INHERITANCE OF COMPETENCIES FOR LEAF DISC REGENERATION, ANTHER CULTURE, AND PROTOPLAST CULTURE IN *Solanum phureja* AND CORRELATIONS AMONG THEM

by

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(ABSTRACT)

Competence for leaf disc regeneration, anther culture, and protoplast culture was tested in the parental, F_1, and F_2 generations of a diploid cultivated primitive potato, *S. phureja* (2n=2x=24). The parental pair consisted of AM3-8, an anther culture derived homozygous diploid, and NBP2, a heterozygous, field selected line. AM3-8 produced embryos in anther culture, and shoots on cultured leaf discs, but its cells did not divide after protoplast isolation. Cells of NBP2 divided to form calli and shoots in protoplast culture, but the clone did not respond to anther culture or leaf disc regeneration. All the individual plants in the F_1 generation were responsive to both anther culture and protoplast culture; however, there was segregation for the ability to regenerate shoots from leaf discs. The F_2 population, the result of a sib-cross, segregated for all three tissue culture competencies. Segregation data fit a one gene model for anther culture competence with the homozygous dominant genotype

expressing the highest response, the heterozygous resulting in a marginal response, and the homozygous recessive resulting in no response. A two-gene model applied to the protoplast culture data, with a dominant allele at both loci required for division to occur after protoplast isolation. Leaf disc regeneration data could only be explained by a two gene model with recessive alleles at each locus required for the highest response, a dominant allele at either of the loci resulting in a marginal response, and dominant alleles at both loci resulting in no response. No significant correlation was found among these traits, implying three separate genetic mechanisms which segregate independently.

Several temperature regimes were used in an attempt to enhance caulogenesis following protoplast isolation during the p-callus growth and regeneration phase of a single F_2 clone from this *S. phureja* population. Each of eight treatments was applied to 120 p-calli in six replications of 20 each. Shoot regeneration was scored at 94, 105, 121, and 131 days after protoplast isolation. P-calli cultured at 30^0C days and 20^0C nights produced significantly more shoots than those cultured at a constant 25^0C. Therefore, the standard 25^0C used for p-calli regeneration in potato may not be optimal; elevated temperatures or simply a diurnal temperature fluctuation may enhance morphogenesis.

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CHAPTER ONE: INTRODUCTION

Potato, *Solanum tuberosum* L., is the most important non-cereal food crop in the world. It is superior to all other crops in protein production per unit area (Ross 1986). In addition to providing all essential amino acids, the potato is a source of vitamins, minerals and trace elements (Li 1985). Therefore, it is not surprising that the potato is ranked fifth in area under cultivation among crop plants for human consumption and fourth for yield (Ross 1986). Despite its wide cultivation in the United States and Europe, the genetic potential of the potato has yet to be fully exploited.

As an autotetraploid (2n=4x=48), the potato offers great potential for genetic enhancement. The autotetraploid nature of potato allows as many as four alleles at each locus. Mendoza and Haynes (1973) have stated that the highest level of heterosis, when the progeny surpasses the value of the best parent for a desired characteristic, is expected only in heteroallelic plants. This is the result of intralocus (overdominant) and interlocus (epistatic) interactions between genes and alleles. Potato has the possibility of tetra-allelic genotypes, where four different alleles are present at each locus, allowing an immense capacity for heterosis.

Wild and primitive cultivated species of potato offer sources of great genetic diversity for many traits. There are 180 tuber-bearing wild species of potato known, most of which are self-incompatible diploids (2n=2x=24) (Ross 1986). Many of these form fertile hybrids when intercrossed. These wild species have a wide range of ecological distribution, from southern Chile to the southern USA, from sea level to 4,500 meters above sea level. In addition, they exhibit a broad spectrum of resistances, including frost, insect, and drought resistance; others are high in starch or protein content. Many of these wild species are already in use in breeding programs. Although commercial production of potato has been limited to the cooler regions of the world, because the genus *Solanum* contains species adapted to a wide range of environments (Li 1985), heat tolerant species could be used as a source of breeding material to expand the range of potato production to the highly populated warmer regions of the world.

Solanum phureja Juz. and Buk., a potato cultivar commonly grown in South America for its small tasty irregular tubers, is adapted to the lower, warm, moist slopes of the Andes. Its lack of dormancy, allowing farmers several plantings each year, makes it a popular crop. As a nutrient source, *S. phureja* is high in protein and vitamin C, and has a stronger flavor than the common potato (Popenoe 1989). Its disease resistance, heat tolerance (Hetherington et al. 1983) and large genetic distance from *S. tuberosum* make it a logical choice for use in breeding programs. By selection of clones expressing a high frequency of unreduced gametes, the

interploid crossing barriers between *S. phureja* (2n=2x=24) and *S. tuberosum* (2n=4x=48) have been overcome (Veilleux et al. 1982).

Simply crossing *S. phureja* with *S. tuberosum* is not sufficient to produce a better strain of potato, although 4x-2x crosses have shown yield potentials equivalent to the tetraploid parent (Veilleux and Lauer 1981; Kidane-Mariam et al. 1985). The advancing field of biotechnology offers improved means of genetically enhancing germplasms, through such methods as genetic transformation, haploid breeding, and the subsequent production of inbred lines. In order to take advantage of biotechnology to make the best use of *Solanum* hybrids, clones responsive to tissue culture procedures must be selected. Three of the most useful are anther culture, protoplast culture and leaf disc regeneration. Generally, representatives of the Solanaceae have been responsive to most tissue culture techniques; however, there is clearly genotypic variation both among and within *Solanum* species for response to these methods. While it is known that competence of a particular clone to a particular tissue culture technique is genetically controlled (Cheng and Veilleux 1991), it is not known if competence for one such trait is related to competence for another.

Tissue culture techniques have long been used in the breeding of the potato (Bajaj 1987); however, selection for clones with tissue culture competence is a prerequisite for utilization of any one such technique. The screening for some tissue culture competencies (TCC), such as leaf disc regeneration, is simple, cheap and quick. Other TCC's, such as anther culture competence and protoplast

competence, require long and expensive screenings. It is known that responsiveness to protoplast and anther culture is genetically controlled (Shepard 1982; Haberlach et al. 1985; Koornneef et al. 1987). If genetic links between tissue culture traits could be found, the process of identifying competent clones would be simplified; i.e., if two or more TCC's are linked, then the easier trait to screen could be used to indirectly identify plants competent for the other(s).

Leaf disc regeneration

The ability to regenerate shoots from leaf discs has been frequently exploited. *Solanum berthaultii* has glandular trichomes on its foliage which confers resistance to aphids, Colorado potato beetles, leaf hoppers, mites and thrips (Gibson 1971; Wright et al. 1985). Attempts to breed this trait into *S. tuberosum* by back-crossing *S. tuberosum* x *S. berthaultii* hybrids to *S. tuberosum* results in the *S. tuberosum* phenotype without the insect resistance (Kalazich 1989). Lentini et al. (1990) regenerated plants from petiole calli of interspecific hybrids of *S. tuberosum* x *S. berthaultii.* The plants regenerated from these hybrids contained genetic changes which overcame the restricted recombination between the two species allowing the insect resistance in *S. berthaultii* to be back-crossed into *S. tuberosum*. Procedures for regenerating shoots from leaf discs generally involve a callus phase preceeding regeneration and the resulting shoots often have an increased ploidy level (Karp et al. 1984; M'Ribu and Veilleux 1990). Monoploids are particularly prone to this type of chromosomal multiplication (endopolyploidization) (Owen et al. 1988). This

makes it possible to double the chromosome number of androgenetically or gynogenetically derived monoploids, producing completely homozygous lines.

Leaf disc regeneration also allows for the recovery of transgenic plants from explants incubated with *Agrobacterium tumefaciens* bearing plasmids with foreign DNA (Visser et al. 1989). By this method, Tavazza et al. (1988b) produced transgenic potatoes which were phenotypically normal and expressed introduced genes. This has also been done with other crops, such as strawberry (Nehra et al. 1990) and tomato (McCormick et al. 1986).

The callus phase prior to shoot regeneration (caulogenesis) can lead to other genetic variations as well. These include increased recombination (Sibi et al. 1984; Singsit et al. 1990; Compton and Veilleux 1991), chromosome breakage, aneuploidy (Ramulu et al. 1985), and unaccounted for somaclonal variation (Evans et al. 1984). Somaclones may be valuable as a source of unique germplasm (Bhojwani and Razdan 1983).

Anther culture

Immature pollen grains (i.e., microspores generally in the late uninucleate or early binucleate stage of development) can be stimulated into an embryonic state through anther culture. For *S. phureja*, flower buds with microspores at the uninucleate stage (anther length approximately 3.0 to 4.0mm) are sterilized, the anthers aseptically removed and placed in a liquid nutrient solution (Uhrig 1985). After four weeks on a shaker, embryos, complete with cotyledons and a radicle, may

develop from the microspores. This procedure has been used to regenerate haploid plants from many species, including *S. phureja* (Veilleux 1990). Monoploids have considerable potential in breeding programs. The innate heterozygosity of the tetraploid commercial potato, *Solanum tuberosum*, makes selection for superior clones among sexually generated seedlings a laborious procedure due to dominance, segregation, crossing-over, epistasis, etc. Monoploids, having only one set of chromosomes, greatly simplify the selection process. The phenotype of the monoploid is a direct reflection of the genotype because dominance and allelic interaction are absent. Although it is sometimes possible to produce inbred lines using conventional methods, especially in self-compatible plants, these methods are time-consuming, and often impossible in self-incompatible species, such as *S. phureja*. Pehu et al. (1987) showed it is possible to identify the desired *S. phureja* haploids based on morphological characteristics without the need to rely on Feulgen-staining. Anther culture promises isogenic diploids on a large scale in minimal time.

Because of the common occurrence of 2n gametes in *S. phureja*, it can readily be crossed to *S. tuberosum*, thus allowing advances made by breeding at the diploid level to be introgressed into *S. tuberosum*. Genetic control of factors responsible for 2n gametes appears to be relatively simple, thus allowing introgression of this trait into diploid germplasm intended for anther culture (Veilleux et al. 1985).

Monoploids are free of all lethal genes due to the effects of the "monoploid sieve" (Wenzel et al. 1979). Pollen grains bearing one or more lethal genes would

not be expected to develop into androgenetic plants due to expression of the lethal genes during some phase of development; such genes would be effectively removed from the population.

Protoplasts

Many techniques for genetic modification of plant cells are dependent upon protoplast competence of the plant line. Somatic hybridization, the fusing of protoplasts from two species that may be sexually incompatible, can result in hybrids not possible by other means (Binding et al. 1986). Binding et al. (1982) incorporated atrazine resistance from *S. nigrum* into *S. tuberosum* by somatic hybridization. Protoplast-fusion-derived cybrids have introduced cytoplasmic male sterility from various diploid potato species into *S. tuberosum* (Perl et al. 1990a; 1990b). These plants are potentially useful as seed-parents for F_1 hybrid seed production. Protoplasts can also take up foreign DNA, cell organelles, bacteria or virus particles, particularly when the membrane is disrupted briefly by an electric current, a process known as electroporation (Binding et al. 1986; Fromm et al. 1986; Tagu et al. 1988). Plants regenerated from protoplasts have frequently exhibited somaclonal variation (Shepard et al. 1980). The calli from protoplasts often undergo genetic changes, including endopolyploidy, aneuploidy, and mutations, which may result in potentially useful somaclonal variation exhibited among regenerated plants. Shepard et al. (1980) tested 800 somaclones derived from protoplasts of Russet Burbank, a cultivar susceptible to *Phytophthora infestans*; 20 showed some resistance.

A later study of plants regenerated from those that had acquired resistance found that these regenerants yielded an even higher frequency of resistant clones (Ayers and Shepard 1981). Protoplast technology is proving to be a powerful tool in modern plant breeding. Therefore protoplast culture competence as a genetic trait has assumed greater importance.

Genetic control of tissue culture traits

Anther culture

Maize (Petolino and Thompson 1987), barley (Dunwell et al. 1986; Powell 1988; Knudsen et al. 1989) and wheat (Agache et al. 1988; Bullock and Baenziger 1982) all show variation among genotypes for anther culture competence. Powell (1987) reported that the frequency of microspore-derived green and albino plant production was under genetic control in barley. Significant reciprocal differences were also detected. Androgenesis in *Solanum tuberosum* was also shown to be under genetic control (Wenzel and Uhrig 1981). Later, Uhrig (1985) reported that androgenetic ability was not only genotype dependent, but could be enhanced in a population by genetic selection. Singsit and Veilleux (1989) crossed anther culture competent *S. phureja* clones with incompetent clones of *S. phureja*, *S. microdontum* and *S. berthaultii* and found that anther culture competence may be under the control of a single dominant gene. Crossing a diploid potato clone with androgentic ability to a root-knot nematode resistant diploid clone which was not androgenetic,

Sonnino et al. (1989) found androgenetic ability among the F_1 generation. Thus, they supported the findings of Singsit and Veilleux (1989) showing that the androgenetic ability in diploid potato clones could be sexually transferred, making it possible to breed anther culture competence into genetic material lacking this ability.

Leaf disc regeneration

Reisch and Bingham (1980) studied the genotypic difference in shoot regeneration from suspension cell cultures in the F_1, F_2 and backcross generations of a diploid alfalfa population. They found segregation ratios suggesting that bud differentiation from callus is controlled by two dominant genes. Being qualitatively controlled, the genes for regeneration should be easily transferred to recalcitrant genotypes.

Somatic embryogenesis and plantlet regeneration from embryo-derived callus were determined to be heritable in maize (Willman et al. 1989). The ability to regenerate plants from leaf explants in *Cucumis sativus* proved to be highly heritable (Nadolska-Orczyk and Malepszy 1989). Plants were classified into three groups: frequently, intermediately and occasionally regenerating. Upon analysis, regeneration was shown to be controlled by dominant genes and probably additive allelic interaction at three loci.

Koornneef et al. (1986) bred the superior regenerating ability of *Lycopersicon peruvianum* into the recalcitrant *L. esculentum* to yield a highly responsive hybrid.

Selfed and backcross progeny were tested for their capacity to regenerate shoots from leaf disc and protoplast derived calli (p-calli). Segregation for regeneration was found to be controlled by two dominant genes; there was no correlation found between callus growth and shoot regeneration (Koornneef et al. 1987). Breeding favorable tissue culture traits into *L. esculentum* will facilitate biotechnological applications to genetic improvement.

M'Ribu and Veilleux (1990) found variation among monoploids derived from different diploid clones and among monoploids from the same diploid clone in *Solanum phureja* for their ability to form shoots from leaf discs. Variation ranged from no shoots to a mean of 45 shoots per explant. Significant variation was also noted in the habit of shoot formation and the capacity to double the chromosome number.

Protoplasts

The genetic component of protoplast competence, defined as the ability of protoplasts to form calli, in Solanaceous species is well-documented (Foulger and Jones 1986; Radke and Grun 1986; Cardi et al. 1990). Tomato has been shown to have both intraspecific (Niedz et al. 1985) and interspecific (Koornneef et al. 1986) variation for protoplast culture. Haberlach et al. (1985) tested 34 clones from five *Solanum* species for protoplast yield, division, calli formation and regeneration. Three clones could not divide, four formed no p-calli and many more were unable to regenerate shoots. Shepard (1982), taking into account the genotypic variation

among *S. tuberosum* cultivars, was able to tailor protoplast isolation techniques to the specific genotypes, although genotypic effects could not be overcome in all cases for shoot morphogenesis. The heritability of this tissue culture trait has been rarely studied. Work with *Petunia* suggests that various stages of protoplast development may be controlled by separate genes (Izhar and Power 1977). The inheritance of specific hormone requirements seemed to be controlled by only a few genes. The model of few genes controlling protoplast competence has also been supported in diploid potato by Cheng and Veilleux (1991), who proposed that protoplast culturability in *S. phureja* is controlled by two unlinked loci with dominant effect.

Environmental effects on protoplast regeneration

The ability to regenerate shoots from p-calli is a prerequisite to using protoplast technology for genetic improvement. However, there are many factors governing p-calli growth and regeneration; genetics (Niedz et al. 1985; Koornneef et al. 1986; Fish and Jones 1988; Wan et al. 1988), pretreatment of the explants (Wright 1985; Kantharajah and Dodd 1990; Rambaud et al. 1990), culture media (Shepard 1982; Li and Constabel 1984; De Vries and Bokelmann 1986; Masson et al. 1987; Tan et al. 1987a) and culture conditions (Shepard and Totten 1977; Robertson et al. 1988; Rambaud et al. 1990).

Pretreatment and culture temperature have been shown to have a significant effect on callus cultures. Cucumber cotyledon protoplast yields were reported to be

maximized when explants were given a 24 hour pretreatment at 30^{0}C (Kantharajah and Dodd 1990). Rambaud and his coworkers (1990) found the plating efficiency of Magdeburg chicory protoplasts to peak at culture temperatures of 30^{0}C. Leathers and Scragg (1989) reported 30^{0}C to be the optimum temperature for *Theobroma cacao* cell suspension cultures.

The conditions for regenerating shoots from *Solanum tuberosum* p-calli were first reported by Shepard and Totten in 1977 using *S. tuberosum* L. cv. Russet Burbank. Most researchers using other species of potato have continued to use the same culture condition, regardless of the climatic preferences of the species.

Comparisons among tissue culture competencies

Links between the ability to regenerate shoots from leaf discs and from protoplast cultures have been reported in both tomato (Tan et al. 1987b) and potato (Fish and Jones 1988). However, there is no consensus. Coleman et al. (1990), extending the work of Fish and Jones (1988), found no correlation between protoplast plating efficiency and any other *in vitro* system tested (nodal multiplication, *in vitro* tuberization and leaf disc regeneration). They stated that previous selection for disease resistance in the genotypes used by Fish and Jones (1988) confounded the data. Coleman used a random sample of diploid potatoes.

Objectives

The first objective of the following reseach was to determine the inheritance of competence for anther culture, protoplast culture and leaf disc regeneration in a population of *Solanum phureja*. The female parent was AM3-8, a doubled monoploid which produced embryos in anther culture, shoots from leaf discs but whose isolated protoplasts did not divide in culture. The male parent, NBP2, did not respond to anther culture or leaf disc regeneration; however,its isolated protoplasts divided in culture and formed abundant p-calli. Segregation for and correlation among these three tissue culture traits were examined in the F_1 and F_2 generations. In a second study, *S. phureja* was tested for its ability to regenerate shoots from p-calli under various temperatures.

CHAPTER TWO: CORRELATIONS AMONG TISSUE CULTURE COMPETENCIES

Introduction

Although differences in the tissue culture competence of potato cultivars and breeding lines have long been recognized, the inheritance of these competencies in segregating populations has only recently been studied. Regeneration of shoots from leaf discs (Fish and Jones 1988; Coleman et al. 1990; M'Ribu and Veilleux 1990), the production of embryos in anther culture (Singsit and Veilleux 1989; Sonnino et al. 1989), and p-calli formation after protoplast isolation (Shepard 1982; Haberlach et al. 1985; Tan et al. 1987a) have all been shown to be genetically controlled.

Finding plant lines that are tissue culture competent is the first step to using these techniques for crop improvement. Screening for competence for either anther or protoplast culture is a long and expensive process, whereas screening for the ability to regenerate shoots from leaf discs is a comparatively quick and easy procedure. Fish and Jones (1988), using a population of potato (*Solanum tuberosum*

L.) preselected for disease resistance, reported that shoot regeneration from leaf discs was indicative of shoot regeneration on p-calli. They suggested screening techniques for regeneration from leaf discs could be used to indicate protoplast culturability. Extending this work to include anther culture competence, we tested a population of the diploid cultivated potato, *S. phureja*, for leaf disc regeneration, anther culture competence and protoplast culture competence to determine if correlations existed among these tissue culture traits.

Materials and methods

Plant materials. The *S. phureja* population was derived from a cross between an anther-derived doubled monoploid, AM3-8, and a heterozygous clone NBP2 (75-21, kindly provided by H. DeJong, Agriculture Canada, Fredericton, NB), selected for adaptation in the field. Both clones can be traced to the diverse adapted population of *S. phureja-S. stenotomum* developed by Haynes (1972). AM3-8, the doubled monoploid (2n=2x=24), was produced by chromosome doubling during shoot regeneration from leaf discs of a monoploid, AM3 (2n=x=12) (Owen et al. 1988) (Fig. 1). It transmits anther culture competence to its progeny (Singsit and Veilleux 1989), but its protoplasts do not divide after isolation and culture (Cheng and Veilleux 1991). Protoplast of NBP2 have been shown to divide, form p-calli, and regenerate shoots after isolation (Haberlach et al. 1985; Cheng and Veilleux 1991). The F_1 population was obtained by crossing AM3-8 with NBP2, using AM3-

8 as the female parent. The F_2 population was generated by crossing two randomly selected individuals ($F_1$21 and $F_1$37) from the F_1 population. Ten individuals from the F_1 population and 20 individuals from the F_2 population were tested in the present study. *In vivo* and *in vitro* copies of the same clones were used for anther culture and leaf disc regeneration studies, respectively.

Leaf disc regeneration. Plants were grown *in vitro* on 15 ml of MS basal (Murashige and Skoog 1962) media in 25 x 150mm culture tubes, at 20^0C, 16 hour photoperiod. The average light intensity was 25 μE $m^{-2}s^{-1}$. Three to four week old leaves were aseptically removed and 5 x 5mm squares were excised to include the midrib. The explants were wounded by slicing the midrib with a scalpel and placed for two weeks on callus induction medium (Karp et al. 1984): MS salts and vitamins, 10μM benzylaminopurine (BAP), 1μM naphthaleneacetic acid (NAA), 3% sucrose, 100 mg/l myo-inositol and 0.6% agar; pH adjusted to 5.8 before autoclaving at 121^0C for 20 minutes at 15 PSI. After two weeks on the callus induction medium, the explants were transferred to a shoot regeneration medium under the same conditions. The shoot regeneration medium was similar to the callus induction medium, except NAA was replaced with 14.4μM gibberellic acid (GA_3) (filter-sterilized). After four weeks, the explants were scored for shoot regeneration using the following scale; 0 for no shoot development, 1 for greater than zero but not more than five shoots (moderately responsive) and 2 for five or more shoots per explant (highly responsive). Each genotype was tested three times in two replications.

Anther culture. The F_1 population and both parents were grown in a greenhouse during the spring of 1990, and the F_2 population was divided into three groups, planted at two month intervals from January to May, 1991. Three plants per clone were grown from tubers in one gallon plastic pots containing a mixture of 1 sand:1 weblite (Weblite Co., P.O. Box 12887, Roanoke, VA):1 promix (Premier Brands, New Rochelle, NY) under greenhouse conditions. All plants received natural lighting; the plants grown from January to May were supplemented with artificial light (mercury vapor lamps) to produce a 16 hour photoperiod to ensure flowering. Plants were fertilized weekly with soluble fertilizer (Peter's Fertilizer Products, W.R. Grace & Co., Fogelsville, PA) containing 20:19:18 (Nitrogen:P_2O_5:K_2O).

Flower buds with microspores at the uninucleate stage (anthers 3.0 to 4.0mm in length) were collected and placed on moist paper towels in plastic Ziplock bags, then refrigerated at 6-8^{0}C for three days in the dark. Buds were then surface sterilized with 100% commercial bleach (Wonder Bleach, 5.25% NaOCl) plus 'Tween 20' for 10 minutes, transferred to 80% ethanol for 30 seconds, rinsed twice in sterile distilled water, and placed on sterile petri plates.

The anthers were aseptically removed and placed in 125 ml Delong Culture Flasks (Bellco Glass Co., Vineland, NJ) containing 15 ml of autoclaved liquid nutrient media (Uhrig 1985) and covered with Magenta 2-way caps (Magenta Plastics, Chicago, IL). Each flask contained 30 anthers. Flasks were incubated on rotary shakers, set at 125 rpm, at room temperature (approximately 25^{0}C). After four weeks, the flask contents were poured through a sterile kitchen strainer and

the anthers and embryos transferred to a petri dish where embryos were counted under an Olympus C011 dissecting microscope. Genotypes were scored for embryo production using the following scale: 0 if none of the flasks contained embryos, 1 if the embryo production fell within the 95% confident interval of the F_1 generation (0.09 ± 0.20 embryos per cultured anther), and 2 of the genotype averaged more than the 95% confident interval of the F_1 generation.

Protoplasts. The protoplast data presented in this study were transformed from previously published data (Appendix 1) (Cheng and Veilleux 1991). A modified procedure of Uijtewaal et al. (1987) for isolation and a modified protoplast culture medium from Schumann and Koblitz (1983) were used. Clones reported to have a plating efficiency (P.E.) greater than 10% were given a rating of 2, those with a P.E. less than 5% were give a rating of 1, and those that did not divide were given a rating of 0.

Results

Leaf disc regeneration. AM3-8 responded by producing more than ten shoots per leaf disc within two weeks; NBP2 did not produce a single shoot. The F_1 population segregated for the ability to regenerate shoots from leaf discs (Table 1). Using the above mentioned scale, a 2:6:2 ratio of unresponsive, moderately responsive and

highly responsive genotypes, respectively, was found. This is not significantly different (X^2=0.3, 0.9 > P > 0.75) from the 1:2:1 ratio expected for segregation of 2 heterozygous loci. The parents for the F_2 generation, $F_1$21 and $F_1$37, were both moderately responsive. The F_2 generation segregated at a ratio of 6:10:5, again not significantly different from 1:2:1 (X^2 =0.4, 0.9 > P > 0.75) (Table 3).

If treated as a qualitative trait, shoot regeneration seems to segregate as two Mendelian genes. Presuming leaf disc regeneration competence is a recessive trait, AM3-8 would be homozygous recessive at both loci. If NBP2 is heterozygous at both loci, it would yield an F_1 generation with a 1:2:1 ratio when crossed with AM3-8. This presumes that genotypes that are homozygous recessive at both loci are maximally responsive, those with a single dominant allele at either locus but not both are moderately responsive, and those with a dominant allele at both loci are nonresponsive. Further supposing $F_1$21 and $F_1$37 are both heterozygous at one locus, a 1:2:1 ratio (unresponsive: moderately responsive: highly responsive) would have appeared in the F_2 generation, as was found (Fig. 2). Leaf disc regeneration has been found to be controlled by two dominant Mendelian genes in tomato (*Lycopersicon peruvianum*) (Koornneef et al. 1987) and diploid alfalfa (Reisch and Bingham 1980).

Anther culture. Of the parents, NBP2 was found to be completely non-responsive to anther culture. AM3-8 was not available for anther culture. However, AM3-4 a plant similarly derived by regeneration on leaf discs of the same monoploid clone

was found to be highly responsive to anther culture. All of the F_1 progeny tested were moderately anther culture competent (Table 2). All of the F_1 progeny scored a 1 (Table 3). Segregation for anther culture competence was observed in the F_2 population, giving a 3:5:7 ratio of unresponsive, moderately responsive and highly responsive genotypes, respectively. For the purpose of hypothesizing a genetic model for anther culture, the genotypes were classified as either responsive if any embyros were produced, or non-responsive, if no embryos were produced. This yields a 3:12 ratio, which is not significantly different from a 1:3 ratio ($X^2 = 0.2$, $0.7 > P > 0.6$), supporting a single gene model with complete dominance (Fig. 3).

Protoplasts. Cheng and Veilleux (1991) reported AM3-8 as unresponsive to protoplast culture while protoplasts isolated from NBP2 divided after three days and formed abundant callus after 30 days. All F_1 plants tested were capable of p-callus formation (Table 3).

Protoplast culturability segregated in the F_2 generation. Among the 20 F_2 individuals tested, four did not respond, four others gave a low plating efficiency and 12 were considered highly responsive (Table 3). Cheng and Veilleux (1991) found protoplast culturability in *S. phureja* to be under the control of two loci with dominant effect, i.e., a dominant allele was required at each locus for good protoplast response (Fig. 4).

Comparisons among tissue culture competencies

Using Pearson's Correlation Coefficient on the SAS (Statistical Analysis Systems) program, no significant correlation was found between any of the tissue culture competencies in the F_2 generation (Table 4). This supports Coleman et al.'s (1990) findings that protoplast culture competence was not correlated with leaf disc regenerability. The present work has added anther culture competence to the list of independently segregating tissue culture traits, suggesting separate genetic controls for each tissue culture competence.

Discussion

The data presented show each of the three tissue culture traits, anther culture, leaf disc regeneration and protoplast culture competence, to be controlled by a small number of genes. However, because no correlation was found among these competencies, each can be considered to be controlled by different genetic mechanisms. Coleman et al. (1990) found similar results, concluding protoplast plating efficiency and shoot regeneration from leaf discs are under separate genetic controls in potato. A lack of correlation between plant regeneration from cotyledon explants and anther culture in *Sinapis alba* was suggested by Jain et al. (1989) when they stated only one of the several cultivars tested responded well to both

procedures. Agache et al. (1988) reported a lack of correlation between anther culture and somatic tissue ability in wheat, and proposed that separate genetic mechanisms controlled these traits. Coleman et al. (1990) also found *in vitro* shoot length and numbers of nodes, microtuber production and protoplast plating efficiency in potato to be controlled by "fewer than many genes." However, the number of "effective factors" for these traits varied among diploids from different cultivars.

Protoplast studies of both potato (Shepard 1982; Fish and Jones 1988) and *Petunia* (Izhar and Power 1977) have shown that genotypic differences controlling protoplast culturability can be overcome to a limited extent by altering the media, especially the content of growth regulators. However, most responsive genotypes performed well over a range of culture conditions, while some genotypes did not respond under any of the culture conditions. This suggests that genotype is the overriding factor in protoplast culturability.

The simple inheritance patterns of the genes controlling these tissue culture competencies facilitate breeding for these characteristics. The data show that tissue culture competence can be sexually transferred to progeny. Unfortunately, due to the lack of correlation among these traits, breeding programs aimed at selecting genotypes responsive to all three of these characteristics will need to screen for each one separately.

Table 1. Mean number of shoots from six leaf discs of parental, F_1 and F_2 clones of *Solanum phureja* after four weeks on regeneration medium.

Genotype	Average number of shoots per explant
Parental generation	
NBP2	0
AM3-8	10+
F_1 generation	
$F_1$1	2.1
$F_1$2	5.5
$F_1$3	10+
$F_1$4	0
$F_1$5	1.3
$F_1$6	0.5
$F_1$7	0
$F_1$8	10+
$F_1$9	3
$F_1$10	3
$F_1$21	4
$F_1$37	3
F_2 generation	
$F_2$1	10+
$F_2$2	0
$F_2$3	9
$F_2$4	0
$F_2$5	0
$F_2$6	10+
$F_2$7	0
$F_2$8	0
$F_2$9	0.5
$F_2$10	4.5
$F_2$11	2
$F_2$12	1
$F_2$13	0
$F_2$14	0.6
$F_2$17	0.8
$F_2$18	3.3
$F_2$19	10+
$F_2$23	10+
$F_2$25	0
$F_2$27	3.1
$F_2$26	2.1

Table 2. Number of anther culture-derived embryos from the parental, F_1, and F_2 generation of a population of *Solanum phureja* clones.

Genotype	Total embryos	Number of cultured anthers	Embryos per cultured anther
Parental			
NBP2	0	300	0
AM3-4	746	330	2.26
F_1 generation			
$F_1$1	25	300	0.08
$F_1$2	37	300	0.12
$F_1$3	12	300	0.04
$F_1$4	41	300	0.13
$F_1$5	26	300	0.08
$F_1$6	20	300	0.06
$F_1$7	47	300	0.16
$F_1$8	27	300	0.09
$F_1$9	19	300	0.06
$F_1$10	34	300	0.11
F_2 generation			
$F_2$1	0	300	0
$F_2$2	145	300	0.48
$F_2$3	31	90	0.34
$F_2$4	0	300	0
$F_2$5			
$F_2$6			
$F_2$7	0	300	0
$F_2$8	17	180	0.09
$F_2$9			
$F_2$10	71	90	0.79
$F_2$11	32	90	0.36
$F_2$12			
$F_2$13			
$F_2$14	43	300	0.14
$F_2$17			
$F_2$18	289	300	0.98
$F_2$19	173	300	0.58
$F_2$21	266	300	0.89
$F_2$23	81	300	0.27
$F_2$25	20	300	0.07
$F_2$27	38	300	0.13

Table 3. Response rating of tissue culture competencies for the parental, F_1 and F_2 generation of a population of *Solanum phureja* clones.

Genotype	Leaf disc	Protoplast	Anther culture
Parental			
NBP2	0	2	0
AM3-8	2	0	
AM3-4			2
F_1 generation			
$F_1$1	1	2	1
$F_1$2	2	2	1
$F_1$3	2	2	1
$F_1$4	0	2	1
$F_1$5	1	2	1
$F_1$6	1	2	1
$F_1$7	0	2	1
$F_1$8	2	2	1
$F_1$9	1	2	1
$F_1$10	1	2	1
F_2 generation			
$F_2$1	2	0	0
$F_2$2	0	2	2
$F_2$3	2	0	2
$F_2$4	0	2	0
$F_2$5	0	1	nt*
$F_2$6	2	0	nt
$F_2$7	0	2	0
$F_2$8	0	2	1
$F_2$9	1	2	nt
$F_2$10	1	1	2
$F_2$11	1	2	2
$F_2$12	1	1	nt
$F_2$13	0	1	nt
$F_2$14	1	2	1
$F_2$17	1	2	nt
$F_2$18	1	2	2
$F_2$19	2	2	2
$F_2$21	1	nt	2
$F_2$23	2	0	1
$F_2$25	0	2	1
$F_2$27	1	2	1

* nt = not tested

Table 4. Correlation coefficients among tissue culture competencies in the F_2 generation of a population of *Solanum phureja* clones. None of the correlation coefficients were significant at $P < 0.05$.

	Leaf disc	Protoplast	Anther
Leaf disc	1.00	-0.41	0.24
Protoplast	-0.41	1.00	0.26
Anther culture	0.24	0.26	1.00

Figure 1. Pedigrees of *S. phureja* plants used for correlating tissue culture competence. A. The development of the homozygous line, AM3-8, through anther culture followed by chromosome doubling during shoot regeneration from leaf disc callus. B. F_1 plants were derived from crosses between AM3-8 and NBP2, a selection from open pollination. C. F_2 generation was produced by crossing two F_1 individuals randomly selected.

A.

PP5 (2n=2x=24)

↓ Anther culture

AM3 (2n=1x=12)

↓ Leaf disc regeneration

AM3-8 (2x=2n=24)

B.

AM3-8 X NBP2

↓

F_1

C.

$F_1$21 X $F_1$37

↓

F_2

Figure 2. Data for shoot regeneration on leaf discs and the proposed model for genetic control of this trait by recessive alleles at two loci in the diploid cultivated potato, *Solanum phureja*.

Phenotypic classification:

0 = no shoots
1 = < 5 shoots per leaf disc
2 = > 5 shoots per leaf disc

Generation	Number of plants in each class.		
	0	1	2
Parents	NBP2		AM3-8
F_1	2	5	3
F_2	7	6	4

Proposed genetic model for inheritance of leaf disc regeneration.

Parental genotypes *aabb* X *AaBb*
Phenotypic classification 2 0

↓

F_1 genotypes 1 *aabb*: 1 *aaBb*: 1 *Aabb*: 1 *AaBb*
Phenotypic classification 2 1 1 0

↓

$F_1$21 and $F_1$37 genotypes *aaBb* X *Aabb*
Phenotypic classification 1 1

↓

F_2 genotypes 1 *aabb*: 1 *aaBb*: 1 *Aabb*: 1 *AaBb*
Phenotypic classification 2 1 1 0

Figure 3. Data for embryo production in anther culture and the proposed model for the genetic control of this trait by one dominant allele in the diploid cultivated potato, *Solanum phureja*.

Phenotypic classification:

0 = no embryos
1 = < 95% confidence interval of F_1 generation
2 = > 95% confidence interval of F_1 generation

Generation	Number of plants in each class		
	0	1	2
Parents	NBP2		AM3-8
F_1		10	
F_2	3	5	7

Proposed genetic model for embryo production in anther cultures.

Parental genotypes	*AA* X *aa*
Phenotypic classification	2 0
	↓
F_1 genotype	all *Aa*
Phenotypic classification	1
	↓
F_2 genotypes	1 *AA*: 2 *Aa*: 1 *aa*
Phenotypic classification	2 1 0

Figure 4. Data for plating efficiency after protoplast isolation and the proposed model for the genetic control of this trait by dominant alleles at two loci in the diploid cultivated potato, *Solanum phureja*.

Phenotypic classification:

0 = 0 % plating efficiency
1 = < 2 % plating efficiency
2 = > 5 % plating efficiency

Generation	Number of plants in each class		
	0	1	2
Parents	AM3-8		NBP2
F_1			7
F_2	4	4	12

Proposed genetic model for protoplast plating efficiency.

Parental genotypes	*aabb* X *AABB*			
Phenotypic classification	0	2		
	↓			
F_1 genotype	all *AaBb*			
Phenotypic classification	2			
	↓			
F_2 genotypes	9 *A-B-*:	3 *A-bb*:	3 *aaB-*:	1 *aabb*
Phenotypic classification	2	1	0	0

CHAPTER THREE: INFLUENCE OF TEMPERATURE ON REGENERATION OF Solanum phureja P-CALLI

Introduction

Shoot regeneration from p-callus is a prerequisite to using protoplast culture as a means of genetic improvement, and a number of factors influencing shoot regeneration from protoplast-derived cell cultures has been studied. Genetic predisposition (Neidz et al. 1985; Koornneef et al. 1986; Fish and Jones 1988; Wan et al. 1988), pretreatment of the explants (Wright 1985; Kantharajah and Dodd 1990; Rambaud et al. 1990), culture media (Shepard 1982; Li and Constabel 1984; De Vries and Bokelmann 1986; Masson et al. 1987; Tan et al. 1987a) and culture conditions (Shepard and Totten 1977; Wright 1985; Robertson et al. 1988; Rambaud et al. 1990) have all been shown to play a role in caulogenesis from p-callus.

Haberlach et al. (1985) found genetic variability among 36 cultivars, breeding lines and accessions from five *Solanum* species in their ability to produce protoplasts that divide, form callus, and produce shoots. Similar differences have been found among cultivars of *S. tuberosum* (Radke and Grun 1986) and within the species, *S. phureja* (Cheng and Veilleux 1991). In an attempt to overcome the genetic factors

in morphogenesis, Shepard (1982) tailored medium to various *S. tuberosum* L. cultivars. While he succeeded in obtaining shoots from most cultivars, 'Bison' remained recalcitrant, suggesting a strong genetic control of caulogenesis from p-calli.

The conditions under which the donor plants are maintained prior to protoplast isolation have proven to affect protoplast yield. Magdeburg chicory required a 16 hour photoperiod in order to obtain a high rate of mesophyll protoplast divisions (Rambaud et al. 1990). Young plants (14 days after germination) were also found to yield protoplasts with a higher plating efficiency (P.E.) than older plants (18-24 days after germination). A dark treatment prior to enzymatic digest has proved important to obtain good protoplast yields and P.E.'s in some crops, such as grape (Wright 1985) and potato (Radke and Grun 1986) but was found to be detrimental in cucumber (Kantharajah and Dodd 1990).

The pretreatment and culture temperature has also been shown to affect P.E. and callus regeneration significantly. Leathers and Scragg (1989) reported 30^0C to be the optimum growth temperature for *Theobroma cacao* cell suspension cultures. Over a range from 10^0C to 35^0C, cucumber cotyledon protoplast yields were reported to be maximized when explants were given a 24 hour pretreatment at 30^0C (Nadolska-Orczyk and Malepszy 1989). While preculture temperatures had no effect on subsequent regeneration from leaf discs, M'Ribu and Veilleux (1990) found that *S. phureja* leaf explants incubated at 20^0C had a higher regeneration frequency and

more shoots than those at 25^{0}C. Rambaud et al. (1990) found the P.E. of Magdeburg chicory protoplasts to peak when the culture temperature was 30^{0}C.

The conditions for regenerating shoots from potato p-calli were first reported by Shepard and Totten (1977) using *S. tuberosum* L. cv. Russet Burbank. They found that culture temperatures above 28^{0}C depressed callus growth and temperatures above 24^{0}C retarded shoot bud development. Therefore, 24^{0}C was adopted as the best temperature under which to regenerate *Solanum tuberosum* p-calli. Many researchers using other species of potato have continued to use culture conditions between 24^{0}C and 26^{0}C. In the present study, p-calli from the diploid (2n=2x=24) cultivated potato, *S. phureja*, were grown under several temperature treatments to determine the temperature effects on shoot regeneration.

Materials and methods

Protoplasts derived from the *Solanum phureja* (2n=2x=24) clone, $F_2$14, are known to form p-calli (Cheng and Veilleux 1991). $F_2$14 protoplasts were isolated and cultured according to Cheng and Veilleux (1991) with some modifications in the enzyme and culture medium. The enzyme medium was prepared using one of 7%, 8%, 9%, 10%, or 11% sugar; all five enzyme media contained 1.8% glucose but mannitol was varied to achieve the desired percent sugar. One ml of enzyme medium was added per gram of macerated *in vitro* leaves, and incubated in the dark

for 16 hours at approximately 25^{0}C. No difference was found for the number of viable protoplasts isolated per gram of leaf tissue among the five enzyme media. Protoplasts were tested for viability using FDA (fluorescein diacetate) stain. Protoplasts which fluoresced green when viewed with UV light were considered viable.

Each of the five cultures was then divided into thirds. One third from each of the five enzyme treatments was incubated in culture media containing either 8%, 9%, or 10% sugar. All three culture media contained 3% glucose and 1% sucrose; sorbitol was varied to achieve the desired percent sugar. Among the resulting 15 plates, no significant difference was found in plating efficiency. Small calli (1mm) were discernible at this stage. A total of 960 calli was placed on greening media, 20 per plate, at 25^{0}C under a 16 hour photoperiod to encourage callus growth. The 48 plates were divided into eight groups of six plates each, and each contained an equal number of calli from the 15 cultures (Table 1). Regeneration requires transfer of calli from culture medium, which has a high auxin to cytokinin ratio to promote callus growth, to greening medium, with a lower auxin to cytokinin ratio, then to regeneration medium, containing gibberellin (GA_3) to encourage shoot elongation. While on the greening medium, each group of calli was placed in a growth chamber at 25^{0}C for one week, then received one of three preregeneration temperature regimes; a heat treatment, 30^{0}C day (13 h) and 20^{0}C night (11 h); a cold treatment, constant 20^{0}C; or constant 25^{0}C. The treatments were applied in similar incubators (Percival model I-60LLVL) set to different temperatures. All groups received a 16

h photoperiod. After two weeks, all groups were subcultured onto regeneration medium, and either placed back into their preregeneration temperature regime (15^0C), or placed into one of the other two regimes (Table 1). All cultures were transferred to fresh regeneration media three times at three week intervals to enhance caulogenesis. Meristems and shoots per callus were counted after seven, eight, nine, and ten weeks on regeneration medium.

Results

As early as eight weeks after placement on regeneration media, groups 2, 4, and 6, all of which received 30^0C/20^0C treatments, produced significantly more meristems per plate than group 1, which received a constant 25^0C. Because many researchers use 25^0C to incubate p-calli, group 1 was used as a control. After nine weeks on regeneration media, groups 2 and 6, both of which received the heat treatment during regeneration, had significantly more meristems and shoots, and the meristems had elongated more than those in group 1. By the tenth week, more segregation among the groups had occurred and a trend was apparent (Table 1). Group 2, given the heat treatment both on the preregeneration and the regeneration media, produced the most shoots per plate. All of the groups which received a heat treatment during either phase produced more shoots than those which did not; however, only two were significantly more productive than the control. Group 1 consistently produced fewer meristems and shoots than any of the other groups, including group 5, which received the cold treatment during both stages. Overall,

the shoots from group 2 were thicker and longer than those in the other groups, characteristics that are indicative of subsequent survival after transfer to basal medium and development into whole plants.

Discussion

Culture temperature has been shown to have a profound effect on P.E. of protoplasts from several crops (grape, Wright 1985; chicory, Rambaud et al. 1990). However, the effect of temperature on caulogenesis has often been overlooked. In the present report, the effect of temperatures on morphogenesis of *S. phureja* p-calli was examined and, of these, 30^{0}C days and 20^{0}C nights during callus growth and shoot development proved to be the most favorable. This is in contrast to Shepard and Totten (1977) who reported reduced shoot yield from *S. tuberosum* p-calli cultured above 24^{0}C.

We realize that the use of only one clone limits the generalizations which can be made. However, the data demonstrate that a constant 25^{0}C is not necessarily ideal for regeneration in potato. Because only the heat treatment had a temperature cycle, it is possible that the improved caulogenesis was due to diurnal fluctuation in temperature rather than to the elevated temperature *per se*. This question is currently being addressed, as the experiment is being replicated. In addition to the heat treatment, and the constant 25^{0}C, a 25^{0}C day and 15^{0}C night treatment is being added.

Table 5. Shoot regeneration from *Solanum phureja* p-calli under different temperature regimes.

Group	Pre-regeneration temperature (^{0}C)	Regeneration temperature (^{0}C)	Mean Shoots	
			9 wks	10 wks
2	30/20	30/20	4.8ab	9.2a*
6	25	30/20	5.2a	7.8ab
7	20	30/20	2.4bc	5.0bc
3	30/20	25	2.3bc	4.1bc
1	25	25	1.5c	1.8c
4	30/20	20	3.4abc	3.4c
8	25	20	3.0abc	2.8c
5	20	20	2.6abc	2.4c

* Means with the same letter are not significantly different, Duncan's multiple range test, $P < 0.05$.

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APPENDIX 1

Protoplast culturability of the parental, F_1, and F_2 generation of a population of *Solanum phureja* clones (from Cheng and Veilleux 1991).

Genotype	% Plating efficiency Liquid	Embedding
Parental		
AM3-8	0	0
NBP2	25	20
F_1 generation		
$F_1$4	11	nt*
$F_1$5	31	19
$F_1$13	14	nt
$F_1$14	17	nt
$F_1$15	12	nt
$F_1$21	19	22
$F_1$37	13	13
F_2 generation		
$F_2$1	0	0
F_{22}	22	18
$F_2$3	0	0
$F_2$4	14	13
$F_2$5	1	1
$F_2$6	0	0
$F_2$7	10	14
$F_2$8	20	nt
$F_2$9	11	13
$F_2$10	2	nt
$F_2$11	13	11
$F_2$12	1	1
$F_2$13	1	1
$F_2$14	17	17
$F_2$17	nt	12
$F_2$18	nt	14
$F_2$19	nt	16
$F_2$23	nt	0
$F_2$25	nt	12
$F_2$27	nt	10

* Not tested.

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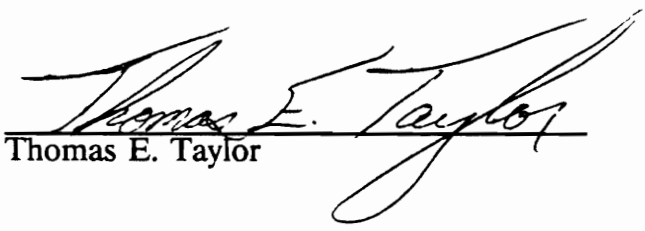

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