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Use of Monoploid *Solanum phureja* in Cell and Tissue Culture Techniques for Potato Improvement

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**USE OF MONOLPLOID SOLANUM PHUREJA IN CELL AND TISSUE
CULTURE TECHNIQUES FOR POTATO IMPROVEMENT**

by

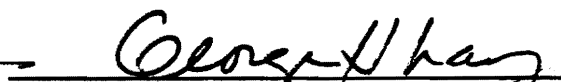
Henry R. Owen, Jr.

Dissertation submitted to the Faculty of the
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Doctor of Philosophy
in
Genetics


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(ABSTRACT)

Monoploid genotypes ($2n = x = 12$), derived by anther culture of a diplandrous genotype of *Solanum phureja*, a South-American diploid potato species, were examined for their utility in germplasm development.

Nine monoploid genotypes and the diploid anther-donor plant were grown in photoperiod chambers at The Southeastern Plant Environment Laboratories (SEPEL) at North Carolina State University to examine the effect of photoperiod on tuber yield and to determine the variability for critical photoperiod for tuberization. Significant differences were found among the monoploid genotypes for total tuber weight and tuber number. Longer photoperiod treatments both decreased and delayed tuberization. Axillary tuber formation from single-node cuttings was used to estimate the onset of tuber induction and demonstrated variability among monoploid genotypes for critical photoperiod for tuberization.

Leaf-disc culture of 24 monoploid genotypes yielded calli which regenerated plants from three genotypes. SDS-polyacrylamide gel electrophoresis of leaf extracts demonstrated variability among diploid and tetraploid calliclones of one monoploid genotype for total protein banding pattern. Absence of stainable pollen and lack of seed set after crosses to diploid species and tetraploid cultivars illustrated infertility among doubled ($2n=2x=24$) and twice doubled ($2n=4x=48$) monoploid-derived lines.

Flow-cytometric analysis of pollen obtained from the diploid anther-donor genotype grown under three photoperiods at SEPEL yielded two populations of pollen based on propidium iodide staining of DNA. These populations corresponded to pollen separation based on size parameters alone, introducing the potential for flow sorting of pollen to increase seed set in $4x-2x$ crosses to tetraploid cultivars.

Protoplast isolation from *in vitro* material and extraction of leaf nuclei both *in vitro* and *in vivo* were performed on the anther-donor plant, one of its anther-derived monoploids, and a diploid and tetraploid plant derived from callus culture of the monoploid genotype. Flow-cytometric analysis of propidium-iodide stained cells and nuclei showed a greater ploidy stability for plant material grown *in vitro* and a limit to endopolyploidization imposed by initial ploidy level.

Flow-cytometric analysis of protoplast-derived nuclei from nine monoploid genotypes derived from anther culture of a single diploid genotype exhibited significant differences for 4C DNA content, but not for 1C DNA content, indicating that ploidy stability, rather than monoploid status *per se*, is influenced by genotype.

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Chapter 1 - Introduction

Historical Background

The cultivated potato, *Solanum tuberosum* L., is a crop species of major economic importance. It ranks fourth in world production, yet it is superior to wheat, corn, and rice in its ability to produce a more nutritious food in less space and in less time. Dependence upon the potato as a world food source is expected to increase into the next century (International Potato Center, 1984).

Much of the increased yield of potato over the last forty years has been the result of optimizing environmental conditions for the limited number of commercially grown cultivars. Future strategies must focus on improving the genetic composition of the crop (Hooker, 1983).

Although the potato has traditionally been vegetatively propagated, production of potatoes from true seed has several advantages over tuber propagation (Ross, 1986). Seed certification, a process designed to screen tubers for disease, is an expensive, time-consuming, and labor intensive process (Callison et al., 1982;

Shepard and Claflin, 1975). Transmission of diseases, particularly viruses, is a serious problem with any vegetatively propagated species, and the potato is particularly vulnerable (Shepard and Claflin, 1975). By comparison, many viruses are not transmitted through botanical seed.

Progenies from sexual crosses of cultivated varieties, however, are generally inferior to either parent, due in part to the multi-allelic nature of tuber yield and in part to the very narrow genetic base of present cultivars (Howard, 1970). This lack of genetic diversity among cultivars is, fortunately, richly compensated by a wealth of native species. Interploid hybridization between tetraploid *Solanum tuberosum* and tuber-bearing, South American, diploid species is one method by which new sources of germplasm can be incorporated into potato cultivars (Ehlenfeldt and Hanneman, 1984; Haynes, 1972; Mok and Peloquin, 1975a, 1975b, 1975c; Ramanna, 1979; Stelly and Peloquin, 1986; Veilleux, 1985; Veilleux and Lauer, 1981).

Solanum phureja ($2n=2x=24$) is a cultivated potato species with tremendous potential for incorporating variability and characteristics such as disease resistance and heat tolerance into potato cultivars. Indigenous to South America, it is cultivated for its regionally superior yield and vigor. It is genetically distant from present-day cultivars and thus represents a source of alleles different from those of *Solanum tuberosum*. Interploid crossing barriers (which are often caused by imbalances in embryo/endosperm ploidy levels) have been overcome via unreduced gametes with the somatic rather than gametic chromosome

number (Hoglund, 1970; Veilleux and Lauer, 1981; Veilleux et al., 1982). Resulting $4x-2x$ hybrids (between a tetraploid cultivar and a diploid, diandrous species) have demonstrated a yield potential equivalent to the tetraploid parent (Kidane-Mariam et al., 1985; Veilleux and Lauer, 1981).

This relatively unadapted, diploid species, however, has many undesirable characteristics for tuber quality (deep eyes, dark skin pigmentation, yellow flesh, small tuber size, many tubers per plant) and for breeding value (self-incompatibility, short photoperiod requirements for tuberization, and variable frequencies of $2n$ pollen production). A population of *Solanum phureja* has been developed through mass selection with tuberization under long days as the only selection criterion (Haynes, 1972). This "semi-adapted" population was developed primarily to facilitate its further study in northern climates. Mass selection is a conventional breeding method for improving heterozygous, self-incompatible species, but it is a time-consuming process. Selection for desirable traits would be simplified by using homozygous lines. This usually is accomplished via inbreeding (Wenzel et al., 1979), a difficult procedure for many potato species, due to self-incompatibility (Abdalla and Hermsen, 1971). A non-conventional method for developing inbred lines which circumvents self-incompatibility is provided by anther culture.

Anther Culture

Embryogenesis and seed development normally occur as a result of the union of haploid gametes, forming a diploid zygote. Androgenesis (embryogenesis from microspores) is a process by which haploid sporophytes may be produced by redirecting the process of microsporogenesis and pollen development (Collins, 1977; Guha and Maheshwari, 1966; Keller and Stringham, 1978; Maheshwari et al., 1980, 1982; Pandey, 1973; Vasil and Nitsch, 1975). Veilleux et al. (1985) derived monoploid genotypes ($2n = x = 12$) from anther cultures of a diandrous ($2n$ pollen-producing), semi-adapted genotype of *Solanum phureja*. The potential for this type of material in a germplasm development program is far-reaching.

Use of Monoploid Genotypes

Monoploid plants are more useful than their diploid counterparts for developmental, genetic, and evolutionary research for several important reasons (Cappadocia et al., 1984; Hermsen, 1984; Maheshwari et al., 1980; Melchers, 1972). Because monoploid plants contain only one set of chromosomes, segregation of alleles and dominance effects (which complicate genotypic selection via phenotypic evaluation) are eliminated. In other words, the phenotype is a direct reflection of the genotype. Recessive alleles which are rarely found in the homozygous state (due to polyploidy or linkage to deleterious genes) can be ex-

pressed in the hemizygous state (Howard, 1973). Similarly, all mutations (spontaneous or mutagen-induced) will be expressed. Direct expression of genotype, along with recent successes in protoplast isolation, fusion, culture, and regeneration, allows plant scientists to manipulate plant cells in much the same manner as microbiologists have manipulated microorganisms.

An added advantage to generating monoploid genotypes is that any genomic combination containing one or more lethal alleles will be eliminated. Wenzel et al. (1979) have labeled this selection process "the monoploid sieve", because it eliminates deleterious combinations prior to regeneration. Thus, screening for desirable monoploid genotypes from among the genotypes that regenerate should be more rapid (because lethals are already eliminated) as well as more direct (because dominance is eliminated). Also, screening can be done early in germplasm development (i.e., prior to either chromosome doubling, if the aim is to create homozygous diploid genotypes, or somatic hybridization, if the aim is to create heterozygous diploid genotypes). These two processes are described below.

Chromosome Doubling

Callus cultures, particularly of monoploid tissues, often are unstable with regard to ploidy level and may undergo one or more cycles of endopolyploidization in culture. Spontaneous chromosome doubling has been reported in callus cultures

of potato (Jacobsen, 1977; Karp, et al., 1985). Maintenance of monoploid status *in vitro* is desirable for somatic hybridization. On the other hand, spontaneous chromosome doubling is desirable if the objective is the production of homozygous diploids. Thus, the occurrence and predictability of endopolyploidization *in vitro* will determine whether genetic manipulation (facilitated by monoploid stability) and restoration of fertility (requiring chromosome doubling) is feasible. Callus cultures may also regenerate somaclonal variants (Smith, 1986). Likewise, the occurrence and type of genetic variation produced by this technique will determine whether clonal propagation (requiring monoploid stability) or creation of novel genetic variation (via somaclonal variation) is possible.

Caulogenesis (shoot regeneration from callus tissue) is influenced by many environmental and physiological factors, including ratio of growth regulators in the culture medium, genotype, explant tissue type and tissue age (Ahloowalia, 1982; Behnke, 1975; Webb et al., 1983). The ability of callus tissue to regenerate whole plants is desirable for storage, maintenance, and multiplication of monoploid genotypes. In addition, regeneration is needed after chromosome doubling to produce homozygous diploid plants.

Sterility in existing potato cultivars is not uncommon (Grun, 1970). Similarly, sterility may occur after chromosome doubling. This can be caused by many factors, including interactions between ploidy levels and self-incompatibility systems, meiotic abnormalities encountered after cell and tissue culture, or se-

vere inbreeding depression (de Jong and Rowe, 1971). However, if they are fertile, calliclones (callus-derived clones) may be useful for later sexual hybridization.

Somatic Hybridization

Fusion of protoplasts from unrelated monoploids may restore fertility by complementation and could produce highly heterozygous diploids for $4x-2x$ hybridization (unilateral sexual polyploidization), $2x-2x$ hybridization (bilateral sexual polyploidization), or somatic fusion to an unrelated, reconstructed diploid (bilateral somatic polyploidization).

Protocols exist for the isolation and purification of viable protoplasts, as well as plant regeneration from tetraploid and dihaploid potatoes (Barsby and Shepard, 1983; Binding et al., 1978; Bokelmann and Roest, 1983; Debnath et al., 1986; Foulger and Jones, 1986; Haberlach et al., 1985; Schumann and Koblitz, 1983; Secor and Shepard, 1981; Shahin, 1984; Shepard, 1980, 1982; Shepard and Totten, 1977; Thomas, 1981). Somatic hybridization between *Solanum* species also has been reported (Austin et al., 1985, 1986; Ehlenfeldt and Helgeson, 1987).

Objectives

The objectives of the following research were to assess the utility of monoploid, anther-derived genotypes of *Solanum phureja* in a genomic reconstruction scheme for potato germplasm development. Initially, the anther-donor genotype (PP5) of *Solanum phureja* was examined for two characteristics of importance to this type of breeding scheme, namely $2n$ pollen production and anther culture response, and the effect of environment (photoperiod) on these two processes was also studied (Chapter 2). Next, nine monoploid genotypes derived from anther culture of PP5 were examined for their inherent variability for an economically important trait (tuber yield) and a yield parameter (critical photoperiod for tuberization), with the intent of selecting superior genotypes (those possessing the highest yield and longest critical photoperiod for tuberization) directly and very early in the reconstruction process (Chapter 3).

Next, callus culture was used to induce chromosome doubling of a monoploid genotype followed by regeneration of an array of clones from callus. Fertility tests and analyses of leaf proteins were conducted simultaneously to determine the somaclonal variation present and the usefulness of this material for later sexual hybridization (Chapter 4). Finally, the utility of monoploids in a somatic hybridization scheme was examined (Chapter 5). This study used a technique relatively new to plant science research (flow cytometric analysis) to reveal the genetic variability for ploidy stability among cell preparations from monoploid

tissues. By this method, genotypes exhibiting a high degree of monoploid stability may be selected for later somatic fusion.

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Chapter 2

Photoperiod effects on $2n$ pollen production, response to anther culture, and net photosynthesis of a diplandrous clone of *Solanum phureja*

Introduction

Interploid hybridization between tetraploid *S. tuberosum* cultivars and diploid potato species is currently being exploited to introduce new germplasm into potato cultivars (Mok and Peloquin, 1975a; Ramanna, 1979; Veilleux, 1985). This technique has relied primarily upon $2n$ pollen formation in a diploid species and subsequent crossing to a tetraploid cultivar, producing a $4x-2x$ hybrid. Tuber yield of such hybrids have approached the tetraploid parent (Veilleux and Lauer, 1981). Unfortunately, seed set in $4x-2x$ crosses is highly variable and generally inferior to intraploid crosses (Kidane-Mariam et al., 1985). Frequency of $2n$ pollen formation has been shown to be affected by physiological (Haynes et al., 1987) and morphological factors (Veilleux and Lauer, 1981), as well as genotypic predispositions (Mok and Peloquin, 1975b).

Estimations of $2n$ pollen frequency have commonly been based on microscopic examination and visual scoring of large pollen grains (Janssen and Hermsen, 1976). Flow-cytometric analysis of pollen samples labeled with a fluorescent DNA stain, on the other hand, is able to offer simultaneous measurement of size and DNA content of a large number of pollen grains with great rapidity and accuracy (Muirhead, 1984). In addition, it is able to display DNA distributions of sub-populations within a sample based on size parameters alone, thus allowing for separate DNA analysis of two size classes, such as large and small pollen grains.

Extraction of monoploid genotypes from diploid species has been envisioned as a useful first step in germplasm development, because it allows for direct phenotypic selection prior to genomic reconstruction (Hermsen, 1984). Reconstruction utilizing monoploid, anther-derived genotypes could be accomplished via somatic hybridization (cell fusion) between two unrelated genotypes which have been selected for superior characteristics, or by sexual hybridization via a $4x-2x$ cross after chromosome doubling and restoration of fertility. As with $2n$ pollen formation, response to anther culture has been shown to vary with environment (Maheshwari et al., 1980) and genotype (Collins, 1977).

Selection for increased photosynthetic capacity has been examined as a method for improving yield of many existing crop species (Huber et al., 1984). In addition to genotype, photosynthetic capacity is strongly influenced by environmental factors (Ma and Hunt, 1983; Casano et al., 1984), and thus selection

efforts require strict environmental control to reduce variability within genotypes. Photoperiod has been shown to influence several developmental and physiological processes in *Solanum* species, most notably tuberization (Hammes and Beyers, 1973; Mendoza and Haynes, 1977) and flowering (Gregory, 1956; Haynes et al., 1987), which generally are favored under short and long day cycles, respectively. Detection of genotypes which demonstrate greater net photosynthesis may depend upon whether vegetative or floral development is favored.

The objectives of this study were to examine the effect of photoperiod on the three aforementioned characteristics of interest to current breeding efforts, i.e. $2n$ pollen production, anther culture response, and net photosynthesis utilizing a diplandrous ($2n$ pollen-producing) clone of *Solanum phureja*. This clone has been selected for enhanced response to anther culture, but has not previously been examined for net photosynthetic rate.

Materials & Methods

The following studies were conducted on plants grown at the Southeastern Plant Environment (Phytotron) Laboratories of North Carolina State University. Tubers from a diploid, diplandrous clone of *Solanum phureja*, P.I. 225669, genotype PP5, were planted in flats containing 1/3 Peat-lite (Redi Earth, W. R. Grace Co.) and 2/3 gravel (standard phytotron substrate), and placed in 3

photoperiod rooms (chamber size B). The chambers were set for 3 photoperiods, 10, 14 and 18 hrs. After the initial 10 hr daylength ($650 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density, or PPFD), low-intensity incandescent lights ($50 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD) were used to extend the photoperiods in the remaining 2 chambers to 14 and 18 hrs. All chambers were maintained at 18°C for the minimum 10 hr light period and 14°C for the remaining 14 hr period. Plants were watered 3 times weekly throughout the experiment with a nutrient solution containing 106 ppm N, 10 ppm P, and 111 ppm K. Sprouted tubers were transplanted into individual 15cm pots 6 wks after planting (6 plants per chamber).

Flow cytometric analyses: Pollen samples from each photoperiod treatment were collected 10 wks after experiment initiation and placed in a dessicator at 4°C . Pollen samples from the 3 photoperiods were placed in 1 ml of buffer solution [882 mg sodium citrate, 419 mg morpholinopropanesulfonic acid (MOPS), 915 mg MgCl_2 , 0.25 ml Triton X-100/250ml]. To each sample, 0.5 ml of RNAase solution (80 mg ribonuclease-A in 100 ml buffer solution) was added and the samples were incubated at 23°C for 30 min. Subsequently, 0.25 ml of propidium iodide (PI) solution (40 mg PI in 100 ml buffer solution) was added to each sample, incubated on ice for an additional 30 min., and analyzed within 3 hrs. Stained samples were filtered through a $37 \mu\text{m}$ nylon mesh and analyzed using an Epics V, Model 752 laser flow cytometer and cell sorter (Coulter Electronics, Hialeah, FL). Laser excitation was 300 mW, 488 nm from a 5 W

Innova 90 Argon Laser (Coherent Inc., Palo Alto, CA). Three parameters were recorded: forward angle light scatter (FALS) and 90 degree light scatter (90LS, 488 nm dichroic filter) for size measurements, and red fluorescence (RFL, 590 nm dichroic, 610 nm long pass filter) for DNA measurements. Multiparameter Data Acquisition and Display System (MDADS) and Easy 88 microcomputer analysis (Coulter Electronics) were used for data collection and analysis. FALS was collected linear integral, 90LS log integral, and RFL linear peak red. Histograms of number of nuclei per fluorescence channel contained 256 channels and were gated on FALS and 90LS dual parameter 64 X 64 channels resolution histograms defining the populations of interest. The fluorescence signal from PI-stained preparations is proportional to the DNA content of particles (pollen grains) passing through the flow sheath. Chicken red blood cells and a microsphere standard (Coulter Electronics) were used as calibration standards. Pollen grain counts were set at 5,000/sample.

Anther cultures: Pre-anthesis floral buds from plants in the 14 and 18 hr photoperiod chambers were collected at 8, 10, 12, and 14 wks, wrapped in moist paper, and kept at 4°C for 3 days prior to anther culture. Flowering in the 10 hr chamber was insufficient for anther culture. Floral buds were disinfested by a 30 sec. dip in 70% ethanol, followed by immersion in a 5.25% sodium hypochlorite solution for 20 min. and 3 rinses in sterile, distilled water. Anthers were dissected from the buds and plated on a solid/liquid bilayer medium (7 ml bottom layer containing MS salts and vitamins (Murashige and Skoog, 1962),

60 g/l sucrose, 5 g/l activated charcoal, 7 g/l agar, and 1 mg/l N⁶-benzylaminopurine (BAP), pH 5.8; 1 ml top layer containing same as bottom, but lacking activated charcoal and agar) as described by Veilleux et al. (1985). Ten anthers were placed in each petri plate and incubated at 25°C under a 16 hr photoperiod for 4 wks. Embryos arising from within the cultured anthers were transferred to 25x150 mm culture tubes containing 20 ml of filter-sterilized embryo medium (MS salts, 100 mg/l inositol, 0.4 mg/l thiamine, 0.1 mg/l gibberellic acid (GA₃), 20 g/l sucrose, 7 g/l agar, pH 5.8). and incubated as above (Wenzel and Uhrig, 1981). After 6 wks, plantlets were transferred to MS basal medium for rooting.

Photosynthesis and yield measurements: After 8 wks of growth, measurements of net photosynthesis (carbon dioxide exchange rate, CER) were taken for all plants during the middle of the light period using an infrared gas analyzer (Anarad, Model AR-500R) equipped with an external reference (ambient CO₂). CER measurements were taken 3 times on fully expanded leaflets from nodes 4, 5, and 6. Measurements were repeated at 10 and 12 wks after experiment initiation. Total tuber weight and tuber number (greater than 1cm diam.) were recorded for each plant at harvest (16 wks after planting).

Results

2n pollen formation: Pollen size was found to correspond reasonably well with DNA content in each of the 3 photoperiods (Fig. 1). As the photoperiod increased, the pollen population shifted to a more obvious bimodal distribution, indicating increasing 2n pollen frequency based on size parameters (Fig. 1a-c) and DNA content (Fig. 1d-f). The major and minor peaks occurred at channels 60 and 90 (out of 256 channels total) and corresponded to small (Fig. 1g-i) and large (Fig. 1j-l) pollen subpopulations, respectively.

In the sample from the 10 hr photoperiod, 62% of the particles which recorded a positive fluorescent event were contained within the major population. Similarly, samples from the 14 and 18 hr photoperiod recorded major populations corresponding to 59 and 62% of their total counts (Figure 1a-c). By contrast, minor populations, corresponding to the larger-sized pollen grains, were 7, 8, and 16% of the total counts from the 10, 14, and 18 hr photoperiods, respectively. Remaining counts fell outside of the populations of interest and consisted mainly of particles (debris, aborted pollen) recording very low size values in the scattergrams. This increased frequency of particles recording large size and DNA values in the 18 hr sample indicates a twofold increase in 2n pollen in the sample taken from plants grown under an 18 hr photoperiod. However, the subpopulation of larger pollen did include a small frequency of 1n pollen, as determined by DNA content (Figure 1, l).

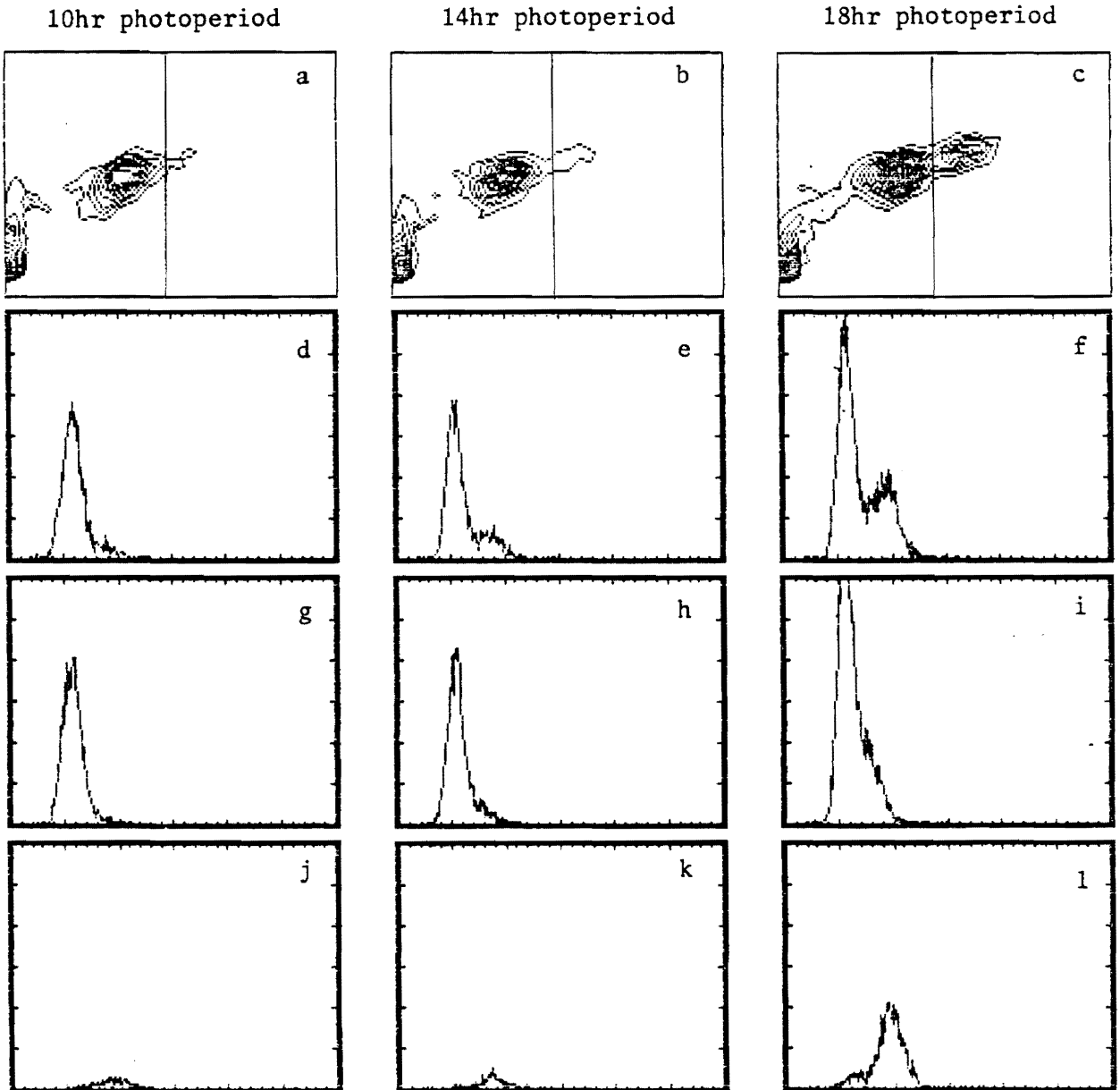


Figure 1. Flow-cytometric analyses of pollen size [measured by forward angle (x -axis) and 90° (y -axis) light scatter] and DNA distribution (measured by fluorescence of propidium iodide) of a $2n$ pollen-producing clone of *Solanum phureja* grown under three photoperiods. After elimination of debris appearing in the lower left of figures a-c, the pollen populations were analyzed for DNA content (d-f). X-axis equals channel number, y-axis equals frequency of nuclei per channel (of 5,000 analyzed). The pollen populations in figures a-c were then divided (vertical line) and the subpopulations reanalyzed for DNA content (g-i, major population of mostly $1n$ pollen and j-l, minor population of mostly $2n$ pollen).

Response to anther culture: The highest response to anther culture was observed on anthers taken from the first flowers on plants in the 14 hr chamber (Table 1). The number of pre-anthesis floral buds formed varied weekly and by photoperiod. Both flowering frequency and duration reached a maximum in the 18 hr chamber. Anthers taken from plants grown under an 18 hr photoperiod produced fewer embryoids and plantlets, even though a greater number of anthers were cultured from this photoperiod. Frequency of embryoids and plantlets was superior from plants grown under a 14 hr photoperiod, as demonstrated by mean embryoids/anther. This increased response, however, was almost entirely the result of anthers cultured at 8 wks. By contrast, anthers cultured from the 18 hr photoperiod did not produce embryoids or plantlets from 8 wk old plants; positive response to anther culture from the 18 hr chamber was both later in occurrence and longer in duration. Maximum response to anther culture also appears to occur later in the 18 hr photoperiod, as indicated by mean plantlets/anther.

Net photosynthetic rate: CER did not vary significantly among the 8, 10, and 12 wk measurements. In addition, no significant interaction was found (5% level) between week and photoperiod treatments (data not shown); thus, weekly measurements of CER were combined for further data analysis. Mean separation of CER by Student-Newman-Keuls' test at the 1% level was significant between photoperiods. Mean CERs were 2.7, 1.3, and 0.8 $\mu\text{l CO}_2 \text{ dm}^{-2} \text{ s}^{-1}$ for the 10, 14, and 18 hr photoperiods, respectively. In addition, significant corre-

Table 1. Anther culture response of *Solanum phureja*, genotype PP5, taken from plants grown under 14 and 18 hr photoperiods for 8, 10, 12, and 14 wks.

Photoperiod	Age of plant	Anthers plated	Embryoids formed	Plantlets regenerated	Embryoids per anther	Plantlets per anther
14	8	40	43	22	1.08	0.55
14	10	300	6	0	0.02	0
14	12	80	0	0	0	0
14	14	0	0	0	0	0
<i>Total</i>		420	49	22	0.12	0.05
18	8	20	0	0	0	0
18	10	340	16	3	0.05	0.01
18	12	200	7	7	0.04	0.04
18	14	200	2	0	0.01	0.01
<i>Total</i>		760	25	10	0.03	0.01

lations (5% level; N = 54) were found between CER and both final tuber weight (0.54) and tuber number (0.74).

Table 2. Mean CO₂ exchange rate (CER), mean tuber number, and mean tuber wt of PP5 at 3 photoperiods.

	<i>Photoperiod</i>		
	10 hrs	14 hrs	18 hrs
CER (ul CO ₂ dm ⁻² s ⁻¹)	2.7	1.3	0.8
Tuber number	7.3	5.7	2.5
Tuber wt. (g)	106.0	95.0	33.4

Discussion

Pollen size was found to be a fairly accurate gauge of DNA content, and allows for estimation of $2n$ pollen frequency based upon visual scoring of pollen preparations. In addition, it makes possible the separation of $2n$ from $1n$ pollen based solely on size parameters. Because flow sorting of pollen samples based upon size does not require fluorescent staining, the potential exists for recovery of viable pollen grains within size classes.

A preliminary experiment to sort fresh, unstained preparations of pollen collected from a diplandrous clone of *Solanum phureja* for later use in a cross to a tetraploid *S. tuberosum* cultivar (Atlantic) was attempted. Sorted samples of $2n$ pollen were collected, centrifuged to remove sheath fluid, and applied to emasculated Atlantic flowers using a 50 μ l pipette. Both unsorted and sorted pollen samples were applied by hand to emasculated Atlantic flowers to compare seed set between the two treatments. Difficulties in removing the sheath fluid from the sorted pollen samples and using moist pollen samples for pollinations may have contributed to senescence of flowers pollinated with sorted samples.

For *S. phureja* genotype PP5, $2n$ pollen frequency was found to increase under an 18 hr daylength. Thus, screening of several genotypes for $2n$ pollen frequency may not give an accurate measurement of maximum frequency unless the genotypes being tested behave similarly for the given photoperiod under which the plants are grown.

For PP5, net photosynthesis and tuberization reached their maximum under short photoperiods. Conversely, flowering and $2n$ pollen formation peaked under long daylengths. Thus, vegetative development was favored under short daylengths, whereas long daylengths stimulated floral development. Response to anther culture in this genotype was greatest from 8 wk plants grown under a 14hr photoperiod. Anther culture itself involves a redirection from floral development (pollen formation) to vegetative development (embryo formation);

therefore, the best physiological and environmental conditions for embryoid formation and plantlet regeneration may be from young anthers grown under a moderate photoperiod.

This work demonstrates that vegetative and floral development *in vivo* and plant regeneration from anther culture each impose their own unique set of environmental conditions for maximum response, including photoperiod exposure. This dichotomization of developmental processes caused by photoperiod may be useful, for example, when determining environmental conditions to maximize either harvest index, seed set, or *in vitro* response.

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Chapter 3

Variability for critical photoperiod for tuberization and tuber yield among monoploid, anther-derived genotypes of *Solanum phureja*

Introduction

Tuberization of potato is a critical process by which photosynthates are redirected from above ground growth to storage tissues. Many factors have been shown to affect its induction and degree. It was first postulated that tuberization was regulated by a symbiotic fungus (Bernard, 1902). Later, an association between tuberization and carbon-nitrogen (C:N) ratio was noted (Werner, 1934). Driver and Hawkes (1943) demonstrated the influence of photoperiod on the degree and timing of tuberization. Under strict environmental controls at California Institute of Technology, Gregory (1956) was able to demonstrate an interaction between photoperiod and temperature on endogenous substances which regulated the process of tuberization. A range of growth substances has been implicated in the tuberization process, including cytokinins and abscisic acid (Palmer and Smith, 1969), gibberellins (Hammes

and Beyers, 1973; Hammes and Nel, 1975), and ethylene (Mingo-Castel et al., 1976). The role of calcium and calcium inhibitors has also been examined (Balamani et al., 1986). The exact controlling mechanism, however, has not been determined and probably consists of a combination of exogenous and endogenous factors, both of which are affected by genotypic predispositions.

Regarding the critical photoperiod for tuberization, *Solanum* species have exhibited a wide range of photoperiodic behaviors, from day-neutral, characteristic of *Solanum tuberosum* cultivars, to strongly short-day regarding the critical photoperiod for tuberization (i.e. will only tuberize under photoperiods shorter than the critical photoperiod), illustrated by many diploid species, both wild and cultivated. Due to the polyploid nature of *S. tuberosum* cultivars, the highly heterozygous nature of the species, and the multigenic nature of tuber yield (Mendoza and Haynes, 1976,1977), conventional breeding methods for exploiting germplasm exhibiting a short critical photoperiod for tuberization (for the development of cultivars adapted to northern climates) have been limited.

Monoploid clones may simplify selection efforts because their phenotypes are a direct reflection of their genotypes. In the present study, variability among nine monoploid genotypes, derived from anther culture of a diandrous clone of *Solanum phureja* Juz. & Buk., was examined with regard to critical photoperiod for tuberization. A modification of Ewing's screening technique (1978b) was used to determine the critical photoperiod for each genotype. By his technique, single-node cuttings are taken from plants grown under successively shorter

photoperiods, placed in moist sand under long days, and examined for root/axillary tuber formation. Cuttings which tuberize indicate that the mother plants were grown under photoperiods shorter than the critical photoperiod necessary for tuber induction. In this study, plants were grown under three photoperiods (10, 14, and 18 hour daylengths) for their entire life cycle to eliminate the confounding effects of plant age on tuberization.

The objectives of this study were twofold: 1) to demonstrate variability among monoploid genotypes extracted from a single, highly heterozygous, diploid genotype for a multigenic trait of economic importance, namely tuber yield, and a morphological trait, namely plant height, and 2) to demonstrate segregation *in both directions* from the anther-donor (i.e. some higher, some lower) among its monoploid genotypes for a yield parameter, namely critical photoperiod for tuberization.

Materials and Methods

Nine monoploid genotypes (AM2, AM3, AM4, AM6, AM7, AM20, AM21, AM26, and AM27) and the anther-donor genotype [PP5, selected from *Solanum phureja* P.I. 225669 (Veilleux et al., 1985)] were grown under greenhouse conditions, harvested simultaneously to minimize any effects of the mother tubers, and stored at 6°C for at least one month to break dormancy. The tubers were transferred to the Southeastern Plant Environment (Phytotron) Laboratories of

North Carolina State University, planted in flats containing 1/3 peat-lite (Redi Earth, W. R. Grace Co.) and 2/3 gravel (standard substrate), and placed in 3 photoperiod rooms (chamber size B).

After the minimum 10 hr daylength ($650 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density, or PPFD), low-intensity incandescent lights ($50 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD) were used to extend photoperiods in 2 of the chambers to 14 and 18 hrs. All chambers were maintained at 18°C for the minimum 10 hr light period and 14°C for the remaining 14 hr period. A nutrient solution (Phytotron Procedural Manual, 1983) containing 106 ppm N, 10 ppm P, and 111 ppm K was applied 3 times weekly throughout the experiment.

Six weeks after planting, sprouted tubers were transplanted into individual 15 cm diam. pots (6 plants per genotype per chamber). Eight weeks after experiment initiation, measurements of node number and plant height were taken for all plants. At 8, 10, 12, and 14 wks, 3 single-node cuttings were taken from each plant, placed in moist sand, and transported to the greenhouse at Virginia Polytechnic Institute and State University in Blacksburg, Virginia. Ambient autumn light conditions were supplemented with incandescent lights to extend the photoperiod to 20 hrs, and the cuttings were placed under intermittent mist. After 3 wks, cuttings were scored on a scale from -1 to +1 for root or axillary tuber formation. Tubers from each plant were harvested at 16 wks. Total tuber weight and tuber number (greater than 1 cm diam.) were recorded for each plant.

Data were analyzed using the General Linear Models procedure of the Statistical Analysis System (SAS Institute, 1982). Mean separation of tuber weight, tuber number, and internode length were by Student-Newman Keuls' test, 5% level.

Results

Response of single node cuttings: Single node cuttings of 8 of the monoploid genotypes, as well as the anther-donor genotype, taken from the 10 hr photoperiod, produced axillary tubers (Figure 1). Therefore, as expected, it can be concluded that most of the genotypes had a critical photoperiod for tuberization which was longer than 10 hrs. Only AM26 demonstrated a consistent rooting response at this photoperiod.

Segregation for critical photoperiod for tuberization among the monoploid genotypes was more apparent at the 14 hr photoperiod (Figure 2). At 8 wks, cuttings from most of the genotypes demonstrated a rooting response, although many formed axillary tubers at later sampling dates. PP5 and AM4 showed a strong rooting response at this photoperiod, suggesting that their critical photoperiods for tuberization were shorter than 14 hrs. AM26 again demonstrated only a rooting response, as it did under the 10 hr photoperiod. AM21 was the only monoploid genotype which demonstrated a tuberizing response at all sampling dates.

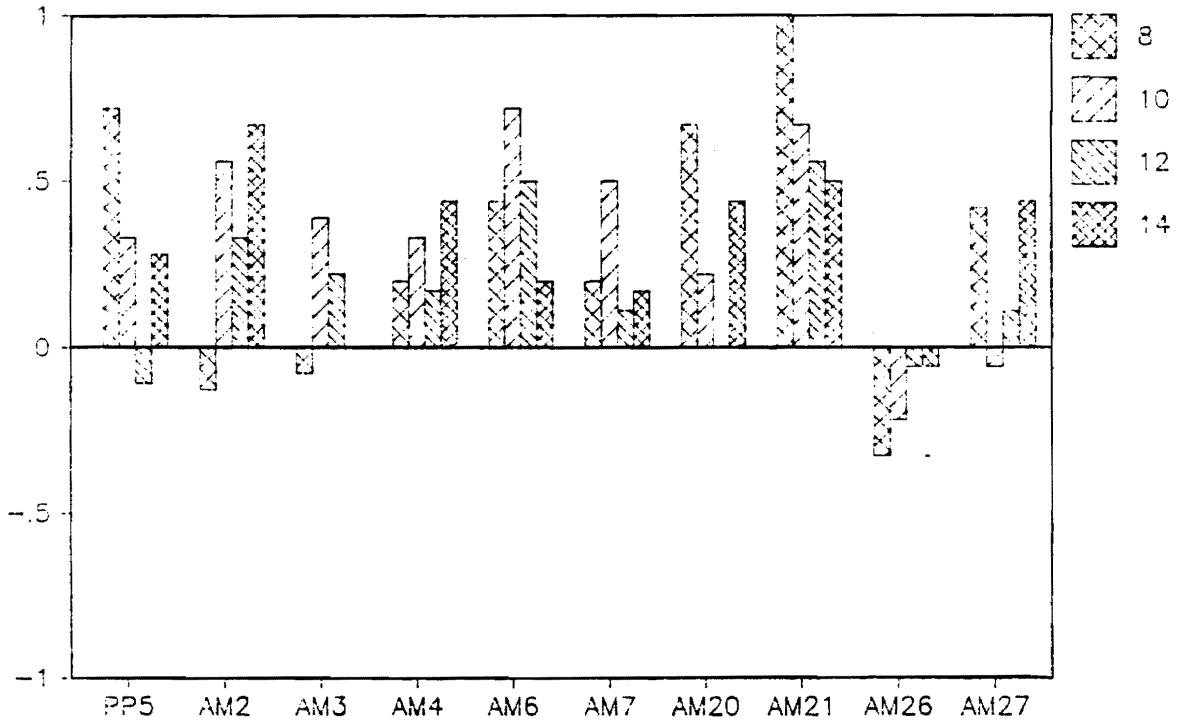


Figure 1. Rooting (-1) vs. axillary tuber formation (+1) of single-node cuttings from nine monoploid (AM) genotypes and their diploid, anther-donor genotype (PP5) of *Solanum phureja* taken from plants after 8, 10, 12, and 14 wks of growth under a 10 hr photoperiod. Zero values indicate equal response; N= 18.

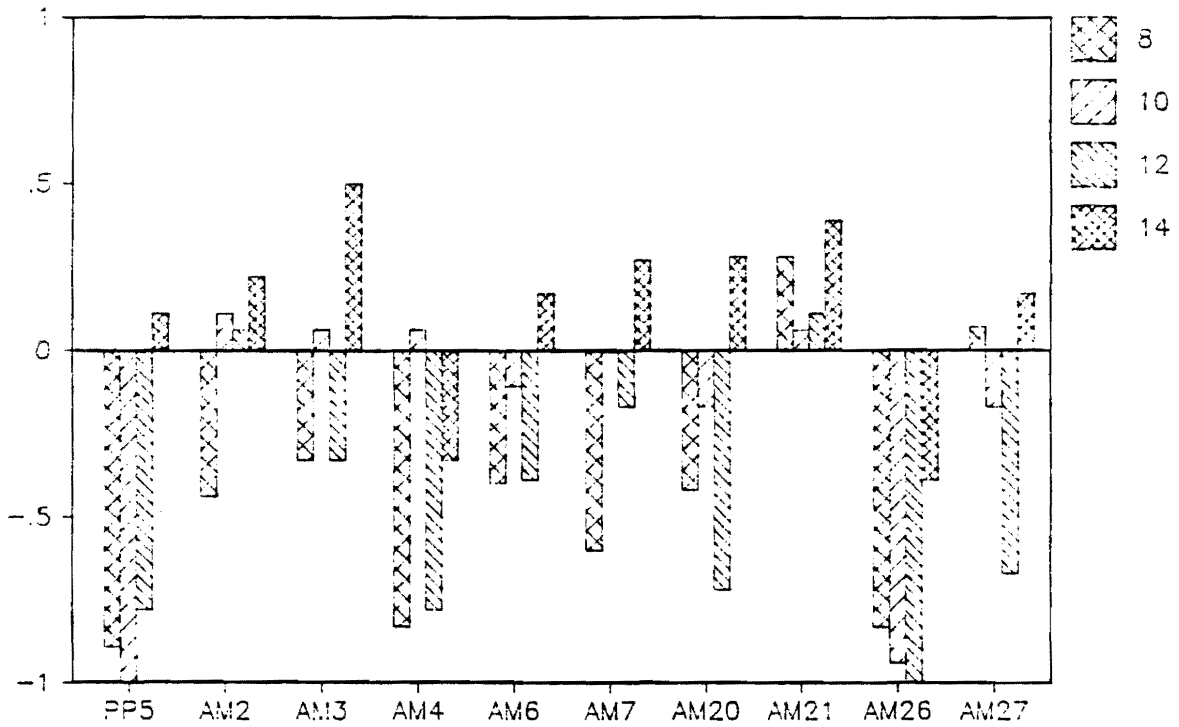


Figure 2. Rooting (-1) vs. axillary tuber formation (+1) of single-node cuttings from nine monoploid (AM) genotypes and their diploid, anther-donor genotype (PP5) of *Solanum phureja* taken from plants after 8, 10, 12, and 14 wks of growth under a 14 hr photoperiod. Zero values indicate equal response; N= 18.

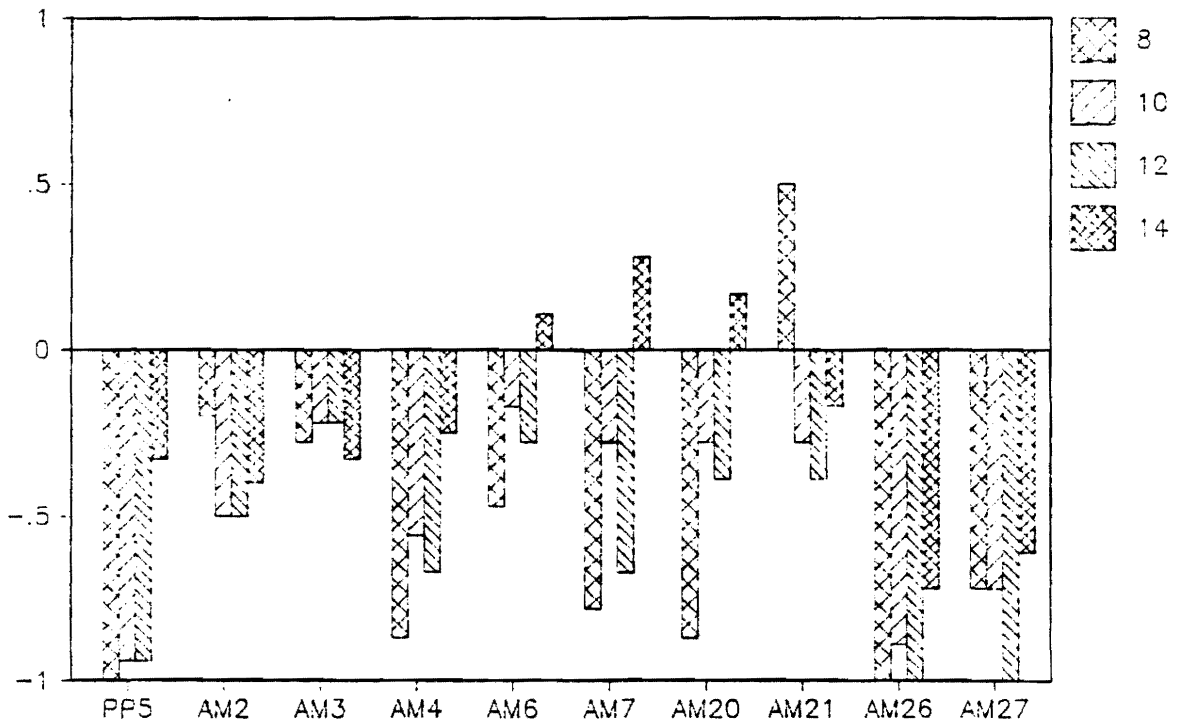


Figure 3. Rooting (-1) vs. axillary tuber formation (+1) of single-node cuttings from nine monoploid (AM) genotypes and their diploid, anther-donor genotype (PP5) of *Solanum phureja* taken from plants after 8, 10, 12, and 14 wks of growth under a 18 hr photoperiod; N = 18.

Under an 18 hr photoperiod, AM2, AM3, and AM27 had switched to a rooting response, placing their critical photoperiods for tuberization between 14 and 18 hrs (Figure 3). By contrast, cuttings from AM6, AM7, AM20, and AM21 still were able to form axillary tubers at one sampling date under this photoperiod, suggesting a longer critical photoperiod for these genotypes.

Tuber yield: With the exception of AM4 grown under an 18 hr photoperiod, all of the monoloids exhibited measurable tuber yield by 16 wks regardless of their critical photoperiod preference. The monoloid genotypes varied significantly for total tuber weight at all photoperiods (Figure 4). PP5 was consistently higher in tuber weight than the monoloid genotypes, but decreased to such an extent under 18 hrs that it no longer significantly differed from AM21, one of the monoloids which showed a tuberization response under an 18 hr photoperiod. In addition, AM21 showed the greatest tuberization response among the monoloids in all photoperiods and was the least affected by increasing photoperiods.

The anther-donor (PP5) characteristically produced a large number of tubers per plant. At 10 hrs, none of the monoloid genotypes had significantly different numbers of tubers per plant from PP5 (Figure 5). However, at longer photoperiods, segregation for tuber number among the monoloids was evident by low (AM4 at 14 hrs) and high (AM21 at 18 hrs) tuber numbers compared with their anther-donor genotype.

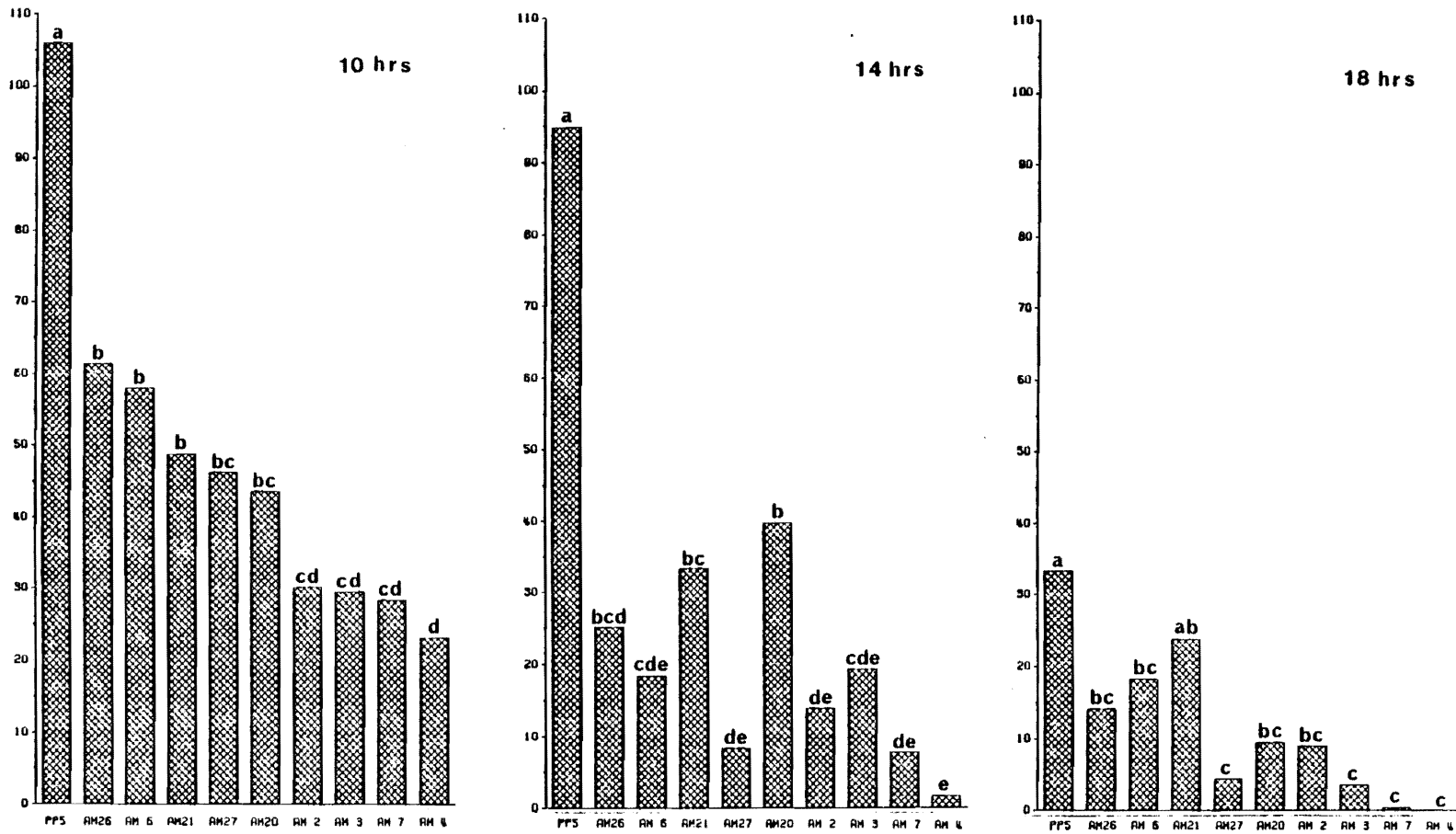


Figure 4. Mean tuber weight (g) of nine monoploid (AM) genotypes and their anther-donor genotype (PP5) of *Solanum phureja* grown under 10, 14, and 18 hr photoperiods. Mean separation by SNK, 5% level; N=6.

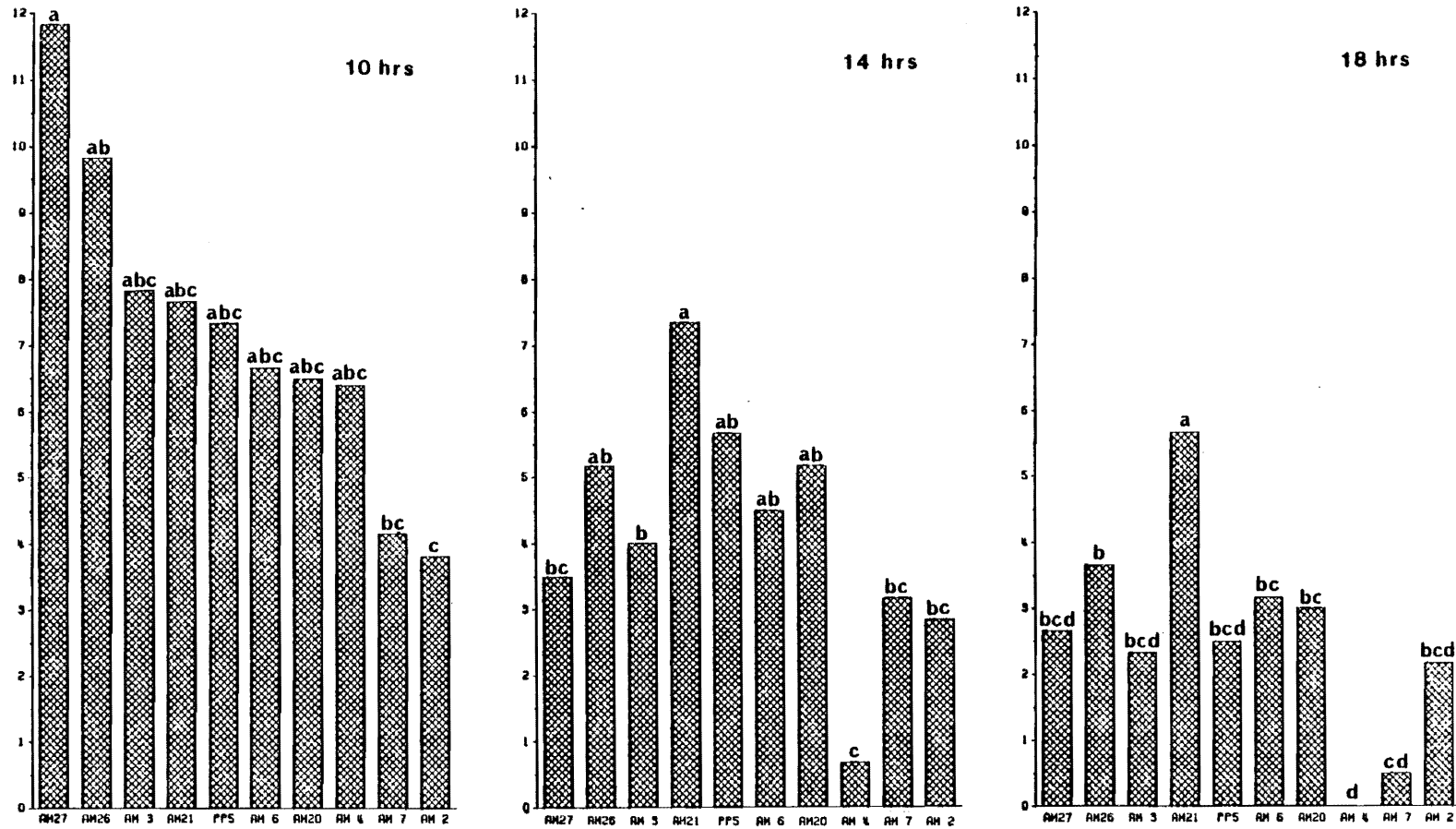


Figure 5. Mean tuber number of nine monoploid (AM) genotypes and their anther-donor genotype (PP5) of *Solanum phureja* grown under 10, 14, and 18 hr photoperiods. Mean separation by SNK, 5% level; N=6.

Plant height: As with critical photoperiod for tuberization, segregation for mean internode length was most apparent at the 14 hr photoperiod (Figure 6). Mean internode length of PP5 was reduced at the 10 hr photoperiod, under which AM6 was significantly taller than PP5. At 14 and 18 hr photoperiods, however, the monoloids were generally shorter than PP5, although there were significant differences among them.

Discussion

Response of single-node cuttings: Segregation for a multigenic trait, such as critical photoperiod for tuberization, may be exposed by analysis of monoloid genotypes and their subsequent study under strict environmental control. Tuber initiation is believed to be a multi-genic trait (Howard, 1970; Mendoza and Haynes, 1976, 1977). *Solanum phureja* is a heterozygous, self-incompatible, diploid species and would be expected to produce a heterogeneous array of genotypes via androgenesis. Results of this experiment confirm the inherent variability of the anther-donor genotype.

Single-node cuttings have been used to estimate the onset of tuberization in a potato plant, while allowing the plant to continue its growth (Ewing, 1978a, 1978b; Lauer, 1977). This method of assessment, however, did not appear to be entirely accurate in this study. For example, AM26 exhibited a consistent rooting response over 4 sampling dates even under the 10 hr photoperiod (Fig-

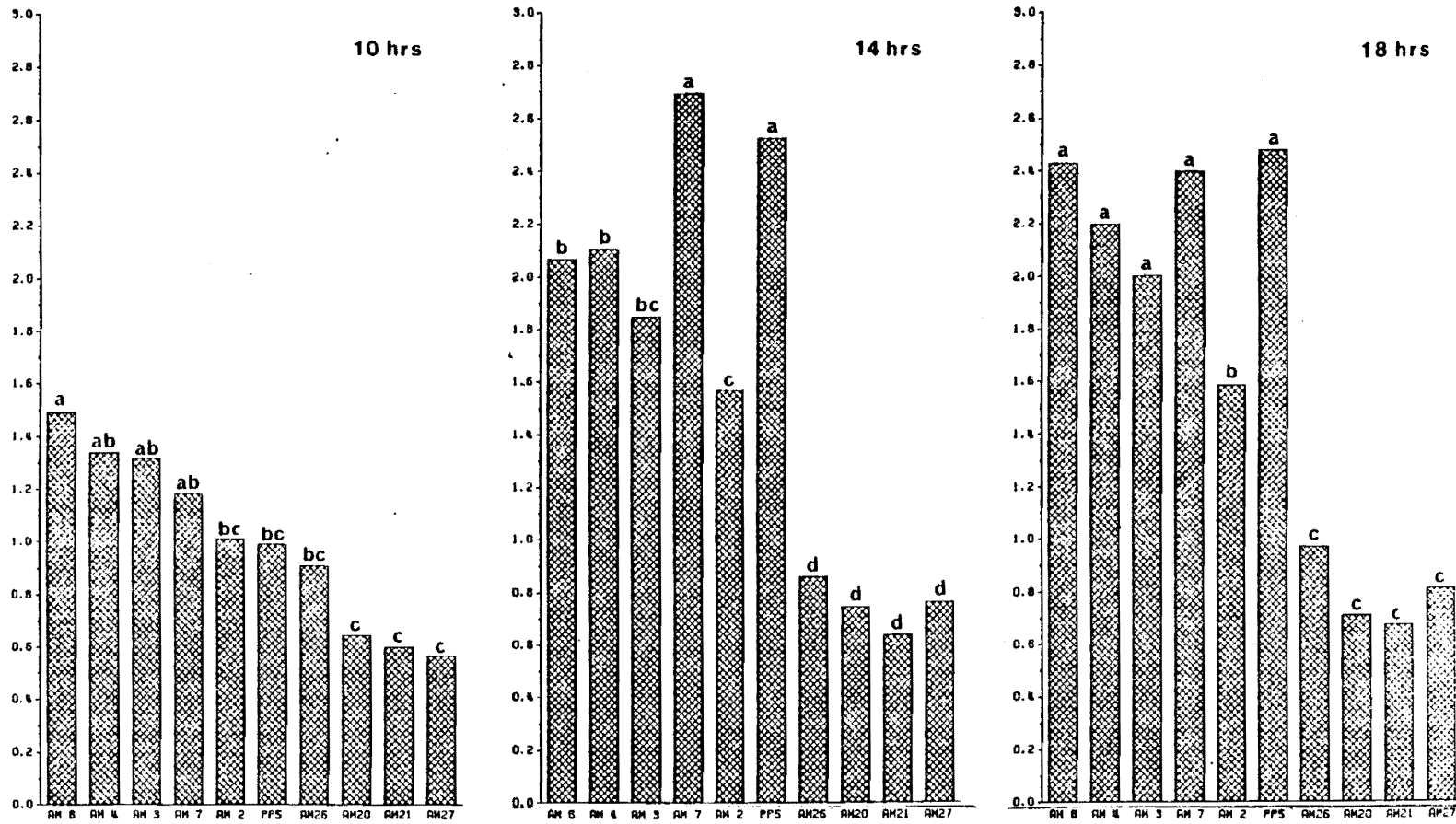


Figure 6. Mean internode length (cm) of nine monoploid (AM) genotypes and their anther-donor genotype (PP5) of *Solanum phureja* grown under 10, 14, and 18 hr photoperiods. Mean separation by SNK, 5% level; N=6.

ure 1), yet it produced a reasonable tuber crop compared with other monoploid genotypes that exhibited strong axillary tuber formation (Figure 4). This suggests that rooting and axillary tuber formation are not mutually exclusive. Removal of single-node cuttings from the environment of the mother plant may cause some genotypes to switch back to a rooting response, prior to axillary tuber formation. In general, however, genotypes which formed a large number of axillary tubers from cuttings of plants exposed to a given photoperiod also produced high tuber yields at that photoperiod. Single-node cuttings of AM21 formed tubers even when taken from plants grown under an 18 hr photoperiod, and the mother plant correspondingly produced a consistently high mean tuber yield. The utility of this technique to screen a large number of genotypes prior to maturity would compensate for its limitations in accuracy.

Physiological age of the plant also affects tuber induction. Plants grown under non-inductive photoperiods will tuberize eventually, but much later than plants grown under inductive conditions. This response is mirrored by the tendency for older single-node cuttings to begin forming axillary tubers even if taken from plants grown under non-inductive photoperiods (see Figure 3). Cuttings from AM6, AM7, and AM20 under 14 and 18 hr photoperiods began to form axillary tubers only when taken from plants which were at least 14 wks old, suggesting that tuber induction in these genotypes occurred much later than on similar plants grown under shorter photoperiods. By contrast, cuttings taken from AM21 eight wks after planting already had been induced to tuberize. This tuberization response of AM21, however, was not demonstrated by subsequent

cuttings. Regardless, for this method to be effective in selecting genotypes possessing higher critical photoperiods for tuberization, cuttings should be taken before the plants have reached physiological maturity.

Tuber yield: Variability for mean tuber yield was apparent among the monoploid genotypes; however, all monoploid genotypes, with the exception of AM21 grown under the 18 hr photoperiod, had lower mean tuber weights than their anther-donor. This was expected, since tuber yield is affected by both ploidy level and genotype (Mendoza and Haynes, 1976). Segregation in both directions was better illustrated by mean tuber number (Figure 5), where PP5 exhibited an intermediate phenotype. Ideally, improvement of tuber yield would require high tuber weight and low tuber number even under a long photoperiod. Unfortunately, the monoploid genotype producing the highest yield at the longest photoperiod (AM21) also produced a mean tuber number which was significantly higher than PP5. It is important, therefore, to assess genotypes for both characteristics simultaneously and at the photoperiod for which the crop is being developed.

Plant height: Photoperiod has been shown to affect internode elongation in tuber-bearing *Solanum* species (Victorio et al., 1986). Potato plants grown under short photoperiods have exhibited a corresponding reduction in internode length. Reduction of internode length may facilitate carbohydrate partitioning to underground tissues by reducing translocation distances, or may be an inde-

pendent response to competition for assimilates (Gregory, 1956). In this experiment, mean internode length was reduced under short photoperiods, but it is unclear whether this was in response to tuberization, a stimulus for it, or an independent event. PP5 exhibited a dramatic decrease in tuber yield (Figure 4) as photoperiod increased, yet its increase in internode length was less dramatic (Figure 6), suggesting independent, though competing, processes.

Variability for multi-genic traits can be exposed directly through the use of monoploid, anther-derived genotypes. The number of characters and the number of genes controlling them, however, will determine the minimum number of regenerants required for detection and recovery of a monoploid genotype possessing all desired traits. This is true for conventional selection efforts as well. Given the segregation ratios for tetraploid cultivars, the incompatibility and sterility which characterize many of them, and the time, space, and labor required for conventional screening, however, monoploid genotypes may reduce selection efforts considerably.

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Chapter 4

Variation and fertility among calliclones of an anther-derived, monoploid genotype of *Solanum phureja*

Introduction

Ploidy stability during cell and tissue culture is an important criterion for determining the usefulness of monoploid plant material for microbial-type manipulations. Aneuploidy, mixoploidy, and endopolyploidy have been reported in callus cultures of monoploid tissues and plants subsequently regenerated from them (Karp et al., 1985; Khvilkovskaya, 1982). Monoploid cell cultures or protoplast preparations are useful for somatic hybridization and mutation selection schemes. For example, monoploid cell preparations simplify selection for stress tolerance and/or disease resistance because their phenotypes are direct reflections of their genotypes. Similarly, detection of useful mutations (either natural or induced) would be more likely if monoploid cells were used. Maintenance of monoploid status *in vitro*, however, is critical during these manipulations.

Whole plant regeneration from potato callus tissue has been accomplished (Ahloowalia, 1982; Behnke, 1975; Wang and Huang, 1975) and has produced regenerants of several ploidy levels, either by spontaneous chromosome doubling (Jacobsen, 1977; Karp et al., 1985; Lu et al., 1985) or colchicine-induced diploidization (Ross et al., 1967). Both methods allow for the production of homozygous euploids which may be fertile, or somaclonal variants which may introduce useful genetic variation not previously encountered.

Many factors have been shown to affect ploidy stability and regeneration from callus tissue, including ratio and type of growth regulators in the medium, tissue type, and genotype (Lam, 1977; Webb et al., 1983). Sterility of homozygous clones is not uncommon, however, due to the deleterious effects of inbreeding to which *Solanum* species are particularly vulnerable (de Jong and Rowe, 1971). Somaclonal variation after regeneration from callus also may occur (Smith, 1986). Thus, screening for fertility and genetic uniformity in calliclones is necessary prior to their further use in germplasm development.

The objectives of the following research were to: 1) examine the effect of hormone ratio and hormone type on callus proliferation and plant regeneration from callus, 2) determine ploidy levels of calliclones, 3) test diploid and tetraploid calliclones for male and/or female fertility, and 4) determine if somaclonal variation existed among calliclones.

Materials & Methods

Leaf-disc culture: All 24 monoploid genotypes were grown under greenhouse conditions for leaf-disc culture. Leaves were disinfested by a 30 s dip in 70% ethanol, followed by immersion in 0.525% sodium hypochlorite for 10 min. and 3 rinses in sterile, distilled water. Using a sterile cork-borer, 5 mm discs containing the midrib were removed from 10 wk old plants and placed abaxial surface down into 25x150 mm culture tubes containing 20 ml agar-solidified Murashige and Skoog (MS) basal medium (1962) and 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). Ten discs were cultured from each genotype and placed on light benches (16 hr photoperiod, 23°C). After 12 wks, callus which formed from the leaf discs was excised and subcultured onto MS medium containing 2 mg/l 2,4-D and 0.5 mg/l N⁶-benzylaminopurine (BAP) for callus proliferation.

Regeneration from callus: Callus from 24 monoploid genotypes was tested for regeneration capacity. Uniform 5 mm³ pieces of callus were excised from cultures of the monoploid genotypes and placed in culture tubes containing 20 ml agar-solidified MS basal medium, 0.1 mg/l naphthalene acetic acid (NAA) and 5 mg/l kinetin (KIN) according to Wang and Huang (1975). Ten tubes per genotype were incubated under light as above.

In addition, a factorial experiment was conducted using callus from monoploid genotype AM13 to test the effect of auxin level, cytokinin level, and cytokinin type on plant regeneration from callus. Three levels of 2,4-D (0.5, 1.0, and 2.0 mg/l), 4 levels of cytokinin (0, 0.5, 1.0, and 2.0 mg/l), and 3 types of cytokinin [KIN, BAP, zeatin (ZEA)] were added to MS basal medium with 10 tubes of AM13 callus cultured for each treatment (360 tubes total). Cultures were placed on light benches and examined after 16 wks for callus proliferation and/or plant regeneration.

Plants regenerated from callus were subcultured onto MS basal medium for rooting and acclimated to greenhouse conditions via one of two procedures. Initially, *in vitro* calliclones were removed from the tubes, planted in moist sand, and placed in the greenhouse under intermittent mist. Some calliclones, however, did not survive this procedure and were transferred from culture tubes to Plantcons (Flow Laboratories, Inc.) containing autoclaved Pro-Mix (Premier Brands, New Rochelle, NY) and allowed to grow to a larger size prior to transfer to the greenhouse. Calliclones were allowed to complete a life cycle in the greenhouse prior to further testing.

Ploidy determination: Ploidy levels of the calliclones were estimated by counting the number of chloroplasts per pair of guard cells from an abaxial epidermal leaf section. Confirmation of the ploidy levels was accomplished by examination of root tip cells. Young root tips of greenhouse-grown plants were fixed in 3:1 ethanol/acetic acid, transferred to 70% ethanol, Feulgen stained, squashed in a

drop of aceto-carmin, and examined microscopically for determination of ploidy level.

Fertility testing: Tuber-propagated calliclones which reached flowering stage were examined for both male and female fertility. Pollen viability was estimated by aceto-carmin staining and microscopic examination. Female fertility was tested by emasculating the flower prior to anthesis and pollinating the stigma with pollen from a fertile, diploid *S. phureja* genotype (NBP-2 and NBDT-5) or tetraploid cultivar (Atlantic), depending on the ploidy level of the calliclone.

Electrophoresis: Leaf tissue extracts of 27 tuber-propagated calliclones, the monoploid callus-donor genotype (AM13), and the diploid anther-donor genotype (PP5), were homogenized in a phosphate buffer, centrifuged, and frozen prior to 10% SDS-polyacrylamide gel electrophoresis. Protein assays (Esen, 1978) were performed on samples to adjust the volume of samples to contain equal protein content. Bovine serum albumin (BSA) samples were included as molecular weight markers. Gels were stained with Coomassie brilliant blue R, destained in 25% ethanol and 10% acetic acid, fixed in 7.5% acetic acid and 5% glycerol, and vacuum dried. GelBond (FMC Corporation, Rockland, ME) plastic support film was used for gel preservation.

Results

Callus production and plant regeneration: All 24 monoploid genotypes produced callus from leaf discs cultured on MS medium containing 2 mg/l 2,4-D. Genotypic differences were evident, however, with respect to the amount of callus produced and its friability. Genotypes AM8, AM9, and AM13 regenerated 6, 5, and 51 calliclones, respectively, after transfer to MS medium containing 0.1 mg/l NAA and 5.0 mg/l KIN.

Effects of auxin level, cytokinin level, and cytokinin type on callus growth of AM13 are listed in Table 1. Few significant differences (5% level) were found between cytokinin types. ZEA at 2 mg/l produced a greater quantity of callus than either KIN or BAP, but only at the lowest level of 2,4-D (0.5 mg/l). There appears to be a trend, however, for increasing callus production with increasing levels of cytokinin, regardless of the cytokinin type. By contrast, increasing levels of 2,4-D did not significantly increase callus fresh weight. Qualitative differences were apparent between cytokinin types; ZEA generally produced a greenish callus, KIN a tan callus, and BAP a brownish callus. Rhizogenesis was not evident in any of the treatments; plant regeneration occurred from only 1 callus section (2 mg/l 2,4-D, 2 mg/l KIN treatment).

Ploidy determinations and fertility tests: Data from ploidy determinations (by chloroplast and chromosome counts) and female fertility tests (by pollinations)

Table 1. Callus fresh weight (mg) of AM13 at 12 wks as influenced by auxin concentration (mg/l 2,4-D), cytokinin concentration, and cytokinin type [kinetin, zeatin, N⁶-benzylaminopurine (BAP)], \pm SE, N = 10.

With 0.5mg/l 2,4-D:			
	Kinetin	Zeatin	BAP
None	0.8 + 1.2	2.4 + 1.3	1.7 + 1.4
0.5 mg/l	2.5 + 1.0	4.4 + 0.9	2.8 + 1.2
1.0	2.8 + 1.2	4.8 + 0.9	2.9 + 0.8
2.0	1.6 + 1.5	5.0 + 0.9	3.0 + 1.0
With 1.0mg/l 2,4-D:			
	Kinetin	Zeatin	BAP
None	1.2 + 1.5	1.3 + 1.4	0.7 + 1.2
0.5 mg/l	2.5 + 1.7	5.1 + 0.5	3.3 + 1.2
1.0	3.6 + 2.1	5.0 + 0.8	3.2 + 1.7
2.0	2.5 + 1.5	5.3 + 0.9	4.1 + 1.0
With 2.0mg/l 2,4-D:			
	Kinetin	Zeatin	BAP
None	0.3 + 0.3	0.6 + 0.8	0.3 + 0.3
0.5 mg/l	1.4 + 1.3	4.2 + 2.0	3.0 + 1.7
1.0	2.1 + 1.6	4.8 + 1.9	2.3 + 1.6
2.0	2.3 + 1.8	5.1 + 1.8	3.5 + 1.4

Table 2. Ploidy determinations (by chloroplast and chromosome counts) and female fertility testing (by pollinations) of calliclones of a monoploid, anther-derived genotype (AM13) of *Solanum phureja*.

Clone	Chloroplasts per guard cell pair ¹	Ploidy level ²	Pollinations ³
AM13- 1	11.5 + 1.6	D	31
AM13- 2	13.2 + 1.1	D	*
AM13- 3	12.6 + 1.2	D	*
AM13- 4	12.1 + 2.0	D	10
AM13- 5	13.5 + 1.4	D	*
AM13- 6	13.5 + 2.4	D	*
AM13- 7	26.1 + 4.4	T	*
AM13- 8	16.1 + 2.1	D	*
AM13- 9	28.7 + 4.7	T	5
AM13-10	19.4 + 3.0	D	14
AM13-11	23.5 + 5.6	T	5
AM13-12	29.1 + 3.7	T	*
AM13-13	26.5 + 3.7	T	*
AM13-14	23.2 + 2.9	T	4
AM13-15	17.6 + 3.6	D	29
AM13-16	25.7 + 3.9	T	*
AM13-17	29.3 + 3.5	T	*
AM13-18	19.6 + 6.0	D	30
AM13-19	17.3 + 2.6	D	20
AM13-20	15.3 + 1.8	D	*
AM13-21	15.3 + 2.3	D	11
AM13-23	19.0 + 2.7	D	*
AM13-27	22.5 + 3.8	D	*
AM13-28	23.0 + 3.3	T	*
AM13-29	18.7 + 2.2	D	10
AM13-30	22.7 + 3.3	T	*
AM13-31	18.1 + 2.8	D	*
AM13-33	15.9 + 1.4	D	*
AM13-35	14.0 + 1.6	D	*
AM13-38	26.6 + 2.2	T	19
AM13-39	29.1 + 4.1	T	36
AM13-43	28.7 + 4.7	T	15

¹ ± s.d., N= 10

²D = diploid, T = tetraploid, by Feulgen staining of root tip cells

³2x pollinators = NBDT-5, NBP-2; 4x pollinator = *S. tuberosum* cv. Atlantic

* did not produce floral buds, or floral buds aborted

of 32 AM13 calliclones are listed in Table 2. Of the 51 clones regenerated, 19 did not survive transfer from culture tubes. Examination of root tip chromosomes (10 counts per clone) confirmed that all calliclones were euploids and had undergone either one or two cycles of spontaneous chromosome doubling. Of the 32 clones which survived greenhouse acclimatization, only 14 produced normal or nearly normal-appearing floral buds necessary for female fertility testing. After pollinations with testers, no seed set was observed on any of the 14 clones tested. In addition, no stainable pollen was detected in these clones, indicating a complete absence of fertility in calliclones derived from AM13.

Electrophoretic banding patterns: SDS-polyacrylamide gel electrophoresis of leaf-protein banding patterns from anther-donor genotype PP5, monoploid genotype AM13, and the first 6 callus-derived clones (AM13-1 through AM13-6) are shown in Figure 1. AM13 exhibited a banding pattern similar its anther-donor (PP5), indicating a high degree of leaf protein conservation. On the other hand, a few of the callus-derived clones showed distinct differences in banding patterns, both among calliclones and between calliclones and their anther-donor and callus-donor genotypes. For example, AM13-2 does not appear to show a band at approximately 50 kd, although it is evident in the other calliclones tested and both the anther-donor and callus-donor genotypes. At approximately the position of a 25 kd protein, a dark band is evident in AM13-3, PP5, and AM13, but not in the other calliclones from the same run.

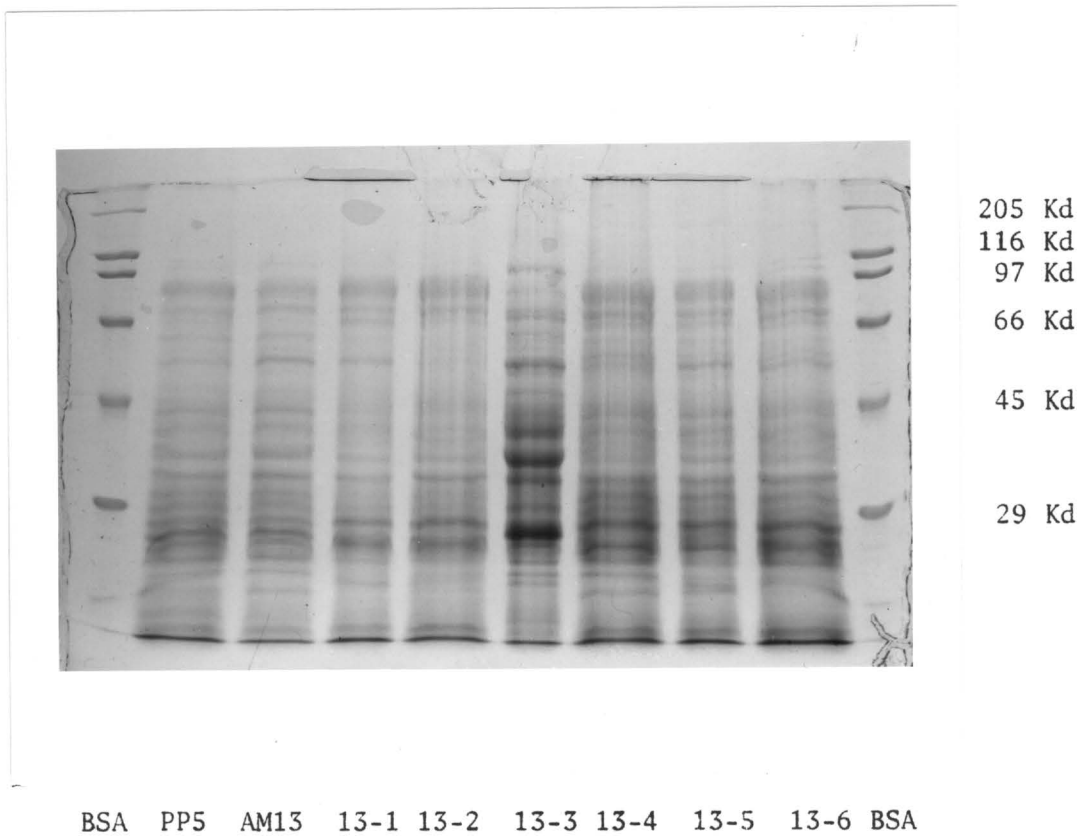


Figure 1. SDS-Polyacrylamide electrophoresis gel stained with Comassie Brilliant Blue R for leaf protein banding patterns from anther-donor genotype (PP5), anther-derived monoploid (AM13), and callus-derived clones (13-1 through 13-6) of *Solanum phureja*.

No relationship between banding pattern and ploidy level was evident among calliclones.

Discussion

Genotype and hormonal composition of regeneration medium were shown to affect the ability of leaf-disc-derived callus to regenerate whole plants. Thus, screening of monoploid genotypes for regeneration capacity may be useful prior to their incorporation into genomic reconstruction programs, especially if regeneration is needed later in the germplasm development protocol.

Monoploid instability was also shown after callus culture, since all calliclones underwent one or two cycles of endopolyploidization. It is encouraging, however, that neither aneuploidy nor mixoploidy was encountered in the calliclones.

Absence of fertility in doubled and twice doubled clones of AM13 is unfortunate, but not surprising, because *Solanum* species are particularly susceptible to the effects of inbreeding depression. Screening of diploid and tetraploid calliclones derived from other genotypes and the utilization of additional pollinator sources may result in generating and detecting fertile clones, respectively.

Electrophoresis of leaf proteins exposed some degree of variation among calliclones of AM13, but lack of fertility in these clones made them unusable for

the incorporation of desired traits to *S. tuberosum* cultivars via 4x-2x hybridization. Therefore, characterization of the specific genetic differences among them by isozyme analysis was abandoned. Production of diploid and tetraploid clones via monoploid callus culture was useful, however, in that it generated a ploidy series representing a single genotype. Separation of genotypic from ploidy influences would be possible from this type of material and, thus, they are being utilized in further studies.

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Chapter 5

Environmental, genotypic, and ploidy effects on endopolyploidization within a genotype of *Solanum phureja* and its derivatives

Introduction

Protoplast fusion is a procedure for producing hybrids which cannot be produced through sexual means (Austin et al., 1985,1986; Hein and Schieder, 1986; Helgeson et al., 1986). By this process, somatic cells, rather than germ cells, fuse and generate an entire organism. The union of somatic cells may result in hybrid cells or organisms with mitotic and meiotic irregularities (Sree Ramulu et al., 1986), which may be due, in part, to the corresponding doubling of the chromosome number in the fusion product. Protoplasts derived from monoploid, anther-derived genotypes may, when fused, result in somatic hybrids which exhibit fewer chromosomal abnormalities because the fusion partners in this instance contain the gametic, rather than the somatic, chromosome complement.

Monoploid genotypes may be useful in potato germplasm development for several reasons. Protoplasts isolated from monoploid tissues would be more suited to microbial techniques, due to their hemizygous state, than their diploid counterparts (Maheshwari et al., 1982; Melchers, 1972). Protoplast fusion of unrelated monoploid genotypes derived from diplandrous donors would produce a heterozygous diploid which, if fertile, could be used in $4x-2x$ (unilateral) sexual polyploidization to create highly heterozygous, tetraploid progeny. If the unreduced gametes of the diploid parent are produced via first division restitution, the heterozygosity inherent in the diploid parent produced by protoplast fusion would be conserved (Veilleux, 1985; Wenzel et al., 1982)

Unfortunately, isolated plant cells and tissues *in vitro* are subject to mitotic irregularities, including chromosomal rearrangement, mutation, and an uncoupling of DNA replication from cell division, a process known as endopolyploidization (Pijnacker et al., 1986; Puite et al., 1986). Plant regeneration from callus or isolated protoplasts would be simplified by using genetically uniform and stable plant material, but this cannot always be maintained, particularly in long term cultures which pass through a callus phase (Karp et al., 1985). Over time, *in vitro* cultures of differentiated or non-meristematic tissues may contain cells with a wide range of ploidy levels. Similarly, plants regenerated from these cells and tissues may contain nuclei of several ploidy levels, creating a polysomatic condition. It has been demonstrated that monoploid plants may not be entirely monoploid; some proportion of cells may undergo one

or more cycles of endopolyploidization *in vivo* (Sree Ramulu and Dijkhuis, 1986; Uijtewaal, 1987).

It is necessary, therefore, to assess the cultural, genotypic and ploidy influences on the process of endopolyploidization in order to select plant material and conditions conducive to monoploid stability. Flow-cytometric analysis has been used to analyze structural and nuclear components in plant cell preparations with great accuracy and rapidity (Alexander et al., 1985; Arndt-Jovin and Jovin, 1977; Galbraith et al., 1984; de Laat and Blaas, 1984; Muirhead et al., 1984). The objectives of the following study were to determine: 1) if the level of endopolyploidization of a monoploid genotype *in vivo* differs from that *in vitro*, 2) if protoplast isolation selects for a particular ploidy level, 3) if initial ploidy level limits endopolyploidization, and 4) if monoploid genotypes, derived from anther culture of a single diploid genotype, differ for their level of endopolyploidization. To address the first three objectives the level of endopolyploidization between *in vivo*, *in vitro*, and protoplast-derived nuclei from plant material of 3 ploidy levels was compared. The last objective was addressed utilizing 9 monoploid genotypes, all derived from anther culture of *Solanum phureja* genotype PP5, a diploid, South American, cultivated potato species possessing several traits useful to breeding programs.

Materials & Methods

Origin of plant material: Monoploid genotypes (AM2, AM3, AM4, AM6, AM7, AM13, AM20, AM21, AM26, AM27) were obtained via anther culture of *Solanum phureja*, P.I. 225669, genotype PP5 (Veilleux et al., 1985). Diploid (AM13-2) and tetraploid (AM13-9) calli clones were generated by leaf-disc culture of AM13 according to Wang and Huang (1975). The plants were acclimated to greenhouse conditions and taken through at least 2 tuber cycles prior to reintroduction of shoot tips and nodal cuttings *in vitro*. Plants were propagated *in vitro* according to Haberlach et al. (1985) for at least 8 wks prior to protoplast isolation.

Protoplast isolation: All steps in protoplast isolation were conducted under sterile conditions. All media (flotation, conditioning, enzyme, and rinse) were filter-sterilized and prepared according to Haberlach et al. (1985). Due to the small size of leaves *in vitro*, both stem and leaf material were removed from 3 Magenta boxes (Carolina Biological Supply Co.), cut into single-node sections, and placed in a 15 cm diam. Petri plate containing a single sheet of filter paper (Whatman #3) and 30 ml of flotation medium. Plates were wrapped in parafilm (American Can Co., Greenwich, CT) and foil and incubated at 23°C for 24 hrs. The plant material was transferred to 250 ml shaker flasks containing 125 ml of conditioning medium and incubated in the dark for an additional 24 hrs at 4°C. The plant material was poured through a sieve to remove the conditioning

medium, placed in a 15 cm diam. glass petri plate, sliced between two scalpels (#10) to form a coarse sample (approximately 2 mm sections) and placed in another 250 ml flask containing 50 ml enzyme medium. The flasks were sealed with parafilm, placed on a oscillating shaker at 40 opm, and incubated for 15 hrs at 23°C under fluorescent light. The enzyme/plant material mixture was poured through a 63 µm filter, transferred to 50 ml Babcock bottles (Kimble Glass), and centrifuged at 500 rpm for 10 min. The band of protoplasts was removed with a Pasteur pipet, dispersed into a Babcock bottle containing rinse medium, and recentrifuged. A 0.25 ml sample of the protoplasts was placed into a microcentrifuge tube containing 1 ml of chopping buffer (882 mg sodium citrate, 419 mg MOPS, 915 mg MgCl₂, 0.1 ml Triton X-100/250 ml) to burst the protoplast membranes prior to RNAase treatment and DNA staining (Sharma et al., 1983).

Preparation of chopped nuclei: One gram of leaf material was chopped on ice for 3 min. with a razor blade in a 6 cm diam. glass Petri plate containing 3 ml chopping buffer. The mixture was poured through a 300 µm filter and then through a 60 µm filter to remove debris. One ml of the filtrate was placed in a microcentrifuge tube for RNAase treatment.

RNA removal and DNA staining: Both protoplast-derived and chopped nuclei were incubated in 0.5 ml RNAase solution (80 mg Sigma R 5503 ribonuclease-A in 100 ml chopping buffer) at 23°C for 30 min. A 0.25 ml aliquot of propidium

iodide (PI) solution (40 mg PI in 100 ml chopping buffer) was added to each sample, incubated on ice for at least an additional 30 min., and analyzed within 3 hrs.

Flow cytometry: Stained samples were filtered through a 37 μ m nylon mesh and analyzed with an Epics V, Model 752 laser flow cytometer and cell sorter (Coulter Electronics, Hialeah, FL). Laser excitation was 300 mW at 488 nm from a 5 W Innova 90 Argon Laser (Coherent Inc., Palo Alto, CA). Three parameters were recorded: forward angle light scatter (FALS) and 90 degree light scatter (90LS, 488 nm dichroic filter) for size and granularity determinations, and red fluorescence (RFL, 590 nm dichroic, 610 nm long pass filter) for DNA measurements. The Multiparameter Data Acquisition and Display System (MDADS) and Easy 88 microcomputer analysis (Coulter Electronics) were used for data collection and analysis. FALS, 90LS, and RFL were collected linear integral, log integral, and linear peak, respectively. Histograms of number of nuclei per fluorescence channel contained 256 channels and were gated on FALS and 90LS dual parameter histograms defining the population of interest. The fluorescence signal from PI-stained preparations is proportional to the DNA content of particles (nuclei) passing through the laser beam. Chicken red blood cells and a microsphere standard (Coulter Electronics) were used as calibration standards. Nuclear counts were set at 10,000/sample. Samples of *in vivo* chopped nuclei, *in vitro* chopped nuclei, and protoplast-derived nuclei from PP5, AM13, AM13-2, and AM13-9 were prepared and analyzed on the same day.

Likewise, a complete set of protoplast-derived nuclei from the 9 monoploid genotypes and PP5 were prepared and analyzed on each of 3 days. DNA measurements of protoplast-derived nuclei from the 9 monoploid genotypes and PP5 were repeated after 7 and 14 days.

Results

DNA histograms of the anther-donor genotype (PP5) from leaf nuclei both *in vivo* and *in vitro* and protoplast-derived nuclei illustrate DNA peaks characteristic of diploid populations (Figure 1). All 3 histograms exhibit a large 2C peak containing approximately 70% of the nuclei, corresponding to G_0G_1 phase, and a smaller 4C peak, which includes $G_2 + M$ phases of the cell cycle. Protoplast-derived nuclei produce cleaner histograms due to the removal of chloroplasts and other cellular components during sample preparation (protoplast flotation and rinsing) and/or the elimination of mechanical shearing of DNA during sample preparation.

Histograms of the monoploid, anther-derived genotype (AM13) contained 1C, 2C, and 4C peaks for both *in vivo* and *in vitro* chopped nuclei. Because the second peak includes both G_2 and M phases, it is not possible to discriminate between a single cycle of endoreplication and DNA replication prior to cytokinesis in this peak. However, because the 4C peak either results from M phase cells of single-cycle endoreplicated cells, or G_2 cells which have undergone

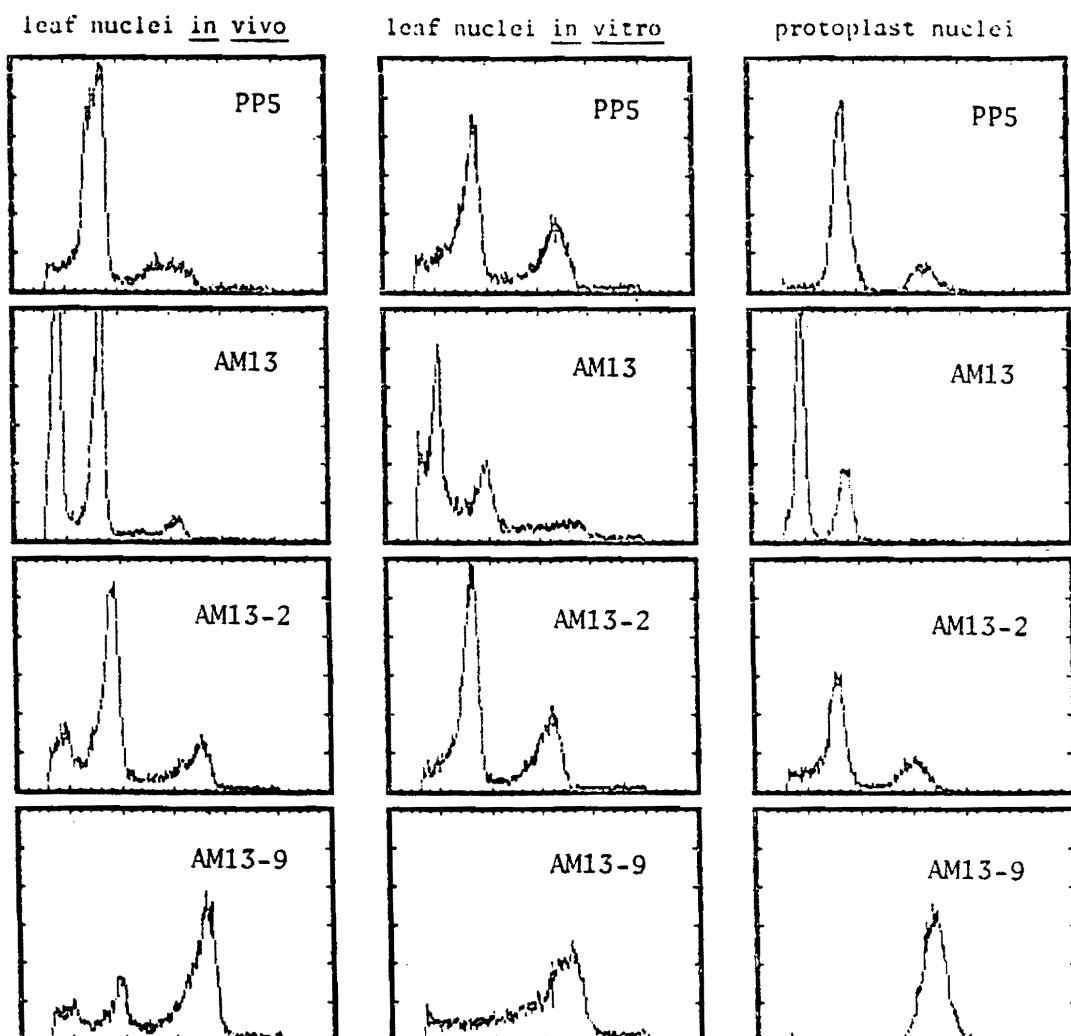


Figure 1. Flow-cytometric analyses of propidium-iodide stained nuclear DNA of 4 genotypes of *Solanum phureja*, PP5 (anther-donor, $2n = 2x = 24$), AM13 (anther-derived monoploid, $2n = x = 12$), AM13-2 (doubled monoploid, $2n = 2x = 24$), and AM13-9 (twice-doubled monoploid, $2n = 4x = 48$). Samples were taken from leaf nuclei *in vivo*, leaf nuclei *in vitro*, and protoplast nuclei extracted from *in vitro* plantlets. The x-axis represents the channel number and the ordinate the frequency of nuclei (of 10,000 counted) in each channel.

two cycles of endoreplication, it is clear that endopolyploidization has occurred in the monoploid preparations. The DNA distribution of protoplast-derived nuclei from monoploid genotype AM13 did not contain a 4C peak, so endopolyploidization is not certain in this sample.

Preparations from diploid (AM13-2) and tetraploid (AM13-9) clones *in vivo* also contain 1C, 2C and 4C peaks, with the peak of highest frequency corresponding to the ploidy level determined for the clone by examination of Feulgen-stained root tip cells. This suggests that endopolyploidization was not complete within the whole plant *in vivo*, creating a mixoploid condition. Preparations of leaf nuclei *in vitro* for both clones did not contain DNA of lower ploidy levels. Because these preparations are from plant material reintroduced *in vitro* from shoot tips or single node cuttings of the *in vivo* plants, endopolyploidization was either complete within the explants, the conditions of plant growth *in vitro* favored replication of the cells of higher ploidy levels.

DNA peaks of nuclei from the 9 monoploid genotypes (Figure 2) show 1C, 2C, and 4C peaks, demonstrating endopolyploidization in protoplast-derived preparations. Chicken red blood cells recorded a peak channel number of 41, which overlapped with the 1C peak of the monoploid genotypes, thus necessitating its use as an external standard only. Variability among the monoploid genotypes for frequency of nuclei exhibiting the 1C DNA content was not evident (Table 1), allowing for analysis of the effect of sampling date on frequency of 1C DNA content by disregarding genotypes. A significant difference was found among

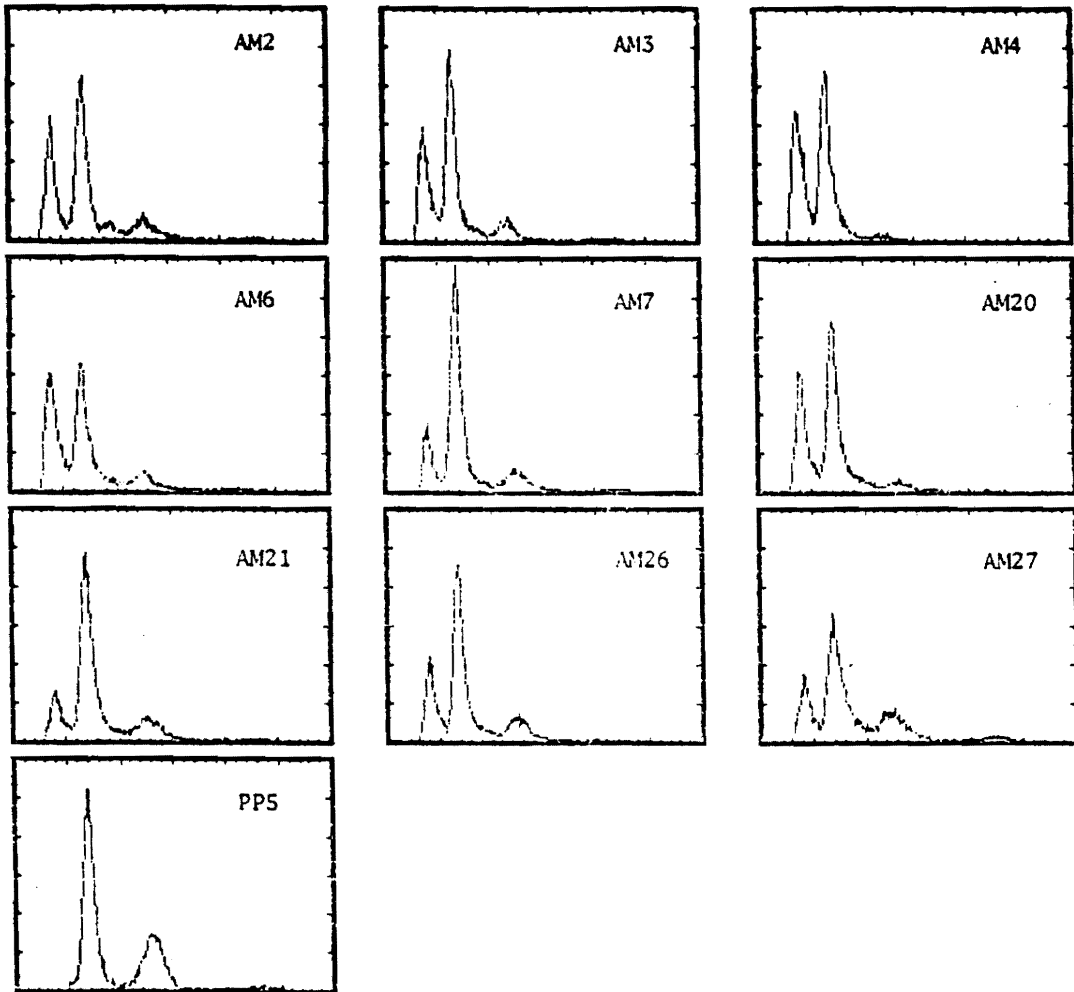


Figure 2. Flow-cytometric analyses of propidium-iodide stained, protoplast-derived nuclear DNA of 9 monoploid ($2n=x=12$), anther-derived genotypes (AM2-AM27) and diploid ($2n=2x=24$), anther-donor genotype (PP5) of *Solanum phureja*. The x-axis represents the channel number and the ordinate the frequency of nuclei (of 10,000 counted) in each channel.

Table 1. Mean fluorescence channel number and percent of protoplast-derived nuclei in 1C, 2C, and 4C DNA peaks for 9 monoploid (AM) genotypes and diploid, anther-donor genotype PP5.

	Channel number			Percent of nuclei \pm s.d.		
	1C	2C	4C	1C	2C	4C
AM 2	41	73	130	39.9 \pm 9.9	44.4 \pm 7.4	8.2 \pm 2.5 cd*
AM 3	38	68	126	25.1 \pm 8.1	53.5 \pm 2.0	12.9 \pm 3.7 bcd
AM 4	39	68	126	31.5 \pm 12.0	59.0 \pm 10.1	5.0 \pm 2.6 d
AM 6	39	69	130	27.3 \pm 11.1	49.8 \pm 5.1	13.4 \pm 4.2 bcd
AM 7	39	69	128	22.8 \pm 7.8	56.7 \pm 12.9	14.7 \pm 3.0 bc
AM20	37	68	127	23.4 \pm 9.0	51.5 \pm 7.2	8.8 \pm 3.3 cd
AM21	40	69	128	19.2 \pm 3.5	61.5 \pm 8.4	14.4 \pm 5.0 bc
AM26	43	73	137	15.8 \pm 11.0	50.4 \pm 6.7	18.8 \pm 7.0 b
AM27	39	70	130	22.6 \pm 7.1	46.4 \pm 1.0	19.7 \pm 0.7 b
PP5		70	130		61.6 \pm 0.7	31.8 \pm 1.6 a

* mean separation of 4C percent by Student-Newman-Keuls' test, 5% level, N = 3.

sampling dates ($P < .0001$), with a mean frequency of 1C nuclei of 42.9, 38.9, and 35.8% for protoplasts extracted on May 21, May 28, and June 4, 1987, respectively. Variability among the monoploid genotypes was demonstrated, however, for frequency of cells exhibiting endopolyploidization at the 4C DNA content, and a significant negative correlation was found between 1C and 4C DNA content (-0.63 , $P < 0.0005$). In addition, the frequency of cells exhibiting the 1C DNA content from the monoploid genotypes was consistently lower (mean = 25.3%) than that for cells with the 2C DNA content of the diploid, anther-donor genotype (mean = 61.6%). Similarly, the frequency of nuclei with the 2C DNA content from the monoploid genotypes was higher than that of nuclei with the 4C DNA content of the diploid, anther-donor genotype (52.6% and 31.8% respectively).

Discussion

As cells and tissues become more differentiated and removed from meristematic zones or growth stages, DNA synthesis and cytokinesis become less synchronous (Pijnacker et al., 1986). For plant cell isolation, manipulation, and regeneration to be useful in germplasm improvement, genetic and genomic stability should be predictable. Current methods for cell and tissue culture, and for genetic manipulation, require the removal of plant cells from natural conditions to synthetic ones. The response of plant cells to new environments may depend upon both internal genetic factors and external environmental factors. The present

study demonstrates that both of these factors affect the process of endopolyploidization.

Nuclei from monoploid protoplast preparations exhibited a range of C values. Sree Ramulu and Dijkhuis (1986) also noted this occurrence in monoploid *S. tuberosum* genotypes and demonstrated variability among tissues for the degree of endopolyploidization. Protoplast preparations from monoploid leaf tissue only, or a combination of leaf and stem tissue, did not differ in their array of DNA values in our investigations.

Genotypes AM13, AM13-2, and AM13-9 differ in ploidy, but except for the possibility of somaclonal variation induced during the culture process, are genetically similar. Thus, they are especially useful for separating ploidy influences from genotypic effects. DNA histograms from the diploid (AM13-2) and tetraploid (AM13-9) clones did not contain C values (i.e. 8C or 16C, respectively) indicative of endopolyploidization, as did the monoploid genotype from which they were derived (AM13). Thus, a limit to endopolyploidization caused by factors other than genotype has been indicated. This limit to endopolyploidization was further supported by the complete absence of an 8C peak in tetraploid preparations from all 3 environments (*in vivo*, *in vitro*, and protoplast-derived nuclei) and from protoplast preparations of 9 monoploid genotypes. The monoploid data suggest that genotype may influence the susceptibility of a cell to undergo endopolyploidization, but that other factors impose limits to this process.

Regarding cell cycle phase, 1C nuclei from monoploid preparations correspond to 2C nuclei from diploid preparations (both representing G_0G_1 phase); similarly, 2C nuclei from monoploid cells correspond to 4C nuclei of diploid cells (both representing $G_2 + M$). However, the 1C monoploid frequencies were consistently lower than the 2C diploid frequency, and the 2C monoploid frequencies were consistently higher than the 4C diploid frequency, suggesting more endopolyploidization in monoploid preparations. Variability among the monoploid genotypes for frequency of 4C nuclei indicates that the degree of endopolyploidization was influenced by genotype. When selecting monoploid genotypes for cell fusion, it may be useful, therefore, to determine the fraction of 4C cells in a sample, since it is the first clear indication of endopolyploidization and a better predictor of a genotype's susceptibility to undergo endopolyploidization during subsequent cell culture and plant regeneration. The negative correlation between 1C and 4C DNA content of the monoploid genotypes suggests that analysis and sorting for higher monoploid status may also select for genotypes having a higher monoploid stability. If monoploid stability in shoot tissue is indicative of ploidy stability during cell and tissue culture, selection of monoploid fusion partners that maintain a high level of monoploidy may produce a higher frequency of diploid heterokaryons and may reduce the frequency of polyploid regenerants from callus.

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Prospectus

Tuber-bearing *Solanum* species pose unique challenges to their improvement. Traditional breeding efforts are confronted with many obstacles: a narrow genetic base among current cultivars, polyploid segregation ratios in progeny, ploidy differences between *S. tuberosum* cultivars and diploid species containing new sources of germplasm, inbreeding depression, disease susceptibility due to clonal propagation, sterility in several economically important cultivars, and self-incompatibility systems at work in many of the diploid, cultivated species. It is no wonder that development of adequate true potato seed has not yet been realized.

Breeding efforts have expanded into biotechnological areas of investigation, including molecular and cellular approaches, cell, tissue and organ culture techniques, and entire genomic reconstruction schemes (Figure 1). The potential for genomic reconstruction in potato germplasm improvement was the focus of the preceding work.

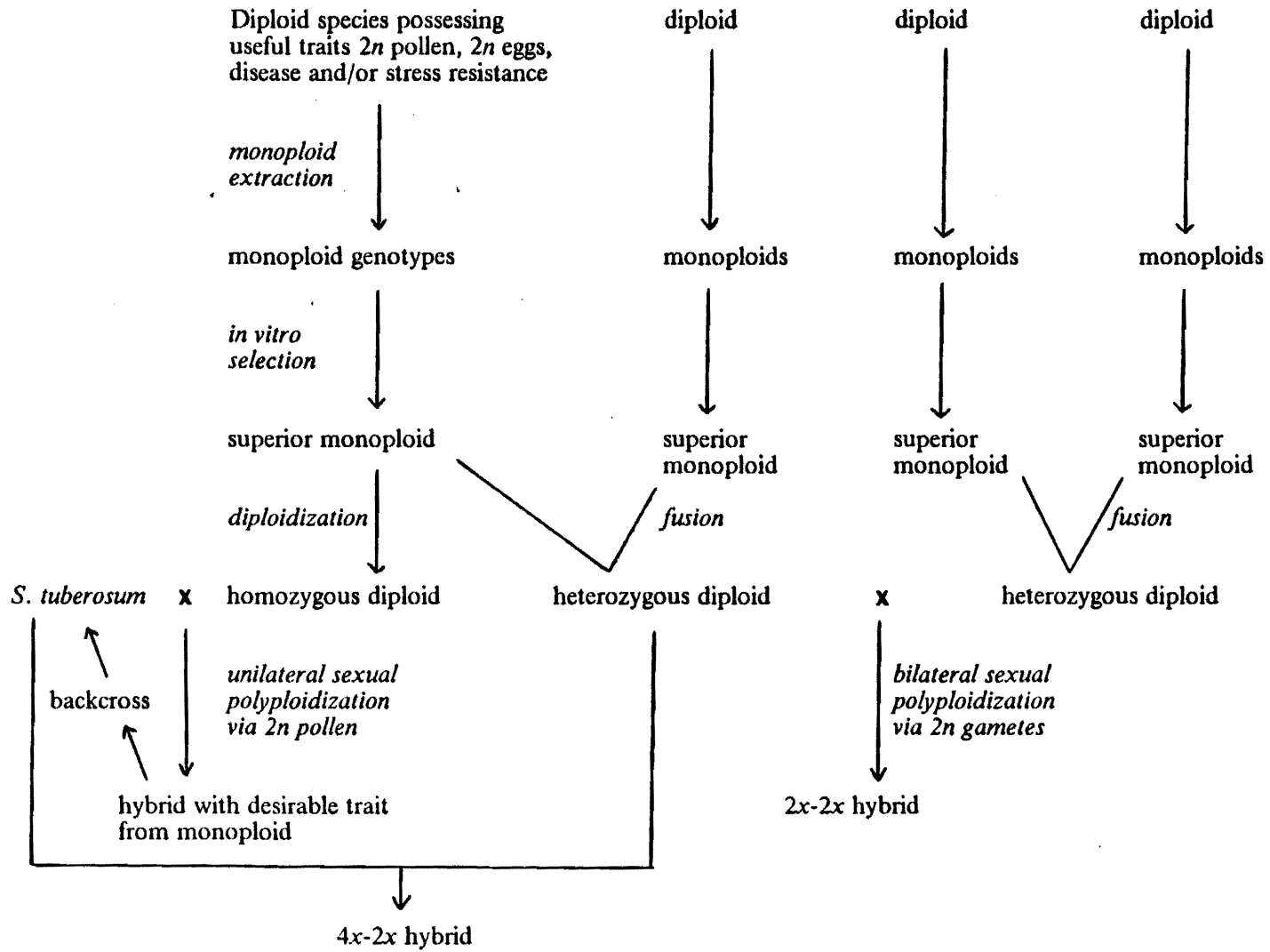


Figure 1. Potential for genomic reconstruction of *Solanum*.

The initial set of experiments was centered on a genotype selected for its capacity to produce unreduced pollen by the genetic equivalent of first division restitution and its ability to regenerate monoploid genotypes via anther culture. Thus, it has the dual ability to produce gametes containing a somatic chromosome complement and whole plants with a gametic chromosome complement. The impact of environment (photoperiod) on these two processes was demonstrated.

In Chapter 3, the effect of photoperiod was again utilized to expose the inherent variability among anther-derived, monoploid genotypes. Reduction to the monoploid level is a critical first step in genomic reconstruction. Monoploids are unique in that they allow for direct phenotypic selection and genetic manipulation prior to diploidization.

Diploidization via chromosome doubling produces a new set of material for study. In Chapter 4, diploid and tetraploid calli clones were examined for the occurrence of fertile homozygotes, useful, for example, in backcrosses to *S. tuberosum* cultivars for the incorporation of traits selected for or induced at the monoploid level. Somaclonal variation may also occur at this point in germplasm development. Its presence in fertile clones would allow for the introduction of new traits into current cultivars by conventional crossing.

In the final chapter, the utility of monoploid genotypes in somatic hybridization schemes was examined. Protoplast fusion between unrelated genotypes would

restore heterozygosity and may restore fertility, again making sexual hybridization possible. Creation of two sets of cell fusion-derived diploids creates the potential for bilateral somatic polyploidization.

It is evident that ploidy reduction and ploidy recombination techniques, combined with sexual hybridization and somatic fusion methods, allow for a multitude of pathways for germplasm development. The potential for this type of technology in crop improvement is far-reaching.

Vita

Henry Robert Owen IV was born on September 29, 1958 in Freeport, New York. He graduated from Plymouth-Salem High School, Plymouth, Michigan in 1976. After attending Henry Ford Community College in Dearborn, Michigan for one year, he transferred to The College of William & Mary in Virginia where he earned a Bachelor of Science degree in Biology in June 1982.

In September of that year, he was granted a teaching assistantship at Virginia Polytechnic Institute & State University and began working toward a Master of Science degree in Horticulture, which he completed in June 1984. He then enrolled in the Genetics program at Virginia Tech to pursue a Doctor of Philosophy degree.

A handwritten signature in black ink that reads "Henry R. Owen, Jr." The signature is written in a cursive style with a large, sweeping initial 'H' and a distinct 'Jr.' at the end.