CHAPTER III: COMPARISON OF ANDROGENIC AND GYNOGENIC MONOPLOID POTATO POPULATIONS

3.1 Introduction

The ability to generate haploid progeny through anther culture and to a lesser extent through various gynogenic mechanisms in a wide variety of species has been heralded as a means to greatly increase efficiency and precision in genetic studies and applied breeding programs. Through haploidization, homozygous lines of any responsive crop, inbreeding or outcrossing, can be produced in a single generation. In self-pollinating crops, these homozygous lines were envisioned as fully developed varieties; in hybrid production as breeding lines to be test-crossed; and in strictly out-crossing crops, as the only means to eliminate deleterious recessive alleles, breed at a hemizygous level, and produce uniform, highly heterozygous hybrids through selection and combination of doubled haploids (Wenzel et al. 1979).

In some applications, haploids have met or exceeded their expectations. Comparison of maize lines derived through single seed descent and anther culture showed that lines derived through anther culture were as vigorous as those derived through single seed descent (Murigneux et al. 1993a). Bjornstad et al. (1993) found that barley populations derived through single seed descent, gynogenesis and androgenesis from each of three F_1 hybrids, exhibited different genotypic arrays. However, the best mechanism differed among the three crosses, so that no single mechanism was consistently favored (Bjornstad et al. 1993). Haploids are now extensively utilized in development of homozygous lines of maize, barley, wheat, rice, and rape seed.

Tobacco was one of the first and most successful crop plants from which haploids were derived through anther culture and is often used as a model system. Tobacco cultivars are traditionally produced by inbreeding a heterozygous population to the extent that segregation is virtually eliminated and plants are phenotypically uniform. After approximately five to ten generations, superior 'homozygous' lines are selected and maintained through self pollination. Anther culture holds great potential for increasing the rate of genetic improvement in tobacco as complete homozygosity can be reached in one generation from an F_1 hybrid, potentially allowing a complete cycle of homozygous line production, testing, and initial selection to occur within a single year.

Early in analysis of the potential of doubled haploid lines for tobacco breeding, several researchers reported that doubled haploid lines derived from certain inbred cultivars and hybrids expressed reduced vigor and variability not present in the anther donor. Burk and Matzinger (1976) found the mean yield of doubled haploids to be 9.5% lower than the source inbred cultivar. Doubled haploids produced 10.6% less cured leaf and were more variable for leaf yield, grade index, total alkaloids, and days to flowering than lines derived from the same F_1 hybrid through single seed descent. It was concluded that single seed descent was superior to androgenesis for line production because the yield of the best line derived through single seed descent was 196 kg per hectare greater than the yield of the best anther-derived line (Schnell et al. 1980). Several possible reasons for inferiority of anther-derived lines have been suggested: residual heterozygosity in the anther donor, linkage bias, mutagenic effects of colchicine utilized in chromosome doubling, gametic selection, cytoplasmic effects, or mutation occurring during androgenesis (Schnell et al. 1980). Chromosome doubling through colchicine was eliminated as

spontaneously doubled androgenic haploids were as variable as those induced through colchicine (Burk and Matzinger 1976) and parthenogenically derived haploids doubled through colchicine displayed none of the negative characteristics present in the androgenic doubled haploids (Kumashiro and Oinuma 1985). Deaton et al. (1982) found that cytoplasmic effects were not a significant factor in doubled haploid performance. Although linkage bias may account for small differences in variability between inbred and anther-derived lines, it would not lead to differences between population means (Schnell et al. 1980). Presence of heterozygosity in the anther donor would necessarily lead to some small reduction of vigor in the completely homozygous doubled haploid progeny, and gametic selection is typically strong in androgenic processes. However, the purported influence of these two factors upon decline in anther-derived progeny has been disproven. Doubled haploid plants derived from an inbred cultivar that had been maintained through selfing for 15 generations expressed variability expected in early segregating generations (Burk and Matzinger 1976). Moreover, a comparison of androgenic and gynogenic doubled haploids and selfed progeny derived from a doubled haploid plant (presumably homozygous) revealed that the gynogenic monoploids and selfed progeny were equivalent, but the androgenic lines were highly variable and generally reduced in vigor (Kumashiro and Oinuma 1985). Therefore, the haploid state itself was not detrimental, rather androgenesis must impose some modification upon the regenerants. When doubled haploid lines were produced from an inbred line of tobacco, yield was reduced by 12-18%. After a second-cycle of anther culture, yields were again generally reduced by 15-17% with an average of 17% yield reduction per anther culture cycle (Brown et al. 1983). Construction of dihaploid synthetics by crossing dihaploids derived from different sources did not restore the productivity of the original anther donors. This

indicated that lack of heterosis was not responsible for the depressed performance of the androgenic lines. It appeared that largely nuclear genetic changes with non-additive action were involved (Brown and Wernsman 1982, Kumashiro and Oinuma 1985).

In *N. sylvestris*, anther-derived lines displayed an abnormal phenotype and reduced growth which was transmitted to successive selfed generations, and further cycles of androgenesis resulted in increased mutation. De Paepe et al. (1981) suggested that differences between the generative and vegetative nuclei gave rise to this systematic mutation. *N. tabacum* doubled haploids exhibited a mean increase in heterochromatin of 12% over the parental value. The nuclear DNA content of the doubled haploids was 10.62 pg, compared to 9.32 pg for the parents (Dhillon et al. 1983).

Studies have shown that haploids of tobacco arise from the microspore vegetative nucleus (Sunderland 1971). Analysis of tobacco microspore vegetative nuclei found that DNA content fluctuates during pollen development, and DNA replication in generative nuclei was accompanied by DNA synthesis in vegetative nuclei (D'Amato et al. 1965). Based upon these observations, Dhillon et al. (1983) suggested that DNA endoreduplication in the vegetative nuclei prior to haploid development was responsible for the consistent occurrence of abnormalities in tobacco doubled haploids. De Paepe et al. (1982) found that nuclear DNA content of *N. sylvestris* doubled haploids was significantly greater than that of the anther donor. This increase in DNA content occurred both in AT-rich and CG-rich highly repetitive regions. Several potential explanations were presented: somaclonal variation, naturally occurring endoreduplication in vegetative nuclei, or the action of 'compensation mechanisms' due to the haploid state (DePaepe et al. 1982).

Whether endoreduplication of the vegetative nucleus is a common phenomenon in plants is not known. However, there is evidence for environmental factors inducing genomic alteration during normal development in a number of plant species including *Pisum sativum* (Cavallini et al. 1996), *Linum usitatissimum* (Durrant 1962, Cullis 1979), and *Nicotiana rustica* (Hill and Perkins 1969). In *Solanum phureja*, an anther-derived monoploid was found to contain a 30% increase in ribosomal DNA sequences. In addition, variation was seen in rDNA that was not present in the anther donor (Pehu 1986).

Direct comparison between source cultivars and doubled haploid lines of potato are distorted by the high level of heterozygosity present in the parental cultivars. Furthermore, this comparison is of little interest, since homozygous lines of potato can only be generated through haploidization and not through conventional means (inbreeding). In potato haploid breeding and genetics, the main issues of concern are whether androgenesis or gynogenesis is the preferred mechanism of haploid derivation and whether haploidization induces undesirable change. It has been suggested that androgenesis is superior in potato as there are greater numbers of microspores than ovules in a given flower (Jacobsen and Sopory 1994), and androgenesis is generally considered more efficient than gynogenesis (Foroughi-Wehr and Wenzel 1993). However, the success of both techniques is genetically controlled and varies widely among genotypes. In addition, larger numbers of gametes do not necessarily give rise to larger numbers of haploids. Efficiency is unquestionably a matter of concern when determining which mechanism of haploid derivation to utilize; but if the populations resulting from androgenesis and gynogenesis are not equivalent on both phenotypic and genotypic levels, one mechanism may be preferred over the alternative, regardless of relative efficiency. Comparisons of

androgenic and gynogenic haploids have been previously reported only in crops which are tolerant of inbreeding. A systematic evaluation of androgenic and gynogenic monoploid populations has not been conducted in potato. Our goals were to begin comparison of androgenic and gynogenic monoploid potato populations which were derived from the same clones under similar environments using a greenhouse study to compare morphological and other growth characteristics.

3.2 Materials and Methods

3.2.1 Greenhouse Experiment-Growth Conditions

Derivation of androgenic and gynogenic monoploid potato populations was described in Chapter II. *In vitro* shoots of 21 androgenic and 21 gynogenic monoploids derived from *S. phureja* 'PP5' were subcultured in baby-food jars containing 40 ml one-half strength Murashige-Skoog basal medium (0.5 × MS salts, 30 g sucrose, and 7 g agar per liter, pH = 5.8) (Murashige and Skoog 1962). Approximately nine three-node shoots of each clone were placed in jars with four to five shoots per jar from September 1-4, 1997 and were rooted for 3 wks in an incubator at 21°C with fluorescent lighting and 16 h daylength. After incubation (roots approx. 1.5-4 cm long) cuttings were transferred to flats with plastic dome lids containing potato soil mix [2 parts Sunshine mix (Sun Gro Horticulture, Inc., Bellevue, Washington) to one part sand] and were acclimated under shade cloth. The plastic covers and shade cloth were gradually removed over a 1-wk period. Acclimated plants were transferred to 3.8 L pots containing potato soil mix and were grown for 6 wks. In order to propagate plants uniformly, six to nine three-node cuttings were taken from each clone, dipped in Rootone rooting powder, placed into flats containing potato soil mix, and rooted under intermittent mist for 3 wks. Rooted cuttings were transplanted

into 1.9 L pots of potato soil mix and grown under temperatures of 15°C (night) and 24°C (day). Natural lighting was supplemented with high intensity sodium lamps to extend daylength to 16 h. The 42 clones were placed in a randomized complete block design with three replications grown on adjacent tables in the same greenhouse.

3.2.2 Data collection

Vegetative data

At 30 days after planting, data were collected on plant height and number of main stems, and a preliminary vigor rating was given on a five-point scale. At 90 days, data were collected with three measurements per plant on internode length, and a final vigor rating was given on a scale where 1 = most vigorous and 5 = least vigorous. The length and width of three fully expanded leaves were measured per plant. The total number of leaflets per leaf was counted for three leaves on each plant. Generally, measurements were taken on the seventh, eighth, and ninth leaves from the shoot apex of each plant.

Floral characteristics

Floral data were taken on number of buds per inflorescence, number of anthers per flower, corolla width, style length, and petal color. Three measurements were taken per plant for all characteristics, except for flower color. Flower color was measured with a colorimeter using approximately six flowers per plant. Regretfully, only the data from number of buds per inflorescence were complete enough for statistical analysis. When possible, at least ten flowers of each plant were emasculated and pollinated with fresh pollen from the diploid clone ID5. ID5 was selected as the pollen parent for its known ability to induce fruit set in crosses with doubled monoploids derived from PP5. Fruit that formed were wrapped in cheesecloth and allowed to

mature for 5-6 weeks. Fully developed fruit were harvested and allowed to ripen at room temperature for 1 wk prior to cleaning.

Tuber Characteristics

Plants were harvested on April 18, and data were taken on number of tubers per plant, total tuber weight per plant, mean weight per tuber, tuber skin color, tuber flesh color, and tuber appearance (rough, moderate, or smooth). Tuber skin color is a complex trait; however, an attempt was made to classify skin color as white, pink, or red. Tuber flesh was classified as either white or yellow and is known to be controlled by a single dominant gene. Analysis of variance was performed on all data collected from the greenhouse study using JMPIN version 3.0 (SAS Institute 1996).

3.2.3 Ploidy Evaluation

Flow cytometry was performed on plants derived from PP5 and BARD1-3 as they became available through anther culture and gynogenesis in order to estimate the ploidy level of the plants and their nuclear DNA content. Since it appeared that some previously identified monoploids derived from PP5, which were included in the greenhouse evaluation, had undergone endopolyploidization; all the PP5 monoploids were re-evaluated with flow cytometry on a single day. Briefly, 0.1-0.5 g *in vitro* plant material was chopped with a razor blade on ice in 1.5 ml buffer (882 mg sodium citrate, 419 mg MOPS, 915 mg $MgCl_2$, 0.1 ml Triton X-100 and distilled H_2O to 250 ml). The macerated plant material was then filtered successively through a 250 µm and a 63 µm mesh sieve to eliminate large debris. From the filtered liquid, 500 µl was transferred to a clean 1.5 ml microcentrifuge tube and 250 µl RNAse-A was added. The samples were incubated at room temperature for 1-2 h, and 125 µl propidium iodide stain was added to

each sample. The samples were stored on ice a minimum of 45 min to a maximum of 2 h prior to analysis with a Coulter Flow Cytometer (Epics-XL) set at an absorbance of 300 (Owen et al. 1988). The flow gates were set using a known monoploid as a standard, and the peak positive values (the median nuclear DNA content of each sample) for the 1*x*, 2*x*, 4*x*, and 8*x* peaks were recorded. All plants with ploidy levels greater than 1*x* were eliminated from further analysis. Androgenic and gynogenic population mean peak positive values for the 1*x*, 2*x*, 4*x*, and 8*x* levels from flow cytometry were compared for three groups of plants (PP5 - various flow dates, BARD1-3 - various flow dates, PP5 - single flow date) by means of Student's T-test with JMPIN version 3.0 (SAS Institute 1996).

3.2.4 RAPD Analysis

DNA extraction and RAPD analysis were conducted as described in Chapter II. Data were collected from a population of 38 to 44 monoploids with 19 RAPD primers. Since RAPDs are dominant markers, all polymorphic bands were expected to segregate in a presence to absence ratio of 1:1 in the monoploid population. The observed ratios were tested using χ^2 analysis to determine the proportion of molecular markers exhibiting skewed segregation.

3.3.1 RESULTS

Greenhouse Experiment

Although there were apparent differences between the androgenic and gynogenic populations for plant height, leaf length, leaf width, and total tuber yield (Table 3.2 and Table 3.6), ANOVA revealed no significant differences between the populations for the 11 traits analyzed (Tables 3.1 and Table 3.5). Tuber flesh color (yellow versus white) is controlled by a single gene (*Y*); yellow flesh is dominant to white. PP5 is known to be heterozygous (*Yy*) at the

Y locus from a previous study (Singsit et al. 198). Therefore, the monoploids extracted from PP5 would be expected to segregate 1:1 for yellow : white flesh. Segregation for tuber flesh color did not differ significantly from the expected 1:1 yellow to white segregation ratio in both populations combined as determined by Chi square analysis ($\chi^2_1 = 0.88$, $p>0.05$); 50% of the androgenic monoploids produced yellow tubers whereas 63% of the gynogenic monoploids were yellow.

Flower color, corolla width, style length, and anthers per bud could not be statistically analyzed due to missing data. Although all clones initiated floral buds, many did not open and abscised prematurely or else were extremely deformed, which hampered data collection. Poor floral development was attributed to the haploid state and aphid infestation.

Variation among clones within a mechanism of derivation was expected, since all genotypes were random gametic samples of a heterozygous parent. Clone within derivation was significant at the 0.05 level for plant height, internode length, vigor rating, leaf length, leaf width, number of leaflets per leaf, total tuber weight, and buds per inflorescence. Replication was also significant for internode length and leaf length indicating the presence of position effects in the greenhouse.

A total of 395 pollinations was performed on androgenic and gynogenic PP5 monoploids (Table 3.4). However, only three androgenic clones set fruit. From crosses between PP5-3L-9 and ID5, six fruit were obtained which contained more than 50 seeds each; however, the clone was later found to be diploid. The morphological appearance of PP5-3L-9 was similar to the monoploids including floral structure abnormality, except morphological features were uncharacteristically large. Crosses between PP5-53 and ID5 yielded three fruit, but the plants

died prematurely, resulting in underdeveloped fruit without seeds, though there appeared to be fragments of aborted ovules present. PP5-53 was later reconfirmed to be monoploid through flow cytometry of *in vitro* plantlets derived from the same source as the plants in the greenhouse. In a second analysis of *in vitro* plant material by flow cytometry, PP5-3S-21 again appeared to be monoploid. When PP5-3S-21 was crossed to ID5, four fruit containing 6-15 seeds apiece were produced.

3.3.2 Ploidy evaluation

Re-analysis of the ploidy level of 21 anther-derived and 22 gynogenic putative monoploids of PP5 revealed that four or 19% of the androgenic putative monoploids were actually diploid. All of the previously-analyzed gynogenic putative monoploids were still monoploid.

Peak positive values resulting from flow cytometry are measurements of the median nuclear DNA content in each peak for a given sample. A monoploid sample generally results in three or four peaks per flow output. The peaks correspond to the 1*x*, 2*x*, 4*x*, and 8*x* cell populations among somatic cells within the tissue sampled. The 2*x* peak consists of cells in the G_2 phase of the cell cycle or cells that have undergone a single round of endopolyploidization, whereas the 4*x* and 8*x* peaks represent endopolyploid cells. Each peak represents a population of cells with approximately the same DNA content. Monoploid peak positive values ranged from 14 to 27 and from 15 to 26 for the androgenic and gynogenic PP5 monoploids analyzed on a single date, respectively. The mean peak positive value of 22.89 for the androgenic population was higher than the mean value for the gynogenic population, 22.36. This trend was evident for the 2*x*, 4*x*, and 8*x* values as well, though the size of the difference between the populations

decreased with increasing ploidy of the peak positive values (Table 3.7). Examination of data from flow cytometry of PP5 and BARD1-3 androgenic and gynogenic monoploids which was conducted on various dates in 1997 and 1998 revealed a similar trend (Table 3.7). Differences between the peak positive values of the androgenic and gynogenic monoploids were statistically meaningful at the 0.05 significance level according to Student's T-test for the BARD1-3 populations analyzed on various dates in 1997 and 1998. Although only one of the differences between the androgenic and gynogenic monoploids in the PP5 populations was significantly different at the 0.05 level, the consistent occurrence of higher DNA content in the androgenic than gynogenic PP5 monoploids provides some evidence for greater nuclear DNA content in androgenic than in gynogenic monoploids (Table 3.7).

3.3.3 RAPD Analysis

With 19 RAPD primers, 53 scorable segregating bands were obtained with a mean of 2.8 and a range of 1-6 bands per primer. In a population ranging from 38 to 45 unique monoploids derived from *S. phureja* BARD1-3, ten or 18.9% of the 53 RAPD markers deviated from the expected 1:1 presence to absence ratio at the 0.05 significance level ($\chi^2_1 > 3.841$, $\alpha < 0.05$); and seven of these were also skewed at the 0.01 significance level ($\chi^2_1 > 6.635$, $\alpha < 0.01$). The segregation ratios and χ^2 values for the ten skewed markers are presented in Table 3.8. Of the loci with skewed segregation, seven deviated toward absence of the marker, whereas three deviated toward presence of the marker. A photograph of an agarose electrophoresis gel generated by RAPD primer OPG-12 can be seen in Figure 3.1. The arrow in the figure points to a band exhibiting skewed segregation among the anther-derived monoploids.

3.4 Discussion

In general, the androgenic monoploids appeared slightly more vigorous and higher yielding than the gynogenic monoploids, though none of the differences was significant due to variability among the monoploid individuals within each population. It remains to be seen whether these results will be applicable to other clones or in different environmental conditions. In view of similar studies in tobacco (DePaepe et al.1983, Dhillon et al. 1983, and Kumashiro and Oinuma 1985), these results were unexpected. Several major differences exist between this study and the aforementioned ones, i.e., other studies utilized an inbred crop and analyzed doubled haploids rather than haploids. In addition, negative effects of androgenesis or gynogenesis were only seen in progeny of particular parental genotypes of tobacco. Possible reasons for the differences between the androgenic and gynogenic populations are differential selection pressures, incorporation of genes from the haploid inducing pollinator in the gynogenic monoploids, and genomic changes inherent in microspore development or the anther culture process.

Although several of the androgenic putative monoploids were later found to be diploid, all gynogenic putative monoploids remained at the 1*x* level. Either mistakes were made in prior analysis of the androgenic clones by flow cytometry, or true monoploids had undergone endopolyploidization. Of the four monoploids which apparently changed, two had been in culture for approximately 5 years and the other two had been derived within 2 years. All of the gynogenic monoploids were derived within the last 2 years. In addition, the gynogenic monoploids were selected based upon morphological markers carried by the haploid inducing

pollinator prior to ploidy level determination with flow cytometry. This may have reduced the number of misidentifications.

PP5 is known to produce a variable frequency (<1% to >5%) of 2*n* male gametes by fusion of second division spindles, a mechanism similar to first division restitution (Veilleux et al. 1985). If the putatively monoploid clones PP5-53 and PP5-3S-21 had not spontaneously doubled, seed set on monoploids would indicate that they produced viable unreduced (1*n*=1*x*=12) female gametes. Unreduced female gametes have been reported to occur in diploid potato germplasm by two mechanisms which are genetically equivalent to first division restitution (FDR). These two FDR mechanisms, delayed meiotic division and synaptic mutation, could give rise to viable unreduced female gametes in monoploid potatoes. In a survey of five diploid potato species, 24% of the 127 plants were found to produce viable 2*n* eggs at a frequency of 4.9-57.3% (Werner and Peloquin 1991). Although three SDR type mechanisms of 2*n* egg formation have also been reported in potato (Werner and Peloquin 1991), they require a normal first meiotic division and therefore would be precluded in monoploids.

Though many (approx. 19%) RAPD loci were skewed in the BARD1-3 anther-derived population, no conclusions can be drawn as to whether the markers were linked to deleterious loci or genes controlling anther culture response. Analysis of the BARD1-3 gynogenic population with the same markers may allow a discrimination. Skewed segregation has been reported in other anther-derived populations. Veilleux et al. (1995) found both SSR and RAPD markers to be skewed in anther culture derived progeny of a diploid hybrid clone, CP2 (*S. chacoense* × *S. phureja* BARD1-3); however, the extent of the segregation distortion could not be precisely characterized due to small population size and heterozygosity of some of the

putatively anther-derived plants. Rivard et al. (1996) found 46% and 70% skewed segregation of RFLP markers in two monoploid populations derived through anther culture of *Solanum chacoense.* Only 10% of the RFLP markers exhibited skewed segregation in F_1 progeny from a cross between the two anther donors, and distorted segregation was found for 30% of the markers in selfed progeny of the anther donors (Rivard et al. 1996).

In general, flow cytometric data revealed 3-8% more DNA per nucleus in the androgenic than in the gynogenic monoploids. If both pathways produced plants with precisely half of the parental chromosome complement, the populations would be expected to be comprised of plants with similar DNA contents. Observations of *Nicotiana tabacum* and *N. sylvestris* have indicated that there is expansion of primarily repetitive regions in anther-derived plants (DePaepe et al.1983; Dhillon et al. 1983). Pehu (1986) achieved similar results and found that there was an increase (30%) in ribosomal DNA in the anther-derived plants. Though this may be caused by the tissue culture process itself, this phenomenon appears unlikely to be somaclonal variation in tobacco as mutations occurred in a systematic and predictable manner rather than at random (Dhillon et al. 1983). It is possible that overreplication of some repetitive sequences, such as rDNA, may enhance monoploid performance by provision of additional copies of essential genes. However, the same overreplication, a compensation mechanism in monoploids, may hinder agronomic performance of doubled monoploids where the presence of homologous chromosomes suffices for optimal gene copy number and the additional DNA merely slows the cell cycle.

It appears that there are unique advantages to both mechanisms of potato haploid induction. Efficiency of derivational processes is primarily controlled by the anther donor or

seed parent genotype. The haploid inducing pollinator also affects the efficiency of gynogenesis, but pollinators have been selected which induce a high frequency of haploids from a wide variety of seed parent species and clones (Hermsen and Verdenius 1973). It is likely that different genes control the responses (the responses appear uncorrelated) so that one mechanism may be much more efficient for a given clone.

Both systems of haploid derivation require a great deal of tedious work. Androgenesis requires an *in vitro* system, in contrast to gynogenesis which can be conducted entirely *in vivo*. Particularly in developing countries, gynogenesis is likely to be the preferred mechanism, as no external inputs are required, less expense is incurred in startup, and little expertise is needed.

For genetic studies, gynogenesis may be preferred due to greatly reduced chances of somaclonal variation. If the vegetative microspore nucleus, which gives rise to anther derived plants, consistently undergoes genetic alteration expressed as increased DNA content and re-arrangement of rRNA in potato (Pehu 1986) then most anther-derived plants will be affected in some way. This advantage of gynogenesis may be outweighed by the potential production of aneuploids *via* gynogenesis and possible incorporation of genetic material from the haploid inducing pollinator.

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