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An Examination and Evaluation of Diphaloid Potatoes of Solanum tuberosum that Produce Mixed Modes of 2n Eggs

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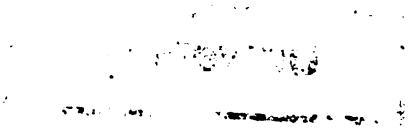

AN EXAMINATION AND EVALUATION OF DIHAPLOID POTATOES OF *Solanum tuberosum* THAT PRODUCE MIXED MODES OF 2N EGGS

By

David Lewis Maas

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ABSTRACT

AN EXAMINATION AND EVALUATION OF DIHAPLOID POTATOES OF *Solanum tuberosum* THAT PRODUCE MIXED MODES OF 2N EGGS

By

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Several first division restitution- (FDR) and second division restitution- (SDR) derived tetraploid subpopulations were constructed using two 2n egg-producing, mixed-mode dihaploids crossed to tetraploid potatoes (*Solanum tuberosum* subsp. *tuberosum* L.). Progeny were separated into subpopulations using *Pgm-2* segregation data. There was no significant difference between the performance of FDR- and SDR-derived subpopulations in multiple crosses over multiple seasons. Inbreeding, as a consequence of the haploidization process, may have negated any advantage of the FDR-derived progenies over the SDR-derived progenies. The effectiveness of the laser scanning confocal microscope (LSCM) is demonstrated in examining megasporogenesis of several *S. tuberosum* dihaploids and diploids. Ethidium bromide was used as a cellular stain (0.5 mg/ml for 15 minutes) to enhance the viewing of ovules. Extremely well-defined images were obtained due to the confocal ability of the microscope. The LSCM is powerful tool for further observation of megasporogenesis in *Solanum*.

"Congress shall make no law respecting an establishment of religion, or prohibiting the free exercise thereof; or abridging the freedom of speech, or of the press; or the right of the people peaceably to assemble, and to petition the government for a redress of grievances."

First Amendment of the Constitution
of the United States of America (1791)

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To my family: thanks for the support!

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CHAPTER I

A STUDY TO COMPARE THE BREEDING VALUE OF FDR AND SDR 2n EGGS IN INTERPLOID CROSSES

INTRODUCTION

The cultivated potato, *Solanum tuberosum* subsp. *tuberosum* L., is an important world food crop, ranking fourth in total crop production (Hawkes 1990). *S. tuberosum* subsp. *tuberosum* L. is a polysomic polyploid (2n=4x=48). *Solanum* species, with ploidy levels ranging from 2x to 6x, extend from South America to North America in a wide range of altitudes (sea level to 4,000 meters) and climates, with the center of diversity in the Andean region of Peru (Hawkes 1990). Wide geographical distribution has given rise to an immense amount of diversity within native potato species. Wild species have adaptations to environmental extremes as well as resistances to insect pests, fungal, bacterial and viral diseases (Hougas and Peloquin 1958; Hermundstad and Peloquin 1985; Hawkes 1990). Other important features of these native species are high dry matter content and low reducing sugars. However, some may contain high levels of toxic glycoalkaloids.

The ultimate goal in a potato breeding program is to produce cultivars with improved horticultural characteristics, such as increased yield, disease and insect resistance, longer storability and desirable processing qualities. However, there are several obstacles in breeding

potato cultivars. Most importantly, the commercial potato is an autotetraploid (2n=4x=48), which leads to complex inheritance patterns (Rowe 1967b). Moreover, the narrow genetic base found in commercial cultivars is exacerbated because many of the initial cultivars could not be used as pollen sources due to a decline in sexual fertility (Bradshaw and Mackay 1994, Grun et al., 1977, Mendoza and Haynes 1974b).

Mendoza and Haynes (1974a) stated that yield in polysomic polyploids is based mainly on intra-locus and inter-locus interactions. The theory of maximum heterozygosity states that hybrid vigor in cross-pollinated polyploid species is maximized by multiple allelic interactions at a locus (overdominance), chromosome segments with complementary alleles (epistasis) or a combination of both (Dunbier and Bingham 1975; Mendiburu and Peloquin 1977a; Bingham 1980; Bonierbale et al., 1993; Bingham et al., 1994). A yield plateau was noted by Mendoza and Haynes (1974b), where no increase in yield had occurred for the preceding twenty years. The idea of a yield plateau was supported by Douches et al., (1996) after comparing yield and performance of numerous cultivars over years of introduction. No increase in yield was observed with new varieties (period 1970-1989) compared to varieties released in the late 19th century.

2n gametes are an effective means of maintaining allelic diversity and desirable epistatic interactions that

are needed for maximum heterozygosity and consequently maximum expression of yield (Iwanaga 1984; Douches and Quiros 1988a, 1988b; Concillo and Peloquin 1991). 2n gametes can be produced through first division restitution (FDR) or second division restitution (SDR). FDR transmits approximately 80% of the parental heterozygosity intact, compared to 40% by SDR (Peloquin, 1983). The use of 2n gametes allows efficient transfer of genes from diploids to tetraploids, as well as broadening the genetic base of the commercial potato (Hougas and Peloquin 1958, Kotch et al., 1992). Unilateral sexual polyploidizations utilizing 2n gametes (4x-2x, 2x-2x, 2x-4x) resulting in tetraploid progeny are well documented (Concillo and Peloquin 1991). Early 4x-2x crossing studies showed a heterotic effect associated with the use of FDR producing haploid-species hybrids (Mok and Peloquin 1975; Mendiburu and Peloquin 1977; Darmo and Peloquin 1990; Concillo and Peloquin 1991, Darmo and Peloquin 1991; Ortiz et al., 1991). The heterotic effect was also noted using dihaploids of *S. tuberosum* (Werner and Peloquin 1991a, 1991c). This observed heterotic effect is contrasted to the lower performance noted for 2x-4x crosses which are primarily SDR. The use of separate genotypes in the current comparison of the performance of FDR to SDR confounds the observation. Dihaploids (haploids derived from tetraploids, 2n=2x=24) of *S. tuberosum* forming 2n eggs by mechanisms genetically equivalent to FDR (delayed meiotic division) and SDR (delayed meiotic division,

omission of second division) have been identified (Werner and Peloquin 1991c). The use of these mixed mode producing dihaploids allows for an unbiased comparison of FDR and SDR.

The objective of this research is to conduct field studies to compare the performance of FDR- and SDR-derived tetraploid progeny populations. With the ability to separate the progeny into SDR- and FDR-derived subpopulations we are able to make unbiased comparisons of the value of differing modes of 2n gamete formation. These evaluations may also give us insights into the role of maximum heterozygosity and the expression of heterosis in autotetraploid potato.

LITERATURE REVIEW

MAXIMUM HETEROZYGOSITY THEORY

Numerous studies have been conducted to study and understand the genetic basis for yield in potato and other polyploid crops. Allopolyploids or disomic polyploids can maintain heterozygosity in two or more divergent genomes. Thus, allopolyploids do not suffer inbreeding depression due to the internal hybridity among their loci. In contrast, autopolyploids or polysomic polyploids must rely upon cross-pollination to ensure intra-locus heterozygosity (Bingham 1980).

Rowe (1967a) examined the performance of diploid potato hybrids (*S. tuberosum* dihaploid x *S. phureja*) and vegetatively doubled diploids. Tuber production of tetraploids was inferior to that of the diploids. Rowe postulated that if heterozygosity influences plant yield and doubling of the diploids produces genotypes with a restricted number of heterozygous loci, inbreeding may result. In later research, Rowe (1967b) compared the performance of 11 diploid and autotetraploid families. The hybrids originated from crosses between diploid potato hybrid (*S. tuberosum* dihaploid x *S. phureja*) and

vegetatively doubled diploids. Diploid performance was less than their parental clones, in contrast, the tetraploids exceeded the midparent value as well as the higher parent (Rowe 1967b). Rowe concluded that for productivity in diploid and tetraploid potatoes, gene action and heterozygosity may be more important than ploidy level. De Jong and Rowe (1971), in additional crossing studies using diploid species *S. phureja*, *S. stenotomum* and dihaploids of *S. tuberosum*, found that S_1, S_2 and S_3 progenies exhibited a linear relationship between the decrease in tuber production and the expected coefficient of inbreeding in successive generations of selfing.

Mendoza and Haynes (1974a) explained heterosis for yield by a model of overdominant gene action instead of dominant gene action based on Rowe's (1967a,b) data. With overdominance (intra-locus interactions), autotetraploid genotypes with high frequencies of tetra- or triallelic loci (more first-order interactions) are superior to those genotypes with higher percentage of di- or monoallelic loci (fewer or no first-order interactions) (Bingham 1980, Bonierbale et al., 1993) (Table 1-1).

To further explore the importance of multiple allelic interactions, researchers in alfalfa (*Megicago sativa* L. *sensu lato*) generated haploid-derived autotetraploids (HD4x) (Dunbier and Bingham 1975). Use of HD4x provides a model system where populations are of a defined genotypic structure. Single crosses (SC) between unrelated HD4x were

Table 1-1. The number of first, second and third order interactions for a locus depending on allelic diversity.

Locus	Number of interactions		
	First	Second	Third
Tetra-allelic ($A_1A_2A_3A_4$)	6	4	1
Tri-allelic ($A_1A_1A_2A_3$)	3	1	
Di-allelic ($A_1A_1A_1A_2$) / ($A_1A_1A_2A_2$)	1		
Mono-allelic ($A_1A_1A_1A_1$)	0		

made, subsequent double crosses (DC) were performed using a random selection of SC plants. Resulting forage yields, seed weight and fertility data show that the DC was consistently greater than the SC. This result supports the importance of multiple allelic interactions and the hypothesis that maximizing heterozygosity maximizes yield in alfalfa.

Alfalfa is a cross-pollinated autotetraploid species, although self-pollination is possible. In alfalfa, vigor and fertility declined more quickly than the theoretical rate of reduction of heterozygosity during selfing (Busbice and Wilsie 1966; Busbice 1968). Studies with inbreeding have shown a decrease of seed yield of half when the inbreeding coefficient was 0.06 and 5/6 when the coefficient was 0.30 (Busbice 1968). Busbice explained these observations by epistatic interactions of recessive lethals masking the effects of fertility genes.

Bingham (1980) proposed the theory that maximum heterozygosity in autopolyploids is due to the presence of multiple alleles at a locus in combination with chromosomes containing linkage blocks (epistasis). Epistasis (inter-locus interactions) occurs when dominant alleles at different heterozygous loci complement each other by masking recessive deleterious alleles. Bonierbale et al. (1993) developed several populations to test the theory. One population consisted of a cross between two adapted *S. tuberosum* cultivars (TT). The second was produced by the

intermating of a Neotuberosum-Tuberosum hybrid by *S. tuberosum* spp. *andigena* (NT). A cross between a *S. tuberosum* advanced selection and a *S. tuberosum* x (*S. phureja*-haploid *S. tuberosum* hybrid) provided the third population (PT). Restriction fragment length polymorphism data showed that the TT population had the highest level of homozygosity (29% vs 24% for NT and 22% for PT). Relative heterozygosity index showed PT to be higher than either NT or TT (50% vs 48% and 47%). Performance trials showed that in adapted x adapted crosses, homozygosity was negatively correlated with tuber yield and maximum heterozygosity was positively correlated to yield for tubers over 6.4 cm. However, crosses between adapted and unadapted parents showed no relationship between yield and heterozygosity. These results are explained by the idea that maximum heterozygosity only influences tuber yield in a favorable direction when adapted parents are combined. An alternate hypothesis could be the effect of negative epistatic interactions. Genes for lack of adaptation could be masking alleles in adapted material producing no increase in mean performance. It has been noted in group *Phureja*, yield increased over generations as populations became long-day adapted (Bradshaw and Mackay 1994).

Another method to examine maximum heterozygosity is through the use of 2n gametes. 2n gametes can be produced by different mechanisms each with different genetic consequences: 1) premeiotic chromosome doubling; 2) first

division restitution (FDR)(parallel spindles, synaptic variant, delayed meiotic division); 3) second division restitution (SDR)(delayed meiotic variation, omission of second division, irregular anaphase II, no second cytokinesis); 4) post meiotic chromosome doubling; 5) apospory (development of the gametophyte from a somatic cell) (Rhodes and Dempsey 1966; Mendiburu and Peloquin 1975; Hermsen 1984; Veilleux 1985; Ramanna 1979; Werner and Peloquin 1991b). Each mode of 2n gamete formation transmits a different amount of parental heterozygosity intact to the progeny (Table 1-2)(Werner 1989). Several meiotic mutants leading to 2n pollen and egg production have been identified in potato (Mok and Peloquin 1975b; Iwanaga and Peloquin 1979; Iwanaga 1984; Stelly and Peloquin 1986; Werner and Peloquin 1987; Douches and Quiros 1988b; Ramanna 1979). Dihaploids of *S. tuberosum* forming 2n eggs by mechanisms genetically equivalent to either FDR (synaptic variant, delayed meiotic division) or SDR (omission of second division, irregular anaphase II, no second cytokinesis, delayed meiotic division) have been identified (Werner and Peloquin 1991c). Use of both forms of 2n gametes in interploidy crosses (2x-4x, 4x-2x and 2x-2x) is well established (Mok and Peloquin 1975a, 1975b; Mendiburu and Peloquin 1977a, 1977b; Peloquin et al., 1989; Darmo and Peloquin 1990; Concillo and Peloquin 1991; Darmo and Peloquin 1991; Ortiz et al., 1991; Ortiz and Peloquin 1991). Studies performed by Peloquin and his co-workers have led to

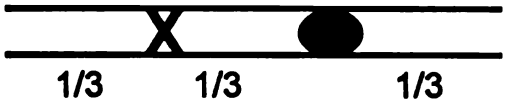
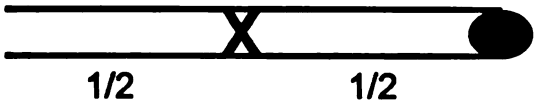
Table 1-2. Genetic consequence of mode of 2n gamete formation measured in terms of average percent parental heterozygosity transmitted intact to progeny.

Mode of 2n gamete formation	Heterozygosity transmitted
Post meiotic chromosome doubling	0%
Second division restitution	40%
Premeiotic chromosome doubling	62%
First division restitution	80%
Apospory	100%

the hypothesis that FDR-derived 2n gametes are superior to SDR-derived 2n gametes with respect to the performance of resulting tetraploid progeny (Mendiburu and Peloquin 1977b; Ortiz et al., 1991; Werner 1989; Werner and Peloquin 1991c). Higher tuber yields have been found in FDR-derived tetraploids than in SDR-derived tetraploids from 4x-2x matings using haploids and haploid-species hybrids (Mendiburu and Peloquin 1977a, 1977b; Chujoy and Peloquin 1986; Darmo and Peloquin 1990; Concillo and Peloquin 1991; Darmo and Peloquin 1991; Ortiz and Peloquin 1991; Werner and Peloquin 1991a, 1991c). Heterotic responses from 4x-2x (FDR) matings with these clones showed progeny yields that surpass midparent values and tetraploid controls in yield trials. Increased yield production of the 4x-2x populations was attributed to the high level of heterozygosity transmission through FDR pollen. FDR 2n gametes transmit approximately 80% of their parental heterozygosity intact to the progeny compared to 40% by SDR 2n gametes (Figure 1-1) (Werner and Peloquin 1987; Peloquin et al., 1989; Concillo and Peloquin 1991; Darmo and Peloquin 1991). In contrast, Mendiburu and Peloquin (1977a), attributed low yield of progeny derived from 2x-4x crosses to the type of 2n egg formation. The primary mechanism of 2n egg production in potato is SDR. This furthered the belief that the breeding value of FDR-derived 2n gametes are indeed superior to SDR-derived 2n gametes.

Bingham et al. (1994) reinterpreted the research of

Figure 1-1. Approximate percentage of parental heterozygosity transmitted by SDR and FDR 2n gametes in four types of chromosomes.

X =crossover ●=centromere	Percentage of Parental Heterozygosity	
	FDR	SDR
 1/4 1/4 1/2	(3/4 x 1)+(1/4 x 1/2) = 7/8	(3/4 x 0)+(1/4 x 1) = 1/4
 1/3 1/3 1/3	(2/3 x 1)+(1/3 x 1/2) = 5/6	(2/3 x 0)+(1/3 x 1) = 1/3
 1/2 1/2	(1/2 x 1)+(1/2 x 1/2) = 3/4	(1/2 x 0)+(1/2 x 1) = 1/2
 1/4 1/4 1/4 1/4	(2/4 x 1)+(2/4 x 1/2) = 3/4	(2/4 x 0)+(2/4 x 1) = 1/2
	Average 80.2%	39.6%

* adapted from Peloquin 1983.

Dunbier and Bingham (1975), attributing apparent multiple allelic interactions in previous studies to linkage disequilibrium. The reexamination of Dunbier and Bingham (1975) and other studies concluded that progressive heterosis in autotetraploids is due to a increase in complementary gene action (Bingham et al. 1994). Populations involving two alleles per locus (chromosome doubled diploids) supported that the accumulated action of favorable alleles not intra-allelic interactions improved performance in alfalfa (Bingham et al. 1994).

HALF TETRAD ANALYSIS

A unique advantage of 2n gametes is the opportunity they offer for half-tetrad analysis (HTA)(Mendiburu and Peloquin 1979; Douches and Quiros 1987; Douches and Quiros 1988a; Werner et al., 1991). HTA is based on the fact that two strands of a bivalent from the diploid parent are recovered in the tetraploid progeny. HTA is feasible in a 2x-4x cross in which a diploid, hetèrozygous for the codominant marker proximal to the centromere, is crossed to a monoallelic (nulliplex) tetraploid. For FDR-derived progeny the markers will all be simplex (Aaaa), if the progeny are SDR-derived one half of the markers will score as nulliplex (aaaa) with the balance duplex (AAaa). HTA is useful in the determination of the ratio of FDR and SDR gamete production in mixed-mode progeny if a marker locus is tightly linked to the centromere. Dihaploids of *S.*

tuberosum forming 2n eggs by mechanisms genetically equivalent to FDR (delayed meiotic division) and SDR (delayed meiotic division, omission of second division) have been identified (Werner and Peloquin 1991c). The use of separate genotypes in previous comparisons of the performance of FDR to SDR confounds earlier observations (Mendiburu and Peloquin 1977a, 1977b; Chujoy and Peloquin 1986; Darmo and Peloquin 1990; Concillo and Peloquin 1991; Darmo and Peloquin 1991; Ortiz and Peloquin 1991; Werner and Peloquin 1991a, 1991c). To avoid confounding, the use of these mixed-mode producing dihaploids allows for an unbiased comparison of FDR and SDR.

MATERIALS AND METHODS

1. Plant Material

Dihaploids (2n=2x=24) previously identified as mixed-mode 2n egg producing, derived from tetraploid *S. tuberosum* (Werner, 1989), were kindly provided by Drs. Werner and Peloquin (University of Wisconsin, Madison)(Table 1-3). Tetraploid clones of potato cultivars (Lemhi Russet, Ranger Russet, Frontier Russet, Ontario, Norgold Russet, Yukon Gold, Chippewa and Desiree) and breeding lines (NDD277-2, ND860-2, and LA12-59) were obtained from the Michigan State University potato breeding program.

2. Isozyme analysis

Crude protein extracts were obtained by crushing 150 mg of fresh juvenile potato leaf tissue from the greenhouse. Before crushing, 150 μl of 0.1 M tris-HCL buffer, pH 7.8, with 2% glutathione was added to the sample to reduce oxidation. Each sample was absorbed into 2 x 10 mm wicks of 3 MM Gel Blot Paper (Schleicher and Schuell, Keene, NH.), placed into plastic racks, then wrapped in Saran Wrap and stored at -20°C overnight.

Electrophoretic assays were performed using a

Table 1-3. Mixed-mode dihaploids obtained from the University of Wisconsin, at Madison.

Dihaploid	Maternal Source
4182-T	W231
H175	Redsen
H57	W231
H69	Merrimack
H76	Merrimack
H109	Merrimack
H115	Merrimack
H161	W760

tris-citrate, pH 8.3 gel buffer system (Vallejos, 1983). The gel matrix consisted of 10 % hydrolysed potato starch (STARCHart, Corp., Smithville, TX). Starch gels were left at room temperature (23°C) overnight and loaded the following morning. Wicks were loaded into refrigerated gels (4°C) along with a marker lane (bromophenol blue) and electrophoresed at 50 mA until the marker had run within 2 cm of the anodal end of gel. Gels were allowed to cool for 10 minutes and sliced. Slices were stained for either phosphoglucomutase (*PGM*) or glutamate oxaloacetate transaminase (*GOT*) according to Vallejos (1983).

3. Parental classification

Two segregating loci were used for HTA: *Pgm-2* and *Got-2* which are each 2.0 cM from the centromere, located on chromosome 4 and 7, respectively (Douches and Quiros 1987). Dihaploids were surveyed to determine their isozyme genotype for these loci. Only dihaploids heterozygous for *Pgm-2* or *Got-2* were selected for use in 2x-4x crosses. Tetraploid cultivars that are nulliplex at least at one of two loci (Douches and Ludlam 1991) were selected for 2x-4x crossing.

4. 2x-4x crosses

Sprouting tubers of *S. tuberosum* dihaploids and tetraploids were planted in the greenhouse for crossing. To promote flowering, petri dishes were put in twelve-liter plastic pots three-fourths full with Baccto mix in the

greenhouse in January of each year. Tubers were covered with Baccto mix until the roots had grown over the petri dish into the lower portion of the pot. The Baccto mix was then washed away from the tubers to expose stolons. Placement of the tubers on the petri dishes allowed for the easy removal of stolons and tubers. This practice promoted continuous flowering. Shoots originating from the tubers were thinned to 1-2 stems, trimmed weekly, and allowed to flower. Pollen from the tetraploid parents was collected in gelatin capsules and stored at 4°C for up to one month until use. From 1990 to 1993, flowers on the dihaploids were emasculated and pollinated with tetraploid pollen from March through May. High pressure sodium lights were used as supplemental lighting (16 hr daylength). The greenhouse nighttime temperature was maintained at 25°C.

Seeds were extracted from mature fruits and dried. Prior to planting seeds were treated with 1500 ppm GA_3 for 24 hours. Treated seeds were placed in 15 cm pots filled with Baccto potting mix and covered lightly. Once germinated seedlings had produced true leaves, they were transplanted into cell packs. Seedlings were assayed for *Pgm-2* and *Got-2* at the four to five leaf stage to classify individuals as SDR- or FDR-derived progeny. The most juvenile leaf was taken from each seedling for crude protein extraction. Seedlings in cell packs were allowed to mature and tubers from each seedling were harvested then held at 4°C until the planting season.

5. Progeny classification

Tetraploid progenies were separated into FDR- and SDR-derived populations based on the isozyme genotype of the *Pgm-2* and *Got-2* loci, (Douches and Quiros 1988a,1988b; Werner et al., 1991). The expectations of the 2x-4x segregation is as follows: the FDR-derived progeny are simplex (Aaaa), while the SDR-derived progeny are either duplex (AAaa) or nulliplex (aaaa).

6. Field studies

Field experiments were conducted from 1991 to 1995 at the Montcalm Research Farm (MRF), Montcalm, MI. Plots were planted with 30 cm spacing between hills with 102 cm between rows. Progeny and parents from the three crosses (Table 1-4) were planted in a randomized complete block design (RCBD). Individuals with sufficient micro-tubers from the progeny classes and parents were planted as four-hill plots with each progeny individual replicated three times and each parent replicated 10 times. Harvested tubers from the current year were used as seed within each population the following year.

7. Cultural

The soil in the experimental plots at MRF is a McBride Sandy Loam with a 2% slope. Initial soil tests showed a very good base nutrition value for the research plots at MSU (Table A-1). Additional fertilizer was applied as needed

Table 1-4. Sizes of progeny populations for 2x-4x crosses. All populations were planted at MRF in a RCBD. Individuals from the progeny classes and parents were planted as four-hill plots with each progeny individual replicated three times and the parents 10 times.

Year	Cross	Progeny Size		Planting Date	Harvest Date	Season Length
		FDR	SDR			
1991	4182-T x NDD277-2	61	120	5-16	9-13	120d
1992	4182-T x NDD277-2	58	110	5-19	9-21	118d
1993	4182-T x NDD277-2	53	100	5-14	9-14	124d
1994	4182-T x ND860-2	21	31	5-12	9-13	124d
1995	4182-T x ND860-2	21	31	5-21	10-3	135d
1995	H175 x Yukon Gold	43	57	5-21	10-3	135d

during the growing season. Approximately 327 kg/Ha 0-0-60(N:P:K) was applied at plowdown, 218 kg/Ha 20-10-20 was applied during planting, 218 kg/Ha 34-0-0 broadcast at emergence, 109 kg/Ha 46-0-0 sidedress at hilling and 130 kg/Ha 28-0-0 through irrigation. Herbicide treatments (metalochlor 2.2 kg/Ha with metrabuzin 0.6 kg/Ha) were applied preemergence. Hilling was done when plants were 25-30 cm in height. Irrigation was applied according to best management practices (Table A-2). Overall total for natural precipitation varied considerably over four years (Table A-3).

8. Harvest

A single row chain harvester was used to lay the tubers on the soil. Tubers were collected and bagged by hand on a per plot basis. For all field plots a red-skinned cultivar (Red Norland or Red Pontiac) was placed between plots to aid in separation at harvest. Vines were killed with Diquat (one pint/acre) two weeks prior to harvest.

9. Yield and Specific Gravity Evaluation

Total tuber yield was measured from tubers of each plot and was weighed using digital scales accurate ± 1 gram (Toledo Scale, Worthington, OH). Stand counts were taken for the four-hill plots one month after emergence. Yield data was adjusted with the stand count data to present yields on a per plant basis. A 0.5 to 4.0 kg sample from

each plot was taken for specific gravity measurement. Specific gravity was calculated as the dry weight of the sample divided by the difference of tuber weight in air minus weight in water.

10. Statistical Analysis

Mean separations (least significant difference) and t-tests of population means and variances for the progeny classes were performed using MSTAT-C (Michigan State University). Skewness and kurtosis values were examined for both yield and specific gravity to characterize the distribution of progeny classes.

RESULTS

1. Isozyme results of dihaploids and tetraploids and 2x-4x progeny

Five of eight dihaploids were heterozygous for *Pgm-2* and 6 of eight were heterozygous for *Got-2* (Table 1-5). Twelve cultivated tetraploid potatoes and breeding lines were homozygous for *Pgm-2*. Four of the twelve were homozygous for *Got-2* locus (Table 1-6).

Crosses were attempted using combinations of a subset of all available dihaploids and tetraploids (Table 1-7). Only seven crosses out of a possible 66 combinations provided viable seed. Progeny from all successful 2x-4x crosses were genotyped for *Pgm-2* and *Got-2*. For *Pgm-2*, all progeny from the dihaploid 4182-T fit a 2:1 ratio of SDR:FDR whereas those from dihaploid H175 had ratios that fit a 1:1 (Table 1-8). For *Got-2*, progeny from H175 x Yukon Gold did not show ratios that supported the *Pgm-2* isozyme data and therefore *Got-2* was not used to classify progeny. Crosses providing sufficient progeny numbers were used in field experiments.

Table 1-5. Isozyme survey of dihaploids used in 2x-4x crosses.

	Locus	
Dihaploid	*Pgm-2*	*Got-2*
H175	23[a]	35[b]
H115	23	35
H69	23	35
H76	22	35
H57	22	35
H161	23	35
4182-T	23	33
H109	23	35

[a] 23 denotes *Pgm* $2^2 2^3$
[b] 35 denotes *Got* $2^3 2^5$

Table 1-6. Tetraploid clones used in 2x-4x crosses along with isozyme genotype.

	Locus	
Tetraploid	*Pgm-2*	*Got-2*
NDD277-2	2222[a]	3355[b]
ND860-2	2222	3355
LA12-59	2222	3355
Lemhi R.	2222	3555
Norchip	2222	3335
Ranger R.	2222	3555
Frontier R.	2222	3555
Ontario	2222	3355
Norgold R.	2222	5555
Yukon Gold	2222	5555
Chippewa	2222	5555
Desiree	2222	5555

[a] 2222 denotes *Pgm-2* $2^2 2^2 2^2 2^2$
[b] 3355 denotes *Got-2* $2^3 2^3 2^5 2^5$

Table 1-7. 2x-4x crosses attempted between dihaploids heterozygous for *Pgm-2* or *Got-2* and tetraploids homozygous for *Pgm-2* or *Got-2*

Dihaploid (2x)		Tetraploid (4x)
4182-T	x	NDD277-2
4182-T	x	ND860-2
4182-T	x	LA12-59
4182-T	x	Lemhi Russet
4182-T	x	Norchip
4182-T	x	Ranger Russet
4182-T	x	Frontier Russet
4182-T	x	Ontario
4182-T	x	Norgold Russet
4182-T	x	Yukon Gold
4182-T	x	Desiree
H175	x	NDD277-2
H175	x	ND860-2
H175	x	LA12-59
H175	x	Lemhi Russet
H175	x	Norchip
H175	x	Ranger Russet
H175	x	Frontier Russet
H175	x	Ontario
H175	x	Norgold Russet
H175	x	Yukon Gold
H175	x	Desiree
H115	x	NDD277-2
H115	x	ND860-2
H115	x	LA12-59
H115	x	Lemhi Russet
H115	x	Norchip
H115	x	Ranger Russet
H115	x	Frontier Russet
H115	x	Ontario
H115	x	Norgold Russet
H115	x	Yukon Gold
H115	x	Desiree

Table 1-7. (Continued)

Dihaploid (2x)		Tetraploid (4x)
H161	x	NDD277-2
H161	x	ND860-2
H161	x	LA12-59
H161	x	Lemhi Russet
H161	x	Norchip
H161	x	Ranger Russet
H161	x	Frontier Russet
H161	x	Ontario
H161	x	Norgold Russet
H161	x	Yukon Gold
H161	x	Desiree
H109	x	NDD277-2
H109	x	ND860-2
H109	x	LA12-59
H109	x	Lemhi Russet
H109	x	Norchip
H109	x	Ranger Russet
H109	x	Frontier Russet
H109	x	Ontario
H109	x	Norgold Russet
H109	x	Yukon Gold
H109	x	Desiree
H69	x	NDD277-2
H69	x	ND860-2
H69	x	LA12-59
H69	x	Lemhi Russet
H69	x	Norchip
H69	x	Ranger Russet
H69	x	Frontier Russet
H69	x	Ontario
H69	x	Norgold Russet
H69	x	Yukon Gold
H69	x	Desiree

Table 1-8. An examination of X^2 values of the ratios of FDR-derived progenies to the SDR-derived progenies in 2x-4x crosses.

PARENTS		OBSERVED RATIOS		EXPECTED RATIOS 1:1		EXPECTED RATIOS 2:1		X^2 VALUE		P VALUE	
Haploid	Tetraploid	SDR[a]	FDR[b]	SDR[c]	FDR[c]	SDR[c]	FDR[c]	1:1	2:1	1:1	2:1
4182-T	Lemhi R.	25	12	18.5	18.5	24.67	12.33	4.57	0.01	0.05	0.9
4182-T	NDD277-2	120	61	90.5	90.5	120.67	60.33	19.23	0.01	0.001	0.9
4182-T	Ranger R.	43	19	31	31	41.33	20.67	9.29	0.20	0.005	0.75
4182-T	ND860-2	31	21	26	26	34.67	17.33	1.92	1.16	0.1	0.6
4182-T	Yukon Gold	42	20	31	31	41.33	20.67	7.81	0.03	0.005	0.9
H175	NDD277-2	13	16	14.5	14.5	19.33	9.67	0.31	6.22	0.65	0.01
H175	Yukon Gold	57	43	50	50	66.67	33.33	1.96	4.21	0.17	0.03

[a] Number of individuals classified as SDR-derived.
[b] Number of individuals classified as FDR-derived.
[c] Number of individuals based on projected ratios.

2. Field experiments

For three years (1991, 1992, 1993) no difference was seen for total tuber yield or specific gravity of FDR- and SDR-derived tetraploid progeny from the cross 4182-T x NDD277-2 (Table 1-9). No difference in yield or specific gravity between the FDR- and SDR-derived populations was observed in the cross 4182-T x ND860-2 in 1994 and 1995. Similar results were found in the H175 x Yukon Gold in 1995. The average yields for the three FDR- and SDR-derived populations approached the mid-parent values during most of the years (Table 1-10). Specific gravity values did not surpass mid-parent values most of the years (Table 1-11).

The distributions of FDR- and SDR-derived populations are demonstrated by the kurtosis values (Tables 1-12, 1-13). A positive value denotes a peaked curve whereas a negative value represents a flatter curve. Kurtosis values for yield (Table 1-12) did not follow any trends. This is typified by the cross 4182-T x NDD277-2 for 1991, 1992 and 1993. In 1991, the SDR-derived progeny had a tighter distribution around the mean. During 1992 there was no difference between the distributions, whereas 1993 showed the FDR-derived progeny with a significantly tighter distribution around the mean. A sample yield distribution is shown for 4182-T x NDD277-2 during 1991 (Figure 1-2). Data for the specific gravity distribution was similar to that of the yield values and did not follow trends (Table 1-13). Figure 1-3 shows the specific gravity distribution for 4182-T x

Table 1-9. T-test values comparing the difference between the means for FDR- and SDR-derived tetraploid progenies.

Year	Cross	Yield[a]	Specific Gravity[a]
1991	4182-T x NDD277-2	0.9210 ns	0.7145 ns
1992	4182-T x NDD277-2	0.1984 ns	0.4796 ns
1993	4182-T x NDD277-2	0.8965 ns	0.3707 ns
1994	4182-T x ND860-2	0.8147 ns	0.0642 ns
1995	4182-T x ND860-2	0.4390 ns	0.4998 ns
1995	H175 x Y. Gold	0.8042 ns	0.6648 ns

[a] Populations within a year are not significantly different from each other at $P \leq 0.05$.

Table 1-10. A summary of average of tuber yields (gms/plant) for three 2x-4x crosses grown at MRF over five years in comparison to mid parent yield values.

Year	Cross	Group	Yield
1991	4182-T x NDD277-2	FDR	1371
		SDR	1527
		Mid Parent	1360
1992	4182-T x NDD277-2	FDR	482
		SDR	542
		Mid Parent	639
1993	4182-T x NDD277-2	FDR	551
		SDR	554
		Mid Parent	414
1994	4182-T x ND860-2	FDR	1085
		SDR	1062
		Mid Parent	1224
1995	4182-T x ND860-2	FDR	1282
		SDR	1179
		Mid Parent	1182
1995	H175 x Y. Gold	FDR	1086
		SDR	1129
		Mid Parent	931

Table 1-11. A summary of average specific gravity for three 2x-4x crosses grown at MRF over five years in comparison to mid parent specific gravity values.

Year	Cross	Group	Specific Gravity
1991	4182-T x NDD277-2	FDR	1.074
		SDR	1.074
		Mid Parent	1.078
1992	4182-T x NDD277-2	FDR	1.076
		SDR	1.075
		Mid Parent	1.082
1993	4182-T x NDD277-2	FDR	1.055
		SDR	1.054
		Mid Parent	1.058
1994	4182-T x ND860-2	FDR	1.065
		SDR	1.062
		Mid Parent	1.076
1995	4182-T x ND860-2	FDR	1.065
		SDR	1.064
		Mid Parent	1.064
1995	H175 x Y. Gold	FDR	1.069
		SDR	1.068
		Mid Parent	1.071

Table 1-12. Skewness and kurtosis values for yield data for 2x-4x crosses at MRF.

Year	Cross	Mode	Skewness[a]	Kurtosis[a]	Range Min	Range Max
1991	4182-T x NDD277-2	FDR	1.5319^{ns}	-0.938^{ns}	678	3029
	4182-T x NDD277-2	SDR	1.8624***	4.447***	720	3932
1992	4182-T x NDD277-2	FDR	0.9108**	0.315^{ns}	539	2292
	4182-T x NDD277-2	SDR	0.7882**	0.659^{ns}	763	3356
1993	4182-T x NDD277-2	FDR	1.2710***	1.854***	230	2740
	4182-T x NDD277-2	SDR	0.3400^{ns}	-0.729^{ns}	420	2680
1994	4182-T x ND860-2	FDR	0.3436^{ns}	-1.048^{ns}	300	3340
	4182-t x ND860-2	SDR	1.4057**	2.801***	390	2280
1995	4182-T x ND860-2	FDR	0.5726^{ns}	-0.448^{ns}	300	1686
	4182-T x ND860-2	SDR	1.1830***	0.928^{ns}	275	1713
1995	H175 x Y. Gold	FDR	0.8095*	0.044^{ns}	281	2511
	H175 x Y. Gold	SDR	0.5571^{ns}	0.725^{ns}	255	2749

[a]ns denotes nonsignificant
* denotes significant at 0.05%
** denotes significant at 0.01%
*** denotes significant at 0.001%

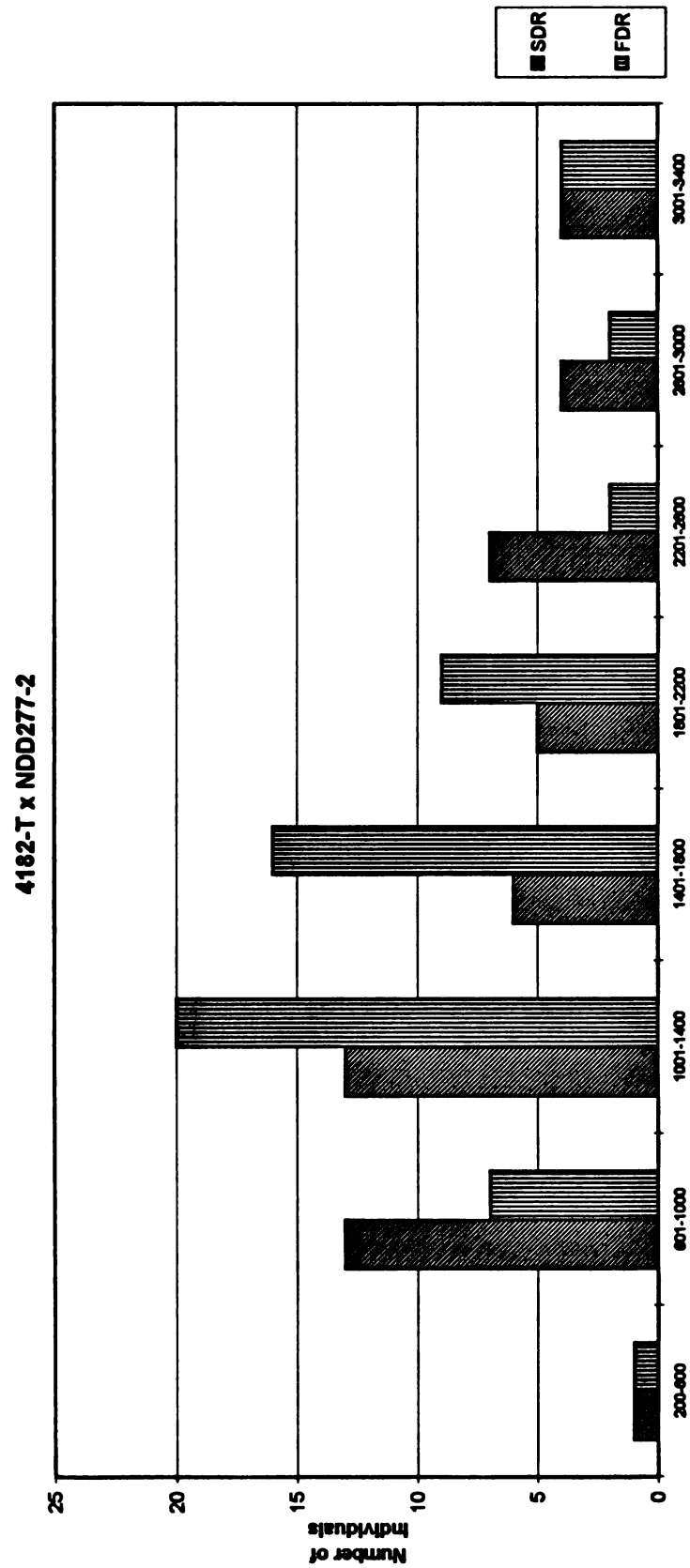


Figure 1-2. Yield data for cross 4182-T x NDD277-2 at MRF for 1991. Yield data is presented on a per plant basis.

Table 1-13. Skewness and kurtosis values for specific gravity data for 2x-4x crosses at MRF.

Year	Cross	Mode	Skewness[a]	Kurtosis[a]	Range	
					Min	Max
1991	4182-T x NDD277-2	FDR	-1.9020***	7.175***	1.049	1.099
	4182-T x NDD277-2	SDR	0.1305ns	0.775ns	1.050	1.102
1992	4182-T x NDD277-2	FDR	1.0474***	1.837**	1.062	1.100
	4182-T x NDD277-2	SDR	-0.1827ns	0.659ns	1.048	1.098
1993	4182-T x NDD277-2	FDR	0.4406ns	0.642ns	1.038	1.080
	4182-T x NDD277-2	SDR	0.8597***	2.023***	1.031	1.083
1994	4182-T x ND860-2	FDR	0.5533ns	0.847ns	1.051	1.084
	4182-T x ND860-2	SDR	0.0619ns	-0.143ns	1.050	1.077
1995	4182-T x ND860-2	FDR	0.4406ns	0.642ns	1.038	1.080
	4182-T x ND860-2	SDR	1.0753**	0.532ns	1.051	1.086
1995	H175 x Y. Gold	FDR	-0.0124ns	0.248ns	1.050	1.088
	H175 x Y. Gold	SDR	0.4578ns	0.726ns	1.051	1.090

[a]ns denotes nonsignificant
* denotes significant at 0.05%
** denotes significant at 0.01%
*** denotes significant at 0.001%

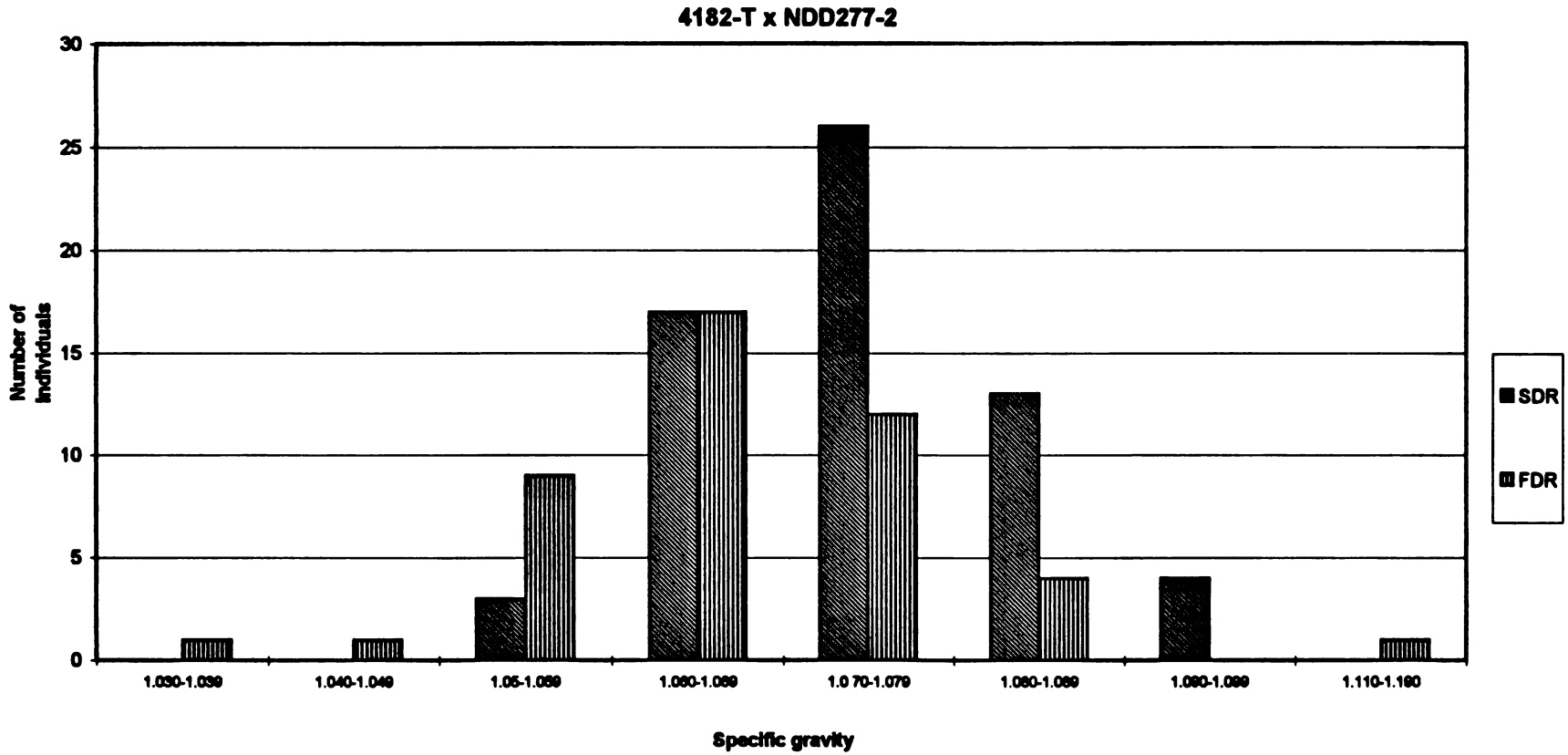


Figure 1-3. Specific gravity data for cross 4182-T x NDD277-2 at MRF 1991. Specific gravity data is shown on a per plot basis.

NDD277-2 during 1991.

Several crosses (4182-T x Lemhi Russet, 4182-T x Ranger Russet, 4182-T x Yukon Gold and H175 x NDD277-2) did not survive the initial field season in large enough numbers to establish a replicated study, therefore were not used further.

DISCUSSION

The lack of superiority of FDR-2n eggs evident in this study contradicts previously published results. Studies examining the performance of FDR-derived 2n eggs in comparison to SDR-derived 2n eggs found that the FDR-derived progeny were superior in 4x-2x crosses utilizing both dihaploids and dihaploid-species hybrids (Mendiburu et al., 1974; Mendiburu and Peloquin 1977b; Chujoy and Peloquin 1986; Tai 1987; Werner 1989; Ortiz et al., 1991; Werner and Peloquin 1991c). In all previous cases the FDR-derived tetraploid progeny came from different crosses than the SDR-derived tetraploid progeny. Thus, the 2x genotype and the mode of 2n egg formation was confounded, producing a bias. If a well adapted FDR pollen producing clone is used in comparison to a less-adapted SDR egg producing clone there may be yield differences masking the superiority of the mode of 2n gamete formation. Through the use of mixed-mode dihaploids we were able to produce both FDR and SDR 2n gametes from the same genotype, allowing us to eliminate confounding by the parental genotype with the mode of 2n gamete formation.

A possible contributing factor for the lack of difference between the FDR- and SDR-derived tetraploids may

be the level of heterozygosity in the dihaploid parent used. Dihaploids produced from *S. tuberosum* tetraploids by haploidization, have a measure of inbreeding. It has been estimated that the haploidization process is equivalent to three generations of inbreeding (Yeh et al., 1964; Howard 1970; Ujtewaal 1987). This inbreeding reduces the level of heterozygosity in the dihaploid and leads to the loss of intra- and inter-locus interactions. As a consequence, drastic yield declines, loss of vigor and fertility can occur (Busbice and Wilsie 1966; Dewey 1966; Levings et al., 1967; Busbice 1968; Dessureaux and Gallais 1969; Rowe 1967a; Mendiburu et. al 1974; Mendoza and Haynes 1974a; Rice and Dudley 1974; Bingham 1980). In all dihaploids, a lack of vigor, low yields and infertility were noted in comparison to tetraploids (data not shown). To determine whether the level of inbreeding in the dihaploid was the factor in not providing the previously observed superiority of haploid and haploid-species hybrid FDR-derived progenies, the allelic diversity of the mixed-mode parents used in 2x-4x crosses should be increased. Crossing the dihaploids to *Solanum* species would increase the allelic diversity of the resulting dihaploid-species hybrids (D-SH) (Hermundstad and Peloquin 1985; Darmo and Peloquin 1990; Hawkes 1990; Concillo and Peloquin 1991; Darmo and Peloquin 1991). These D-SH could be used to construct genetically diverse 2x-4x populations. A series of mixed-mode producing *Solanum* species previously identified by Werner (1989) would be

suitable for this purpose (Table 1-14). An alternative to constructing D-SH is to increase the allelic diversity of the tetraploid clones prior to producing dihaploids. Molecular evidence has provided information on levels of allelic diversity in potato. A survey of 10 isozyme loci from 40 *Solanum* species showed wide diversity between species (5.7 alleles/locus)(Douches et al., 1989). In contrast, isozyme analysis of North American potato cultivars for the same loci show that allelic variation is 2.15 alleles/locus and that the majority of loci in the cultivars are diallelic (Douches and Ludlam 1991). The possibility of low levels of allelic variation in polyploids is supported by research in blueberries (Hokanson 1996). Potato restriction fragment length polymorphism (RFLP) analysis showed that *S. tuberosum* was the least heterozygous when compared to *S. tuberosum* spp. andigena and *S. phureja* (Bonierbale et al., 1993). The fact that few tri- or tetra-allelic loci have been observed in cultivated potato, yet heterosis is seen regularly, may suggest epistatic gene interaction might be contributing to performance. If we assume that tetraploid potato is diallelic at most loci, this leaves only one first order interaction between alleles. In this light, heterosis would more likely come from the effect of complementary gene action, supporting Bingham's reevaluation (1994).

Pgm-2, a proximal locus located 2.0 cM from the centromere, was used to separate the progeny classes. A

Table 1-14. The 2n egg producing wild species available for haploid-species hybrids.

Solanum Species	Accession Number[a]	Modes
S. brevicaule	233961	FDR/SDR
S. sparsipilum	230502	FDR/SDR
S. sparsipilum	310933	FDR/SDR
S. tarijense	414148	FDR/SDR

[a] P.I. Numbers from Potato Introduction Station, Sturgeon Bay, Wisconsin.

certain degree of misclassification is expected due to recombination. With a population size of 181 (4182-T x NDD277-2) assuming a 2% misclassification rate, 1 FDR-derived and 2 SDR-derived progenies would be misclassified (Werner et al., 1991). If a second proximal locus is available for HTA the amount of misclassification can be reduced. Dihaploid parents 4182-T and H175 are heterozygous for *Pgm-2* with H175 also heterozygous for the *Got-2* locus. Tetraploid progeny from the cross H175 x Yukon Gold were assayed for both *Pgm-2* and *Got-2*. *Got-2* results indicate a more distal gene-centromere estimate then with the previous gene-centromere mapping estimate (Douches and Quiros 1988). The previous gene-centromere map distance for *Got-2* was determined from a haploid-species hybrid. Gene-centromere distances may have been underestimated due to reduced recombination in the hybrid (Douches and Quiros 1988). Therefore, *Got-2* segregation data was not used to classify the 2x-4x progeny in this study.

The ratio of FDR-derived progenies to the SDR-derived progenies observed for the 2x-4x crosses in the experiment was dependent on the dihaploid parent used (Table 1-7). 4182-T always produced a 2:1 (SDR:FDR) ratio, however, H175, had a 1:1 (SDR:FDR) ratio. These observed ratios may be important if one mode of 2n gamete formation from the dihaploid is desired over another. 4182-T produces approximately one-third FDR-derived progeny out of the total compared to one-half with H175 (Table 1-15).

Table 1-15. Percentage of FDR- and SDR-derived progeny in 2x-4x crosses.

Parents		Percent[a]	
Haploid	Tetraploid	FDR	SDR
4182-T	NDD277-2	34	66
4182-T	ND860-2	40	60
4182-T	Lemhi Russet	32	68
4182-T	Ranger Russet	31	69
4182-T	Yukon Gold	32	68
H175	NDD277-2	55	45
H175	Yukon Gold	47	53

[a] Based on *Pgm-2* isozyme separation.

The variability of ratios between genotypes suggests that the formation of FDR and SDR gametes ratios are dependent on genotype; however, more crosses are needed to support this observation.

In our study we saw that the yields of both FDR-derived and SDR-derived progenies approached the midparent value for both populations. Yield reductions were seen each year but the 2x-4x progenies tended toward the midparent values. There were no trends in the distribution of values for total yield and specific gravity for either population. A tighter distribution might be expected with a higher percentage of parental genome transferred intact resulting in a more uniform gamete production in the FDR-derived tetraploid population. The lack of consistent difference in distribution between FDR- and SDR-derived progeny also supports the idea of inbreeding in the dihaploid.

Performance of the 2x-4x progeny in this study support an additive genetic model. Werner and Peloquin (1991c) and other researchers have determined that non-additive effects contribute to yield (Mendoza and Haynes 1974; Mendiburu and Peloquin 1977a; Bingham et al., 1994). Therefore, our results suggest that total yield in potato may also be due to additive genetic effects.

The steady decline in yield over the three years for the 4182-T x NDD277-2 population, may implicate other non-genetic factors in the production of total tuber yield. First of all, year to year variation in yield may be related

to weather conditions during the field season. During the year with the highest yields (1991) within the 4182-T x NDD277-2 population, total moisture was the second highest of the three years, growing degree days accumulated were highest and average temperature was highest of three years. It is known that higher temperatures increase the rate of utilization of carbohydrates produced by photosynthesis. Consequently the amount of carbohydrates produced is dependent on the rate of photosynthesis and length of time that it occurs (Sieczka and Thornton 1993). Secondly, since the experiment was conducted for three years using the previous seasons tubers there was an opportunity for virus accumulation. In 1992, the level of aphid infestation at MRF was abnormally high. Large populations of aphids may have led to increased virus vectoring. A random sample of 15 plants from each group (SDR-derived progenies, FDR-derived progenies, 4182-T and NDD277-2) showed high virus titers (data not shown) at harvest in 1993 according to DAS-ELISA (Agdia, Inc., Elkhart, Indiana) results. Since it is known that virus will lower yield, the virus titer buildup in these populations may have reduced overall productivity. With the virus problem widespread, we can assume that it affected the total field experiment randomly. Despite virus infection levels in the seed potatoes, there was never any significant difference between SDR- and FDR-derived progenies.

Fruit set in the 2x-4x crosses was limited (data not

shown) to the first half of April during the crossing season. It has been observed in some studies that the production of 2n eggs is dependent on temperature and photoperiod (Hermsen 1984; Mooney and Peloquin 1992; E. Jongedijk personal communication). Although the greenhouse temperature was maintained at a maximum of 25°C during the winter months, the daily maximum temperature climbed higher as summer approached in spite of greenhouse shading. These temperature spikes may have been a factor in fruit abortion in the majority of crosses.

Through the use of dihaploids producing both FDR and SDR 2n eggs we provided some insights into the question of heterosis and the theory of maximum heterozygosity in autopolyploids.

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CHAPTER II

USE OF THE LASER SCANNING CONFOCAL MICROSCOPE TO EXAMINE MEGASPOROGENESIS IN POTATO

INTRODUCTION

Cytological analyses have been valuable in potato *(S. tuberosum)* and related diploid species for studying the mechanisms of 2n gamete formation in both microsporogensis and megasporogenesis. Initial efforts focused on microsporogensis because of the ease of examination. Megasporocyte meiosis in synaptic mutants of *Solanum* sp. were largely unstudied, with conformation of the existence of mutants relying the upon success of pollinations (Stelly et al., 1984). The lack of early cytological information is largely due to laborious preparation procedures involved with viewing ovules. Early studies of *Solanum* sp. ovule and megagametophyte development required fixing of the ovule followed by imbedding in paraffin (Herr 1971).

The embedding-sectioning technique has several limitations. First, the process of producing slides is tedious and time consuming for large studies. Second, because of physical sectioning, 3-D structures are distributed over several slides. Final interpretation of the object requires meticulous reconstruction of multiple sections. Stelly et al., (1984) developed a high contrast stain-clearing technique for *Solanum* sp. which negated the need for embedding.

Recent advances in technology have provided researchers in the field of microscopy with some very powerful resources (Czymmek et al., 1994). Confocal microscopy is not a new discovery, but combined with advanced laser technology, laser scanning confocal microscopy (LSCM) becomes a valuable tool for examination of female gametophytes (Frederikson 1990, 1991, 1992). The confocal ability of the LSCM allows extremely detailed images to be gathered from within a cell without physical sectioning. Confocal imaging provides non-destructive optical sections similar to that of physical sections, but without destroying the object. Since there is no damage to the tissue, images obtained from the laser scanning confocal microscope are not altered by physical sectioning and will retain true-to-life aspects. Research in the field of developmental biology has expanded due to the availability of the LSCM (Paddock 1994).

The objective of this research project is to examine megasporogenesis in potato (*Solanum* sp.) through the use of LSCM. Laser scanning confocal microscopy provides an excellent opportunity to study megasporocyte meiosis without traditionally tedious techniques.

LITERATURE REVIEW

CLEARING TECHNIQUES

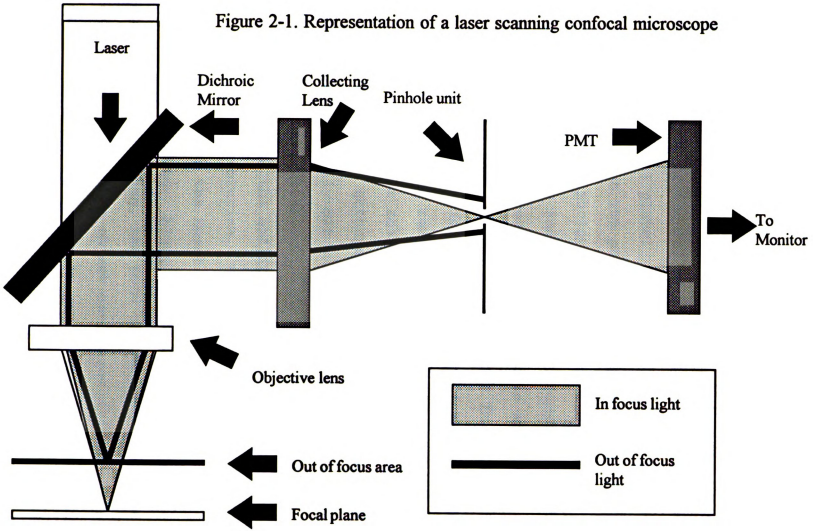
In attempts to avoid imbedding and sectioning processes, many ovule clearing techniques were developed. A 10% sucrose solution was used by Poddubnaya-Arnoldi (1960) to clear ovules in several species of orchid. An ovule clearing technique developed by Herr (1971) included lactic acid, chloral hydrate, phenol, clove oil and xylene. Ovules were placed in the 2:2:2:2:1 clearing solution for 24 hours, then placed between a coverslip and slide with pressure applied gradually. Herr's technique allows the ovule to break apart without disrupting the integrity of the individual cells. Herr's clearing method usually required 4 days to perform from fresh flower to prepared ovary tissue. Stelly (1984) followed with a method that utilized Mayer's-hemalum followed by clearing. Mayer's-hemalum is a positive stain specific for chromatin and nucleoli. Stelly's protocol was a modification from Herr's in that the complex solution was replaced by methyl salicylate (oil of wintergreen) for clearing. A new step in Stelly's procedure was to utilize whole ovules. The use of whole ovules reduces the number of lost and damaged ovules. Stelly's process also requires five days to fix and clear ovule

tissue for use. Jongedijk (1987a) improved on the clearing method by inserting ovules into pure methyl salicylate for 30 minutes after 24 hours in a fixing solution. Jongedijk's protocol reduced the time required to approximately 1 1/2 days. Observations were performed on non-squashed whole ovules. Jongedijk (1987b) further modified the process by using pectinase to degrade cell walls to separate the cells, followed by acetocarmine stain. Jongedijk's pectinase protocol was shown to be very useful in viewing species with small megasporocytes and small chromosomes such as potato.

CONFOCAL PRINCIPLES AND LSCM

There are many methods of viewing objects through a light microscope. One of these is through the use of a confocal microscope. Confocal microscopy images the same plane as the illuminated plane. Confocal is defined as having the same foci, meaning all out of focus light from above and below the focal plane is excluded from the final image. Advancement of laser technologies has allowed for the refinement of stage-scanning confocal microscopes (Minsky 1988). A laser creates a uniform, monochromatic, coherent beam of light. The attributes of laser light combined with the confocal ability of microscopes gives the laser scanning confocal microscope (LSCM) even more resolution. The LSCM can produce several types of images: laser transmitted, reflected and fluorescent images. An example of the light path in a LSCM is shown in Figure 2-1.

Figure 2-1. Representation of a laser scanning confocal microscope



The laser is focused in a plane within the sample by the objective lens. Fluorescent light is emitted from the illuminated spot, some of which will return through the objective. As the light is returned through the objective lens the dichroic mirror redirects it to a collecting lens. Returning light is focused by the collecting lens toward a pinhole in a metal plate. In-focus light from the image that passes through the pinhole is allowed to expand and is then detected by a photomultiplier tube. All of the in-focus light from the image is passed through the pinhole, whereas most of the out-of-focus light is excluded. Out-of-focus light has it focal point either in front or behind the pinhole, only in-focus light can pass through. A confocal pinhole can remove up to 95% of the out-of-focus light. As the laser scans across the image, light from a number of areas is gathered and processed into an image. The image is usually 512 x 512 or 1024 x 1024 pixels in size (Czymmek et al., 1994).

The LSCM has the ability to produce optical sections with enhanced resolution. Optical sections are formed from light coming from the focal plane and are similar to mechanical sections. Along with the ability to do horizontal sectioning, the LSCM can collect images on the z axis. For example, an object can be partitioned into a series of optical sections at preset intervals and thicknesses.

The resolution of the microscope is dependent on several factors which include, but are not limited to, the objective's numerical objective (NA), length of light path, the emission wavelength of the selected laser, the laser spot size and sensitivity of the detector. The spot size for a 488-nm laser with a high NA (1.4) is about 250 nm in diameter (Bertero 1990; Czymmek et al., 1994). The video monitor may also play a role in the resolution of the final image. The number of pixels available on the video monitor for viewing may be the limiting factor in resolution.

APPLICATION OF THE LSCM

The flexibility of the LSCM in different research areas has been demonstrated (Carlsson 1990; Boyde 1994; Czymmek et al., 1994; Paddock 1994). The optical sectioning capability combined with the z-series ability is the basis of more advanced capabilities found in the LSCM (Czymmek et al., 1994). A z-series can be examined as a gallery with all of the optical sections displayed side by side. The LSCM has the ability to recombine the z-series into stereo pairs. Multiple z-series can also be transferred to a graphics workstation for 3-D reconstruction and production of rotational models.

In 1990, Frederickson described the use of LSCM to observe whole orchid ovules. Ovules were fixed in FPA_{50} (formalin:propionic acid: 50% ethanol, 5:5:90) for 24 hours. To make the ovules transparent a few drops of Herr's

clearing fluid was applied (Herr, 1971). Prepared ovules were viewed under a 488 nm laser which caused the ovules to autofluoresce. Fredrickson (1990, 1991, 1992) found that optical sections produced by the LSCM are thin and without artifacts caused by embedding, cutting or staining.

Three-dimensional reconstructions have been performed using LSCM optical sections (Sheppard et al., 1990; Bertero et al., 1990). van Spronsen et al., (1989) utilized the LSCM to make optical sections of chloroplast and then made 3-D reconstructions to explore the structure. Easily assembled three-dimensional rotational models allow for the exploration of the chloroplast and other structures with little effort.

MATERIALS AND METHODS

1. PLANT MATERIAL

Four diploid potato clones were used for the cytological analysis. H175 and 4182-T are dihaploids of *S. tuberosum* while 84S10 is a selection from *S. phureja*. W5295.7 is a hybrid between *S. tuberosum* and *S. phureja*. H175, 4182-T and W5295.7 were supplied by Drs. S. Peloquin and J. Werner, University of Wisconsin, Madison. H175 and 4182-T were previously identified as producers of 2n eggs (Werner and Peloquin 1991c) whereas 84S10 produced 2n pollen by the parallel spindle mechanism. W5295.7 produces both 2n eggs and 2n pollen. Sprouting tubers were placed on petri dishes in three gallon plastic pots with Baccto mix in the greenhouse in January, using high pressure sodium lights (16 hr daylength) as supplemental lighting. To enhance the flowering period stolons were removed using a modification of the "brick technique". Plants were thinned to 1-2 stems, trimmed weekly and allowed to flower. The nighttime greenhouse temperature was maintained at 25°C.

2. BUD PREPARATION

Unopened flower buds of various maturity stages were collected from the greenhouse during the months of February

through April. The size range of the flower buds was 5 mm to 1 mm. The flower buds were fixed for 24 hours in Farmers solution (3:1, 95% ethanol:glacial acetic acid).

3. STAINING PROCEDURE

Ovules were stained with ethidium bromide (EtBr) and rinsed with distilled H_2O. Several different stain concentrations (5, 1, 0.5, 0.05 mg/ml at 1, 5, 15, 45 minutes) and rinse time combinations (1, 2, 3 times the stain period) were tried.

4. SLIDE PREPARATION

The EtBr-treated ovaries were divided in two with one half stored in sterile dH_2O and the remainder used for slide study. Ovules were teased out of remaining ovary tissue and placed into a drop of glycerol on a slide. A coverslip was applied over the ovules with gentle pressure for viewing.

5. SLIDE VIEWING

Ovules were examined with a LSCM (Zeiss 210, Carl Zeiss, Inc., Thornwood, NY) under differing magnifications (5x, 10x, 40x, 40x oil and 100x oil). Ovules were viewed in fluorescence mode using a 488 nm Argon laser in combination with a long pass barrier filter (LP520) to remove light below 520 nm from the image. Contrast and brightness were adjusted until a satisfactory image was obtained. Magnification of the scanned image from 20x to 80x was

through use of LSCM software. One ovule at a time was scanned to identify stages of megasporogenesis. Ovule samples that displayed clear images were Z-sectioned into a 40 image series. Images were printed using a video printer (Sony, Inc., Japan), saved to disk, photographed (Matrix, Multicolor, Orangeberg, NY) or transferred to the Silicon Graphics Workstation (Personal Iris, Silicon Graphics, Inc., Mountain View, CA). The Z series images were processed using VoxelMath™ (Vital Images, Inc., Iowa). The edited images were then transferred to VoxelView™ (Vital Images, Inc., Iowa) to produce a 3-D rotational image.

RESULTS

1. STAINING AND VIEWING

Serial dilutions were performed with the EtBr and dH_2O washes (data not shown). The best staining occurred when the ovules were placed into 0.5 mg/ml EtBr for 15 minutes followed by three distilled water washes of 15 min each. The 40x oil and 100x oil objectives were found to be the most effective to examine megasporogenesis. The 40x oil was used for rapid scanning of the slide to identify areas containing ovules. Once the ovules were identified the objective was changed to 100x oil and ovules were examined further.

2. STAGES OBSERVED

Several optical sections of whole EtBr-stained *Solanum* ovules under going normal meiosis are shown in Figures 2-2 to 2-11. In Figure 2-2, an ovule from 4182-T the MMC in interphase. The chromatin is becoming distinct around the nucleolus. Pachynema stage of prophase shows chromosomes more distinct and recognizable in 84S10 (Figure 2-3). Diplonema is characterized by the separation of bivalents at points along their length (Figure 2-4). During diakinesis

Figure 2-2. Ovule from 4182-T in interphase. Nucleolus is visible and the chromatin is becoming distinct. Key: o=ovule, m=megaspore mother cell and n=nucleolus. 3000x.

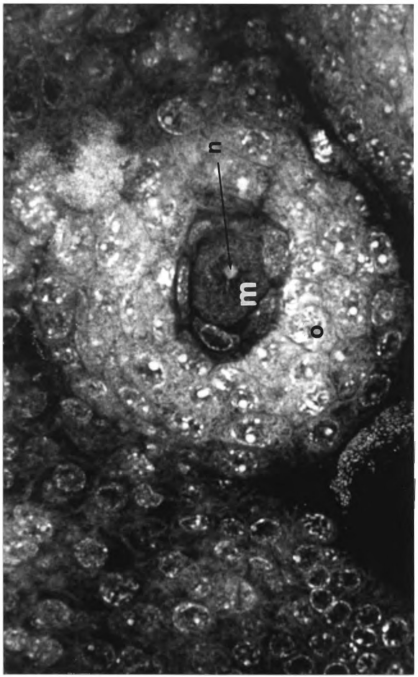

n
E
o

Figure 2-3. Pachynema stage of prophase shows chromosomes more distinct and recognizable in 84S10. 9000x.

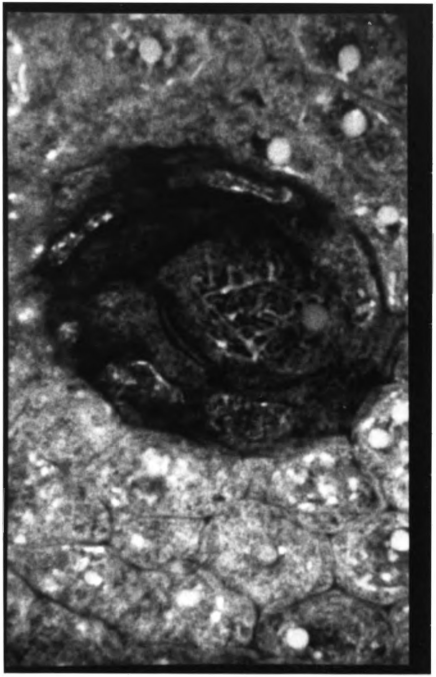
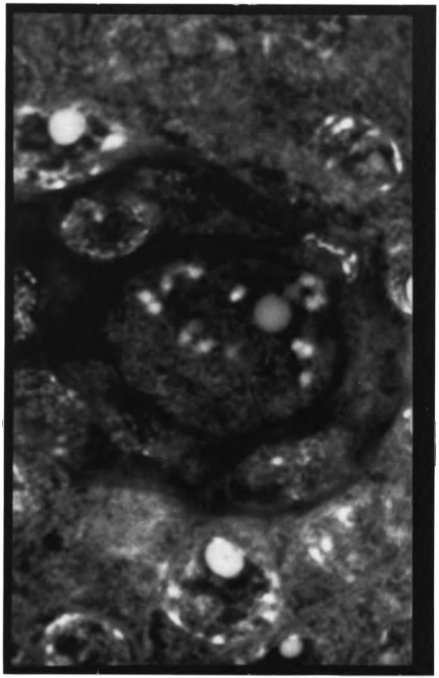


Figure 2-4. Diplonema is characterized by the separation of bivalents at points along their length. 10,000x



chromosomes are aligned in a circle on a plane with the nucleolus of W5295.7 (Figure 2-5). Figure 2-6 and 2-7 are of the same megaspore. In Figure 2-6, anaphase I, chromosomes have begun separation to opposite poles of the cell (W5295.7). A second optical section shows anaphase I at a different depth in W5295.7 (Figure 2-7). A composite made using the numerous optical sections provides a view of telophase I stage in 4182-T (Figure 2-8). A stereo reconstruction was made using the numerous optical sections provides a 3-D relief view of telophase I stage in 4182-T (Figure 2-9). Figure 2-10 shows a dyad produced from 4182-T. One functional megaspore mother cell and three non-functional daughter cells from 4182-T is observed (Figure 2-11).

Figure 2-5. During diakinesis chromosomes are aligned in a circle on a plane with the nucleolus of W5295.7. 8000x

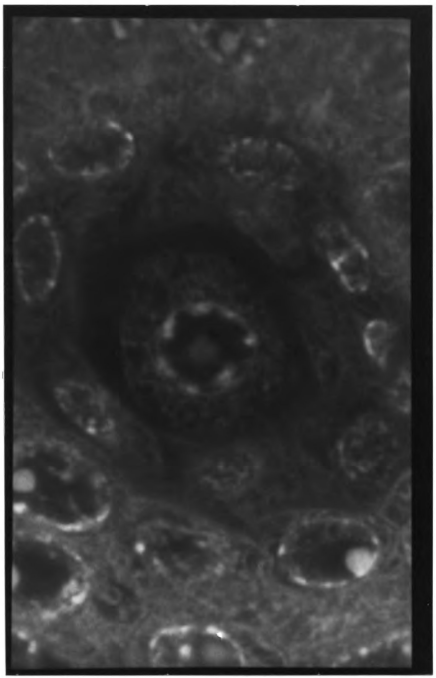


Figure 2-6. Anaphase I, chromosomes have begun separation to opposite poles of W5295.7. 2000x.

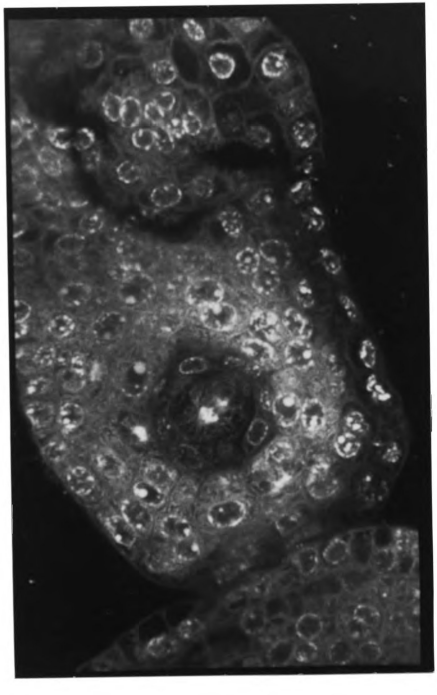


Figure 2-7. Different optical section of anaphase I shows progression of separating chromosomes in W5295.7. 6000x.

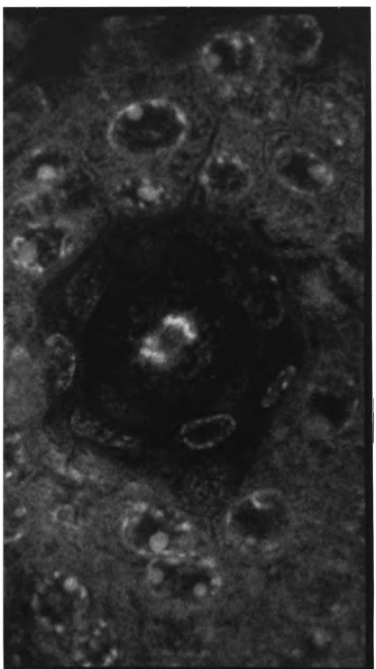


Figure 2-8. A composite was made using the numerous optical sections provides a view of telophase I stage in 4182-T. 6000x.

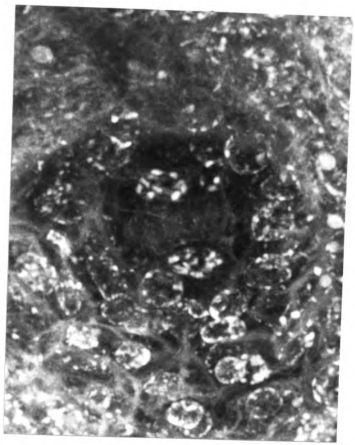


Figure 2-9. A stereo reconstruction was made using the numerous optical sections provides a view of telophase I stage in 4182-T. 2000x.

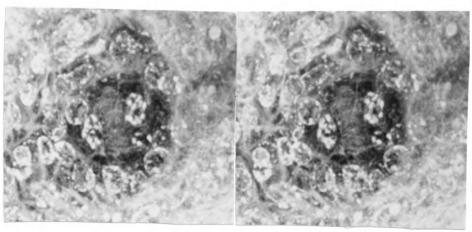


Figure 2-10. Dyad produced from 4182-T. 8000x.

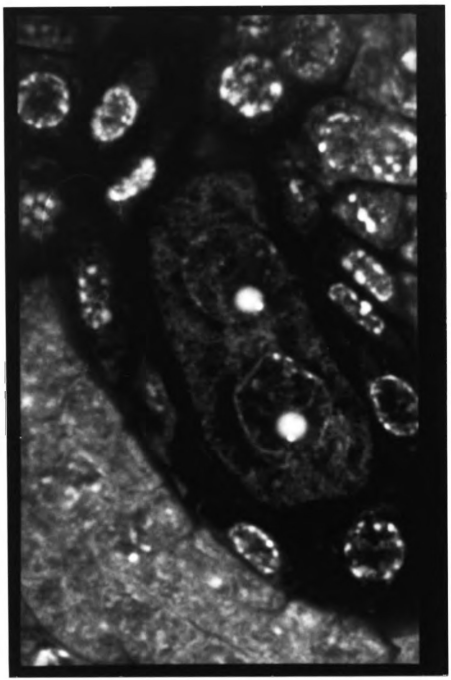
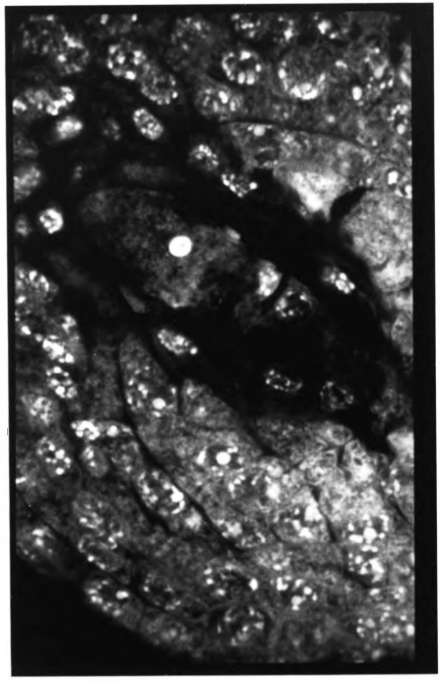


Figure 2-11. Functional megaspore and three non-functional daughter cells from 4182-T is observed. 8000x.



DISCUSSION

The ease by which *Solanum* spp. ovules can be stained, optically sectioned and photographed is demonstrated by our results. A reduction in time and labor utilizing the LSCM-Etbr method is noted over traditional physical sectioning and squash techniques. Utilizing the LSCM-EtBr procedure, 26 hours were required for fixing, staining and final rinse. This surpassed Stelly's protocol (1984) which required 4-5 days for fixing and viewing. Jongedijk's procedure (1987a) required approximately the same time period for preparation (27 hrs), but called for mounting and physical sectioning of ovary samples. Early squash techniques impeded the examination of small ovules by loss and destruction of samples. Jongedijk (1987b) stated that only 40% of the megasporocytes were usable for examination after squash preparation techniques. The LSCM procedure compares to Herr's clearing method (1984) in which whole ovules were processed. In the LSCM-EtBr procedure no clearing technique is applied, the confocal ability of the microscope allows the observation of the MMC through numerous cell layers (Figure 2-8). Optical sectioning by the LSCM does not subject the ovule to any mechanical pressure, providing a accurate image with excellent definition.

EtBr having an affinity for chromosomes and spindle

fibers provided an ideal stain for this technique. Preliminary surveys (data not shown) of fixed buds showed a faint autofluorescence similar to that noted by Fredrikson (1992), but not sufficient to view chromosomes clearly. Staining was accomplished with a single 0.5 mg/ml EtBr stain and triple rinse in sterile ddH_2O. Using higher concentrations stained the outer layers heavily, but inner layers had absorbed little EtBr. Stronger concentrations needed less time to stain, but required longer rinse times to reduce background fluorescence and bleeding of EtBr into the glycerol. Individual ovules physically separated from the ovary could be stained using a lower concentration of EtBr for a shorter time with minimal background fluorescence. Since the EtBr-stained cellular material fluoresces brightly on a dark background, the images from the LSCM are in reverse of conventional images. Whereas Fredrikson (1992) reversed the LSCM images to make them more comparable to microscope images, ours are not. It may be possible to utilize photophores that would stain selectively for different parts of the cell (Entwistle and Noble 1992), and thus acquire more information on the activities of the cellular components during meiosis.

Optical images obtained by the LSCM can be manipulated in many ways before saving to provide the best definition. The contrast and brightness of the image can be adjusted as well as enhanced using numerous digital filters. To obtain desired levels of contrast for *Solanum* ovules Stelly et al.

(1984) was required to use various film development regimes. To obtain similar results with the LSCM, contrast adjustments are made via a knob, providing an instant change, as compared to a tedious film development schedule. Since microscope images are stored digitally on magnetic media they can be recalled at any time for manipulation or to be rephotographed. The use stored digital images is extremely advantageous over traditional microscope photography techniques. A photographed image is stored on a fragile negative, if the negative is scratched the image quality is severely reduced. Subsequent negatives can be reproduced from existing pictures, but they will be lacking the quality of the original. An image saved digitally can be recalled and reproduced with the original clarity. Several desktop imaging programs can be used to adjust the optical images away from the microscope. Digital microscope images can be imported into slide preparation programs to construct presentations. These images can be distributed as tools for classroom instruction to anyone with a desktop computer for viewing.

The LSCM has the ability to create stereo pairs from a z-series of optical sections from a single object. Stereoscopic images viewed with a stereoscope produce a 3-D effect which allow for the viewing of objects in relief. Subsequent transfer of a z-series to the graphics workstation can be completed quickly. Subsequent reassembly of unaltered optical sections is done using VoxelView™.

VoxelView™ can quickly produce a 3-D rotational model. If changes were made to the images such as trimming, altering the opacity of an object, adding color or masking; the final product may require several additional hours of work. Reconstructions of rotational 3-D images from ovule optical sections present yet another method to examine megasporogenesis. The method of reconstruction used with the potato ovule (data not shown) was similar to that of van Spronsen (1989). van Spronsen used 16 optical sections in the reconstruction, whereas the potato ovules required at least 40 sections. The reassembled 3-D image can be adjusted to emphasize certain areas of the ovule that may not have been apparent with 2-D studies.

Meiotic cells were easily to distinguish from the surrounding somatic cells by their large size and distinctive appearance (Figures 2-2, 2-6). All stages of Meiosis I were readily identified in ovary samples. LSCM images of meiosis were similar in structures to that of Jongedijk's (1987a). Jongedijk (1987a) noted that within plant species such as potato, female meiosis is highly synchronized, entailing a search of numerous ovaries to identify all stages. In this study, meiosis II stages were not identified and may be more transient and difficult to detect. The appearance of 2n eggs has not been confirmed, other than the possible production of a dyad (Figure 2-10). Due to the stage of the megasporocyte it was not possible to determine whether the megaspore was FDR- or SDR- derived.

Production of 2n eggs in *Solanum* is under genetic control, however the environment can influence frequency. It was noted that production of 2n eggs might be dependent on temperature and photoperiod conditions of the source plant (Mooney and Peloquin 1992; E. Jongedijk personal communication).

The use of the LSCM-Etbr technique provide an excellent tool to expand exploration of other species with small chromosomes like potato. An immense savings in preparation time allows for the quick examination of large numbers of ovules. The resulting images are very clear and suitable for many uses. The LSCM advances our ability to examine megasporogenesis in potato efficiently and effectively and justifies further experimentation.

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APPENDIX A

Table A-1. Summary of average maximum and minimum temperatures (in C°) during the growing season at Montcalm Research Farm, Montcalm, MI.

Year	April		May		June		July		August		September		6-Month Average	
	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max	Min
1991	16	4	22	8	28	15	27	16	27	14	21	8	23	10
1992	11	1	21	6	24	10	24	12	24	11	21	8	21	8
1993	12	1	20	7	23	13	27	16	26	16	18	7	21	10
1994	14	1	19	9	26	13	26	16	24	13	23	11	22	10
Avg.	13	2	21	7	25	12	26	15	25	13	21	9	21	9

Table A-2. Summary of precipitation (in centimeters per month) recorded during the growing season at the Montcalm Research Farm, Montcalm, MI.

Year	April	May	June	July	August	September	6-Month Average
1991	4.76	3.68	4.03	5.73	1.75	1.50	21.45
1992	3.07	0.47	1.18	3.51	3.20	3.90	15.33
1993	3.47	3.27	4.32	2.58	6.40	3.56	23.60
1994	3.84	2.63	6.04	5.16	8.05	1.18	26.90
Ave.	3.79	2.52	3.89	4.25	4.85	2.53	21.82

Table A-3. Summary of soil test for the general plot area for the growing season at Montcalm Research Farm, Montcalm, MI.

Year	pH	P_2O_5	K_2O	Ca	Mg	C.E.C
1991	6.1	449	219	800	152	5.3 me/100 g
1992	5.6	383	152	610	124	5.8 me/100 g
1993	6.2	395	177	762	137	2.7 me/100 g
1994	6.0	452	222	838	151	2.7 me/100 g

Table A-4. The four year summary of growing degree days (base 10 C°) during the season at Montcalm Research Farm, Montcalm, MI.

Year	May	June	July	August	September
1991	452	1014	1632	2185	2491
1992	282	718	1210	1633	1956
1993	261	698	1348	1950	2153
1994	231	730	1318	1780	2148