# INHERITANCE OF PROTOPLAST CULTURABILITY AND IMPROVEMENT IN POLLEN DEVELOPMENT BY PROTOPLAST MANIPULATION IN SOLANUM

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Thesis submitted to the Faculty of the

Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Genetics

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May, 1990

Blacksburg, Virginia

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(ABSTRACT)

Genetic improvement of the potato through classical breeding has been limited by its tetraploid nature, the narrow genetic variability within cultivars, and interploidy barriers between tetraploid cultivars and diploid germplasm. Breeding at reduced ploidy levels has been proposed as a solution to these problems. Because of sterilities, somatic hybridization via protoplast fusion has been considered an alternative to sexual polyploidization for resynthesizing superior diploids from selected monoploids, and tetraploids from selected diploids and dihaploids.

Successful application of somatic hybridization largely depends upon protoplast culturability and regenerability of a plant. The ability of callus formation and plant regeneration from protoplasts varies among plants. To understand the genetic basis for this variation, the mode of inheritance for protoplast culturability, defined as the ability to develop calli from cultured protoplasts, was

studied in the diploid potato species, *Solanum phureja.* Based upon data from $F_2$ as well as from $F_1$ and backcross progenies, it was found that protoplast culturability in this potato species was controlled by two unlinked loci with dominant effect. In addition, there was quantitative variation for protoplast plating efficiency among culturable genotypes.

Male sterility in cultivars of *Solanum tuberosum* ssp. *tuberosum* results from nuclear-cytoplasmic interactions. 'Donor-recipient' protoplast fusion and regeneration were conducted between a sterile *S. tuberosum* ssp. *tuberosum* cultivar, Russet Burbank, and fertile selections of *S. tuberosum* ssp. *andigena* which have a non-sensitive cytoplasm and were used as the cytoplasmic donor. Sixteen regenerated plants possessed nuclear background and chloroplast DNA of Russet Burbank. However, two of these regenerants had improved pollen stainability. The possible causes for the improvement of pollen stainability are discussed.

In the last chapter, allelic polymorphism in a monoploid population derived from anther culture of a clone of *S. phureja* was assessed by isozyme electrophoresis. Fourteen monoploids and their anther donor were examined for six enzymes. No allozyme variation was detected in these plants. However, genetic variability among these monoploids was manifested by variations in some growth characters and general morphology. The limitation of enzymatic markers in detecting allelic polymorphism in these monoploids is discussed.

# Acknowledgements

The author is grateful to Prof. Richard E. Veilleux, chairman of the advisory committee, for his guidance, assistance, and support during the author's study at VPI & SU. The author also would like to thank the following persons who have served as members of the advisory committee, for their advice, assistance, and encouragement: Profs. Carole L. Cramer, Asim Esen, David N. Radin, Mosbah M. Kushad, and Thomas M. Starling. The author received training in starch gel electrophoresis from Dr. D. N. Radin, genomic DNA isolation from Dr. C. L. Cramer, and chloroplast DNA isolation and restriction from Dr. Khidir W. Hilu. Their assistance is appreciated. Finally, the author wants to express his gratitude to his wife Xiaolin and son Zhaoyuan for their steady support and understanding.

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# Chapter 1: Introduction

## *Economic importance of the potato*

Among the world's food crops, the potato ranks in the top five in tonnage. It yields more edible energy and protein per hectare per unit of time than any other crop. It contains high-quality protein and substantial amounts of essential vitamins and trace elements. It is grown in more countries than any other crop except maize. Over the last two decades, the growth rate of potato production in developing countries has exceeded that of other food crops (International Potato Center, 1984; Horton, 1987).

## *Potato systematics and breeding strategies*

Cultivated potato includes four diploid ($2n = 2x = 24$), *Solanum ajanhuiri* Juz. et Buk., *S. goniocalyx* Juz. et Buk., *S. phureja* Juz. et Buk., *S. stenotomum* Juz. et Buk., two triploid ($2n = 3x = 36$), *S.* ×*chauca* Juz. et Buk., *S.* ×*juzepczukii* Buk., two tetraploid ($2n = 4x = 48$), *S. tuberosum* L. ssp. *tuberosum, S. tuberosum* L. ssp. *andigena* Hawkes, and one pentaploid species ($2n = 5x = 60$), *S.* ×*curtilobum* Juz. et Buk.. There are over two hundred wild potato species which are mostly diploid and occur in an extremely wide habitat range. These wild species have great potential for improvement of cultivated potato, because of their resistances to diseases, pests, and various environmental stresses (Hawkes, 1978a; Hawkes and Hjerting, 1989). In the Northern Hemisphere, commercially important potato cultivars are tetraploid *S. tuberosum* ssp. *tuberosum*. It is believed that this subspecies evolved from *S. tuberosum* ssp. *andigena*, which originated from the central Andes of South America, in southern Bolivia and northern Peru (Hawkes, 1978b).

Classical potato breeding is carried out by intercrossing tetraploid parental clones followed by stepwise selection for desirable genotypes in the progenies. Genetic improvement of the potato has been limited mainly due to: 1) limited genetic variability within tetraploid cultivars (Mendoza and Haynes, 1974; Mendoza, 1989); 2) tetraploid nature (Chase, 1963); 3) common occurrence of male sterility among cultivars (Howard, 1970); and 4) interploidy barriers be-

tween diploid germplasm and the tetraploid cultivars. The so-called analytic breeding scheme has been proposed to overcome the disadvantages of tetraploid breeding. In this scheme, ploidy level is first reduced from $4x$ to $2x$ through parthenogenesis induced by haploid-inducing pollinators. Then, breeding is carried out at the diploid level. Finally, two selected diploids are combined to reconstruct a tetraploid either through sexual polyploidization via unreduced gametes or through somatic hybridization via protoplast fusion (Chase, 1963; Wenzel et al., 1979; Neele and Louwes, 1986; Deimling et al., 1988). A different strategy for breaking interploidy barriers has been through $4x$-$2x$ crosses via $2n$ pollen from diploid selections (Mok et al,. 1975; De Jong et al., 1981; McHale and Lauer 1981; Veilleux and Lauer, 1981; Hermsen, 1984). Further reduction of ploidy level to the monoploid level ($2n = x = 12$) through parthenogenesis or anther culture would eliminate deleterious alleles from diploid potato stocks which are generally cross-pollinated, thus accelerating the development of diploid germplasm (Wenzel et al., 1979).

## *Objectives*

*S. phureja* Juz. et Buk. is a cross-pollinated diploid potato species cultivated in South America. An adapted population that tuberizes under long days has been selected (Haynes, 1972). Further selection within this population has yielded genotypes capable of $2n$ pollen production by the genetic equivalent of first divi-

sion restitution (Veilleux, 1985). Because of selection only for tuberization under long days and for 2*n* pollen production, general improvements for horticultural traits are needed before interploid hybridization can be expected to yield agronomically viable progeny. The success of monoploid regeneration from anther culture for some genotypes of this species provides the opportunity to conduct monoploid breeding on these 2*n*-pollen producers (Veilleux et al., 1985). Doubled monoploids have been generated through shoot regeneration from leaf explants of some monoploids. Protoplast fusion is the only way to resynthesize diploid hybrids from selected monoploids, because crosses between the doubled monoploids are prevented by male sterility. However, these 2*n*-pollen-producing genotypes of *S. phureja* and their monoploid derivatives have been found to lack ability for callus regeneration from protoplasts; thus, hybridization between selected monoploids via protoplast fusion cannot be realized. To solve this dilemma, studies were conducted on: 1) sexual transmission of protoplast culturability, which is defined here as capability of callus regeneration from protoplasts isolated from *in vitro* grown shoots; and 2) mode of inheritance for this trait in this diploid potato species. A separate chapter is devoted to the improvement of pollen fertility of Russet Burbank, a leading cultivar in the potato industry in the United States, via 'donor-recipient' protoplast fusion.

## *References*


Chase, S. S., 1963. Analytic breeding in *Solanum tuberosum* L. A scheme utilizing parthenotes and other diploid stocks. Can. J. Genet. Cytol. 5:359-363.

Deimling, S., J. Zitzisperger, and G. Wenzel, 1988. Somatic fusion for breeding of tetraploid potatoes. Plant Breeding 101:181-189.

De Jong, H., G. C. C. Tai, W. A. Russell, G. R. Johnson, and K. G. Proudfoot, 1981. Yield potential and genotype-environment interactions of tetraploid-diploid ($4x$-$2x$) potato hybrids. Am. Potato J. 58:191-199.

Hawkes, J. G., 1978a. Biosystematics of potato. *In*: Harris, P. M. (eds.), The potato crop, Halsted Press, New York, pp. 15-69.

Hawkes, J. G., 1978b. History of potato. *In*: Harris, P. M. (eds.), The potato crop, Halsted Press, New York, pp. 1-14.

Hawkes, J. G., and J. P. Hjerting, 1989. The potatoes of Bolivia. Oxford Science Publications, pp. 1-7.

Haynes, F. L., 1972. The use of cultivated diploid *Solanum* species in potato breeding. *In*: French, E. R. (ed.), Prospects for the potato in the developing world: an international symposium on key problems and potentials for greater use of potato in the developing world, International Potato Center, Lima, Peru.

Hermsen, J. G. Th., 1984. The potential of meiotic polyploidization in breeding allogamous crops. Iowa J. Res. 58:435-448.

Horton, D., 1987. Potatoes: production, marketing, and programs for developing countries. Westview Press, Boulder, pp. 5-8.

Howard, H. W., 1970. The genetics of potato. Springer-Verlag Inc., New York, pp. 20-23.

International Potato Center, 1984. Potatoes for the developing world. Lima, Peru, pp. 13-20.

McHale, N. A., and F. I. Lauer, 1981. Breeding value of $2n$ pollen diploid from hybrids and phureja in $4x$-$2x$ crosses in potatoes. Am. Potato J. 58:365-374.

Mendoza, H. A., 1989. Population breeding as a tool for germplasm enhancement. Am. Potato J. 66:639-653.

Mendoza, H. A., and F. L. Haynes, 1974. Genetic relationship among potato cultivars grown in the United States. HortScience 9:328-330.

Mok, D. W. S., and S. J. Peloquin, 1975. Breeding value of 2*n* pollen (diplandroids) in tetraploid × diploid crosses in potatoes. Theor. Appl. Genet. 46:307-314.

Neele, A. E. F., and K. M. Louwes, 1986. The analytic breeding method: possibilities for potato breeding. *In*: Beekman, A. G. B., K. M. Louwes, L. M. W. Dellaert, and A. E. F. Neele (eds.), Potato research for tomorrow, Pudoc, Wageningen, pp. 107-114.

Veilleux, R. E., 1985. Diploid and polyploid gametes in crop plants: mechanisms of formation and utilization in plant breeding. Plant Breed. Rev. 3:253-288.

Veilleux, R. E., J. Booze-Daniels, and E. Pehu, 1985. Anther culture of 2*n* pollen producing clones of *Solanum phureja* Juz. and Buk. Can. J. Genet. Cytol. 27:559-564.

Veilleux, R. E., and F. I. Lauer, 1981. Breeding behavior of yield components and hollow heart in tetraploid-diploid vs. conventionally derived potato hybrids. Euphytica 30:547-561.

Wenzel, G., O. Schieder, T. Przewozny, S. K. Sopory, and G. Melchers, 1979. Comparison of single cell culture derived *Solanum tuberosum* L. plants and a model for their application in breeding programs. Theor. Appl. Genet. 55:49-55.

# Chapter 2: Literature review

## *Protoplast culture in potato*

The term protoplast refers to all the components of a plant cell excluding the cell wall (Evans and Bravo, 1983). Without a cell wall, protoplasts can take up foreign macromolecules and cellular components under certain conditions, thus providing opportunities for genetic and cellular manipulation (Bhojwani and Razdan, 1983; Galun and Aviv, 1983; Potrykus et al., 1985; Cocking and Davey, 1987; Glimelius, 1988). In addition, novel characters could be generated among plants developed from protoplasts (Matern et al., 1978; Karp et al., 1989).

Among food crops, potato is the most amenable to protoplast techniques. Successful plant regeneration has been achieved with a wide range of cultivars and breeding stocks of *S. tuberosum*, including both tetraploids and dihaploids

(Shepard and Totten, 1977; Binding et al., 1978; Gunn and Shepard, 1981; Thomas, 1981; Bokelmann and Roest, 1983; Schumann and Koblitz, 1983; Haberlach et al., 1985; Foulger and Jones, 1986; Radke and Grun, 1986; Tavazza and Ancora, 1986; Masson et al., 1987). In addition, plants have been regenerated from several cultivated diploid and wild potato species (Binding and Nehls, 1977; Nelson et al. 1983; Schumann and Koblitz, 1983; Haberlach et al. 1985; Dai et al., 1987). Recently, protoplasts from monoploids ($2n = 1x = 12$) derived through parthenogenesis of various diploid clones have been regenerated into plants (Uijtewaal et al., 1987a).

The essential components of protoplast culture and regeneration in potato include: preparation of donor plants, protoplast isolation, protoplast culture to form p-calli, shoot regeneration from the p-calli, and shoot elongation and rooting (Shepard, 1982b; Shahin, 1984).

Potato protoplasts could be isolated either from pot-grown plants (Shepard and Totten, 1977; Shepard, 1980; Radke and Grun, 1986) or from *in vitro* plants (Binding et al., 1978; Thomas, 1981; Shahin, 1984). The cultural conditions for donor plants have been found to be critical for protoplast yield and growth. For pot-grown plants, it is necessary to grow donor plants in well-defined nutritional and environmental conditions and precondition the leaf tissue before isolation (Shepard, 1980; Shepard et al., 1980). For *in vitro* plants, photoperiod, light intensity, temperature, sucrose concentration in the propagation medium, and

inhibition of ethylene production have been found to influence protoplast yield and quality (Foulger and Jones, 1986; Masson et al., 1987; Perl et al., 1988).

Usually, potato cell wall can be digested by a combination of cellulase and pectinase (Shepard and Totten, 1977; Binding et al., 1978). For digesting leaf tissue, cellulase at concentrations of 0.5% to 2.0%, and pectinase at concentrations of 0.1% to 0.3% have been used (Shepard and Totten, 1977; Binding et al., 1978). For suspension cells, cell wall digestion requires driselase and higher concentrations of cellulase and pectinase (Schumann et al., 1980). Either 0.3 M sucrose, or 0.5 M mannitol or sorbitol have been used as the osmoticum in the digestion solution (Shepard and Totten, 1977; Binding et al., 1978; Schumann et al., 1980; Thomas, 1981).

Protocols for protoplast purification vary depending upon researchers. Some groups have used relatively higher speed of centrifugation (350 × g) and 0.35 M sucrose solution in Babcock bottles to purify protoplasts (Shepard and Totten, 1977; Shepard, 1982b; Haberlach et al., 1985), whereas others have developed lower-speed centrifugation (50-100 × g) and using 0.5 M mannitol or 0.5 M sucrose as purifying solution (Binding et al., 1978; De Vries and Bokelmann, 1985; Uijtewaal et al., 1987b).

For protoplast culture, both bilayer (Shepard and Totten, 1977; Haberlach et al. 1985) and liquid culture have been used (Thomas, 1981; Bokelmann and

Roest, 1983; Schumann and Koblitz, 1983). The composition of culture media also varies among researchers. Generally, MS medium without ammonium ion (Shepard and Totten, 1977; Haberlach et al., 1985; Tavazza and Ancora, 1986) or V-KM medium (Binding and Nehls, 1977; Bokelmann and Roest, 1983; Foulger and Jones, 1986; Uijtewaal et al., 1987b) has been employed. The types and concentrations of growth regulator supplements to the culture media have been similar: usually α-naphthaleneacetic acid (NAA) at 1-2 mg/l and 6-benzylaminopurine (BAP) or zeatin at 0.5 mg/l. In some cases, 2,4-dichlorophenoxyacetic acid (2,4-D) at 0.2-0.4 mg/l was added in addition to NAA. Sucrose or glucose have been the most frequently used sugars as the energy source, and mannitol or sorbitol have generally been used to adjust the osmolarity of culture media (Shepard and Totten, 1977; Schumann and Koblitz, 1983; Shahin, 1984; Haberlach et al., 1985; Uijtewaal et al., 1987b). It has been reported that culture media enriched with various sugars and sugar alcohols improved protoplast plating efficiency (Foulger and Jones, 1986).

Shoot regeneration from p-calli requires media with a higher ratio of cytokinin to auxin, or without auxin (Shepard, 1982a; Shahin, 1984; Taylor and Secor, 1988). Low concentrations of sucrose (1-5 g/l), low light intensity (about 30 $\mu$E $m^{-2}$ $s^{-1}$), and lower temperature (approximately 20°C) have been found to promote shoot regeneration (Shepard, 1982a; Lillo, 1989).

Successful results have been dependent primarily upon plant genotype, growth conditions and physiological status of the source plants, and control of cryptic contamination (Shepard and Totten, 1977; Thomas, 1981; Shepard, 1982b; Haberlach et al., 1985; Debnath et al., 1986; Foulger and Jones, 1986; Radke and Grun, 1986; Tavazza and Ancora, 1986; Masson et al., 1987).

Phenotypic variation has been frequently reported among plants regenerated from protoplasts (Karp et al., 1989). Secor and Shepard (1981) observed significant variation for growth habit, photoperiod response, maturation date, disease response, tuber shape, size, and skin color among more than one thousand protoclones of the cultivar Russet Burbank grown in the field. Phenotypic variation has been reported with protoclones from other potato cultivars and dihaploid breeding stocks (Thomas et al., 1982; Ramulu et al., 1983, 1986; Thomson et al., 1986). Numerical and structural changes of chromosomes have often been associated with this phenotypic variation (Karp et al., 1982; Creissen and Karp, 1985; Gill et al., 1986, 1987; Nelson et al., 1986; Ramulu et al., 1986, 1989). Most of these variants have been either useless or undesirable (Karp et al., 1989) but, in a few cases, useful characters such as resistance to phytotoxins (Matern et al., 1978), earlier maturation date, and more desirable tuber characteristics (Shepard et al., 1980) have been reported.

## *Somatic hybridization and cybridization in potato breeding*

Protoplast fusion has various applications to potato breeding. In analytic breeding, somatic hybridization through protoplast fusion has been recommended as the route for returning to tetraploid level, because many dihaploid potato plants suffer from reduced fertility. Even though some dihaploids are fertile, production of unreduced gametes sufficient for sexual polyploidization is limited to only a few selected clones (Howard, 1970; Wenzel et al., 1982; Deimling et al., 1988). Plants have been regenerated from fusion between dihaploid potato clones or between a dihaploid and a diploid potato species (Austin et al., 1985a; Hein and Schieder, 1986; Puite et al., 1986; Debnath and Wenzel, 1987; Deimling et al., 1988; Waara et al., 1989). The somatic hybrids manifested hybrid vigor for both growth rate and tuber yield (Austin et al., 1985a; Debnath and Wenzel, 1987; Deimling et al., 1988).

Another use of protoplast fusion in potato breeding is to introduce disease resistance from distantly related *Solanum* species. Through somatic hybridization, resistances to potato leafroll virus, potato virus Y, and late blight have been incorporated into breeding stocks from *S. brevidens* which is a non-tuber-bearing *Solanum* species and is sexually incompatible with *S. tuberosum* (Austin et al., 1985b; Helgeson et al., 1986; Fish et al., 1988; Gibson et al., 1988). The maternally inherited resistance to the herbicide atrazine has been introduced into *S.*

*tuberosum* from a biotype of *S. nigrum* by interspecific protoplast fusion and plant regeneration (Binding et al., 1982).

'Monoploid sieve' has been proposed as a means to eliminate deleterious genes from cross-pollinated diploid species within a single generation. By combining two distantly related monoploids, hybrid vigor should occur in the resulting diploid (Wenzel et al., 1979; Uijtewaal and Hermsen, 1986). Monoploids ($2n = x = 12$) have been produced from diploid potato clones ($2n = 2x = 24$) either by parthenogenesis (Uijtewaal et al., 1987a) or by anther culture (Veilleux et al., 1985). Protoplast fusion can be used to advantage for this purpose because doubled monoploids of potato are often male sterile. Protoplast fusion between monoploid potato clones has been achieved, but only tetraploid and triploid somatic hybrids could be recovered due to ploidy instability of the monoploids (Uijtewaal et al., 1987b).

## *Genetic control over in vitro growth and development*

Although plant cells are considered totipotent, that is, capable of regenerating whole plants from single cells, there are great differences in the capability for protoplast regeneration among plants tested. Generally, solanaceous plants have been more amenable to protoplast culture than monocots and legumes (Bhojwani and Razdan, 1983; Evans and Bravo, 1983; Webb, 1988). Recent progress in a

few of the recalcitrant species has been limited to suspension or callus derived protoplasts (Davey and Power, 1988; Myers et al., 1989; Shillito et al., 1989; Wang et al., 1989).

Even among solanaceous plants, intrageneric and intraspecific variation in the capability for protoplast regeneration have been demonstrated (Izhar and Power, 1977; Shepard, 1982a; Haberlach et al., 1985; Debnath et al., 1986; Foulger and Jones, 1986; Koornneef et al., 1986, 1987; Radke and Grun, 1986). In Haberlach's studies of protoplast culture characters among six potato species including 36 clones, three clones could not divide; among clones capable of division, four could not undergo sustained cell division to form p-calli, and still others, which formed p-calli, were unable to regenerate shoots from these calli (Haberlach et al., 1985). A study of genetic control over p-callus development was carried out in *Petunia* (Izhar and Power, 1977). Five phenotypes were identified according to differences in their progress toward p-callus development. These phenotypes were the result of interactions between genotypes and plant growth regulators in the culture media. Protoplasts from some lines were capable of p-callus formation in media supplemented with NAA, whereas protoplasts from other lines required 2,4-D for p-callus formation. Still others did not respond to either NAA or 2,4-D. Using the data from $F_1$ and backcross progenies, the authors concluded that auxin-specificity was under the control of a few nuclear genes, the phenotypes corresponding to attainment of different develop-

mental stages in culture were controlled by different genes, and intergenic complementation could result in formation of genotypes with no auxin-specificity.

Genetic control over *in vitro* growth and development has been more frequently reported in other *in vitro* culture systyms with various species, e.g., callus induction from embryos in maize, wheat and rice (Sears and Deckard, 1982; Nesticky et al., 1983; Tomes and Smith, 1985; Abe and Futsuhara, 1986), callus growth rate in maize, wheat, and tomato (Nesticky et al., 1983; Koornneef et al., 1987; Kaleikau et al., 1989), somatic embryogenesis from callus in maize and alfalfa (Brown and Atanassov, 1985; Hodges et al., 1986; Willman et al., 1989), organogenesis on cultured callus in alfalfa, wheat, rice, maize, cucumber, and tomato (Reisch and Bingham, 1980; Sears and Deckard, 1982; Abe and Futsuhara, 1986; Koornneef et al., 1987; Nadolska-Orczyk and Malepszy, 1989; Peng and Hodges, 1989; Willman et al., 1989), androgenesis in tobacco, potato, barley, maize and wheat (Jacobson and Sopory, 1978; Foroughi-Wehr et al., 1982; Subhashini and Venkateswarlu, 1985; Deaton et al., 1987; Petolino and Thompson, 1987; Singsit and Veilleux, 1989; Sonnino et al., 1989). Several different models have been proposed to describe genetic control over *in vitro* growth and development. In alfalfa, bud formation from callus was found to be under the control of two dominant genes (Reisch and Bingham, 1980). In tomato, shoot regeneration from leaf explants was also found to be controlled by two dominant genes. (Koornneef et al., 1987). The genetic model proposed by Kaleikau et al., (1989) for tissue culture response (TCR) in wheat was more complicated, with

TCR controlled by one major and two minor genes; the expression of these three genes was proposed to be regulated by another major gene. Using ditelosomic and nullisomic-tetrasomic wheat lines, the chromosomal locations of these genes were determined. In cucumber, interaction among genes from three loci was proposed as the cause for differences in regeneration of embryoidal callus and plantlets from leaf explants (Nadolska-Orczyk and Malepszy, 1989). Cytoplasmic effect on *in vitro* growth and development also has been observed. Peng and Hodges (1989) reported that both nuclear and cytoplasmic factors were important in controlling plant regeneration from immature embryo-derived callus in rice. Cytoplasmic effect was also observed in androgenesis in wheat (Bullock et al., 1982), barley (Foroughi-Wehr et al., 1982), triticale (Charmet and Bernard, 1984), and potato (Singsit and Veilleux, 1989).

There is a tendency for different *in vitro* characters to be independently inherited. The frequency of callus or embryo-regenerating anthers and the frequency of plant-regenerating callus were found to be separately controlled in barley and wheat (Foroughi-Wehr et al., 1982; Lazar et al., 1984; Agache et al., 1988). No correlation was observed between plant regeneration and callus growth rate in *Zea mays* and tomato (Duncan et al., 1985; Koornneef et al., 1987). In potato, no significant correlation coefficients were found between plant regeneration from p-calli and protoplast plating efficiency or p-callus formation (Debnath et al., 1986). On the other hand, correlation between plant regeneration and induction

of embryoidal callus from young leaf explants was recently reported in cucumber (Nadolska-Orczyk and Malepszy, 1989).

## *Male sterility in potato*

Male sterility is common within the cultivated tetraploid potato (*S. tuberosum* ssp. *tuberosum*) and, as a result, many desirable crosses cannot be obtained (Mullin and Lauer, 1966; Howard, 1970; Staub et al., 1982; Deimling et al., 1988). Hybrids between cultivars and other potato species, such as *S. phureja, S. stenotomum, S. vernei*, and *S. tuberosum* ssp. *andigena*, display various phenotypic abnormalities associated with male sterility, especially when the cultivars have been used as female parents (Grun, 1974, 1979; Sanford and Hanneman, 1979; Hanneman and Peloquin, 1981). These phenotypic abnormalities include: indehiscence of anthers, arrested sporads in meiosis, shrivelled microspores, thin anthers, and anther-style fusion (Grun, 1979). It has been proposed that male sterilities in the cultivated potato are due to nuclear-cytoplasmic interactions, and that *S. tuberosum* ssp. *tuberosum* has a cytoplasm sensitive to dominant nuclear genes specifying various phenotypes of male sterility, whereas *S. tuberosum* ssp. *andigena, S. stenotomum, S. vernei* and *S. phureja* possess non-sensitive cytoplasms (Grun, 1979).

Based on chloroplast DNA restriction patterns, the cytoplasms of cultivated potato and sixteen wild potato species have been classified into seven different types and the cytoplasm of *S. tuberosum* ssp. *tuberosum* was indeed found to be unique (Hosaka, 1986).

Some cultivars of *S. tuberosum* ssp. *tuberosum* are male fertile. Their male fertility was attributed to the presence of male fertility restorer genes in their nuclear genomes (Hanneman and Peloquin, 1981; Ortiz et al., 1989). Reciprocal crosses between these male fertile cultivars and *S. tuberosum* ssp. *andigena* have demonstrated that male fertile cultivars still have sensitive cytoplasm (Staub et al., 1982).

The multiple phenotypes for male sterility in *S. tuberosum* cultivars and their general responsiveness to protoplast culture and regeneration make this crop a particularly interesting target for cytoplasmic engineering using protoplast fusion. By substituting the sensitive cytoplasm with non-sensitive via somatic cybridization, it may be possible to improve the fertility of male sterile cultivars without altering their nuclear composition.

# *References*


Abe, T., and Y. Futsuhara, 1986. Genotypic variability for callus formation and plant regeneration in rice (*Oryza sativa* L.). Theor. Appl. Genet. 72:3-10.

Agache, S., J. D. Buyser, Y. Henry, and J. W. Snape, 1988. Studies of the genetic relationship between anther culture and somatic tissue culture abilities in wheat. Plant Breeding 100:26-33.

Austin, S., M. A. Baer, M. Ehlenfeldt, P. J. Kazmierczak, and J. P. Helgeson, 1985a. Intra-specific fusion in *Solanum tuberosum.* Theor. Appl. Genet. 71:172-175.

Austin, S., M. A. Baer, J. P. Helgeson, 1985b. Transfer of resistance to potato leaf roll virus from *Solanum brevidens* into *Solanum tuberosum* by somatic fusion. Plant Sci. 39:75-82.

Bhojwani, S. S., and M. K. Razdan, 1983. Plant tissue culture: theory and practice. Elsevier, pp. 237-260.

Binding, H., and R. Nehls, 1977. Regeneration of isolated protoplasts to plants in *Solanum dulcamara* L. Z. Pflanzenphysiol. 85:279-280.

Binding, H., R. Nehls, O. Schieder, S. R. Sopory, and G. Wenzel, 1978. Regeneration of mesophyll protoplasts isolated from dihaploid clones of *Solanum tuberosum.* Physiol. Plant. 43:52-54.

Binding, H., S. M. Finger, G. Mordhorst, R. Nehls, and J. Gressel, 1982. Somatic hybridization of an atrazine biotype of *Solanum nigrum* with *Solanum tuberosum.* Theor. Appl. Genet. 63:273-277.

Bokelmann, G. S., and S. Roest, 1983. Plant regeneration from protoplasts of potato (*Solanum tuberosum cv.* Bintje). Z. Pflanzenphysiol. 109:259-263.

Brown, D. C. J., and A. Atanassov, 1985. Role of genetic background in somatic embryogenesis in *Medicago.* Plant Cell Tissue Organ Cult. 4:111-122.

Bullock, W. P., P. S. Baenziger, G. W. Schaeffer, and P. J. Bottino, 1982. Anther culture of wheat (*Triticum aestivum* L.) $F_1$'s and their reciprocal crosses. Theor. Appl. Genet. 62:155-159.

Charmet, G., and S. Bernard, 1984. Diallel analysis of androgenetic plant production in hexoploid triticale (*X. triticosecale*, Wittmack). Theor. Appl. Genet. 69:55-61.

Cocking, E. C., and M. R. Davey, 1987. Gene transfer in cereals. Science 236:1259-1262.

Creissen G. P., and A. Karp, 1985. Karyotipic changes in potato plants regenerated from protoplasts. Plant Cell Tissue Organ Cult. 4:171-182.

Dai, C., D. Mertz, and V. Lambeth, 1987. Improved procedures for the isolation and culture of potato protoplasts. Plant Sci. 50:79-84.

Davey, M. R., and J. B. Power, 1988. Aspects of protoplast culture and plant regeneration. Plant Cell Tissue Organ cult. 12:115-125.

Deaton, W. R., S. G. Metz, T. A. Armstrong, and P. N. Mascia, 1987. Genetic analysis of the anther-culture response of three spring wheat crosses. Theor. Appl. Genet. 74:334-338.

Debnath, S. C., R. Schuchmann, and G. Wenzel, 1986. Regeneration capacity of protoplasts isolated from single cell derived donor plants. Acta Bot. Neerl. 35:233-241.

Debnath, S. C., and G. Wenzel, 1987. Selection of somatic fusion products in potato by hybrid vigor. Potato Res. 30:371-380.

Deimling, S., J. Zitzisperger, and G. Wenzel, 1988. Somatic fusion for breeding of tetraploid potatoes. Plant Breeding 101:181-189.

Duncan, D. R., M. E. Williams, B. E. Zehr, J. M. Widholm, 1985. The production of callus capable of plant regeneration from immature embryos of numerous *Zea mays* genotypes. Planta 165:322-332.

De Vries, S. E., G. S. Bokelmann, 1986. Regeneration of callus and plants from cell suspension protoplasts of diploid potato (*Solanum tuberosum* L.). J. Plant Physiol. 122:199-203.

Evans, D. A., and J. E. Bravo, 1983. Protoplast isolation and culture. *In*: Evans, D. A., W. R. Sharp, P. V. Ammirato, and Y. Yamada (eds.) Handbook of plant cell culture Vol. 1, Macmillan Publishing Co., New York, pp 124-165.

Fish, N., S. H. Steele, and M. G. K. Jones, 1988. Field assessment of dihaploid *Solanum tuberosum* and *S. brevidens* somatic hybrids. Theor. Appl. Genet. 76:880-886.

Foulger, D., and M. G. K. Jones, 1986. Improved efficiency of genotype-dependant regeneration from protoplasts of important potato cultivars. Plant Cell Rep 5:72-76.

Foroughi-Wehr, B., W. Friedt, G. Wenzel, 1982. On the genetic improvement of androgenetic haploid formation in *Hordeum vulgare* L. Theor. Appl. Genet. 62:233-239.

Galun, E., and D. Aviv, 1983. Cytoplasmic hybridization: Genetic and breeding applications. *In*: D. A. Evans, W. R. Sharp, P. V. Ammirato, and Y. Yamada (eds) Handbook of plant cell culture, Volume 1. Macmillan Publishing Co. New York, pp 358-392.

Gibson, R. W., M. G. K. Jones, N. Fish, 1988. Resistance to potato leaf roll virus and potato virus Y in somatic hybrids between dihaploid *Solanum tuberosum* and *S. brevidens*. Theor. Appl. Genet. 76:113-117.

Gill, B. S., L. N. W. Kam-Morgan, and J. F. Shepard, 1986. Origin of chromosomal and phenotypic variation in potato protoclones. J. Hered. 77:13-16.

Gill, B. S., L. N. W. Kam-Morgan, and J. F. Shepard, 1987. Cytogenetic and phenotypic variation in mesophyll cell-derived tetraploid potatoes. J. Hered. 78:15-20.

Glimelius, K., 1988. Potentials of protoplast fusion in plant breeding programs. Plant Cell Tissue Organ Cult. 12:163-172.

Grun, P., 1974. Cytoplasmic sterilities that separate the Group *Tuberosum* cultivated potato from its putative tetraploid ancestor. Evolution 27:633-643.

Grun, P., 1979. Evolution of the cultivated potato: a cytoplasmic analysis. *In*: Hawkes, J. G., R. N. Lester, and A. D. Skelding (eds.), The biology and taxonomy of the *Solanaceae*. Linnean Society Symposium No 7, Academic Press, London, pp 655-665.

Gunn, R. E., and J. F. Shepard, 1981. Regeneration of plants from mesophyll-derived protoplasts of British potato (*Solanum tuberosum* L.) cultivars. Plant Sci. Lett. 22:97-101.

Haberlach, G. T., B. A. Cohen, N. T. Reichert, M. A. Baer, L. E. Towill, and J. Helgeson, 1985. Isolation, culture, and regeneration of protoplasts from potato and several related *Solanum* species. Plant Sci. 39:67-74.

Hanneman, R. E.Jr., and S. J. Peloquin, 1981. Genetic-cytoplasmic male sterility in progeny of 4*x*-2*x* crosses in cultivated potatoes. Theor. Appl. Genet. 59:53-55.

Hein, T., and O. Schieder, 1986. An improved culture method of mechanically isolated heterokaryons of potato. Plant Breeding 97:255-260.

Helgeson, J. P., G. J. Hunt, G. T. Haberlach, and S. Austin, 1986. Somatic hybrids between *Solanum brevidens* and *Solanum tuberosum*: expression of a late blight resistance gene and potato leaf roll resistance. Plant Cell Rep. 3:212-214.

Hodges, T. K., K. K. Kamo, C. W. Imbrie, and M. R. Becwar, 1986. Genotype specificity of somatic embryogenesis and regeneration in maize. Bio/Technology 4:219-223.

Hosaka, K., 1986. Who is the mother of the potato? Restriction endonuclease analysis of chloroplast DNA of cultivated potatoes. Theor. Appl. Genet. 72:606-618.

Howard, H. W., 1970. The genetics of potato. Springer-Verlag, New York Inc. pp 20-23.

Izhar S., and J. B. Power, 1977. Genetical studies with *Petunia* leaf protoplasts. I. Genetic variation to specific growth hormones and possible genetic control on stages of protoplast development in culture. Plant Sci. Lett. 8:375-383.

Jacobsen, E., and S. K. Sopory, 1978. The influence and possible recombination of genotypes on the production of microspore embryoids in anther culture of *Solanum tuberosum* and dihaploid hybrids. Theor. Appl. Genet. 52:119-123.

Kaleikau E. K., R. G. Sears, and B. S. Gill, 1989. Control of tissue culture response in wheat (*Triticum aestivum* L.). Theor. Appl. Genet. 78:783-787.

Karp, A., M. G. K. Jones, D. Foulger, N. Fish, and S. W. J. Bright, 1989. Variability in potato tissue culture. Am. Potato J. 66:669-684

Karp, A., R. S. Nelson, E. Thomas, and S. W. J. Bright, 1982. Chromosome variation in protoplast-derived potato plants. Theor. Appl. Genet. 63:265-272.

Koornneef, M., C. J. Hanhart, M. Jongsma, I. Toma, R. Weide, P. Zabel, and J. Hille, 1986. Breeding of a tomato genotype readily accessible to genetic manipulation. Plant Sci. 45:201-208.

Koornneef, M., C. J. Hanhart, and L. Martinelli, 1987. Genetic analysis of cell culture traits in tomato. Theor. Appl. Genet. 74:633-641.

Lazar M. D., P. S. Baenziger, and G. W. Schaeffer, 1984. Combining abilities and heritability of callus formation and plantlet regeneration in wheat (*Triticum aestivum* L.) anther cultures. Theor. Appl. Genet. 68:131-134.

Lillo, C., 1989. Effects of media components and environmental factors on shoot formation from protoplast-derived calli of *Solanum tuberosum*. Plant Cell Tissue Organ Cult. 19:103-111.

Masson, J., M. Lecerf, P. Rousselle, P. Perennec, and G. Pelletier, 1987. Plant regeneration from protoplasts of diploid potato derived from crosses of *Solanum tuberosum* with wild *Solanum* species. Plant Sci. 53:167-176.

Matern, U., G. Strobel, and J. Shepard, 1978. Reaction to phytotoxins in a potato population derived from mesophyll protoplasts. Proc. Natl. Acad. Sci. 75:4935-4939.

Mullin, R., and F. I. Lauer, 1966. Breeding behavior of $F_1$ inbred potato clones. Proc. Am. Soc. Hort. Sci. 89:449-455.

Myers, J. R., P. A. Lazzeri, and G. B. Collins, 1989. Plant regeneration of wild *Glycine* species from suspension culture-derived protoplasts. Plant Cell Rep. 8:112-115.

Nadolska-Orczyk A., and S. Malepszy, 1989. In vitro culture of *Cucumis sativus* L. 7. Genes controlling plant regeneration. Theor. Appl. Genet. 78:836-840.

Nelson, R. S., G. P. Creissen, and S. W. J. Bright, 1983. Plant regeneration from protoplasts of *Solanum brevidens*. Plant Sci. Lett. 30:355-362.

Nelson, R. S., A. Karp, and S. W. J. Bright, 1986. Ploidy variation in *Solanum brevidens* plants regenerated from protoplasts using an improved culture system. J. Exp. Bot. 37:253-261.

Nesticky, M., F. J. Novak, A. Piovarci, M. Dolezelova, 1983. Genetic analysis of callus growth of maize (*Zea mays* L.) in vitro. Z. Pflanzenzuchtg. 91:322-328.

Ortiz, R., M. Iwanaga, and S. J. Peloquin, 1989. Genetic-cytoplasmic male sterility and $2n$ pollen in $4x$-$2x$ and $4x$-$4x$ progenies. Am. Potato J. 66:

Peng J., and T. K. Hodges, 1989. Genetic analysis of plant regeneration in rice (*Oryza sativa* L.). In Vitro Cell & Develop. Biol. 25:91-94.

Perl, A., D. Aviv, and E. Galun, 1988. Ethylene and in vitro culture of potato: suppression of ethylene generation vastly improves protoplast yield, plating efficiency and transient expression of an alien gene. Plant Cell Rep. 7:403-406

Petolino, J. F., and S. A. Thompson, 1987. Genetic analysis of anther culture response in maize. Theor. Appl. Genet. 74:284-286.

Potrykus, I., R. D. Shillito, M. W. Saul, and J. Paszkowski, 1985. Direct gene transfer: state of the art and future potential. Plant Mol. Biol. Rep. pp. 117-128.

Puite, K. J., S. Roest, and L. P. Pijnacker, 1986. Somatic hybrid potato plants after electrofusion of diploid *Solanum tuberosum* and *Solanum phureja*. Plant Cell Rep. 5:262-265.

Radke, S., and P. Grun, 1986. Isolation, culture, and regeneration of leaf mesophyll protoplasts of selected clones of *Solanum*. Potato Res. 29:451-462.

Ramulu, K. S., P. Dijkhuis, and S. Roest, 1983. Phenotypic variation and ploidy level of plants regenerated from protoplasts of tetraploid potato (*Solanum tuberosum* L. cv. Bintje). Theor. Appl. Genet. 65:329-338.

Ramulu, K. S., P. Dijkhuis, and S. Roest, 1989. Patterns of phenotypic and chromosome variation in plants derived from protoplast cultures of monoploid, dihaploid and diploid genotypes and in somatic hybrids of potato. Plant Sci. 60:101-110.

Ramulu, K. S., P. Dijkhuis, S. Roest, G. S. Bokelman, and B. De Groot, 1986. Variation in phenotype and chromosome number of plants regenerated from protoplasts of dihaploid and tetraploid potato. Plant Breeding 97:119-128.

Reisch B., and E. T. Bingham, 1980. The genetic control of bud formation from callus cultures of diploid alfalfa. Plant Sci. Lett. 20:71-77.

Sanford, J. C., and R. E. Hanneman, Jr., 1979. Reciprocal differences in the photoperiod reaction of hybrid populations of *Solanum tuberosum*. Am. Potato J. 56:531-540.

Schumann, U., and H. Koblitz, 1983. Studies on plant recovery from mesophyll protoplasts of *Solanum tuberosum* L. and *Solanum phureja* Juz. and Buk. Biologia Plant. 25:180-186.

Schumann, U., H. Koblitz, Z. Opatrny, 1980. Plant recovery from long term callus cultures and from suspension culture-derived protoplasts of *Solanum phureja*. Biochem. Physiol. Pflanz. 175:670-675

Sears, R. G., E. L. Deckard, 1982. Tissue culture variability in wheat: callus induction and plant regeneration. Crop Sci. 22:546-550.

Secor, G., and J. F. Shepard, 1981. Variability of protoplast-derived potato clones. Crop Sci. 21:102-105.

Shahin, E. A., 1984. Isolation, culture, and regeneration of potato leaf protoplasts from plants preconditioned *in vitro*. *In*: I. K. Vasil (ed.) Cell culture and somatic cell genetics of plants, Vol. 1., Academic Press, Inc., pp. 381-390.

Shepard, J. E., 1980. Mutant selection and plant regeneration from potato mesophyll protoplasts. *In*: Genetic improvement of crops: emergent techniques. I. Rubenstein, B. Gengenbach, R. L. Phillips, and C. E. Greens (eds.). Univ. of Minnesota Press, Minneapolis, pp. 185-219.

Shepard, J. F., 1982a. Cultivar dependant cultural refinements in potato protoplast regeneration. Plant Sci. Lett. 26:127-132.

Shepard, J. F., 1982b. Regeneration of potato leaf cell protoplasts. *In*: E. D. Earle and Y. Demarly (eds.), Variability in plants regenerated from tissue culture. Praeger Scientific, New York, pp. 47-57.

Shepard, J. F., and R. E. Totten, 1977. Mesophyll cell protoplasts of potato. Plant Physiol. 60:313-316.

Shepard, J. F., D. Bidney, and E. Shahin, 1980. Potato protoplasts in crop improvement. Science 208:17-24.

Shillito, R. D., G. K. Carswell, C. M. Johnson, J. DiMaio, and C. T. Harms, 1989. Regeneration of fertile plants from protoplasts of elite inbred maize. Bio/Technology 7:581-587.

Singsit, C., and R. E. Veilleux, 1989. Intra- and interspecific transmission of androgenetic competence in diploid potato species. Euphytica 43:105-112.

Sonnino, A., S. Tanaka, M. Iwanaga, and L. Schilde-Rentschler, 1989. Genetic control of embryo formation in anther culture of diploid potatoes. Plant Cell Rep. 8:105-107.

Staub, J. E., P. Grun, and V. Amoah, 1982. Cytoplasmic evaluations during substitution backcrossing in *Solanum*. Potato Res. 25:299-319.

Subhashini, U., and T. Venkateswarlu, 1985. Genotypes and their response to in vitro production of haploids in lines of *Nicotiana tabacum*. Theor. Appl. Genet. 70:225-226.

Tavazza, R. T., and G. Ancora, 1986. Plant regeneration from mesophyll protoplasts in commercial potato cultivars (Primura, Kennebec, Spunta, Desiree). Plant Cell Rep. 5:243-246.

Taylor, R. J., and G. A. Secor, 1988. A shoot induction procedure altered for increased shooting efficiency of potato protoplast-derived calli (cvs. Crystal and Norgold Russet). Potato Res. 31:651-657.

Thomas, E., 1981. Plant regeneration from shoot-culture derived protoplasts of tetraploid potato (*Solanum tuberosum cv.* Maris Bard). Plant Sci. Lett. 23:84-88.

Thomas, E., S. W. J. Bright, J. Franklin, V. Lancaster, B. J. Miflin, and R. Gibson, 1982. Variation amongst protoplast-derived potato plants (*Solanum tuberosum* cv. Maris Bard). Theor. Appl. Genet. 62:65-68.

Thomson, A. J., R. E. Gunn, G. J. Jellis, R. E. Boulton, and C. N. D. Lacey, 1986. The evaluation of potato somaclones. *In*: J. Semal (ed), Somaclonal variation and crop improvement. Martinus Nijhoff Publishers, Dordrecht / Boston / Lancaster, pp 236-243.

Tomes, D. T., O. S. Smith, 1985. The effect of parental genotype on initiation of embryogenetic callus from elite maize (*Zea mays* L.) germplasm. Theor. Appl. Genet. 70:505-509.

Uijtewaal, B. A., and J. G. Th. Hermsen, 1986. Monohaploids in potato: pseudogamic induction and potential use in genetic and breeding research. *In*: Beekman, A. G. B., K. M. Louwes, L.M.W. Dellaert, and A. E. F. Neele (eds), Potato research for tomorrow. Pudoc, Wagneningen, pp 143-146.

Uijtewaal, B. A., E. Posthumus, L. C. J. M. Suurs, E. Jacobsen, and J. G. Th. Hermsen, 1987a. Relative performance of monohaploid potato clones and their diploid parents at plant level and after protoplast isolation and subsequent fusion. Theor. Appl. Genet. 74:451-458.

Uijtewaal, B. A., L. C. J. M. Suurs, and E. Jacobsen, 1987b. Protoplast fusion of monohaploid ($2n = x = 12$) potato clones: identification of somatic hybrids using malate dehydrogenase as a biochemical marker. Plant Sci. 51:277-284

Veilleux, R. E., J. Booze-Daniels, and E. Pehu, 1985. Anther culture of a $2n$ pollen producing clone of *Solanum phureja* Juz. and Buk. Can. J. Genet. Cytol. 27:559-564.

Waara, S., H. Tegelstrom, A. Wallin, and T. Eriksson, 1989. Somatic hybridization between anther-derived dihaploid clones of potato (*Solanum tuberosum* L.) and the identification of hybrid plants by isozyme analysis. Theor. Appl. Genet. 77:49-56.

Wang, D., P. D. Miller, and M. R. Sondahl, 1989. Plant regeneration from protoplasts of Indica type rice and CMS rice. Plant Cell Rep. 8:329-332.

Webb K. J., 1988. Recent development in the regeneration of agronomically important crops from protoplasts. Plant Cell Tissue Organ Cult. 12:127-131.

Wenzel, G., C. Meyer, T. Przewozny, H. Uhrig, and O. Schieder, 1982. Incorporation of microspore and protoplast techniques into potato breeding programs. *In*: E. D. Earle and Y. Demarly (eds.), Variability in plants regenerated from tissue culture. Praeger Publications, pp. 290-302.

Wenzel, G., O. Schieder, T. Przewozny, S. K. Sopory, and G. Melchers, 1979. Comparison of single cell culture derived *Solanum tuberosum* L. plants and a model for their application in breeding programs. Theor. Appl. Genet. 55:49-55.

Willman M. R., S. M. Schroll, and T. K. Hodges, 1989. Inheritance of somatic embryogenesis and plantlet regeneration from primary (type 1) callus in maize. In Vitro Cell & Develop Biol. 25:95-100.

# Chapter 3: Genetic analysis of protoplast culturability in Solanum phureja

## *Summary*

Protoplast culturability, defined as the ability to develop calli from cultured protoplasts, was studied in the diploid potato species *Solanum phureja*. A non-culturable, androgenetically derived homozygous line, AM3-8, was crossed with a culturable selection, NBP2. All seven $F_1$ plants tested were capable of forming abundant p-calli. $F_2$ progeny was produced by crossing two randomly selected $F_1$ individuals ($F_1$21 × $F_1$37). Segregation for protoplast culturability occurred in the $F_2$ generation. Among twenty $F_2$ plants repeatedly tested, twelve were culturable, and eight non-culturable. Backcrossing $F_1$37 to both AM3-8 and NBP2 generated BCA and BCN populations. Eight plants from each backcross were studied. All eight plants from the backcross to NBP2 were culturable,

whereas only two plants from the backcross to AM3-8 were culturable. In addition, quantitative variation for protoplast plating efficiency among culturable genotypes was observed. Based upon chi-square analyses for segregation in the $F_2$ as well as upon the data from $F_1$ and the backcrosses, it was proposed that protoplast culturability in this potato species is controlled by two independent loci with complete dominance. The two loci were designated as *pcd-1* and *pcd-2* for *p*-*c*allus *d*evelopment 1 and 2, respectively. Variation in plating efficiency among culturable genotypes was discussed.

## *Introduction*

Protoplast culture and regeneration are useful tools for various genetic manipulations, such as somatic hybridization (Glimelius 1988), cybridization (Galun and Aviv 1983), direct gene transfer (Cocking and Davey 1987), and development of novel characters via somaclonal variation (Karp et al. 1989). Nevertheless, protoplast culture is an artificial system which imposes various stresses upon the cultured protoplasts, and later cells. There is great variation in response to protoplast culture among higher plants. Generally, solanaceous plants are amenable to protoplast culture, whereas monocots have been found to be recalcitrant to this *in vitro* system (Bhojwani and Razdan 1983; Evans and Bravo 1983; Webb 1988).

Even among solanaceous plants, there is intrageneric and intraspecific variation in response to protoplast culture and regeneration (Izhar and Power 1977; Shepard 1982; Haberlach et al. 1985; Debnath et al. 1986; Foulger and Jones 1986; Radke and Grun 1986). In Haberlach's study on testing for protoplast culture characters involving six *Solanum* species and 36 clones, three clones could not divide; among clones which were capable of dividing, four could not undergo sustained cell division to form p-calli; still others, which could form p-calli, were unable to regenerate shoots (Haberlach et al. 1985). In *Petunia* protoplast culture, interactions between genotypes and growth regulators in the development of cell colonies were observed (Izhar and Power 1977). Protoplasts from some lines grew better in media containing α-naphthaleneacetic acid (NAA), whereas protoplasts from other lines preferred 2,4-dichlorophenoxyacetic acid (2,4-D). Some lines did not respond to either NAA or 2,4-D. Using the data from $F_1$ and backcross progenies, the authors suggested that auxin-specificity was under the control of a few nuclear genes. In our previous studies aimed at germplasm development using *in vitro* techniques, we encountered both responsive and non-responsive selections of *Solanum phureja* to protoplast culture. The non-responsive plants either could not divide or could divide but were unable to undergo sustained cell division to form p-calli under several established procedures.

An understanding of the genetic basis for response to protoplast culture can lead to more efficient application of protoplast technology (Ettinger-Paltin et al.

1984), and provide a means to introduce gene(s) for protoplast regeneration into recalcitrant genotypes (Izhar and Power 1977; Koornneef et al. 1986). The present paper reports the mode of inheritance for protoplast culturability, which is defined here as the ability to develop calli from cultured protoplasts, in the diploid potato species, *Solanum phureja.*

## *Materials and Methods*

AM3-8 is a doubled monoploid ($2n = 2x = 24$) derived from chromosome-doubling during shoot regeneration from leaf disc callus of an androgenetically developed monoploid, AM3 ($2n = x = 12$) (Veilleux et al. 1985). The anther donor, PP5, is a clone of adapted *Solanum phureja* (Haynes 1972) selected for *2n* pollen production and anther culture competence (Veilleux et al. 1985). PP5 and its androgenetic derivatives, AM3 and AM3-8, have been repeatedly tested in our preliminary studies for protoplast culturability and characterized as protoplast non-culturable. NBP2 (75-21), another selection of *S. phureja*, was obtained from Henry DeJong, Agriculture Canada, Frederickton, NB. It has been reported as protoplast culturable and plants were regenerated from protoplasts (Haberlach et al. 1985). $F_1$ plants were produced by crossing AM3-8 with NBP2, using AM3-8 as the female parent. $F_2$ plants were generated by crossing two individuals ($F_1$21 and $F_1$37) randomly selected from the $F_1$ population. Backcrossing of $F_1$37 to both AM3-8 and NBP2 yielded BCA and BCN backcross populations.

Their pedigree is illustrated in Fig. 1. Seven $F_1$ plants, twenty $F_2$ plants, eight BCA plants, and eight BCN plants were randomly selected and tested for protoplast culturability.

Botanical seeds were surface-sterilized in 80% ethanol for five seconds, washed in 30% commercial bleach (containing 5.25% sodium hypochlorite) for 20 minutes, and rinsed three times with sterilized distilled water. Sterilized seeds were planted on MS basal medium (Murashige and Skoog 1962) containing 20 g/l sucrose in 25 × 150 mm glass tubes. Selected seedlings were propagated by culturing nodal cuttings on MS basal medium supplemented with 30 g/l sucrose, 170 mg/l potassium phosphate (monobasic), 500 mg/l casein hydrolysate in 25 × 150 mm glass tubes. *In vitro* plants were maintained in a growth chamber with a temperature regime of 20°C day and 18°C night, and a 16-hour photoperiod. The average light intensity was 28 $\mu$E $m^{-2}$ $sec^{-1}$ provided by cool white fluorescent tubes. Prior to protoplast isolation, plants were placed in the dark for 48 hours.

Protoplast isolation was done using a modified procedure of Uijtewaal et al. (1987). In vitro shoots and leaves (0.5-1 g) from 3-week-old cultures were chopped into fine pieces and digested with 1% Cellulase R-10 "Onozuka" (Yakult Honsha Co., Ltd., Japan) and 0.1% Macerozyme R-10 (Yakult Honsha Co., Ltd., Japan) solution containing 0.4 M mannitol and 0.1 M glucose as osmotica. Digestion proceeded in the dark on a reciprocal shaker (60 rpm) for 16 hours. Upon digestion, the digests were diluted with an equal volume of 0.3 M KCl sol-

ution and sieved through a 63-$\mu$m screen. The filtrate was centrifuged at 50 × g for 5 minutes. Pellets were resuspended in 10 ml of 0.5 M sucrose solution, and then 1 ml of 0.3 M KCl solution was layered on the top of the protoplast suspension. After centrifuged at 50 × g for 10 minutes, intact protoplasts were collected at the interface of the sucrose and KCl solutions. Protoplasts were rinsed in 10 ml 0.3 M KCl and pelleted again. The pH of the isolation solutions was adjusted to 5.8 with 0.1 N KOH and HCl.

Purified protoplasts were cultured in 35 × 10 mm petri dishes using a modified protoplast culture medium of Schumann and Koblitz (1983) supplemented with 1.25 mg/l NAA, 0.25 mg/l 2,4-D, and 1 mg/l zeatin. Each dish contained 1 ml of culture with a protoplast density of $3 \times 10^5$. Two slightly different culture methods were applied: 1) liquid culture, in which protoplasts were cultured initially in liquid medium, and after 10 days embedded into culture medium solidified with 0.3% agarose (type VII, low gelling temperature, Sigma); 2) embedding culture, in which protoplasts were cultured initially in the culture medium solidified with 0.3% agarose. The cultures were incubated at 25°C in the dark for one week, then under cool white fluorescent light with an average light intensity of 16 $\mu$E $m^{-2}$ $sec^{-1}$. After cell colonies had formed, the agarose blocks with embedded cell colonies were transferred into a 60 × 15 mm petri dish containing 2 ml liquid culture medium. After another two to three weeks, these colonies were transferred onto solid culture medium containing 0.6% agarose for callus growth.

Plating efficiency was examined 7 days after culture initiation with a Zeiss inverted microscope. Callus formation was observed 30 to 60 days after culture initiation depending upon the genotype. Each genotype was tested in at least two independent experiments. For each experiment, a minimum of one culturable genotype was used as the control.

For shoot regeneration, p-calli approximately 3 mm in diameter were transferred onto shoot regeneration medium composed of MS salts and vitamins, 2 mg/l zeatin, 0.01 mg/l indoleacetic acid, 0.2 mg/l gibberellic acid ($GA_3$), 10 g/l sucrose, 36 g/l mannitol, 100 mg/l myo-inositol, 100 mg/l casein hydrolysate, 100 mg/l adenine sulfate, 100 mg/l glutamine, 100 mg/l potassium phosphate, and 7 g/l agar, at pH 5.8. Cultures were grown at 25°C under cool white fluorescent light with an average light intensity of 46 $\mu E\ m^{-2}\ sec^{-1}$ and a 16-hour day length. Shoot regeneration was attempted only on NBP2 and $F_1$ plants.

## *Results*

Most protoplasts from AM3-8 burst after 7 days in culture. A few remained intact and regenerated cell wall, but they never divided (Fig. 2). Protoplasts isolated from NBP2 started dividing after 3 days in culture (Fig. 3). The average protoplast plating efficiency was 25% when examined on the 7th day (Fig. 4).

Callus formation was rapid and abundant. After approximately thirty days in culture, calli visible to the naked eye were formed in large quantity (more than two hundred per plate, Fig. 5). P-calli from these protoplasts were fast-growing and highly organogenic with a shoot regeneration rate of 95%.

All seven $F_1$ plants tested were capable of p-callus formation. However, their plating efficiencies varied from 11% to 31% in liquid culture (Table 1). Shoot regeneration was achieved on four of five individuals tested (Fig. 6).

Segregation for protoplast culturability occurred in the $F_2$ generation (Table 2). Among the twenty $F_2$ individuals tested, four ($F_21$, $F_23$, $F_26$, and $F_223$) had protoplast performance similar to that of AM3-8. Protoplasts isolated from the remaining seventeen $F_2$ plants were capable of dividing. Among these individuals, four ($F_25$, $F_210$, $F_212$, and $F_213$) had very low plating efficiencies (1-2%) and could not undergo sustained cell divisions to form p-calli. Their cell division ceased after a few cell cycles, except that in one of the four experiments, $F_25$ and $F_213$ formed 6 and 11 p-calli respectively. Only twelve $F_2$ individuals consistently produced abundant p-calli (more than two hundred per culture plate). Plants with very low plating efficiencies (0-2%) and unable to form p-calli, were classified as the non-culturable phenotype, and those individuals with high plating efficiencies and capable of p-callus formation, were classified as the culturable phenotype. Like the $F_1$ plants, these culturable $F_2$ individuals also varied for plating efficiency (Table 2).

All plants from the backcross to NBP2 were culturable but their plating efficiency varied (Table 3). Seven plants had plating efficiencies (16-24%) comparable to that of NBP2, whereas one individual (BCN8) had a lower plating efficiency (9-10%). In the backcross to AM3-8, protoplasts from two individuals could not divide; protoplasts from another four individuals divided but could not undergo sustained cell division to form p-calli; only two plants (BCA8 and BCA9) were able to undergo sustained cell division and formed large amounts of p-calli. However, these two plants had plating efficiencies much lower than the culturable plants from other populations (Table 4).

The differences in plating efficiency between the two cultural methods was negligible. However, agarose embedding either at the initiation of protoplast culture or after 7 days was required for reproducible p-callus formation.

## *Discussion*

The data presented in this paper demonstrate the existence of genetic factors controlling protoplast culturability, i.e., the ability of cultured protoplasts to form calli, and the inheritance of this character in this diploid potato species. Lack of segregation for protoplast culturability among the $F_1$ and BCN plants indicates that the culturable is dominant over the non-culturable. The observed segre-

gation ratio (12 culturable vs. 8 non-culturable) in the $F_2$ generation suggests that the number of loci involved could be either one (the expected ratio thus would be 3:1, assuming complete dominance) or two (the expected ratio would be 9:7, assuming complete dominance at both loci and epistasis of either recessive homozygote to the effects of dominant alleles at the other locus). Although the one-locus hypothesis could not be unequivocally rejected (Table 5), the results of chi-square test for goodness of fit between the observed values and expected values favor the two-locus hypothesis (Table 6). The segregation data (2 out of 8 were culturable) from the backcross to the non-culturable parent, AM3-8, also support this hypothesis. Because the exact cellular and biochemical functions of these genes are not known, the two loci are tentatively designated as *pcd-1* and *pcd-2*, respectively, and the symbol *pcd* stands for p-callus development. Variation for plating efficiency among culturable individuals implies that additional minor loci influence this character. The results of this study are generally similar to those found in genetic studies of *Petunia* p-callus development (Izhar and Power, 1977), in which genotypes having requirements for specific types of auxin (either NAA or 2,4-D) were identified. Using inbred lines and their $F_1$ and backcrosses to both parents, it was concluded that only a few genes were involved, different stages of p-callus development were controlled by different genes, and intergenic complementation between the NAA type and the 2,4-D type could lead to a genotype with no auxin specificity.

Genetic studies on *in vitro* growth and development have been reported in various *in vitro* systems with several species (Reisch and Bingham 1980; Hodges et al. 1986; Deaton et al. 1987; Koornneef et al. 1987; Petolino and Thompson 1987; Kaleikau et al. 1989; Nadolska-Orczyk and Malepszy 1989; Peng and Hodges 1989; Willman et al. 1989), whereas genetic studies of p-callus development from protoplast culture have been infrequent (Izhar and Power 1977). One of the reasons for this is the technical limitation on the sample size that can be handled in each experiment. In the present study, twenty individuals representing the $F_2$ population were repeatedly tested for protoplast culturability. According to a widely accepted rule of thumb for application of chi-square tests, which requires that the smallest expected number be larger than five (Snyder et al. 1985), the proposed two-loci model has a sound statistical basis.

Diploid potato species are generally self-incompatible, and homozygous lines are not available conventionally (Cipar et al. 1964). The use of a doubled androgenic monoploid, AM3-8, as the non-culturable parent greatly reduced the complexities of this genetic analysis. Because AM3-8 is completely homozygous, variation in the $F_1$ generation reflected variation only in the culturable parent, NBP2, which is a selection from open pollination. NBP2 thus would be expected to be homozygous dominant at the two major loci, but heterozygous at some of the minor loci. The results that all individuals from the backcross to NBP2 were culturable and had variation for plating efficiency further supports this inference. Plating efficiencies of the two culturable individuals in the backcross to AM3-8

were low, indicating that the majority of the minor loci in AM3-8 have alleles with negative effect.

The fact that two $F_2$ individuals with a plating efficiency of 1% ($F_2$5 and $F_2$13) formed six and eleven p-calli, respectively, in one of the four independent experiments could be due to mutations at the *pcd* loci during culture. Protoplast culture has been reported to generate high frequencies of mutations (Secor and Shepard 1981; Karp et al. 1989). Culturable cells resulted from mutation at the key locus or loci are likely to be selected by protoplast culture itself.

It was reported that plants with an *in vitro* history had increased protoplast plating efficiencies and shoot regeneration (Glimelius 1984; Robertson et al. 1988). In the present study, the parental line, AM3-8, was derived from shoot regeneration in leaf disc culture of an androgenic monoploid; yet it had no protoplast culturability. This fact also indicates that the loci *pcd-1* and *pcd-2* identified in this study are not important for androgenesis in anther culture and organogenesis in leaf explant culture. A lack of correlation between different *in vitro* responses has been reported by several authors working on various plant species. In barley and wheat, the frequency of embryoid or callus-regenerating anthers and the frequency of plant-regenerating calli were reported to be unlinked (Foroughi-Wehr et al. 1982; Lazar et al. 1984; Agache et al. 1988). In maize and tomato, callus growth rate and plant regeneration from immature embryos or from leaf explants were not correlated (Duncan et al. 1985; Koornneef et al.

1987). In potato, non-significant correlation coefficients between plant regeneration from p-calli and protoplast division or number of p-calli per culture plate were obtained (Debnath et al. 1986). However, it was recently reported in cucumber that plant regeneration and induction of embryoidal calli were positively correlated (Nadolska-Orczyk and Malepszy 1989).

The genetic system described in this study is potentially useful for developing schemes for selecting somatic hybrids after protoplast fusion by genetic complementation. Based upon this genetic model, breeding for protoplast culturability in this species can also be achieved. Further studies on the molecular and biochemical aspects of these loci would lead to a better understanding of the process of p-callus development, which is poorly understood at the present time. The possibility of isolating genes for protoplast culturability and regenerability, and introducing them into crop species or cultivars, which currently cannot be reached by genetic engineering because of their lack of both protoplast culturability and compatibility with transformation by agrobacteria, would make them accessible to genetic manipulation via protoplasts.

## *References*


Agache S, De Buyser J, Henry Y, Snape JW (1988) Studies of genetic relationship between anther culture and somatic tissue culture abilities in wheat. Plant Breeding 100:26-33

Bhojwani SS, Razdan MK (1983) Plant tissue culture: theory and practice. Elsevier Amsterdam-Oxford-New York-Tokyo, pp 237-260

Cipar SS, Peloquin SJ, Hougas RW (1964) Inheritance of incompatibility in hybrids between *Solanum tuberosum* haploids and diploid species. Euphytica 13:163-172

Cocking EC, Davey MR (1987) Gene transfer in cereals. Science 236:1259-1262

Deaton WR, Metz SG, Armstrong TA, Mascia PN (1987) Genetic analysis of the anther-culture response of three spring wheat crosses. Theor Appl Genet 74:334-338

Debnath SC, Schuchmann R, Wenzel G (1986) Regeneration capacity of protoplasts isolated from single cell derived donor plants. Acta Bot Neerl 35:233-241.

Duncan DR, Williams ME, Zehr BE, Widholm JM (1985) The production of callus capable of plant regeneration from immature embryos of numerous *Zea mays* genotypes. Planta 165:322-332

Ettinger-Paltin R, Izhar S, Swartzberg D, Tabib Y (1984) Growth hormones as a selection tool for somatic hybridization in *Petunia*. Plant Sci Lett 35:231-235

Evans DA, Bravo JE (1983) Protoplast isolation and culture. *In*: Evans DA, Sharp WR, Ammirato PV, Yamada Y (eds) Handbook of plant cell culture Volume 1. Macmillan Publishing Co. New York, pp 124-165

Foroughi-Wehr B, Friedt W, Wenzel G (1982) On the genetic improvement of androgenetic haploid formation in *Hordeum vulgare* L. Theor Appl Genet 62:233-239

Foulger D, Jones MGK (1986) Improved efficiency of genotype-dependent regeneration from protoplasts of important potato cultivars. Plant Cell Rep 5:72-76

Galun E, Aviv D (1983) Cytoplasmic hybridization: Genetic and breeding applications. *In*: Evans DA, Sharp WR, Ammirato PV, Yamada Y (eds) Handbook of plant cell culture Volume 1. Macmillan Publishing Co. New York, pp 358-392

Glimelius K (1984) High growth rates and regeneration capacity of hypocotyl protoplasts in some Brassicaceae. Physiol Plant 61:38-44

Glimelius K (1988) Potentials of protoplast fusion in plant breeding programs. Plant Cell Tissue Organ Cult 12:163-172

Haberlach GT, Cohen BA, Reichert NA, Baer MA, Towill LE, Helgeson JP (1985) Isolation, culture and regeneration of protoplasts from potato and several related *Solanum* species. Plant Sci 39:67-74

Haynes FL (1972) The use of cultivated diploid *Solanum* species in potato breeding. *In*: French ER (ed) Prospects for the potato in the developing world: an international symposium on key problems and potentials for greater use of potato in the developing world. Lima, Peru. July 17-29. International Potato Center, Lima, Peru.

Hodges TK, Kamo KK, Imrie CW, Becwar MR (1986) Genotype specificity of somatic embryogenesis and regeneration in maize. Bio/Technology 4:219-223

Izhar S, Power JB (1977) Genetical studies with *Petunia* leaf protoplasts. I. Genetic variation to specific growth hormones and possible genetic control on stages of protoplast development in culture. Plant Sci Lett 8:375-383

Kaleikau EK, Sears RG, Gill BS (1989) Control of tissue culture response in wheat (*Triticum aestivum* L.). Theor Appl Genet 78:783-787

Karp A, Jones MGK, Foulger D, Fish N, Bright SWJ (1989) Variability in potato tissue culture. Am Potato J 66:669-684

Koornneef M, Hanhart C, Jongsma M, Toma I, Weide R, Zabel P, Hille J (1986) Breeding of a tomato genotype readily accessible to genetic manipulation. Plant Sci 45:201-208

Koornneef M, Hanhart CJ, Martinelli L (1987) A genetic analysis of cell culture traits in tomato. Theor Appl Genet 74:633-641

Lazar MD, Baenziger PS, Schaeffer GW (1984) Combining abilities and heritability of callus formation and plantlet regeneration in wheat (*Triticum aestivum* L.) anther cultures. Theor Appl Genet 68:131-134

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473-497

Nadolska-Orczyk A, Malepszy S (1989) In vitro culture of *Cucumis sativus* L. 7. Genes controlling plant regeneration. Theor Appl Genet 78:836-840

Peng J, Hodges TK (1989) Genetic analysis of plant regeneration in rice (*Oryza sativa* L.). In vitro Cell & Develop Biol 25:91-94

Petolino JF, Thompson SA (1987) Genetic analysis of anther culture response in maize. Theor Appl Genet 74:284-286

Radke S, Grun P (1986) Isolation, culture and regeneration of leaf mesophyll protoplasts of selected clones of *Solanum*. Potato Res 29:451-462

Reisch B, Bingham ET (1980) The genetic control of bud formation from callus cultures of diploid alfalfa. Plant Sci Lett 20:71-77

Robertson D, Earle ED, Mutschler MA (1988) Increased totipotency of protoplasts from *Brassica oleracea* plants previously regenerated in tissue culture. Plant Cell Tissue Organ Cult 14:15-24

Schumann U, Koblitz H (1983) Studies on plant recovery from mesophyll protoplasts of *Solanum tuberosum* L. and *Solanum phureja* Juz. and Buk. Biologia Plant 25:180-186

Secor GA, Shepard JF (1981) Variability of protoplast-derived potato clones. Crop Sci 21:102-105

Shepard, JF (1982) Cultivar dependent cultural refinements in potato protoplast regeneration. Plant Sci Lett 26:127-132

Snyder LA, Freifelder D, Hartl DL (1985) General genetics. Jones and Bartlett Publishers, Inc. Boston, pp 52-58

Uijtewaal BA, Suurs LCJM, Jacobsen E (1987) Protoplast fusion of monohaploid ($2n = x = 12$) potato clones: identification of somatic hybrids using malate dehydrogenase as a biochemical marker. Plant Sci 51:277-284

Veilleux RE, Booze-Daniels J, Pehu E (1985) Anther culture of a *2n* pollen producing clone of *Solanum phureja* Juz. & Buk. Can J Genet Cytol 27:559-564

Webb KJ (1988) Recent development in the regeneration of agronomically important crops from protoplasts. Plant Cell Tissue Organ Cult 12:127-131

Willman MR, Schroll SM, Hodges TK (1989) Inheritance of somatic embryogenesis and plantlet regeneration from primary (type 1) callus in maize. In Vitro Cell & Develop Biol 25:95-100

**Table 1. Protoplast culturability of AM3-8, NBP2 and 7 plants randomly selected from the cross of AM3-8 × NBP2[1]**

| Genotype | Liquid | | | Embedding | | |
|---|---|---|---|---|---|---|
| | No. of tests | P.E.[2] % ± STD | Callus[3] | No. of tests | P.E. % ± STD | Callus |
| AM3-8 | 4 | 0 | — | 3 | 0 | — |
| NBP2 | 4 | 25 ± 3.5 | + | 2 | 20 ± 1.7 | + |
| $F_1$4 | 2 | 11 ± 1.0 | + | 0 | nt[4] | nt |
| $F_1$5 | 3 | 31 ± 6.0 | + | 4 | 19 ± 4.2 | + |
| $F_1$13 | 2 | 14 ± 2.3 | + | 0 | nt | nt |
| $F_1$14 | 2 | 17 ± 2.0 | + | 0 | nt | nt |
| $F_1$15 | 2 | 12 ± 2.0 | + | 0 | nt | nt |
| $F_1$21 | 2 | 19 ± 1.0 | + | 2 | 22 ± 1.0 | + |
| $F_1$37 | 5 | 13 ± 1.4 | + | 5 | 13 ± 3.5 | + |

[1] For some genotypes, two slightly different culture methods were used: 1) liquid culture; and 2) embedding, in which protoplasts were embedded in the culture medium containing 0.3% agarose on the day of culture initiation

[2] Plating efficiency (P.E.) was examined 7 days after culture initiation

[3] Callus formation. The symbols '+' and '—' represent more than 200 p-calli formed per plate and no p-callus formation, respectively

[4] Not tested

**Table 2. Protoplast culturability of 20 randomly selected $F_2$ ($F_1$21 × $F_1$37) plants[1]**

| Genotype | Liquid | | | Embedding | | |
|---|---|---|---|---|---|---|
| | No. of tests | P.E.[2] % ± STD | Callus[3] | NO. of tests | P.E. % ± STD | Callus |
| $F_2$1 | 3 | 0 | — | 2 | 0 | — |
| $F_2$2 | 2 | 22 ± 2.5 | + | 2 | 18 ± 1.5 | + |
| $F_2$3 | 3 | 0 | — | 2 | 0 | — |
| $F_2$4 | 2 | 14 ± 1.0 | + | 2 | 13 ± 1.0 | + |
| $F_2$5 | 2 | 1 ± 0.5 | —[5] | 2 | 1 ± 0.3 | — |
| $F_2$6 | 4 | 0 | — | 2 | 0 | — |
| $F_2$7 | 2 | 10 ± 2.0 | + | 2 | 14 ± 1.5 | + |
| $F_2$8 | 2 | 20 ± 2.0 | + | 0 | nt[4] | nt |
| $F_2$9 | 2 | 11 ± 1.0 | + | 2 | 13 ± 1.3 | + |
| $F_2$10 | 2 | 2 ± 0.5 | — | 0 | nt | nt |
| $F_2$11 | 2 | 13 ± 2.5 | + | 2 | 11 ± 1.5 | + |
| $F_2$12 | 3 | 1 ± 0.4 | — | 2 | 1 ± 0.3 | — |
| $F_2$13 | 3 | 1 ± 0.5 | —[5] | 2 | 1 ± 0.3 | — |
| $F_2$14 | 2 | 17 ± 2.0 | + | 2 | 17 ± 1.0 | + |
| $F_2$17 | 0 | nt | nt | 2 | 12 ± 1.0 | + |
| $F_2$18 | 0 | nt | nt | 2 | 14 ± 2.3 | + |
| $F_2$19 | 0 | nt | nt | 2 | 16 ± 1.5 | + |
| $F_2$23 | 0 | nt | nt | 2 | 0 | — |
| $F_2$25 | 0 | nt | nt | 2 | 12 ± 2.0 | + |
| $F_2$27 | 0 | nt | nt | 2 | 10 ± 2.0 | + |

[1] For some genotypes, two slightly different cultural methods were used: 1) liquid culture; and 2) embedding, in which protoplasts were embedded in the culture medium containing 0.3% agarose on the day of culture initiation

[2] Plating efficiency (P.E.) was examined 7 days after culture initiation

[3] Callus formation. The symbols '+' and '—' represent more than 200 p-calli formed per plate and no p-callus formation in each test, respectively

[4] Not tested

[5] Less than 12 p-calli formed only in one of four tests

**Table 3. Protoplast culturability of 8 randomly selected BCN (NBP2 × $F_1$37) plants[1]**

| Genotype | Liquid | | | Embedding | | |
|---|---|---|---|---|---|---|
| | No. of tests | P.E.[2] % ± STD | Callus[3] | No. of tests | P.E. % ± STD | Callus |
| BCN1 | 0 | nt[4] | nt | 2 | 16 ± 3.0 | + |
| BCN2 | 2 | 22 ± 1.5 | + | 2 | 19 ± 2.5 | + |
| BCN4 | 2 | 24 ± 2.0 | + | 2 | 25 ± 2.5 | + |
| BCN5 | 0 | nt | nt | 2 | 18 ± 5.0 | + |
| BCN6 | 2 | 22 ± 2.3 | + | 2 | 23 ± 3.5 | + |
| BCN8 | 2 | 9 ± 2.0 | + | 2 | 10 ± 1.7 | + |
| BCN12 | 2 | 15 ± 4.5 | + | 2 | 20 ± 2.3 | + |
| BCN14 | 2 | 19 ± 3.5 | + | 2 | 23 ± 2.8 | + |

[1] For some genotypes, two slightly different cultural methods were used: 1) liquid culture; and 2) embedding, in which protoplasts were embedded in the culture medium containing 0.3% agarose on the day of culture initiation

[2] Plating efficiency (P.E.) was examined 7 days after culture initiation

[3] Callus formation. The symbols '+' and '—' represent more than 200 p-calli formed per plate and no p-callus formation in each experiment, respectively

[4] Not tested

**Table 4. Protoplast culturability of 8 randomly selected BCA (AM3-8 × $F_1$37) plants[1]**

| Genotype | Liquid | | | Embedding | | |
|---|---|---|---|---|---|---|
| | No. of tests | P.E.[2] % ± STD | Callus[3] | No. of tests | P.E. % ± STD | Callus |
| BCA3 | 0 | nt[4] | nt | 2 | 2 ± 0.5 | — |
| BCA6 | 0 | nt | nt | 2 | 2 ± 1.0 | — |
| BCA8 | 2 | 9 ± 2.5 | + | 2 | 8 ± 1.5 | + |
| BCA9 | 2 | 3 ± 0.5 | + | 2 | 5 ± 0.5 | + |
| BCA10 | 0 | nt | nt | 2 | 0 | — |
| BCA11 | 2 | 5 ± 3.8 | — | 2 | 5 ± 3.5 | — |
| BCA12 | 2 | 7 ± 2.5 | — | 2 | 6 ± 2.8 | — |
| BCA13 | 2 | 0 | — | 2 | 0 | — |

[1] For some genotypes, two slightly different cultural methods were used: 1) liquid culture; and 2) embedding, in which protoplasts were embedded in the culture medium containing 0.3% agarose on the day of culture initiation

[2] Plating efficiency (P.E.) was examined 7 days after culture initiation

[3] Callus formation. The symbols '+' and '—' represent more than 200 p-calli formed per plate and no p-callus formation in each experiment, respectively

[4] Not tested

**Table 5. Chi-square test for the hypothesis of one segregating locus with complete dominance for protoplast culturability in the $F_2$ generation**

| Callus formation | Observed number | Expected number | Deviation from expected number |
|---|---|---|---|
| + | 12 | 15 | −3 |
| − | 8 | 5 | +3 |

$\chi^2 = 2.4000 \rightarrow p < 0.2$

**Table 6. Chi-square test for the hypothesis of two independently segregating loci with complete dominance for protoplast culturability in the $F_2$ generation**

| Callus formation | Observed number | Expected number | Deviation from expected number |
|---|---|---|---|
| + | 12 | 11.25 | +0.75 |
| — | 8 | 8.75 | —0.75 |

$\chi^2 = 0.1143 \rightarrow p > 0.5$

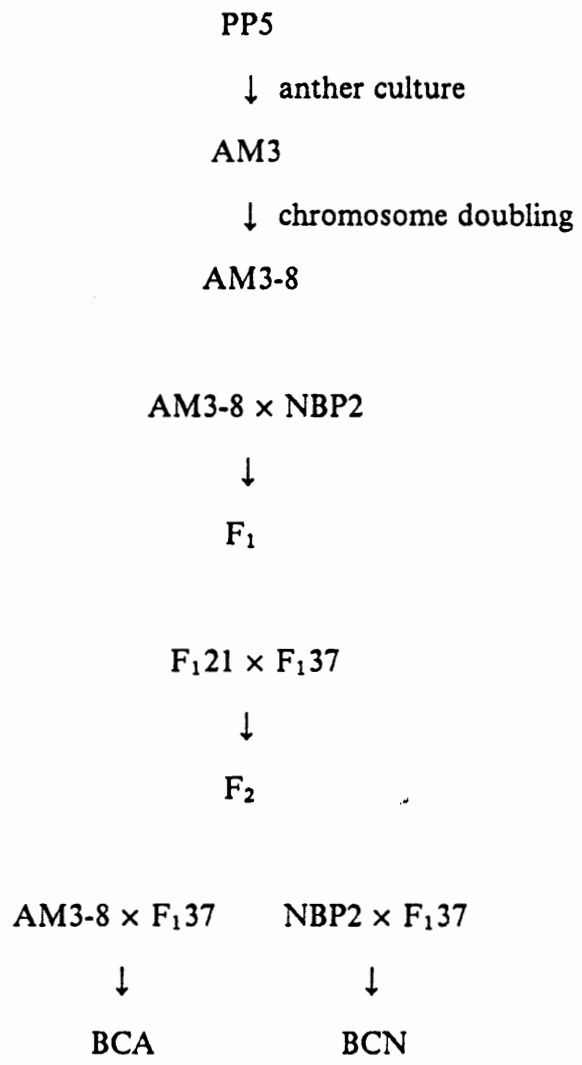


Figure 1. Pedigrees of S. phureja plants used for the genetic analysis of protoplast culturability

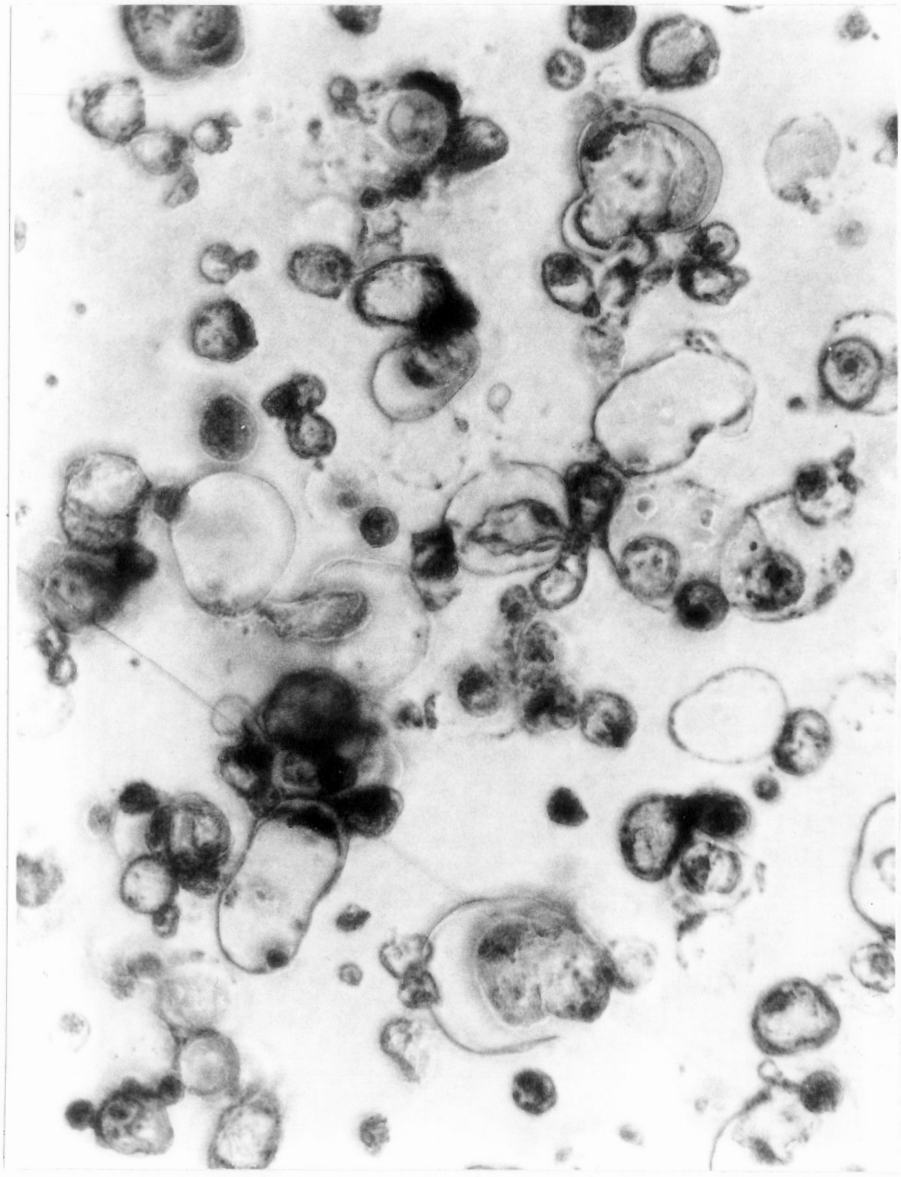

Figure 2. Protoplasts of AM3-8 after seven days in culture: most protoplasts have burst. A few protoplasts have formed cell wall which can be identified by the non-spherical shape of the cells

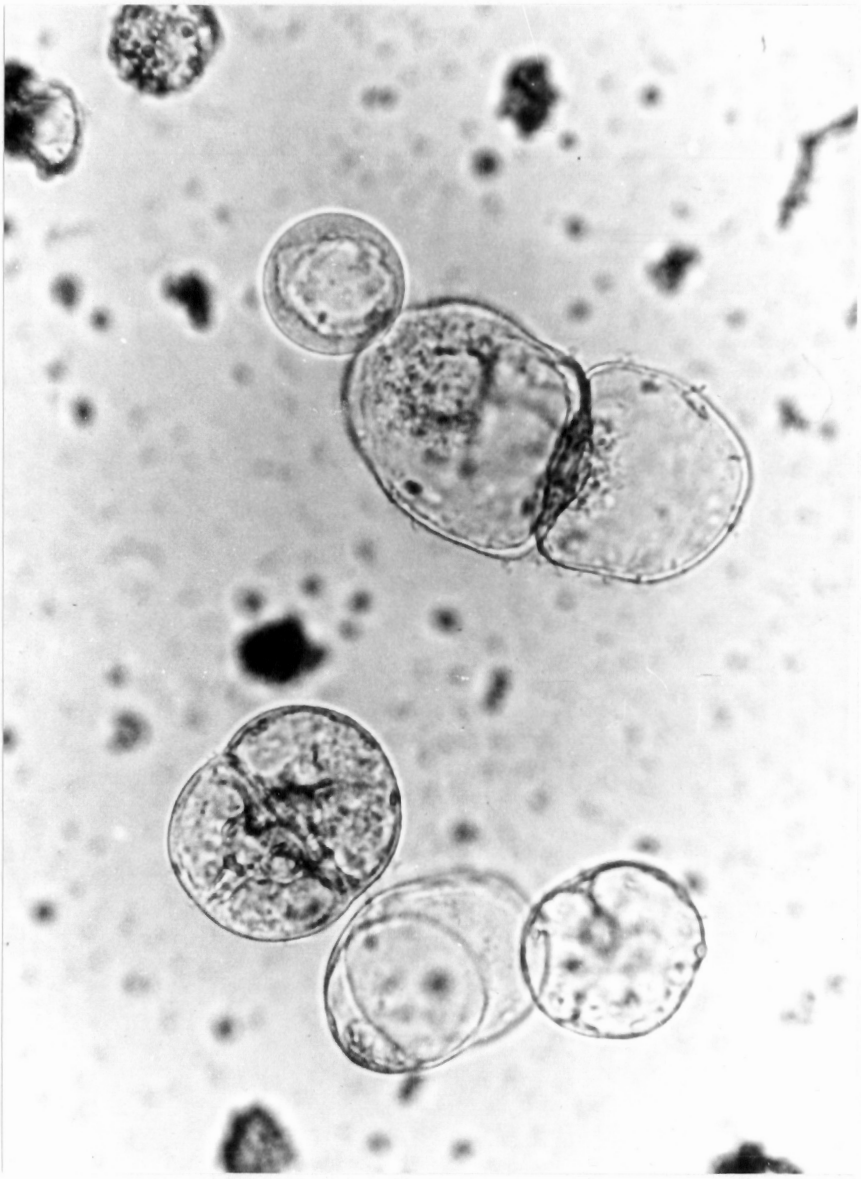

Figure 3. First mitotic division observed in protoplast culture of NBP2 three days after culture initiation

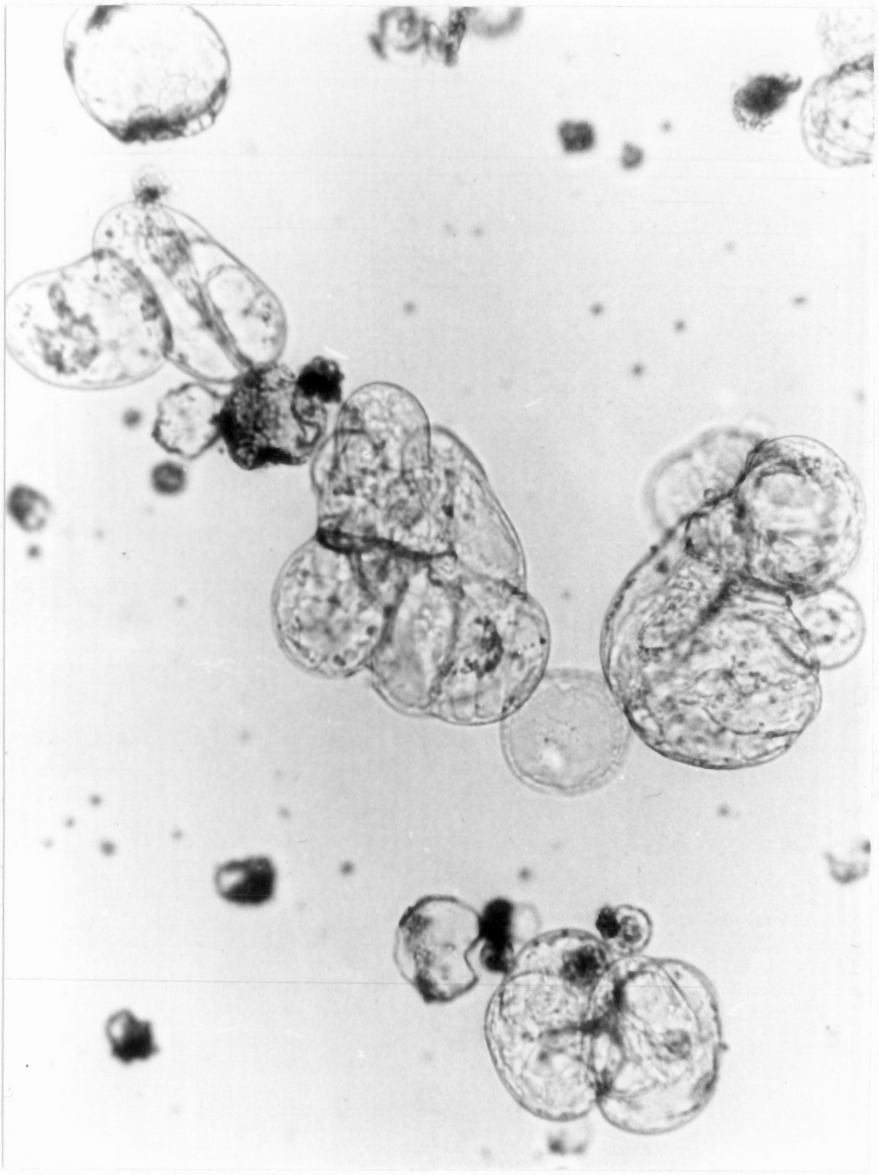

Figure 4. High plating efficiency in protoplast culture of NBP2: the cells were photographed seven days after culture initiation

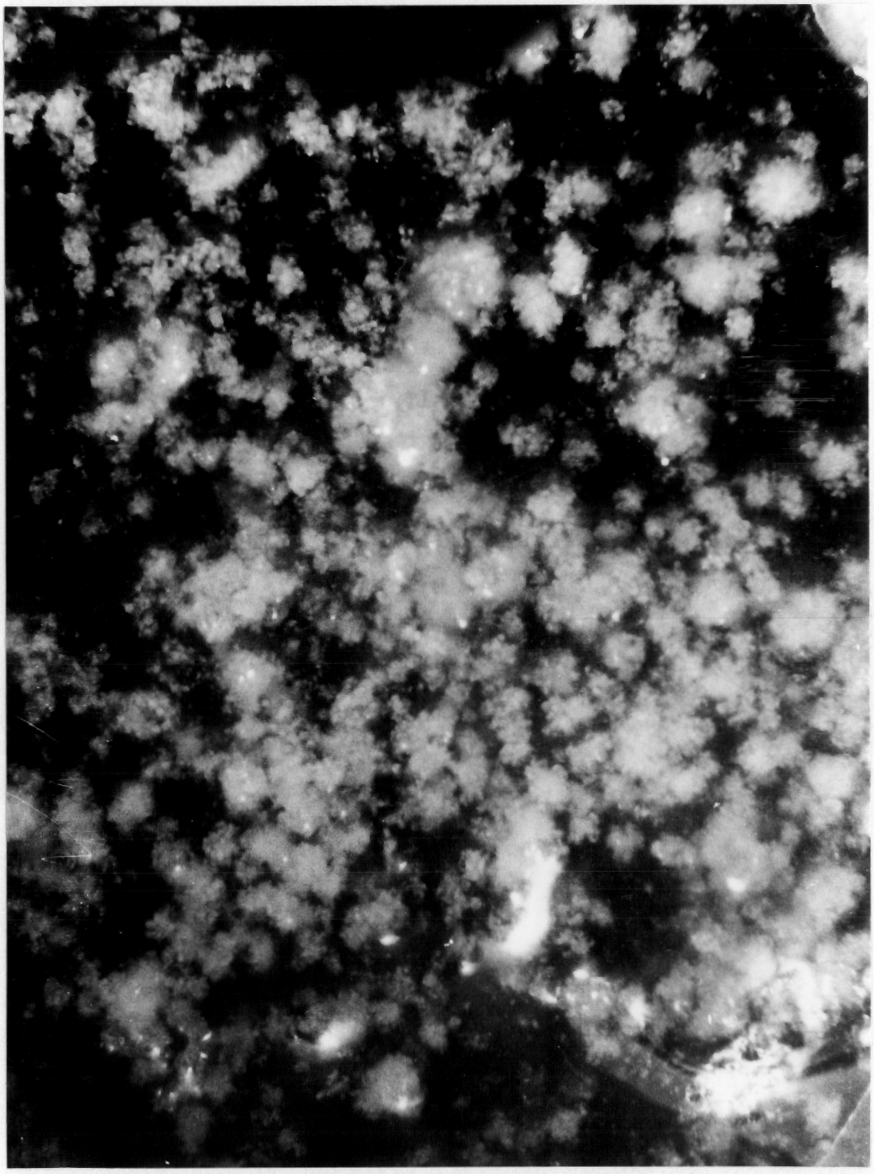

Figure 5. P-calli formed from protoplasts of NBP2

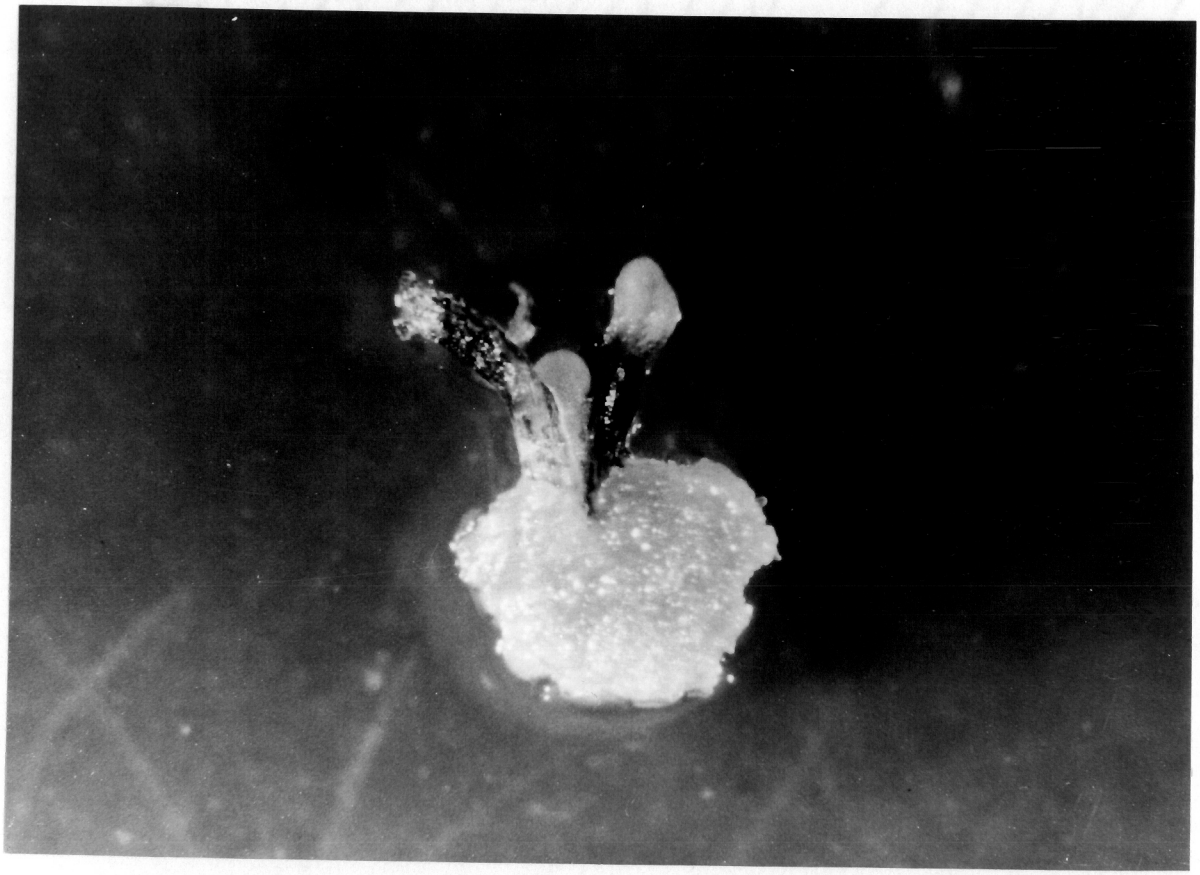

Figure 6. Shoot regeneration from a p-callus of $F_1 5$

# Chapter 4: Improvement in pollen development of a sterile potato cultivar by protoplast manipulation

## *Summary*

'Donor-recipient' protoplast fusion was conducted between a sterile potato cultivar Russet Burbank and fertile selections of *Solanum tuberosum* ssp. *andigena* using the selections of *andigena* as the cytoplasmic donor. Differences in chloroplast DNA between the cytoplasmic donor and the recipient were detected. Dosage effect of X-ray irradiation on cell division was tested. Fusion frequency was checked with three different concentrations of polyethylene glycol; 16 plants were regenerated after fusion. Two of the regenerated plants were found to have improved pollen stainability, though they possess chloroplast DNA and nuclear background of the male sterile cultivar.

# *Introduction*

Male sterility is common among cultivars of *Solanum tuberosum* ssp. *tuberosum* and this sterility is caused by interaction between sensitive cytoplasm and dominant nuclear genes (Grun, 1974, 1979). It has been postulated that the sensitive cytoplasm of *S. tuberosum* L. ssp. *tuberosum* arose during its evolution from *S. tuberosum* L. ssp. *andigena* Hawkes, which has a non-sensitive type cytoplasm (Grun, 1974). Hybrids between cultivars and other potato species are often male sterile when cultivars are used as female parents (Mullin and Lauer, 1966; Sanford and Hanneman, 1979; Hoopes et al., 1980; Hanneman and Peloquin, 1981). Therefore, many breeding schemes, designed to introduce useful genes into male sterile cultivars from other potato species through recurrent backcrosses, cannot be realized.

Substitution of the sensitive cytoplasm of *S. tuberosum* ssp. *tuberosum* cultivars with non-sensitive from *S. tuberosum* ssp. *andigena* and other diploid species has been attempted through recurrent backcrossing using male fertile cultivars as the recurrent pollen parents (Staub et al., 1982). Cytoplasmic influence on male sterilities was demonstrated in the $F_1$ and $BC_1$ generations. In later backcross generations, this influence was not observed (Staub et al., 1982; Amoah and Grun, 1988). The obscurity of the cytoplasmic role in male sterilities in the later backcross generations may be caused by the effects of male fertility restorer

genes from the male fertile cultivars which have to be used as the recurrent pollen parents for such cytoplasmic substitution.

Somatic cybridization via 'donor-recipient' protoplast fusion offers great opportunities for cytoplasmic engineering to manipulate organellar genomes (Galun and Aviv, 1983; Kumar and Cocking, 1987; Kemble and Barsby, 1988). Using this technique, cytoplasmic male sterile tobacco plants were converted into male fertile (Aviv and Galun, 1980). On the other hand, male fertile rapeseed plants were made male sterile by cytoplasmic substitution (Pelletier et al., 1983; Menczel et al., 1987). Restoration of male fertility by fusion of protoplasts from two different cytoplasmic male-sterile cybrids has also been reported (Aviv and Galun, 1986).

*S. tuberosum* cv. Russet Burbank is a leading cultivar in the potato industry in North America. Because of male sterility, its use is limited in breeding programs. Fortunately, it is amenable to protoplast culture and regeneration (Shepard and Totten, 1977; Shepard et al., 1980). In this study, we aimed to improve male fertility of Russet Burbank via 'donor-recipient' protoplast fusion and subsequent plant regeneration.

# *Materials and methods*

## *Plant materials*

*S. tuberosum* ssp. *tuberosum* cv. Russet Burbank was used as the cytoplasmic recipient. It was acquired in the form of certified seed tubers from the Wisconsin Seed Potato Farm, Rhinelander, Wis. Three fertile selections of *S. tuberosum* ssp. *andigena*, A140-11, C14-343 and C1-884, were used as the cytoplasmic donor. These selections were from the Neo-tuberosum collection (Plaisted et al., 1987) and were received as *in vitro* plantlets from Dr. Robert Plaisted, Dept. of Plant Breeding and Biometry, Cornell University, Ithaca, New York.

## *Chloroplast DNA analysis*

Chloroplast DNA (cpDNA) was prepared from purified chloroplasts isolated from young leaves of greenhouse-grown plants using a modified procedure of Kemble (1987). Five grams of leaves were ground with liquid nitrogen in isolation buffer, then filtered through 4 layers of cheesecloth and 2 layers of miracloth. Chloroplasts were purified by sucrose gradient centrifugation at 81,900 × g for 40 minutes. The purified chloroplasts were lysed in 2% Sarkosyl and 0.012% proteinase K solution with 0.2 M ammonium acetate. The cpDNA was purified twice with phenol-chloroform solution and precipitated with 95% ethanol at -20°C overnight.

Restriction endonuclease fragments were produced by digesting 1.5 $\mu$g cpDNA with *Eco*RI, *Bam*HI, *Ava*I and *Dra*I for 3 hours separately. DNA electrophoresis was carried out using 1% agarose gel in tris-borate buffer (Maniatis et al., 1982) at 1.6 V/cm for 14 hours with a horizontal DNA electrophoretic apparatus. After electrophoresis, the gel was stained with ethidium bromide (EtBr) solution (50 $\mu$l EtBr/500 ml water) for 25 minutes and photographed under UV light.

For regenerated plants only, leaves from two different individuals (3 grams from each) were pooled together for cpDNA isolation in order to enhance the efficiency of cpDNA screening.

***Protoplast isolation***

To obtain axenic plant material for protoplast isolation from Russet Burbank, green sprouts were cut from tubers which had been sprouted under cool white fluorescent light at room temperature. Surface sterilization was done by immersing the sprouts in 80% ethanol for five seconds and then washing in 20% commercial bleach (containing 5.25% sodium hypochlorite) for 20 minutes, followed by three rinses with sterilized distilled water. Nodal cuttings from sterilized sprouts of Russet Burbank and *in vitro* plantlets of *andigena* were planted on MS basal medium (Murashige and Skoog 1962) supplemented with 30 g/l sucrose, 170 mg/l potassium phosphate (monobasic), and 500 mg/l casein

hydrolysate in 25 × 150 mm glass tubes. The cultures were incubated in a growth chamber with a temperature regime of 20°C day and 18°C night, and a 16-hour photoperiod. The average light intensity was 28 $\mu E\ m^{-2}\ sec^{-1}$ provided by cool white fluorescent tubes. Just before isolation, plants were treated in the dark for 48 hours.

Protoplast isolation was done using a modified procedure of Uijtewaal et al. (1987). Three-week-old *in vitro* shoots and leaves (approximately one gram) were chopped into fine pieces. Shoots and leaves from the three selections of *andigena* were pooled into one sample. The cell wall digesting solution was composed of 1% Cellulase R-10 "Onozuka" (Yakult Honsha Co., Ltd., Japan), 0.1% Macerozyme R-10 (Yakult Honsha Co., Ltd., Japan), half-strength MS salts and vitamins, 0.4 M mannitol and 0.1 M glucose. Digestion was carried out in the dark on a shaker (60 rpm) for 16 hours. Upon digestion, the digests were diluted with an equal volume of 0.3 M KCl solution and sieved through a 63-$\mu$m wire mesh. The filtrate was centrifuged at 50 × g for 5 minutes. Pellets were resuspended in 0.5 M sucrose solution and 1 ml 0.3 M KCl solution was layered upon the top of the protoplast suspension. After centrifugation at 50 × g for 10 minutes, intact protoplasts were collected at the interface of the sucrose and KCl solution. Protoplasts were rinsed and pelleted again with 0.3 M KCl solution. The pH of all isolation solutions was adjusted to 5.8 with 0.1 N KOH and HCl solutions.

*X-ray irradiation*

Freshly isolated and purified protoplasts from *andigena* selections were transferred to 60 × 15 mm petri dishes in 0.3 M KCl solution at a density of 1 × $10^6$/ml and irradiated in an X-ray cabinet (Minishot X-ray cabinet, TFI Co., West Haven, Conn.). Three different dosages at a rate of 740 R/minute were tested.

***Protoplast fusion and plant regeneration***

Protoplasts of Russet Burbank were mixed at 1:1 ratio with the composite sample of the three selections of *andigena* which had been treated with 8 Kr X-ray irradiation. Protoplast density of the fusion partners was adjusted to 1 × $10^6$/ml before they were mixed. Fusion was conducted using polyethylene glycol (PEG 4,000) according to the procedure of Bottino (1981).

***Protoplast culture and plant regeneration***

After fusion treatment, protoplasts were cultured in 60 × 15 mm petri dishes using a modified protoplast culture medium of Schumann and Koblitz (1983) supplemented with 1.25 mg/l α-naphthaleneacetic acid, 0.25 mg/l 2,4-dichlorophenoxyacetic acid, and 1 mg/l zeatin. The cultures were incubated at 25°C initially in the dark for one week, then under white fluorescent light with

an average light intensity of 16 $\mu$E $m^{-2}$ $sec^{-1}$. After three weeks, 1 ml fresh culture medium was added to each culture dish and the culture was divided into two dishes. When p-calli reached the size of approximately 3 mm, they were transferred to shoot regeneration medium, which contains MS salts and vitamins, 2 mg/l zeatin, 0.01 mg/l indoleacetic acid, 0.2 mg/l gibberellic acid ($GA_3$), 10 g/l sucrose, 36 g/l mannitol, 100 mg/l myo-inositol, 100 mg/l casein hydrolysate, 100 mg/l adenine sulfate, 100 mg/l glutamine, 100 mg/l potassium phosphate, 7 g/l agar, at pH 5.8. Cultures were grown at 25°C under white fluorescent light with an average light intensity of 46 $\mu$E $m^{-2}$ $sec^{-1}$ and a 16-hour daylength.

Regenerated shoots, approximately 10 mm long, were cut from the p-calli and transferred to MS basal medium for rooting. Rooted plants were transplanted to Jiffy peat pellets and grown under similar environmental conditions as for the *in vitro* nodal cuttings. After acclimation in the growth chamber for 2 weeks, the plants were transferred to the greenhouse and established in soil.

***Pollen staining***

Mature anthers were dissected on a microscope slide. Pollen was squeezed out and stained in a drop of 0.5% acetocarmine for 3 minutes before observation under a Zeiss microscope.

# *Results*

### *Cytoplasmic differences between the fusion partners*

Chloroplast DNA restriction fragment patterns produced by all four types of restriction endonuclease showed no difference among the three *andigena* selections (Fig. 7). However, restriction patterns generated by each of the four enzymes revealed differences between cpDNA of Russet Burbank and that of *andigena*. A minimum of 8 cpDNA markers were identified (1 in the *Eco*RI digestion, 3 in the *Bam*HI digestion, 1 in the *Ava*I digestion, and 3 in the *Dra*I digestion). These markers could be used to distinguish cytoplasm of Russet Burbank from that of *andigena*.

### *X-ray irradiation*

The effectiveness of X-ray irradiation on inactivation of cell division was tested using three different dosages of X-ray (Table 6). Plating efficiency was examined 7 days after irradiation. Without irradiation, the average plating efficiency for the donor protoplasts was 49%. After approximately four weeks in culture, more than 200 p-calli per culture plate were formed (Table 6). With an irradiation dosage of 3 Kr, plating efficiency was greatly reduced to 5.5%. P-calli formation was still profuse. Further increase of irradiation dosage to 5 Kr resulted in no countable plating efficiency on the 7th day, but a few p-calli were formed after

prolonged culture. When the dosage was increased to 8 Kr, cell division was completely prevented, and no callus was formed at all. Therefore, 8 Kr dosage was used to irradiate the cytoplasmic donor protoplasts (i.e., protoplasts of *andigena*) in the subsequent fusion experiments.

***Influence of PEG concentration on fusion frequency***

Fusion frequency was observed 20 minutes after addition of PEG solution by counting the number of fusing protoplasts (Fig. 8) against the total protoplast number counted under a Zeiss inverted microscope. Fusion frequency may be somewhat overestimated because, after dilution with $CaCl_2$ solution, only some of the half-fused protoplasts will become truly fused and others may separate from each other and return to unfused status. With 15% PEG, only a few fused figures were observed. Increasing PEG concentration to 25% led to a 19% fusion frequency. Further increasing PEG concentration resulted in fewer fused figures (Table 7).

***Flowering and pollen stainability of fusion partners and regenerated plants***

Sixteen plants were regenerated and established in the greenhouse in the first year. Tubers from regenerated plants and fusion partners were planted as three blocks in a randomized complete block design in the following year. After approximately 40 days, flower buds emerged on the fusion partners and some of the

regenerated plants. However, bud abscission occurred on Russet Burbank and most of the regenerated plants, so that only the three *andigena* selections and three regenerated plants (Reg8, Reg19, Reg21) flowered. Although Russet Burbank did not flower in the second year, it flowered in the first year. Pollen of Russet Burbank was shrivelled and no stainable pollen grain could be observed. In contrast, pollen stainability for the *andigena* selections was 51.4%, 57%, and 32.4% for A140-11, C14-343, and C1-885 respectively (Table 8). Two regenerated plants, Reg19 and Reg21, had 5.9% and 4.3% stainable pollen, respectively (Fig. 9). No stainable pollen was found in Reg8.

***Leaf morphology, tuber skin morphology and cpDNA of regenerated plants***

Because the three selections of *andigena* had been selected for morphological resemblance to *S. tuberosum* ssp. *tuberosum* (Plaisted et al., 1987), there is no marked difference in morphology between Russet Burbank and the selections of *andigena*. However, their tuber skin morphology is distinctly different: the three selections of *andigena* possess a smooth white tuber skin, but Russet Burbank has the characteristic rough russet tuber skin. All the regenerated plants had the same rough russet tuber skin as Russet Burbank (Fig. 10), indicating that they all have the nuclear background of Russet Burbank. Most of the regenerated plants, including one stainable-pollen bearer (Reg19), were normal in general morphology and resemble Russet Burbank. The other regenerant with stainable pollen (Reg21) had smaller, lighter green leaves and grew slowly.

Chloroplast DNA of the regenerated plants was tested with 3 restriction endonucleases. Results show all the regenerated plants, including the two having improved pollen stainability, had the same type of cpDNA as Russet Burbank (Fig. 11).

## *Discussion*

The cpDNA restriction fragment patterns effectively revealed differences in cytoplasm between Russet Burbank and the selections of ssp. *andigena*. Hosaka (1986) identified seven types of chloroplast genome in 44 clones of 16 wild and 7 cultivated *Solanum* species by five restriction enzymes. According to his classification, ssp. *tuberosum* has type T cpDNA, and ssp. *andigena* has type A cpDNA (Hosaka, 1986; Hosaka et al., 1988). The results of cpDNA analysis in the present study is in good agreement with their reports. Moreover, the use of restriction enzymes *Ava*I and *Dra*I added more information to the differences in cpDNA between the two subspecies.

X-ray irradiation can prevent cell division while retaining the metabolic activities of the cell (Raveh et al., 1973). Galun and Raveh (1975) applied 1.5 Kr doses of X-ray (750 R/min) to diploid and haploid tobacco protoplasts on the day of isolation, and were able to prevent 93% of p-callus formation. In the present

study, twice that dosage (3 Kr) was required to achieve 95% inactivation of cell division in tetraploid potato on the day of protoplast isolation. It was reported that protoplasts of haploid tobacco were more sensitive to X-ray irradiation than those of diploids; protoplasts on the day of isolation were more tolerant of X-ray irradiation than on the second and third day after isolation (Galun and Raveh, 1975). The X-ray delivery rate also affects inactivation of cell division. At a slower rate of 250 R/min, 10 Kr was required to completely supress p-callus formation in tobacco (Kumashiro et al., 1988).

Acetocarmine stains for chromatin, RNA, and various undefined cytoplasmic constituents (Heslop-Harrison et al., 1984). It has been conventionally used to detect the development of cytoplasmic contents of ungerminated pollen grains (Knox and Williams, 1986). Although acetocarmine-stainable pollen may not be necessarily viable and fertile, this staining is able to distinguish cytoplasm-deficient pollen grains from well-developed, cytoplasm-dense pollen grains (Janssen and Hermsen, 1976; Knox and Williams, 1986). Because no pollen from Russet Burbank could be stained, the stainable pollen grains observed in the two regenerants (Reg19 and Reg21) represent an improvement in pollen development.

There are several possible mechanisms which could lead to the improved pollen stainability of the two regenerated plants. Despite the fact that they have the cpDNA and nuclear background of Russet Burbank, they could be cybrids with mitochondrial DNA completely or partially from the cytoplasmic donors.

Discriminative sorting of chloroplast DNA is common in fused cells. In most cases, somatic hybrids or cybrids possess only one type of parental cpDNA (Flick and Evans, 1982; Kemble et al., 1986; Morgan and Maliga, 1987; Robertson et al., 1987; Sidorov et al., 1987; Rosen et al., 1988). But mitochondrial genomes in somatic hybrids and cybrids could be either recombinant types (Kemble et al., 1986; Morgan and Maliga, 1987; Robertson et al., 1987), or completely from one parent (Rosen et al., 1988). Alternatively, the improved pollen stainability could be the result of mutational events that occurred to the mitochondrial DNA of Russet Burbank during protoplast culture and regeneration. Kemble and Shepard (1984) reported that, in protoclones of Russet Burbank, an additional low molecular weight DNA species appeared in their mitochondrial genome, but their cpDNA remained unchanged. Similarly, in cultured cells of *Brassica campestris*, rearrangement, amplification and loss of mitochondrial DNA were detected, while no cpDNA change was found (Shirzadegan, et al., 1989). Reversion of cytoplasmic male sterility to male fertility occured in maize plants regenerated from tissue culture and this reversion was attributed to mutations in a 6.6 Kb fragment of the mitochondrial DNA (Gengenbach and Connelly, 1981; Umbeck and Gengenbach, 1983). Finally, mutational events during protoplast culture and regeneration in the nuclear genome of Russet Burbank could be a possible cause. Any mutation which has positive effects on microsporogenesis would lead to improved pollen stainability of the two regenerated plants. Spontaneous mutations in nuclear genomes have frequently been found among *in vitro* regenerated plants (Edallo et al., 1981; Evans and Sharp, 1983; Landsmann and

Uhrig, 1985; Brown and Lorz, 1986). In plants regenerated from protoplasts of Russet Burbank, extensive variation in morphology and vegetative growth have been reported (Secor and Shepard, 1981).

## *References*


Amoah, V. and P. Grun, 1988. Cytoplasmic substitution in *Solanum*: 1. Seed production, germination and sterilities of reciprocal backcross generations. Potato Res. 31:113-119.

Aviv, D. and E. Galun, 1980. Restoration of fertility in cytoplasmic male sterile (CMS) *Nicotiana sylvestris* by fusion with X-irradiated *N. tabacum* protoplasts. Theor. Appl. Genet. 58:121-127.

Aviv, D. and E. Galun, 1986. Restoration of male fertile *Nicotiana* by fusion of protoplasts derived from two different cytoplasmic male-sterile cybrids. Plant Mol. Biol. 7:411-417.

Bottino, P. J., 1981. Methods in plant tissue culture. Kemtec Educational Corporation, pp. 50-55.

Brown, P. T. H. and H. Lorz, 1986. Molecular changes and possible origin of somaclonal variation. *In*: J. Semal (ed.), Somaclonal variations and crop improvement. Martinus Nijhoff Publishers, Dordrecht/Boston/Lancaster, pp. 148-159.

Edallo, S., C. Zucchinali, M. Perenzin and F. Salamini, 1981. Chromosomal variation and frequency of spontaneous mutation associated with *in vitro* culture and plant regeneration in maize. Maydica 26:39-56.

Evans, D. A. and W. R. Sharp, 1983. Single gene mutations in tomato plants regenerated from tissue culture. Science, 221:949-951.

Flick, C. E. and D. A. Evans, 1982. Evaluation of cytoplasmic segregation in somatic hybrids of *Nicotiana*: tentoxin sensitivity. J. Hered. 73:264-266.

Galun, E. and D. Aviv, 1983. Cytoplasmic hybridization: genetic and breeding applications. *In*: Evans, D. A., W. R. Sharp, P. V. Ammirato and Y. Yamada (eds.), Handbook of plant cell culture, Vol. 1. Macmillan Publishing Co. pp. 358-392

Galun, E., and D. Raveh, 1975. *In vitro* cultures of tobacco protoplasts: survival of haploid and diploid protoplasts exposed to X-ray radiation at different times. Radiation Bot. 15:79-82.

Gengenbach, B. G. and J. A. Connelly, 1981. Mitochondrial DNA variation in maize plants regenerated during tissue culture selection. Theor. Appl. Genet. 59:161-167.

Grun, P., 1974. Cytoplasmic sterilities that separate the group *tuberosum* cultivated potato from its putative tetraploid ancestor. Evolution 27:633-643.

Grun, P., 1979. Evolution of the cultivated potatoes: a cytoplasmic analysis. *In*: Hawkes, J. G., R. N. Lester, and A. D. Skelding (eds.), The biology and taxonomy of the Solanaceae. Linnean Society Symposium No. 7, Academic Press, London, pp. 655-665.

Hanneman, R. E., Jr., and S. J. Peloquin, 1981. Genetic-cytoplasmic male sterility in progeny of 4x-2x crosses in cultivated potatoes. Theor. Appl. Genet. 59:53-55.

Heslop-Harrison, J., Y. Heslop-Harrison, and K. R. Shivanna, 1984. The evaluation of pollen quality, and a further appraisal of the fluorochromatic (FCR) test procedure. Theor. Appl. Genet. 67:367-375.

Hoopes, R. W., R. L. Plaisted and A. G. Cubillos, 1980. Yield and fertility of reciprocal-cross *tuberosum-andigena* hybrids. Am. Potato J. 57:275-284.

Hosaka, K., 1986. Who is the mother of the potato? Restriction endonuclease analysis of chloroplast DNA of cultivated potatoes. Theor. Appl. Genet. 72:606-618.

Hosaka, K., G. A. De Zoeten and R. E. Hanneman, Jr., 1988. Cultivated potato chloroplast DNA differs from the wild type by one deletion - evidence and implications. Theor. Appl. Genet. 75:741-745.

Janssen, A. W. B., and J. G. Th. Hermsen, 1976. Estimating pollen fertility in *Solanum* species and haploids. Euphytica 25:577-586.

Kemble, R. J., 1987. A rapid, single leaf, nucleic acid assay for determining the cytoplasmic organelle complement of rapeseed and related *Brassica* species. Theor. Appl. Genet. 73:364-370.

Kemble, R. J. and T. L. Barsby, 1988. Use of protoplast fusion systems to study organelle genetics in a commercially important crop. Biochem. Cell Biol. 66:665-676.

Kemble, R. J., T. L. Barsby, R. S. C. Wong and J. F. Shepard, 1986. Mitochondrial DNA rearrangements in somatic hybrids of *Solanum tuberosum* and *Solanum brevidens*. Theor. Appl. Genet. 72:787-793.

Kemble, R. J. and J. F. Shepard, 1984. Cytoplasmic DNA variation in a potato protoclonal population. Theor. Appl. Genet. 69:211-216.

Knox, R. B., and E. G. Williams, 1986. Pollen, pistil, and reproductive function in crop plants. Plant Breeding Reviews 4:9-97.

Kumar, A. and E. C. Cocking, 1987. Protoplast fusion: a novel approach to organelle genetics in higher plants. Am. J. Bot. 74:1289-1303.

Kumashiro, T., T. Asahi, and T. Komari, 1988. A new source of cytoplasmic male sterile tobacco obtained by fusion between *Nicotiana tabacum* and X-irradiated *N. africana* protoplasts. Plant Sci. 55:247-254.

Landsmann, J. and H. Uhrig, 1985. Somaclonal variation in *Solanum tuberosum* detected at the molecular level. Theor. Appl. Genet. 71:500-505.

Maniatis, T., E. F. Fritsch, and J. Sambrook, 1982. Molecular cloning. Coldspring Harbor Laboratory, pp. 156

Menczel, L., A. Morgan, S. Brown and P. Maliga, 1987. Fusion-mediated combination of Ogura-type cytoplasmic male sterility with *Brassica napus* plastids using X-irradiated CMS protoplasts. Plant Cell Rep. 6:98-101.

Morgan, A. and P. Maliga, 1987. Rapid chloroplast segregation and recombination of mitochondrial DNA in *Brassica* cybrids. Mol. Gen. Genet. 209:240-246.

Mullin, R. and F. I. Lauer, 1966. Breeding behavior of $F_1$ inbred potato clones. Proc. Am. Soc. Hort. Sci. 89:449-455.

Murashige, T., and F. Skoog, 1962. A rivised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant 15:473-497.

Pelletier, G., C. Primard, F. Vedel, P. Chetrit, R. Remy, P. Rouselle and M. Renard, 1983. Intergeneric cytoplasmic hybridization in Cruciferae by protoplast fusion. Mol. Gen. Genet. 191:244-250.

Plaisted, R. A., H. A. Mendoza, H. D. Thurston, E. E. Ewing, B. B. Brodie, and W. M. Tingey, 1987. Broadening the range of adaptation of *andigena (Neo-tuberosum)* germplasm. Circular, International Potato Center, 15(2):1-5

Raveh, D., E. Huberman, and E. Galun, 1973. *In vitro* culture of tobacco protoplasts: use of feeder techniques to support division of cells plated at low densities. In Vitro 9:216-222.

Robertson, D., J. D. Palmer, E. D. Earle and M. A. Mutschler, 1987. Analysis of organelle genomes in a somatic hybrid derived from cytoplasmic male-sterile *Brassica oleracea* and atrazine-resistant *B. campestris*. Theor. Appl. Genet. 74:303-309.

Rosen, B., C. Hallden and W. K. Heneen, 1988. Diploid *Brassica napus* somatic hybrids: characterization of nuclear and organellar DNA. Theor. Appl. Genet. 76:197-203.

Sanford, J. C. and R. E. Hanneman, Jr., 1979. Reciprocal differences in the photoperiod reaction of hybrid populations of *Solanum tuberosum*. Am. Potato J. 56:531-540.

Schumann, U. and H. Koblitz, 1983. Studies on plant recovery from mesophyll protoplasts of *Solanum tuberosum* L. and *Solanum phureja* Juz. and Buk. Biologia Plant. 25:180-186.

Secor, G. A., and J. F. Shepard, 1981. Variability of protoplast-derived potato clones. Crop Sci. 21:102-105.

Shepard, J. F., and R. E. Totten, 1977. Mesophyll cell protoplasts of potato: isolation, proliferation, and plant regeneration. Plant Physiol. 60:313-316.

Shepard, J. F., D. Bidney, and E. Shahin, 1980. Potato protoplasts in crop improvement. Science 208:17-24.

Shirzadegan, M., M. Christey, E. D. Earle and J. D. Palmer, 1989. Rearrangement, amplification and assortment of mitochondrial DNA molecules in cultured cells of *Brassica campestris*. Theor. Appl. Genet. 77:17-25.

Sidorov, V. A., M. K. Zubko, A. A. Kuchko, I. K. Komarnitsky and Y. Y. Gleba, 1987. Somatic hybridization in potato: use of $\gamma$-irradiated protoplasts of *Solanum pinnatisectum* in genetic reconstruction. Theor. Appl. Genet. 74:364-368.

Staub, J. E., P. Grun and V. Amoah, 1982. Cytoplasmic evaluations during substitution backcrossing in *Solanum*. Potato Res. 25:299-319.

Uijtewaal, B. A., L. C. J. M. Suurs and E. Jacobsen, 1987 Protoplast fusion of monohaploid ($2n = x = 12$) potato clones: identification of somatic hybrids using malate dehydrogenase as a biochemical marker. Plant Sci. 51:277-284.

Umbeck, P. F. and G. Gengenbach, 1983. Reversion of male-sterile T-cytoplasm maize to male fertility in tissue culture. Crop Sci. 23:584-588.

**Table 7. X-ray dosage effect on protoplast plating efficiency (P.E.) of andigena selections used as cytoplasmic donors in fusion**

| Dosage | P.E. (% ± S.E.)[1] | Callus formation |
|---|---|---|
| 0 Kr | 49 ± 4.0 | > 200/plate |
| 3 Kr | 5.5 ± 1.5 | > 200/plate |
| 5 Kr | 0[2] | < 200/plate |
| 8 Kr | 0 | 0 |

[1] Plating efficiency was examined after protoplasts had been cultured for 7 days

[2] No dividing cell was observed at the 7th day, but calli were formed after prolonged culture

**Table 8. Mean frequency of fusing protoplasts at three concentrations of PEG[1]**

| PEG Concentration | Frequency (% ± S. E.) |
|---|---|
| 15% | 7 ± 1.0 |
| 25% | 19 ± 2.5 |
| 35% | 12 ± 2.0 |

[1] Frequency of fusing protoplasts was counted 20 minutes after addition of PEG solution

**Table 9. Morphology, cpDNA and pollen stainability of Russet Burbank, three selections of andigena, and three regenerated plants**

| Plant ID | General morphology | Tuber skin | Type of cpDNA | Stainable pollen (% ± S.D.)[1] |
|---|---|---|---|---|
| R.B. | normal | rough | T | 0 |
| A140-11 | normal | smooth | A | 51.4 ± 15.4 |
| C14-343 | normal | smooth | A | 57.0 ± 2.0 |
| C1-885 | normal | smooth | A | 32.4 ± 1.7 |
| Reg8 | normal | rough | T | 0 |
| Reg19 | normal | rough | T | 5.9 ± 0.2 |
| Reg21 | abnormal | rough | T | 4.3 ± 0.5 |

[1] Pollen was stained with 0.5% acetocarmine

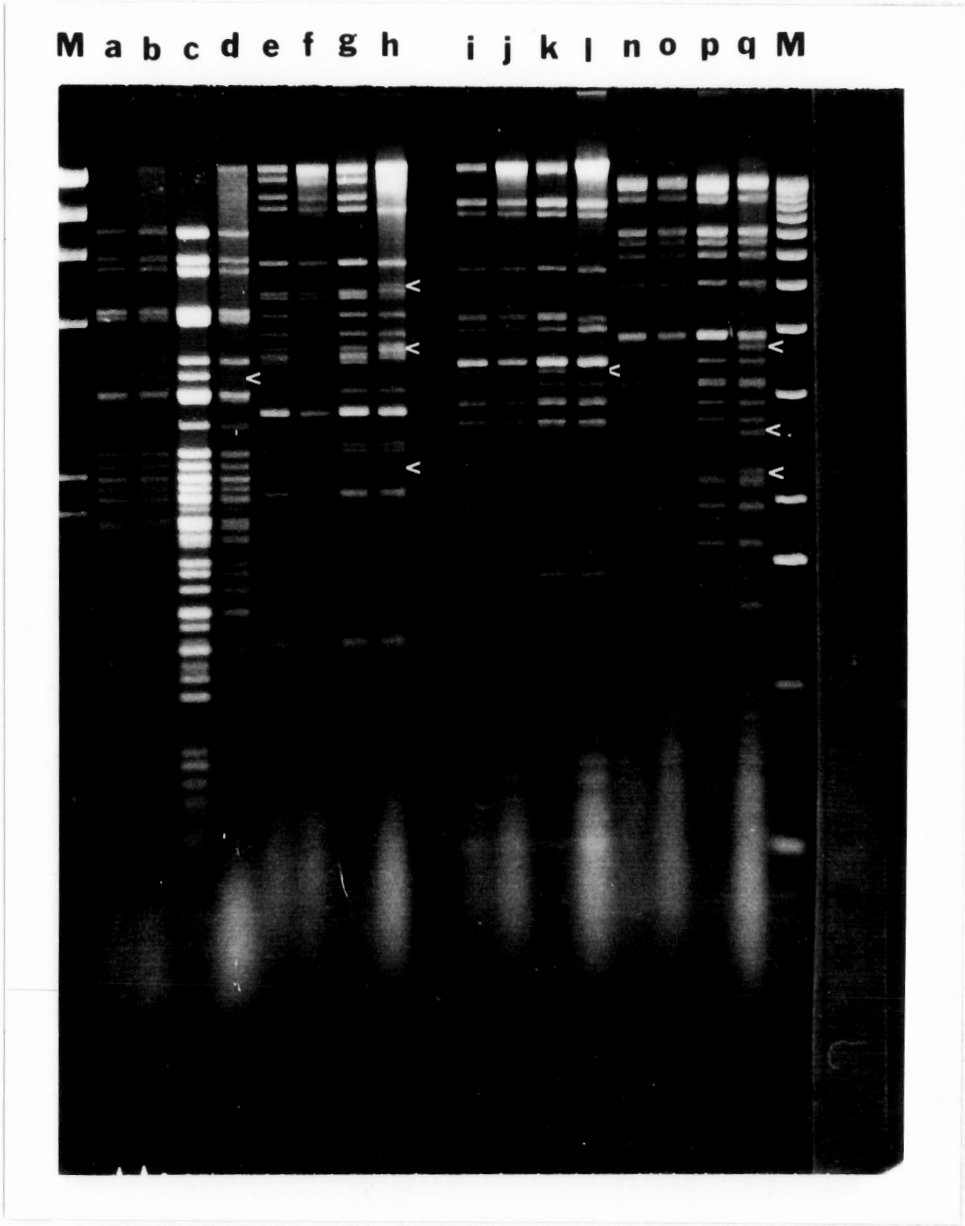


Figure 7. Electrophoresis in 1% agarose gel of cpDNA from the fusion partners: lane d, h, l, q, cpDNA from Russet Burbank; lane a, b, c, e, f, g, i, j, k, n, o, p, cpDNA from three selections of *andigena*. DNA was digested with *Eco*RI (lane a, b, c, d), *Bam*HI (lane e, f, g, h), *Ava*I (lane i, j, k, l) and *Dra*I (lane n, o, p, q). Lanes M are *Hind*III digest of $\lambda$ DNA (left) and the 1 kb ladder markers (right). Positions where differences exist between Russet Burbank and *andigena*, are pinpointed by the symbol '<'

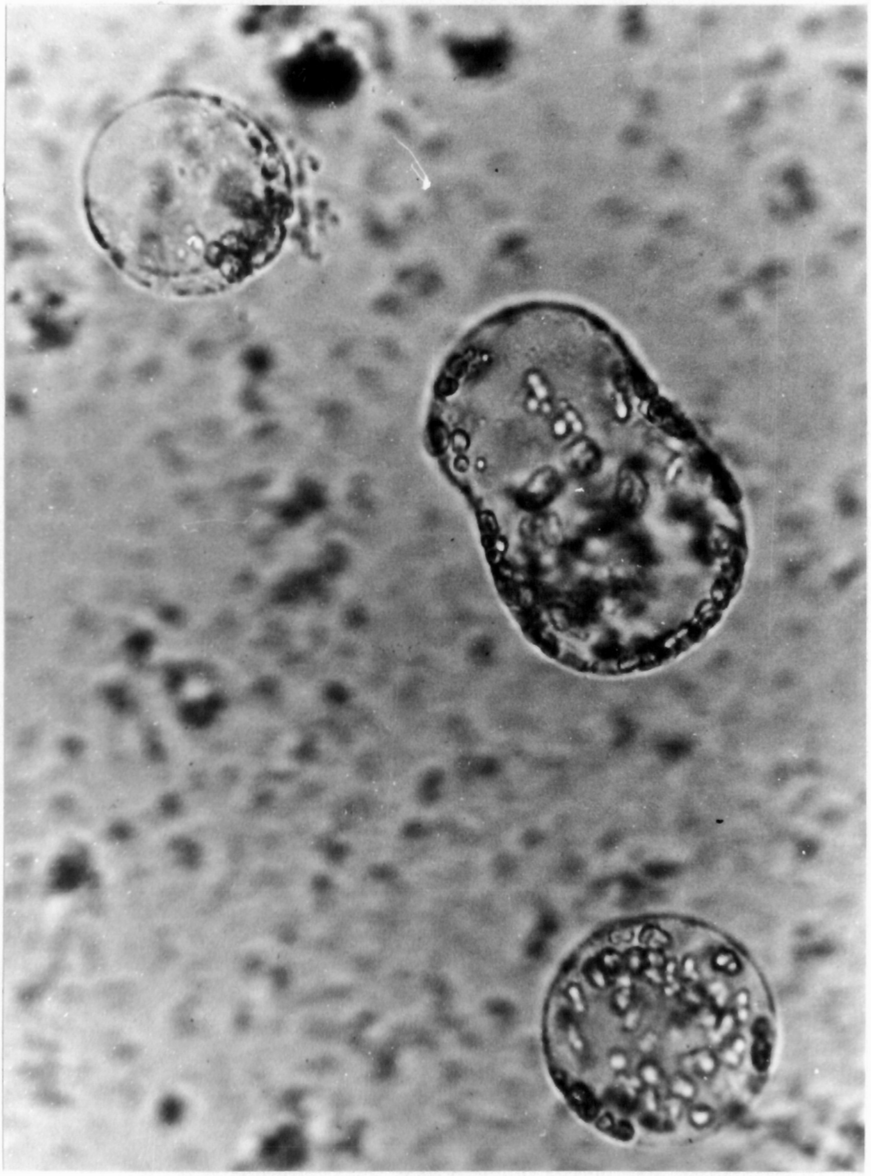

Figure 8. Fusing protoplasts: protoplasts were photographed 20 minutes after addition of 25% (w/v) PEG solution

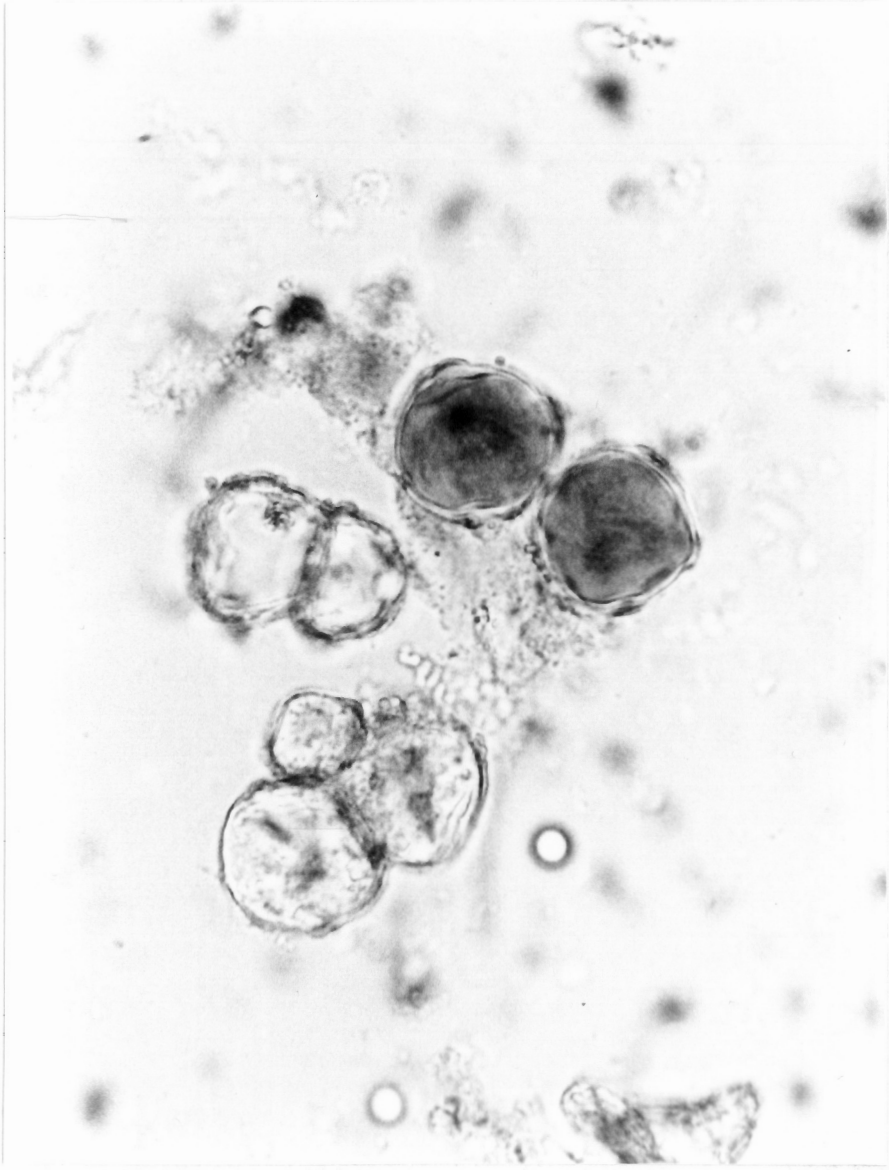

Figure 9. Stainable and non-stainable pollen from Reg19: pollen was stained with 0.5% acetocarmine

Figure 10. Tuber-skin morphology: Russet Burbank (right), *andigena* (left), and Reg21

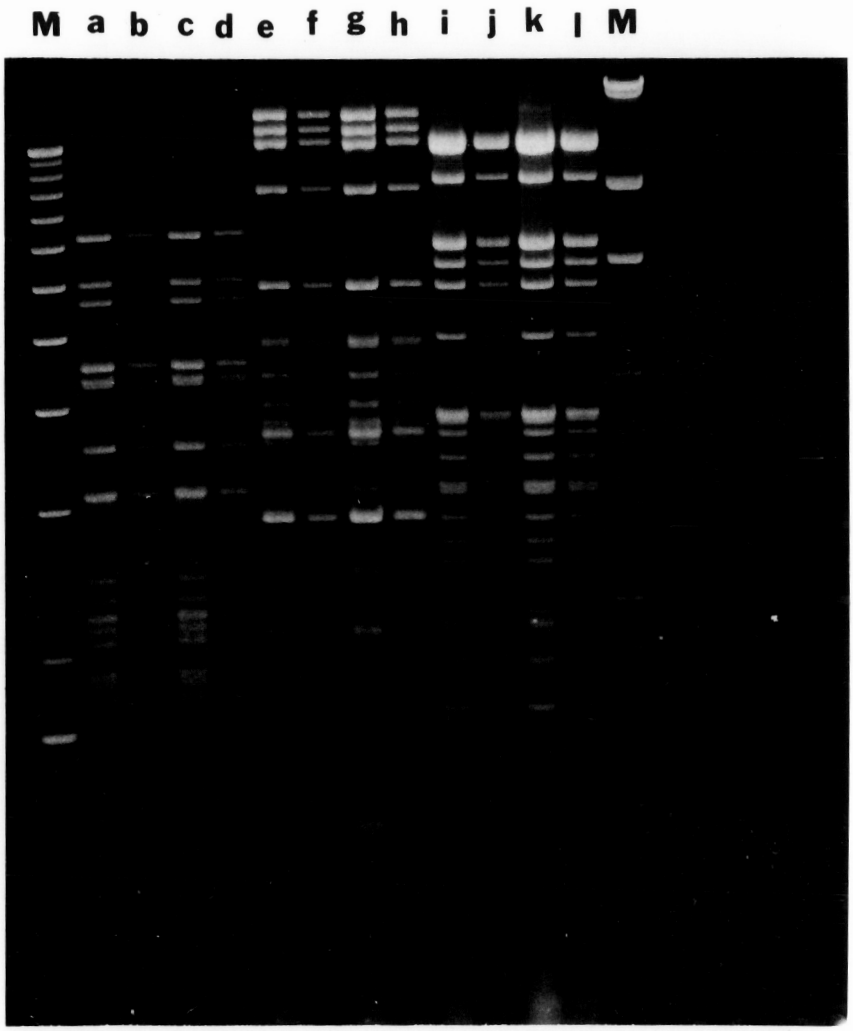


Figure 11. Electrophoresis in 1% agarose gel of cpDNA from 8 regenerated plants: each lane was loaded with cpDNA from two regenerated plants. Lanes a, e, i each contain cpDNA from Reg13 and Reg17; lane b, f, j, Reg19 and Reg12; lanes c, g, k, Reg8 and Reg24; lanes d, h, l, Reg21 and Reg22. DNA was digested with *Eco*RI (lane a, b, c, d), *Bam*HI (lane e, f, g, h), and *Dra*I (lane i, j, k, l). Lanes M are *Hind*III digest of $\lambda$ DNA (right) and the 1 kb ladder markers (left)

# Chapter 5: Limitation of enzymatic markers for assessing genetic variability in monoploids derived from a diploid potato clone

Monoploid production through either anther culture or parthenogenesis has been proposed as a means of eliminating deleterious genes from cross-pollinated plants in a single generation. This kind of genomic purge has been termed 'monoploid sieve' (Wenzel et al., 1979; Uijtewaal et al., 1986). Monoploids have been produced through anther culture of PP5, a clone of *Solanum phureja* which has been selected only for adaptation to long day conditions and *2n* pollen production (Haynes, 1972; Veilleux et al., 1985). To investigate whether or not any part of the genome from the anther donor has been preferentially eliminated by the 'monoploid sieve', fourteen regenerated monoploids and the anther donor

were screened for allozyme variation using horizontal starch gel electrophoresis as described by Douches and Quiros (1988).

Six enzymes including 6-phosphogluconate dehydrogenase (6-PGDH), shikimic acid dehydrogenase (SDH), malate dehydrogenase (MDH), isocitrate dehydrogenase (IDH), diaphorase (DIA), and phosphogluconate isomerase, were examined. For each of these enzymes, only one type of zymogram was found in these fourteen monoploids and the anther donor. These results suggest that the anther donor may be homozygous at the loci for all six enzymes. Further electrophoretic examinations of the anther donor by Dr. D. S. Douches at Michigan State University confirmed the homozygosity of this diploid potato clone at these isozyme loci.

However, genetic variability among these monoploids has been manifested by variations in growth, vigor, photoperiod response, tuber yield, ploidy stability, *in vitro* shoot regeneration, and general morphology (Owen et al., 1988a,b; M'Ribu and Veilleux, 1990). The absence of allozyme variation and the presence of variations in other characters among these monoploids indicates the limitation of enzymatic markers for assessing genetic variability in a monoploid population derived from a single clone. Although allozyme variation is widespread in natural populations of virtually all species, the number of loci studied by electrophoresis is very small compared to the total number of loci in any organism (Snyder et al., 1985). Based upon extensive electrophoretic data, it was estimated that only 7%

of enzymatic loci were heterozygous among 15 plant species (Nevo, 1978). In contrast to this, DNA markers are much more powerful in detecting genetic variability even within a single plant. Rivard et al. (1989) reported DNA restriction fragment length polymorphism in plants regenerated from anther culture of a diploid potato clone, whereas no allozyme variation could be detected among these plants.

From this study and the above discussion, it is concluded that: 1) enzymatic markers are limited in detecting heterozygosity of an individual plant and genetic variability among plants androgenetically or parthenogenetically derived from a single genotype; and 2) the technique of DNA restriction fragment length polymorphism can overcome these limitations.

## *References*


Douches, D. S., and C. F. Quiros, 1988. Additional isozyme loci in tuber-bearing Solanums: inheritance and linkage relationships. J. Hered. 79:377-384.

Haynes, F. L., 1972. The use of cultivated diploid *Solanum* species in potato breeding. *In*: French, E. R. (ed.), Prospects for the potato in the developing world: an international symposium on key problems and potentials for greater use of potato in the developing world, International Potato Center, Lima, Peru.

M'Ribu, H. K., and R. E. Veilleux, 1990. Effect of genotype, explant, subculture interval and environmental conditions on regeneration of shoots from *in vitro* monoploids of a diploid potato species, *Solanum phureja* Juz. & Buk.. (in review).

Nevo, E., 1978. Genetic variation in natural populations: patterns and theory. Theor. Pop. Biol. 13:121-177.

Owen, H. R., Jr., R. E. Veilleux, F. L. Haynes, and K. G. Haynes, 1988a. Variability for critical photoperiod for tuberization and tuber yield among monoploid, anther-derived genotypes of *Solanum phureja*. J. Am. Soc. Hort. Sci. 113:755-759.

Owen, H. R., Jr., R. E. Veilleux, D. Levy, and D. L. Ochs, 1988b. Environmental, genotypic, and polyploidization within a genotype of *Solanum phureja* and its derivatives. Genome 30:506-510.

Rivard, S. R., M. Cappadocia, G. Vincent, N. Brisson, and B. S. Landry, 1989. Restriction fragment length polymorphism (RFLP) analyses of plants produced by in vitro anther culture of *Solanum chacoense* Bitt. Theor. Appl. Genet. 78:49-56.

Snyder L. A., D. Freifelder, and D. L. Hartl, 1985. General genetics. Jones and Bartlett Publishers, Inc. Boston, pp 501-502.

Uijtewaal, B. A., and J. G. Th. Hermsen, 1986. Monohaploids in potato: pseudogamic induction and potential use in genetic and breeding research. *In*: Beekman, A. G. B., K. M. Louwes, L.M.W. Dellaert, and A. E. F. Neele (eds.), Potato research for tomorrow. Pudoc, Wageningen, pp 143-146.

Veilleux, R. E., J. Booze-Daniels, and E. Pehu, 1985. Anther culture of a 2*n* pollen producing clone of *Solanum phureja* Juz. and Buk. Can. J. Genet. Cytol. 27:559-564.

Wenzel, G., O. Schieder, T. Przewozny, S. K. Sopory, and G. Melchers, 1979. Comparison of single cell culture derived *Solanum tuberosum* L. plants and a model for their application in breeding programs. Theor. Appl. Genet. 55:49-55.

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