol	Roots	Shoots	Embryogenesis
A1	8.3 a	2.0 a	0.0 c
A2	2.5 b	1.9 a	3.0 b
A3	4.7 b	3.4 a	0.0 c
B	8.4 a	0.1 b	5.0 a
lsd	2.8	1.6	1.6
p>F	0.0001	0.0016	0.0001

Table 6. Mean values of the percentage of sweet potato cultures undergoing regeneration in four distinct populations.[z]

Population	Roots	Shoots	Embryogenesis
North Carolina	7.9 a	2.2	5.0
Philippines	2.3 bc	1.6	1.0
Taiwan	0.9 c	0.2	2.1
Puerto Rico	4.8 ab	1.9	3.1
lsd	3.6	2.1	2.1
p>F	0.0001	0.247	0.116

[z] - Means followed by different letters are significantly different at the p = 0.01 level. This table counts only the two successful treatments for embryogenesis - if all four trt. were included, the values would be halved.

This survey of genotypes and tissue culture protocols for propensity for morphogenesis indicates that both these factors are critical. Embryogenesis is the most useful type of regeneration from secondary culture, and cultures capable of producing a large number of embryos would be valuable in germplasm enhancement programs. Somatic embryos obtained in this study germinated normally and produced plantlets within a few weeks. However data in this study, which encompassed 360 genotypes, indicate that for this important trait, *in vitro* protocol overshadows genotype in importance. Shoot regeneration could be of similar importance if a large number of shoots could be obtained from a single culture. Sweet potato is unusual in that shoot regeneration may also occur from adventitious roots produced in culture (CARSWELL and LOCY, 1984). In this study root regeneration rates were generally higher than rates of shoot or embryo regeneration so this avenue of morphogenesis or somatic embryogenesis.

Table 7. Correlation of callus growth characteristics with regeneration in secondary cultures - All four populations combined.

Response variable	Sub x Protocol	Root regen. A	Root regen. B	Shoot Regen. A	Shoot Regen. B	Embryogenesis A	Embryogenesis B
Growth	2	-0.28**	ns	-0.16**	ns	ns	ns
Friability	2	-0.17**	ns	ns	ns	ns	ns
Orange	2	ns	ns	ns	ns	ns	ns
Green	2	ns	ns	ns	ns	ns	ns
Dark	2	ns	0.14**	ns	ns	ns	0.15**
Snow	2	ns	ns	ns	ns	ns	ns
Growth	3	-0.27**	-0.17**	-0.14**	ns	ns	ns
Orange	3	ns	0.12*	ns	ns	ns	ns
Green	3	ns	ns	ns	ns	ns	ns
Dark	3	ns	ns	ns	ns	-0.11*	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.

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