Summer 8-16-2024

Assessing Haploid Induction Rates From Diploid Solanum tuberosum Selections

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ASSESSING HAPLOID INDUCTION RATES FROM DIPLOID *SOLANUM TUBEROSUM* SELECTIONS

by Noah Williams

B.A. Skidmore College, 2022

A THESIS

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Science

(in Botany and Plant Pathology)

The Graduate School

The University of Maine

August 2024

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An Abstract for the Thesis Presented in Partial

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Abstract

Potato (*Solanum. tuberosum*) is the fourth most cultivated crop worldwide, and the most grown vegetable crop in Maine. Currently, all available commercial potato varieties developed at the University of Maine and in the United States are autotetraploids ($2n = 4x = 48$). However, potato and its wild relatives exist in a range of ploidy states. In contrast to commercial varieties in the US, many South American landraces and related tuber-bearing Solanum species are diploid ($2n = 2x = 24$). As a highly heterozygous polyploid, inbreeding in conventional tetraploid potato breeding cycles is impractical, and has prevented breeders from establishing an F1 hybrid breeding system for potatoes. Unlike tetraploid potatoes, diploid potatoes that can be self-fertilized may be used to create inbred lines. With additional ploidy manipulation, inbred potato

development can be expedited. In this work, we test potato haploid inducers (PL-4 and IvP48) to create potato monoploids ($2n = 1x = 12$) by crossing to selected diploid lines. Five monoploids from three families were produced and identified. Chromosome doubling has been attempted using colchicine treatments to generate a fully inbred diploid potato in one generation. Previous doubled monoploids have been developed with this method, but poor fertility and agronomic traits have hindered their utility in breeding programs. Developing doubled monoploids from improved diploid germplasm can produce more fertile, fully inbred lines to accelerate diploid hybrid potato breeding.

ACKNOWLEDGEMENTS

All thanks to Ek Han Tan & Paul Collins for getting me this far, Greg Kornelis for help crossing and maintaining plants and Diana Spencer for rescuing ME1x0032-27. This work was made possible by the public and collaborative nature of potato research, especially Jeff Edelman's R package PolyBreedR which enabled me to complete this project in a two year timeframe.

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1. Literature Review

1.1 *Importance of potato as a vegetable crop*

Potato (*Solanum tuberosum*) is the most grown vegetable crop worldwide and the fourth most cultivated food crop. It is highly water, labor and land efficient on a per calorie basis, and provides a more complete nutrient profile than the three most cultivated grain crops (wheat, rice, maize) (Nunn & Qian, 2011). Potato consumption when supplemented with vitamins A and D, traditionally through dairy, can provide a complete nutrition for the average person (Burgos et al., 2007). There are several potato landraces particularly dense in sources of essential nutrients such as zinc and iron (Angé et al., 2018; Burgos et al., 2007). Potato consumption is largely responsible for a population boom following its establishment as a crop in the Old World, with up to a quarter of the Old World population growth between 1700 and 1900 being attributable to the crop (Nunn & Qian, 2011), and continues to play a key role in feeding the growing world population, with potato acreage increasing in African and South Asian countries (Walker et al., 1999).

One market surrounding potato production is seed potatoes, which are vegetative material from tubers that are used for propagation, ideally or implicitly expected to have a low disease load (VanderZaag et al., 2021). North America saw a major transition in potato production following the first major outbreak of Late Blight (*Phytophthora infestans*), responsible for the European Potato Failure. Outbreaks of disease spurred concerted breeding efforts of new varieties, first by individuals, then taken up by the public sector. Disease load and new varieties prompted interest in the seed potato supply. The 1910's saw the rise of several state-level seed

potato programs which contributed to the distribution of improved cultivars, reduction of disease load and the steep increase in yield during the post-war period (Campbell & Frutchey, 1956). The United States alone exported 2.1 Billion USD worth of potatoes in 2021 showing the fruits of these efforts (Connon, 2023).

Seed potato production successes in Western countries have failed to produce comparable results in many tropical regions, where demand is growing. Higher disease load puts greater strain on local seed potato programs, while limited infrastructure, government management problems, and unequal trade relations exacerbate seed shortages and their effects (VanderZaag et al., 2021). Several alternative methods were developed to work around these limitations, namely apical cuttings and the development of true potato seed (TPS). The International Potato Center (CIP) worked on developing tetraploid true potato seed between 1977 and 2000 to ameliorate disease load issues in planting material. The demand for potatoes, the need for clean sources of potato planting material and logistic constraints that drove the push for tetraploid TPS remain in place, an alternative is needed. To address this a shift in the crop's ploidy has been proposed by a collective of academic, public sector and private industry researchers (Bradshaw, 2022; Jansky et al., 2016).

1.2 *Ploidy manipulation of potato and potato wild relatives*

Potato (*S. tuberosum*) and other tuber-bearing Solanum species exist in a range of ploidies—the majority of commercial cultivars are tetraploid ($2n = 4x = 48$) while many landraces and related tuber-bearing *Solanum* species are diploid ($2x$) (Uijtewaal et al., 1987). Some species naturally exist in a range of ploidy states, such as the wild species *Solanum microdontum* which has populations with triploid ($2n = 3x = 36$) and diploid individuals ($2n = 2x$

= 24) (Okada, 1981 ; Kozub, 2021). In cultivated *S. tuberosum* from Group Tuberosum are primarily autotetraploid (4*x*) while Group Phureja are generally considered diploid (2*x*). In plants, polyploidy is a common occurrence in angiosperms with approximately 70% being polyploids and many diploids deriving from polyploid ancestors (Stupar et al., 2007; Meyer et al., 2012). Tuberising members of the Solanum species due to their propensity for shifting ploidy and hybridization have challenged taxonomists. Ploidy was initially weighed heavily in taxonomy, new genetic studies have challenged this reclassifying several species as groups within the cultivated potato species including the predominantly diploid Group Phureja (Huaman & Spooner ; Jansky & Spooner, 2018).

Diploids have had a longstanding place in potato breeding with diploid male to tetraploid female upcrosses present in the pedigrees of Yukon Gold and Atlantic (Johnston & Rowberry, 1981 Webb et al., 1978). Upcrossing has played a role in introducing resistance genes from wild species and other desirable traits into the cultivated tetraploid genepool. These crosses rely on 2*n* pollen being present in the diploid male through incomplete meiosis. Approximately 5% of pollen needs to be 2*n* for normal seed set, in one selection of Phureja/Stenontonum crosses roughly 27% of plants met this threshold, which is both exceptionally high and low enough to constrain breeding efforts(Haynes, 2001). This inefficiency and sometimes complete absence of 2*n* pollen constrains the effectiveness of upcrossing.

Ploidy manipulation has a long history in potato, since at least the 1950's applications of colchicine to seed or tissue has been used to double the ploidy of *Solanum spp*. This has been used to mitigate barriers between wild species and *S.tuberosum.* The Scottish Crop Research Institute used this method to double the ploidy of *S.vernei* and through this introduced potato

cyst nematode (*Globodera. pallida*) to the commercially successful cultivar Lady Balfour and several others (Bradshaw, 2009). The Scottish program often had to implement backcrossing to compensate for the wild varieties attributes several times, extending the time between the initial cross and variety release. Colchicine has also been used to replicate the autotetraploidization event in *S. tuberosum,* three lines of monoploid ($2n = 1x = 12$) were doubled to a diploid state and redoubled to create fully homozygous tetraploids (Uijtewaal et al., 1987).

Recently, diploid commercial varieties have been introduced in the United Kingdom and Japan, as well as serving an important economic role in the Andean States (Kobayashi et al., 2008; Bradshaw, 2009; Devaux et al., 2023). In Japan the diploid variety Inca-No-Mezame was released in 2001. In the U.K. the Scottish Crop Research Institute developed six Phureja-based diploid varieties which were added to the national register beginning in 2001 with Mayan Gold. The cross Mayan Gold was derived from, took place in 1986 (Bradshaw, 2009). These long development intervals are in line with the pace of tetraploids in the program. The value of diploid germplasm in the form of desirable traits and contributions to economically successful cultivars has been recognized and is in use by breeding programs. Use has transcended market class with significant contributions to chipper, table and specialty varieties and with transnational utilization. Inefficiency of crosses and inheritance prediction to and at the tetraploid level remain limiting factors in integrating desirable traits. Still, outside of the Andean region, widespread adoption of diploid varieties has yet to occur.

Reticence to adopt diploid varieties is in part influenced by the assumption that tetraploids are inherently higher yielding as a consequence of their ploidy. The first wave of germplasm distributed to Europe was tetraploid and heavy contributions from the all tetraploid

Chilotanum group from the lowlands of Chile are found in modern cultivars (Jansky & Spooner, 2018). Early breeding work in Utica, NY increased this influence in North American cultivars (Campbell & Frutchey, 1956). Historical inertia and a widespread belief in higher yields in tetraploids, has led to a focus on this tetraploid stock, but there is evidence yield can be agnostic to ploidy (Jansky & Spooner, 2018; Manrique-Carpintero et al., 2018; Paz & Veilleux, 1997). In a comparison of five genotypes at a fully homozygous diploid state and a fully homozygous tetraploid state the vigor increase in tetraploids was small, for both the heterozygous diploid parent outperformed the homozygous genotypes (Uijtewaal et al., 1987). In 2007, a comparison study showed reduced vigor when going from a diploid to a tetraploid state (Stupar et al., 2007).

1.3 *Diploid breeding models based on true seed*

Diploid models in grain crops such as maize (*Zea mays)* rely on a process of inbreeding, then outcrossing to create uniform F1 seed with hybrid vigor (Bradshaw, 2022). In diploid potatoes, it theoretically takes eight generations of self-fertilization to reach 99% homozygosity, but in practice may be greater, where inbreeding depression reduces the efficiency of crosses. Several *S. tuberosum* Group *phureja* accessions have been demonstrated to, when crossed as the male parent, produce haploid progeny with no male inheritance, these accessions are referred to as haploid inducers (Ordoñez et al., 2021; Van Breukelen et al., 1977; Hermsen, 1973; Peloquin & Hougas, 1958). The haploid progeny of diploids are monoploids with a single set of chromosomes. These monoploids demonstrate male and female sterility, as well as poor agronomic traits. However, when their chromosomes are chemically doubled using colchicine, fertility can be restored and the result is a 100% homozygous diploid produced in a single generation (M'Ribu & Veilleux, 1992; Zhang et al., 2022). This process expedites inbreeding by

seven generations and produces a fully inbred final product. The aim of this study is to test selected diploids with improved genotypes through haploid induction crosses for monoploid production in potato and to characterize monoploids and their doubled monoploid counterparts. Current potato research in diploid models is hampered by limited availability of fertile inbred lines. Heterosis and mapping studies rely on an inbred self-compatible *S.chacoense* as the male parent (Endelman & Jansky, 2016, Hosaka & Sanetomo, 2024).

1.4 Haploid induction in potato

Haploid induction is the process of creating progeny with half the chromosome sets of the parent. In potato, there are two methods for haploid induction "in vitro" through anther culture and "in vivo" crosses with selected *Phureja* inducers as the male parent (Ordoñez et al., 2021, Amundson et al., 2020). Haploid induction crosses are also referred to as prickle pollination (Peloquin & Hougas, 1958). The mechanism of haploid induction in haploid induction crosses is contended, and limited mechanistic understanding impedes efforts to increase efficiency. Parthenogenesis has been suggested as well as post-fertilization elimination of haploid inducer chromosomes (Van Breukelen et al., 1977). Haploid inducer DNA has been observed in some dihaploid progeny (<1%) lending credence to the latter theory of haploid induction (Amundson et al., 2021; Bamberg et al., 2016).

Identification of inducer inheritance is aided by the presence or absence of dominant anthocyanin markers which present as a dark embryo spot on seeds (Peloquin & Hougas, 1958). IvP48, PL-4 and related IVP inducers are homozygous for this trait, which is rarely found in other varieties (Van Breukelen et al., 1977). Presence of this spot indicates male inheritance. The

anthocyanin marker is also present as a nodal band on seedlings and mature plants. This can be used to screen out false negatives and progeny from ambiguous seeds (Ordoñez et al., 2021).

One gene (StDMP) expressed in potato pollen has been identified as a contributor to haploid induction. Twelve monoploids were successfully produced in crosses with a stDMP positive male parent. Further genetic sources or mechanisms of haploid induction in potato inducers are currently unknown and the role of stDMP is still unclear (Zhang et al., 2022). IvP48 is one of the earliest identified haploid inducers developed, as it has high male fertility and pollen shed. PL-4 (CIP596131.4) is a novel haploid inducer that was introduced in 2021. In tests of haploid induction using a diverse set of tetraploid parents having members with cytoplasm types D,T and W, PL-4 outperformed inducers IVP101 and IVP35 in overall haploid induction rates and dihaploid production per 100 fruit. This increased induction efficiency across cytoplasm types and genotypes in tetraploids would increase the efficiency and feasibility of wider monoploid production, in the event that it behaves comparably in diploid induction crosses (Ordoñez et al., 2021).

1.5 *Cytoplasm types for potato breeding*

Cytoplasm types or cytotypes are clusters of the associated chloroplast and mitochondrial genomes of an organism. In potato, there are six commonly associated cytotypes: A, P, W, M and T are identified through chloroplast markers and type D is determined through mitochondrial markers. Type T is the most common in modern cultivated *S. tuberosum* having supplanted earlier A-type varieties following the European Potato Failure, A-type is still found in varieties such as Irish Lumper (Hosaka & Sanetomo, 2012, Smyda-Dajmund et al., 2020, Sood et al., 2021).

Cytoplasm type is primarily associated with male sterility and has an unknown relationship to haploid induction. W-type has been associated with malformed pollen when interacting with S.tuberosum nuclear DNA, causing tetrad male sterility (Sanetomo et al., 2022). D-type is associated with morphologically normal but sterile pollen under the same conditions, while T-type is associated with poor male fertility.

The variety Atlantic, the maternal source of the ME2x0005 family, has been reported as having T-type cytoplasm (Sood et al., 2021). Caribou Russet, the maternal source of ME2x0001 has no publicly available information on its cytoplasm type or that of its maternal line. Families ME2x0005 and ME2x0001 are derived from maternal dihaploids of tetraploid varities crossed as the female with W2x001-22-45 and are expected to have the same cytoplasm type as their maternal ancestors. *S. microdontum* accessions from the Plant Breeding and Acclimatization Institute—National Research Institute were reported to have wild type (W) cytoplasm (Smyda-Dajmund et al., 2020). *S.stenontonum* accessions have been reported as types P, W and in one accession T (Hosaka, 1995). Recent publications classify the species as type P exclusively (Hosaka & Sanetomo, 2014). *S.tuberosum* Group *Phureja* accessions are expected to have type P cytoplasm. Inca-No-Mezame was also reported as type P (Hosaka & Sanetomo, 2012).

Chapter 2

2. Report of a spontaneous potato monoploid resulting from a biparental diploid potato cross

2.1 *Abstract*

Reduction to a haploid state followed by chromosome doubling is an established method for the production of inbred lines for diploid-hybrid true seed production. In potato, the focus on haploid induction has been to use haploid inducers to develop primary dihaploids from tetraploid breeding clones via maternal haploid induction. Further reduction of diploid breeding lines into true monoploids via haploid induction is also possible, but has not been explored extensively. PL-4, a diploid potato line developed as an improved haploid inducer, was crossed as female in a limited number of crosses with a diploid breeding line derived from *Solanum tuberosum* Group Phureja and *Solanum stenotomum*. Embryo rescue resulted in two offspring with low guard cell counts, and SNP analysis identified one offspring as a monoploid of PL-4.

2.2 *Introduction*

Cultivated potato is a highly heterozygous autotetraploid and six decades of research have explored converting commercial potato breeding to an inbred diploid hybrid system based on true seed (Jansky et al., 2016). Tuber-bearing *Solanum spp.* naturally exist in a wide range of ploidies among which some wild species, landraces and cultivated varieties are diploid and can be taken advantage of if a diploid breeding system is employed (Bradshaw, 2022). For diploid

species, reduction to the monoploid state ($2n = 1x$) followed by chromosome doubling is an established method of producing inbred lines for hybrid seed production in crops such as maize (Chase, 1952). Replicating this process for diploid potato breeding continues to be of interest today (Hougas et al. 1958., Jansky et al., 2016, Bradshaw, 2022).

The first two recorded haploids from plants were derived from *Solanaceae* (Blakeslee et al., 1922, Clausen & Mann, 1924, Jorgensen, 1928). A decade later, the first haploid in *Solanum tuberosum* was discovered (Lamm, 1938). Selections made from diploid *Solanum tuberosum* Group *Phureja* identified a number of potato haploid inducers that have been well characterized and developed to facilitate potato breeding (Hougas & Peloquin, 1957, Van Breukelen et al 1975. Van Breukelen et al., 1977, Jacobsen, 1978). These inducers are typically used as the male parent (pollen donor), creating maternal haploids in a process termed maternal haploid induction.

In these lines, homozygous dominant anthocyanin markers have been developed, specifically to screen out paternal inheritance in maternal haploid induction crosses, by the Plant Breeding Institute (I.v.P.) (Van Breukelen et al., 1977). These markers appear as an embryo spot on true seed and a dark pigmentation on the nodal band, allowing for two stages of screening. Not all studies have relied on the same anthocyanin marker and two stages of screening. Notable exceptions include the first study on potato haploids, some studies focused on interspecific crosses, and the recent introduction of fluorescent markers. All studies relied on readily distinguishable phenotypes indicative of paternal inheritance. (Hougas et al., 1958, Singsit & Hanneman, 1991, Zhang et al., 2022).

Paternal haploid induction involves using a haploid inducer as female, and creating haploids of the male parent (pollen donor) in the haploid inducer cytoplasm. Paternal haploid induction via centromere-mediated genome elimination has been recorded in wheat, maize, broccoli and *Arabidopsis thaliana*, and offers a convenient way of swapping cytoplasm type (Bortiri et al., 2024, Lv et al., 2020 ,Wang et al., 2023, Han et al. 2024). Reviewing the literature, it appears that the potato haploid inducers currently in use have not been tested for paternal haploid induction.

In order to determine the effectiveness of PL-4 as a female parent for paternal haploid induction and its potential to induce paternal haploids, a limited number of crosses were performed with BD1250-1 as male. The line PL-4 used as a female parent is a recently described haploid inducer line developed at the International Potato Center (Ordoñez et al., 2021). In their study, PL-4 performed better overall and had a more even effectiveness as a haploid inducer for maternal haploid induction in tetraploid breeding lines compared to its parents, IvP101 and IvP35. The USDA-ARS breeding line BD1250-1, used as the male parent, is a long-day adapted diploid. BD1250-1 was developed through the open pollination of BD1050-4 as part of a long-term mass selection diploid potato breeding project. The initial population of 30 *S. tuberosum* Group *Phureja* clones and 30 *S. stenontonum* clones underwent many cycles of mass selection for long-day adaptation and dormancy, beginning in 1972, with additional germplasm integrated throughout the trials (Haynes, 1972, Haynes, 2018).

Limited developmental knowledge of some of the diploid germplasm used and observations of wide phenotypic ranges around the nodal band led to reduced selection ability compared to other applications. As a result, a clone which later developed clear nodal bands was screened for haploid status. The haploid passed three additional screening phases focused on ploidy—plastid counts from guard cells, genetic analysis using a single nucleotide polymorphism array and cytogenetic chromosome counts from a root squash agree on the monoploid status of the haploid as being derived from PL-4.

2.3 *Methods*

2.3.1 *Crossing*

During the course of the 2023 crossing season at the USDA-ARS New England Plant, Soil, and Water Laboratory, a limited number of paternal haploid induction crosses were performed between diploids PL-4 and BD1250-1. Eight PL-4 flowers from three clusters were emasculated and pollinated with fresh BD1250-1 pollen, each flower was only pollinated once. One fruit formed and was harvested 48 days post pollination.

2.3.2 *Seed Sorting*

Seeds were visually and tactilely assessed and sorted into three categories with the aid of a dissecting microscope. Tactile assessment focused on seed depth as part of determining if the seed was shriveled. A seed was scored as spotted if it had a visible anthocyanin embryo spot on

one or both sides of the seed. A seed was scored as unspotted if the seed had no visible spot and was fully intact. A seed was marked as “shriveled” if it was shriveled, blackened or otherwise appeared damaged.

2.3.3 *Embryo Rescue*

Seed processing and embryo rescues were performed on the shriveled seeds. Seeds were placed in a 1.5 mL tube with a 1500 ppm Gibberellic Acid (GA_3) solution (Jansky, 2016). Tubes were taped to a spin plate and shaken for an hour. Under a hood, the GA_3 solution was pipetted out. A solution of 50% bleach was pipetted in and tubes were inverted for ten minutes. Following inversion, the bleach solution was pipetted out. Sterilized water was pipetted in and the tubes were inverted once then the water was removed. This step was repeated twice. Seeds were plated on a germination media and sealed with 3M micropore tape. Plates are stored in a dark room-temperature location for one week to stratify. Plates were moved to a growth chamber maintained between 18-21°C following stratification.

Germination media used was prepared as a solution of 500 mL Milli-Q water, 100 mL Coconut Water (VitaCoco® pure coconut water), 1.1 g MS media + Gamborg vitamins (MSP06-50LT, Caisson Lab), 10 g Sucrose and 0.1 g MES. This solution was titrated to a pH between 5.5-5.7 using 1M KOH and 3.5 g of Phyto Agar (9002-18-0 PhytoTech Labs) was added following titration. The solution was autoclaved, stirred until no particulates were visible, then poured into petri-dishes at a thickness of approximately 0.5 cm while still fully liquid.

2.3.4 *Ploidy Determination*

Plants with no, late forming or ambiguous nodal banding were retained. Any reasonable doubt when compared to a check of PL-4 was grounds for retention and further screening.

The protocol for plastid counting within a pair of guard cells was adapted from (Ordoñez et al., 2017). Plantlets were grown in potting media under 16 hour light and leaves developed in tissue culture were excluded from the analysis. Leaves greater in size than 1.8cm were preferentially sampled to ensure leaf maturity. A slide was prepared with two drops of iodine-potassium iodide solution near the center of the slide. A portion of the epidermis was peeled with tweezers and a scalpel from the underside of the leaf excluding the epidermis of the vein. Peels were immediately placed on top of the solution on the slide. Another two drops of the iodine-potassium iodide solution was placed over the sample followed by the coverslip. The solution was given a minimum of five minutes to stain before excess solution was dried off with a Kimwipe. Examinations used an optical microscope at 400x. All plastids within a pair of guard cells were counted from a total of ten stomata. Ploidy status was assigned based on the average of ten guard cell pairs. (Frandsen, 1968). A score of eleven plastids or fewer per guard cell pair or 5.5 per guard cell was considered a putative monoploid.

DNA was extracted from potato tissue according to the Edwards protocol (1991). Extracted DNA of putative monoploids was sent to the University of Minnesota Genomics Center with two replicates. Data obtained from UMNGC includes a final report of the SolCAP Infinium array

containing the fluorescent value of the X and Y of 31,190 single-nucleotide-polymorphisms which were used for the ploidy analysis. The geno function of PolyBreedR (V3.4) produced dosage calls for 11,000 SNPS with values between 0 and 4 (Endelman et al., 2024). Monoploids were expected to only have values of 0 and 4. Relatedness of the haploid to its parents was determined using the check_trio function of PolyBreedR, an error rate greater than 1.5% was used as grounds for rejecting the tested paternity.

Root squash procedures were adapted from Ordoñez et al., 2017. Roots were collected from plants that had achieved a height greater than 5 cm. 10 mm of material was removed from the root terminal. Roots were stored in flasks of distilled water for one hour. Roots were transferred to a pre-fixative consisting of a chilled aqueous solution of 0.01% pyrethrin and refrigerated for at least 24 hours at 4℃. Roots were transferred to 60℃ 1 M hydrochloric acid, roots were incubated at temperature for ten minutes, followed by rinsing with deionized water. Roots were then stained at room temperature for ninety minutes in a solution of lacto-propionic orcein. Stained roots were placed on a slide with a drop of the lacto-propionic orcein solution. A cover slip was placed over the root and a strike from a pencil eraser was used to squash the root. Root squashes were imaged using brightfield microscopy to determine chromosome counts.

2.4 *Results*

From eight pollinations, one fruit formed. Seeds from the unspotted and shriveled classes were grown out. Of the unspotted class, sixteen plants developed to a stage where leaf peels could be performed and determined to have plastid counts consistent with being diploid . Of the shriveled seeds which underwent embryo rescue, only eight seeds germinated (Table 1). Of those five failed to thrive, one died, and two developed to a stage where leaf peels could be performed. Plastid counts from leaf peels for both lines ME1x0036-22 and ME1x0036-27, were conducted along with PL-4 and BD1250-1 (Table 2). ME1x0036-22 had a much lower average of ~3, about half the count of all the other lines that were counted. Figure 1a shows a pair of guard cells from ME1x0036-22 with 3 plastids per guard cell.

As part of Potato 2.0, the two parents and the offsprings were genotyped using the SolCAP Infinium array and dosage analyses were performed to ascertain ploidy (Endelman, 2024). Figure 2 shows the frequency of dosage calls between 0 and 4 with each dosage frequency being represented by a column in the histogram. Tetraploids are expected to have dosages in all columns, diploids are expected to have dosages only in columns 0, 2 and 4, and monoploids are expected to have dosages only in columns 0 and 4.

The histogram from a tetraploid variety, Caribou Russet (Fig. 1a) had all expected dosage calls. Diploid potato lines with dosage calls 0, 2, and 4 shown include PL-4 (Fig. 2b), BD1250-1 (Fig. 2c) and ME03-0007 (Fig. 2d), which is a primary dihaploid of Caribou Russet. ME1x0036-27

(Fig. 2e) had dosage calls of 0, 2, and 4 and could be identified as a diploid while ME1x0036-22 (Fig. 2f) only had dosage calls of 0 and 4, consistent with what is expected of a monoploid. Next, the PolyBreedR function check_trio was performed on ME1x0036-22 and indicated that it inherited DNA solely from PL-4, while ME1x0036-27 was indicated to be a hybrid of PL-4 and BD1250-1. Finally, cytogenetic images of the mitotic metaphase root squash from ME1x0036-22 (Fig. 1b) shows that it has 12 chromosomes. The plastid count from guard cells, dosage analysis, parentage analysis as well as chromosome count confirms ME1x0036-22 as a maternal monoploid haploid of PL-4. The full breeding process is in figure 3.

Table 1. Classification of true potato seeds extracted from the PL-4 x BD1250-1 cross.

	Shriveled Seeds	Unspotted Seeds	Spotted Seeds
Seeds	55	21	213
Germinated Seedlings	8	20	Not Germinated

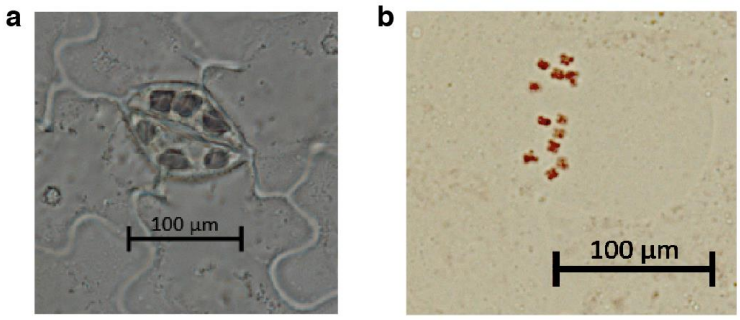

Figure 1. Brightfield image of a stained stomata and metaphase root squash from ME1x0036-22 at 630x magnification. (a) Leaf peel of ME1x0036-22 showing a pair of guard cells with six plastids stained by potassium-iodine. (b) Root squash of ME1x0036-22 with 12 metaphase chromosomes.

Table 2. Average (n=10) number of plastids per guard cell with standard deviation.

Line	Average Number of Plastids Per Guard Cell
PL-4	5.55 ± 1.04
BD1250-1	5.75 ± 1.16
ME1x0036-22	2.95 ± 0.55
ME1x0036-27	5.35 ± 1.16

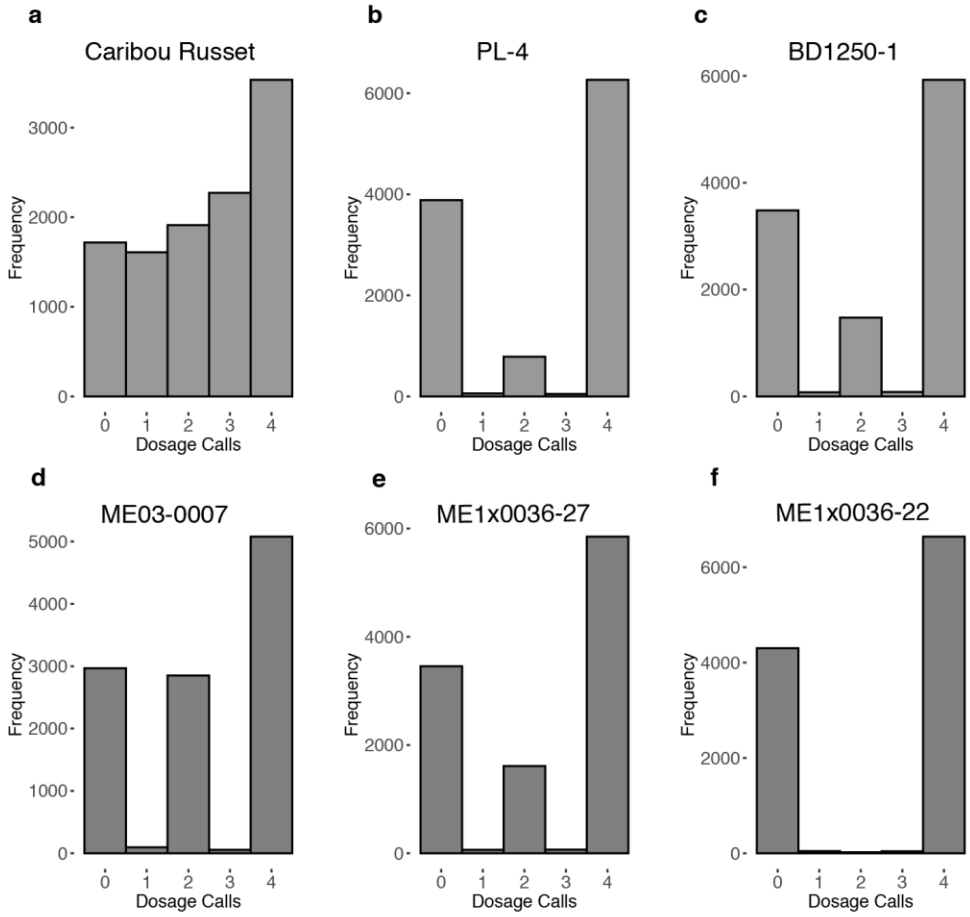

Figure 2. Histograms of tetrasomic dosage call frequencies from tetraploid, diploid and monoploid potato lines derived from the SolCAP Infinium array. Shown here are the dosage classes from tetraploid Caribou Russet (a) exhibiting all five classes across the genome. Next, diploid parents PL-4 (b) and BD1250-1 (c), primary dihaploid derived from Caribou Russet ME03-0007 (d), and diploid hybrid of PL-4 and BD1250-1, ME1x0036-27 (e) showing only dosages of 0, 2 and 4. Lastly, monoploid line ME1x0036-22 (f) shows a fully homozygous genotype, exhibiting only dosages of 0 and 4.

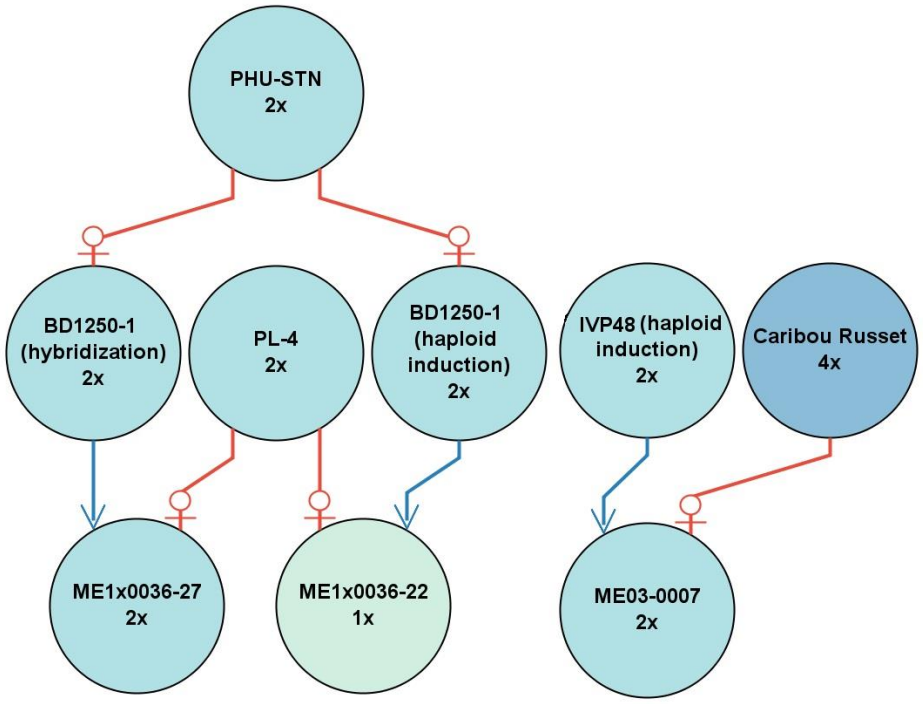

Figure 3. Pedigree chart of ME1x0036 family and ME03-0007, a dihaploid derived from Caribou Russet.

2.5 *Discussion*

Three hypotheses have been proposed for the mechanism of haploid induction in potatoes: parthenogenesis, parent specific chromosome elimination and egg-pseudo fertilization (Wangenheim et al. 1960; Clulow et al. 1991; Amundson et al. 2021). The circumstances of producing ME1x0036-22 raise several possibilities. Parthenogenetic production could occur in PL-4 agnostic to the specific pollinator or as a response to something specific to BD1250-1. PL-4

could have selfed before or during emasculation and acted as a haploid inducer on itself. The male *Phureja-stenotomum* parent BD1250-1 could also have potentially acted as a haploid inducer generally.

Because ME1x0036-22 was identified from a pool of only fifty-five seeds, it is possible that this may be a relatively common phenomenon. Embryo rescue may be a route for increasing monoploid production or PL-4 could frequently produce haploid offspring. Identifying ME1x0036-22 was the result of several deviations from the standard haploid induction process using IvP-derived lines. First, PL-4 was used as a female parent. Second, embryo rescue of shriveled seeds was performed, and third, offspring exhibiting ambiguous or late-forming nodal banding were not eliminated during the screening process.

Further test crosses can be performed, towards the production of a population of PL-4 monoploids for embryological analysis. A population of monoploids from a known haploid inducer may have use in genetic mapping for haploid induction. Embryological analysis of diploid by diploid induction crosses, and crosses with known inducers such as done with tetraploid by diploid induction crosses may also shed light on the process (von Wangenheim et al., 1960). Ongoing efforts at developing hybrid diploid potatoes in the U.S. are challenged by severe inbreeding depression. A doubled monoploid approach could facilitate inbred development and improve the biological understanding of the haploid induction mechanism.

Chapter 3

3. Assessing maternal haploid induction rates from diploid *Solanum tuberosum* selections

3.1 *Abstract*

The development of improved germplasm derived from *S. tuberosum* primary dihaploids and a partially inbred, self-compatible diploid breeding clone W2x001-22-45 offers a promising population for revisiting the production of maternal monoploids. Another improved population of adapted diploids with promising fertility traits including documented high levels of self compatibility and inter-ploidy compatibility was trialed. Haploid induction crosses were performed within these germplasm categories as well as the wild species *S.microdontum* and other diploid clones to test if they might be an effective source for monoploids. After haploid induction crosses, putative monoploid offspring were screened using a two-tiered phenotyping system followed by guard cell counts, single nucleotide polymorphism analysis and root squashes for ploidy confirmation. Four maternal monoploids were identified from the adapted diploid population, with one confirmed by root squash. Efforts to effectively double the identified monoploids to obtain 100% homozygous doubled monoploids are underway.

3.2 *Introduction*

Improving diploid potato breeding is the goal of Potato 2.0, a USDA Specialty Crop Research Initiative grant-aimed at reinventing the potato as a diploid inbred hybrid system. The strategy focused on developing primary dihaploids derived from tetraploid cultivars. Next, the dihaploids are introgressed with germplasm with useful fertility traits such as the self-incompatibility

inhibitor gene (Sli) sourced from *S. chacoense*. The current goal of Potato 2.0 is to develop inbred lines that align with these four broad criteria for a successful diploid potato breeding system: they must be self fertile, homozygous enough to produce uniform F1 seeds, produce offspring superior to existing cultivars and be fecund enough to produce seed at a commercial level (Bradshaw, 2022). This project is currently in a nascent stage, focused on the introgression of self-compatibility and maintaining male and female fertility. Towards this goal a self-compatible and agronomically sound W2x001-22-45 is being used currently as a male Sli donor for crosses with primary dihaploids (Song & Endelman, 2022). An alternative to relying on multiple rounds of selfing for inbreeding and introgression of the Sli gene, is reduction to the monoploid stage followed by chromosome doubling, producing a fully inbred in one generation. Determining the best pool of germplasm for reduction to the monoploid stage is difficult with limits in our mechanistic understanding of the process. Testing a diverse array of diploids may help focus future efforts. Previous efforts at doubled monoploid development predate new diploid breeding clones derived from dihaploids, commercially released diploid varieties and other germplasm has been underexplored, such as adapted diploids and several wild diploid species such as *S.microdontum.*

To develop the improved diploid breeding population using Group Tuberosum material, primary dihaploids derived from Caribou Russet and Atlantic were crossed with W2x001-22-45, and selections from each first generation population was made based on yield, uniformity, and overall tuber size from 2022 field trials at the USDA farm in Chapman, Maine. Two clones, W2x001-22-45 developed by the University of Wisconsin and MSEE815-06 developed by Michigan State University (a *S.tuberosum* Group *tuberosum* with some *S.chacoense* background)

were also included as an alternative pool of S.tuberosum Group Tuberosum diploid material (Douches et al., 2019.). Next, the *Solanum tuberosum* Group *Phureja-stenotomum* population, which comprises the bulk of the adapted diploids, have several unusual fertility traits including high rates of self compatibility (Haynes & Guedes, 2018). In addition, Inca-No-Mezame was selected for trials (Kobayashi et al., 2008). It was selected on the basis of its outbred pedigree, uniformity of yield, popularity in Japanese markets, and female fertility. It is not known to be male fertile. The diploid cultivar was released in Japan in 2001 and this variety has distinct orange flesh with high carotenoid content. However, total yield from Inca-no-mezame was insufficient for meeting market demand as a result of low market suitable yield and its popularity. The popularity of its distinct flavor allows it to fetch a premium and remain commercially viable despite difficulties (Kobayashi et al., 2008). Inca-No-Mezame was included as an alternative commercially successful diploid with a background in between the primary categories.

Besides determining the effectiveness of different genetic backgrounds for haploid induction, we also considered cytoplasm type. The relationship of cytoplasm type to haploid induction has not been established, although notable monoploids such as DM1-3 are from *S.tuberosum* Group *Phureja* and are presumed to have P-type cytoplasm (Bradshaw, 2022). Cytoplasm type is important for hopes of male fertility in doubled monoploids. With the most common cytoplasm type in *S.tuberosum* Group *tuberosum,* T-type, conferring reduced male fertility while D- and W-types conferring male sterility. Therefore, cytoplasm type and its relation to haploid induction is a consideration in a diploid potato breeding system (Smyda-Dajmund et al., 2020, Sanetomo et al., 2022). Comparison of haploid induction rates in improved diploid germplasm, retesting of

established and experimentation with monoploid identification methods, and chromosome doubling offers a path towards the goals of Potato 2.0.

3.3 *Methods*

3.3.1 *Greenhouse Production*

Plants were grown in the USDA-ARS facility between January and May 2023. Greenhouse conditions were maintained between 60 – 85°F with a light cycle of sixteen hours on, eight off. Plants were grown in one gallon pots spaced approximately one inch from each other. Fertilizer was administered three times a week with every watering to increase true seed yield (Bamberg et al., 2017) using Dyna-Gro Foliage (9-3-6) at a concentration of 1.5tsp/gal from emergence to the first budding, followed by Dyna-Gro Bloom (3-12-6) for the remainder of the season with the exception of late May when the plants were senescing.

3.3.2 *Pollination*

Open flowers and flowers in stages 8-10 as defined by Bohórquez-Quintero et al. (2022) were emasculated and pollinated with fresh inducer pollen from either IvP48 or PL-4. Pollen was extracted from inducer anthers with an electric engraver on to glass slides, pollen was applied to the stigma directly from the slide. Each flower was only pollinated once, fused stigmas were counted as a single pollination.

3.3.3 *Seed Extraction*

Approximately ten days post-pollination, the initial fruit set was assessed. If fruit formed the cluster was bagged, ambiguous pollinations were reassessed weekly. Fruit ripeness was assessed

six weeks from the first observed fruit set (Jansky et al., 2012). Fruit with a sweet smell and a modest amount of give when squeezed were considered mature as well as any fallen fruit, some varieties lack both phenotypes in their mature fruit and were evaluated on an individual basis. Seed extraction was performed by halving or quartering berries and massaging them in a bowl of room temperature water. Seeds were separated from the water by straining over cheesecloth. Pulp was manually removed at both stages to ensure seeds are clean and not attached to other seeds. Seeds were air dried on the cloth for the greater of 24 hours or until fully dry then placed in paper envelopes which were stored in a low humidity environment.

3.3.4 *Seed Sorting*

Seeds were visually assessed using a dissecting microscope and tactically examined for thickness and defects. Seeds were marked as spotted if they had a visible anthocyanin embryo mark on one or both sides or if they had multiple darkened regions. Seeds were marked as spotted, unspotted or "shriveled". Seeds were unspotted if they had no visible mark and were fully intact. All other seeds that were shriveled, blackened or otherwise damaged were considered shriveled.

3.3.5 *Germination*

Unspotted seeds were prepared in groups of no more than fifty with crosses exceeding fifty being subdivided. Seeds were placed in a 1.5ml tube with 20% bleach, taped to a spin plate and spun for ten minutes. The bleach solution was pipetted out followed by two washes of 1 mL of deionized water and mixed by inversion each time. Tubes were then filled with a pre-prepared 1500 ppm Gibberellic Acid (GA_3) Solution. GA Solution was made by dissolving 1.5 grams of GA_3 in 10mL absolute ethanol, and 200mL of DI water. Once GA_3 was fully dissolved, DI water

was added to reach a total volume of 1L (Jansky et al., 2012). Tubes were taped to a spin plate and spun for an additional hour.

Germination chambers for unspotted seeds consisted of twelve layers of paper towel folded and laid flat in an enclosed plastic container. The container was soaked in deionized water for half an hour and drained until only a film of water was mobile when angled to the side.

Following GA_3 treatment, seeds were poured over a sieve and rinsed with deionized water before being manually placed on the paper towel surface of pre-prepared germination chambers. Seeds were spaced to avoid direct contact with other seeds. The chambers were then sealed and stratified in a dark space at room temperature for one week. Seedlings were moved to a grow chamber maintained between 18-21°C following stratification.

Two boxes of 50 tetraploid true potato seeds from a cross between Norgleam and a USDA-ARS potato breeding line, were used to confirm the viability of the germination method and provide a baseline before germinating diploids and putative monoploids. 79 out of 100 seeds germinated. 79% germination was considered acceptable and provided a baseline.

3.3.6 *Embryo Rescue*

Seed processing and embryo rescues were performed on the shriveled seeds. Seeds were placed in a 1.5 mL tube with a 1500 ppm Gibberellic Acid (GA_3) solution. Tubes were taped to a spin plate and shaken for an hour. Under a hood, the GA solution was pipetted out. A solution of 50% bleach was pipetted in and tubes were inverted for ten minutes. Following inversion, the bleach solution was pipetted out. Sterilized water was pipetted in and the tubes were inverted once then the water was removed. This step was repeated twice. Seeds were plated on a germination media

and sealed with 3M micropore tape. Plates are stored in a dark room-temperature location for one week to stratify. Plates were moved to a growth chamber maintained between 18-21°C following stratification.

Germination media used was prepared as a solution of 500 mL Milli-Q water, 100 mL Coconut Water (VitaCoco® pure coconut water), 1.1 g MS media + Gamborg vitamins (MSP06-50LT, Caisson Lab), 10 g Sucrose and 0.1 g MES. This solution was titrated to a pH between 5.5-5.7 using 1M KOH and 3.5 g of Phyto Agar (9002-18-0 PhytoTech Labs) was added following titration. The solution was autoclaved, stirred until no particulates were visible, then poured into petri-dishes at a thickness of approximately 0.5 cm while still fully liquid.

3.3.7 *Ploidy Determination*

Nodal band procedures varied across groups. Outside of *S.tuberosum* Group *tuberosum*, some maternal parents displayed environment specific nodal banding, dark nodal banding or their developmental patterns were unknown. The protocol for plastid counting within a pair of guard cells was adapted from Ordoñez et al., 2017. Plantlets were grown in potting media under 16 hour light and leaves developed in tissue culture were excluded from the analysis. Leaves greater in size than 1.8cm were preferentially sampled to ensure leaf maturity. A slide was prepared with two drops of iodine-potassium iodide solution near the center of the slide. A portion of the epidermis was peeled with tweezers and a scalpel from the underside of the leaf excluding the epidermis of the vein. Peels were immediately placed on top of the solution on the slide. Another two drops of the iodine-potassium iodide solution was placed over the sample followed by the coverslip. The solution was given a minimum of five minutes to stain before excess solution was

dried off with a Kimwipe. Examinations used an optical microscope at 400x. All plastids within a pair of guard cells were counted from a total of ten stomata. Ploidy status was assigned based on the average of ten guard cell pairs (Frandsen, 1968). A score of eleven plastids or fewer per guard cell pair or 5.5 per guard cell was considered a putative monoploid.

Extracted DNA of putative monoploids was sent to the University of Minnesota Genomics Center with two replicates. Data obtained from UMNGC includes a final report of the SolCAP Infinium array containing the fluorescent value of the X and Y of 31,190 single-nucleotide-polymorphisms which were used for the ploidy analysis. The geno function of PolyBreedR (V3.4) produced dosage calls for 11,043 SNPS with values between 0 and 4 (Endelman et al., 2024). Monoploids were expected to only have values of 0 and 4.

Root squash procedures were adapted from Ordoñez et al., 2017. Roots were collected from plants that had achieved a height greater than 5 cm. Roots were preferentially harvested from rooted cuttings over plants that developed roots in tissue culture. 10 mm of material was removed from the root terminal. Roots were stored in flasks of distilled water for one hour. Roots were transferred to a pre-fixative consisting of a chilled aqueous solution of 0.01% pyrethrin and refrigerated for at least 24 hours at 4℃. Roots were transferred to 60℃ 1 M hydrochloric acid, incubated for ten minutes, followed by three rinses with deionized water. Roots were then stained at room temperature for ninety minutes in a solution of lacto-propionic orcein. Stained roots were placed on a slide with a drop of the lacto-propionic orcein solution. A cover slip was

placed over the root and the root was squashed with a strike from a pencil eraser. Images were assessed at 630x magnification using brightfield microscopy.

3.3.8 *Germplasm Evaluation*

Leaf samples from selected parents were taken during the course of the 2023 crossing season. DNA extraction was using the standard Edwards Buffer protocol (Edwards 1991). Primers, PCR and gel electrophoresis for cytoplasm-typing multiplex PCR were adapted from Hosaka & Sanetomo (2012 & 2014). The initial run was prepared with 10µl of Go Taq Long PCR Master Mix 2x (M402A Promega), 7.5µl of nuclease free water and 1µl of the multiplex primer mix as described in Hosaka & Sanetomo 2014. The solution was centrifuged, mixed by light tapping, and re-centrifuged. The 10µl of Go Taq Long PCR Master Mix 2x (M402A Promega) was replaced with 10µl of NEBNext Q5 Hot Start HiFi PCR Master Mix (M0543S). DNA content from 1.5µl to 10µl was added to the solution and was centrifuged, mixed by light tapping, and re-centrifuged.

In a Bio Rad T100 Thermal Cycler samples were incubated at 95°C for 10 minutes, 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 90 seconds with 34 repeats and at 72°C for 5 minutes Following the PCR 5µl of digestion solution was introduced to the PCR product, centrifuged, mixed by light tapping, and re-centrifuged. The digestion solution consisted of .1µl Purified BSA 100x (New England BioLabs B9001S), 1.5µl of Bam H I (New England BioLabs R0136S), 1µl of NEBuffer for Bam H I (New England BioLabs BOI 365) and 2.6µl of nuclease-free water. The solution was digested overnight at 37°C.

A 3% agarose gel was prepared with LE Quick Dissolve Agarose (GeneMate E-3119-500) and 50 ml of 1x TAE. Repeated microwaving and stirring every four seconds for a total of two minutes was performed until there were no particulates left. 1.5 ul of ethidium bromide was added to the solution and mixed in. The gel was poured and allowed to harden for one hour before running. 3.5 µl GeneRuler 1kb Plus DNA Ladder (Thermo Scientific SM1333) was inserted into the first lane.15µl of the post digestion solution was used for each lane, and run for one hour at 143 volts.

3.3.9 *Pollen Shed*

Flowers at anthesis were removed and placed on a high contrast surface. Flowers were emasculated with tweezers and anthers were placed back on the high contrast surface. Tweezers and fingernails were inspected for pollen. If no pollen was observed, an electric toothbrush or metal engraving dremel was applied to the tip of the anther, then applied up the length of the anther. If no pollen was observed the anthers would be collected in a 1.5ml tube and vortexed for 45 seconds. A genotype was determined to have no pollen shed if >5 flowers had no observed pollen.

3.3.10 *Chromosome Doubling*

Diploid potato plants exceeding a height of 30 cm had their axillary buds removed. Cotton balls were soaked in an aqueous solution of 0.25% colchicine and placed over the wound and meristem. A ziplock bag was positioned over the cotton ball to prevent evaporation. The cotton

ball was re-soaked at the 24 hour mark and removed after 48 hours as described in Langton et al. 1974.

To increase potential regenerants the protocol was altered to align closer to Ross et al. 1967. The apical bud of each shoot was removed 24 hours in advance of treatment. Immediately before treatment lateral stems were removed. Pieces of cotton balls were taped over the apical bud and nodes, with an average of three nodes per cotton ball. Using a pipette 2 mL of 0.25% colchicine solution was applied to each cotton ball, saturating the ball. A ziplock bag was placed over the entire plant to reduce evaporation.

Media for colchicine doubling was prepared in a batch of 250 mL adapted from Zhang et. al (2022) and unpublished Tan lab protocols. 5 grams of sucrose, 0.55 grams of MS salts + Gamborg Vitamins (MSP06-50LT Caisson Labs), 0.075 grams of MES, and 0.5mL of plant preservation mixture were dissolved in deionized water. The solution was brought up to 250mL with the addition of deionized water and titrated to a pH of 5.7 using 1M KOH. 1.8 grams of agar (9002-18-0 PhytoTech Labs) was then added to the solution along with 10 ml of 2.5mg/mL colchicine (Cat#C3915, Sigma). The solution was autoclaved, stirred until no particulates were visible, then poured evenly across three Magenta boxes (# 30930007-1 bioWorld)

Regenerated shoots after developing a minimum of three nodes were cultured as cuttings in soil (Pro-Mix Potting Mix American Formula). Shoots were evaluated through root squashes and guard cell pair chloroplast counts to screen out chimeras and determine ploidy states.

3.4 *Maternal haploid induction from improved, adapted and wild diploid germplasm*

3.4.1 *Outcomes from haploid induction crosses*

In the first generation *S. tuberosum* Group *tuberosum* population 172 pollinations were performed; 101 with the inducer line IvP48 and, 71 with the inducer line PL-4. From the IvP pollinations, there were 5,386 seeds of those 775 were shriveled or unspotted. From PL-4 there were 582 seeds, 88 were shriveled or unspotted. No monoploids were identified from either group (table 3).

In the adapted diploid category, 510 pollinations were performed. IvP48 was used as the haploid inducer for 235 pollinations and for PL-4 275. From the IvP pollinations there were 13,368 seeds, of which 1,203 were unspotted or shriveled. From the PL-4 pollinations there were 6,338 seeds of which 1,119 were unspotted or shriveled. In the adapted diploid population four monoploids were identified from two families. PL-4 had an overall success rate of 0.18% of unspotted or shriveled seeds resulting in a monoploid, IvP-48 had a success rate of 0.16% (Table 4).

For the variety Inca-No-Mezame pollinated with IvP48, there were 110 pollinations resulting in 1 harvested fruit. There were a total of -29 seeds. Out of these, 16 seeds were unspotted or shriveled. When pollinated with PL-4 there were 58 pollinations resulting in 5 harvested fruits. There were a total of 351 seeds. Out of those, 122 seeds were unspotted or shrivelled. No monoploids were identified from either cross. Crossing *S.microdontum* (PI 218223) with haploid inducer IvP48, 46 pollinations resulted in 4 harvested fruits. There were 9

seeds extracted, 8 of which were unspotted or shriveled. No monoploids were identified. With PL-4, 56 pollinations were performed resulting in 8 fruits set. No seeds were extracted. With W2x001-22-45 crossed with IvP48, 37 pollinations resulted in no harvested fruit, with PL-4 39 pollinations resulted in no harvested fruit. The clone MSEE815-06 was only pollinated with IvP48. All seven pollinations resulted in harvested fruit. 844 seeds were extracted, of which 266 were unspotted or shriveled. No monoploids were identified from this cross (table 5).

Table 3. Haploid induction metrics of first generation Group Tuberosum diploids.

Female	Male	Pollinations	Fruit Set	# Fruit Harvested	Total seeds	Unspotted + Shriveled seeds	Monoploids isolated (% screened)
All genotypes	IvP48	101	37	36	5386	775	0 (0%)
All genotypes	PL-4	71	6	6	582	88	0 (0%)
ME2x0001	IvP48	55	6	6	989	99	0 (0%)
ME2x0001	PL-4	11	0	0	0	0	0 (0%)
ME2x0005	IvP48	46	31	30	4397	676	0 (0%)
ME2x0005	PL-4	60	6	6	582	88	0 (0%)

Table 4. Haploid induction metrics of adapted Phu-Stn diploids.

Female	Male	Pollinations	Fruit Set	# Fruit Harvested	Total seeds	Unspotted + Shriveled seeds	Monoploids isolated (% screened)
All Genotypes	IvP48	235	74	72	13368	1203	2 (0.16%)
All Genotypes	PL-4	275	58	58	6338	1119	2 (0.18%)
BD1222-1	IvP48	10	2	2	111	43	0 (0%)
BD1222-1	PL-4	15	4	4	322	157	2 (1.27%)
BD1250-1	IvP48	68	25	25	5169	244	2 (0.81%)
BD1250-1	PL-4	59	12	12	2553	200	0 (0%)

Table 5. Haploid induction metrics of other diploid clones and species.

Female	Male	Pollinations	Fruit Set	# Fruit Harvested	Total seeds	Unspotted + Shriveled seeds	Monoploids isolated (% screened)
Inca-No-Mezame	IvP48	110	2	1	29	16	0 (0%)
Inca-No-Mezame	PL-4	58	5	5	351	122	0 (0%)
Solanum microdontum PI 218223	IvP48	46	5	4	9	8	0 (0%)
Solanum microdontum PI 218223	PL-4	56	19	8	0	0	0 (0%)
W2x001-22-45	IvP48	37	0	0	0	0	0 (0%)
W2x001-22-45	PL-4	39	0	0	0	0	0 (0%)
MSEE815-06	IvP48	7	7	7	884	266	0 (0%)

3.4.2 *Nodal Band Screening*

Figure 4. Potato seedling from haploid induction cross displaying anthocyanin nodal bands.

Treatment of seedlings with nodal bands varied by germplasm. Some seedlings displayed clear dark purple anthocyanin nodal bands indicative of inheritance from haploid inducers as shown in Figure 4, others were ambiguous. Mortality and phenotypic variance made absolute counts of nodal band positive seedlings untenable. To help clarify the guard cell count threshold for monoploids some putative diploids with clear nodal bands were evaluated. In the first generation S.*tuberosum* Group *Tuberosum* population, seedlings with nodal bands were discarded without guard cell evaluation, although fifteen were arbitrarily retained for guard cell counts as diploid controls. In the adapted diploid population, seedlings with nodal bands were discarded, except for an arbitrary selection of 34 as diploid controls. All *S. microdontum* seedlings from HI crosses were retained due to ignorance of the accession's nodal band coloration. All Inca-No-Mezame seedlings from HI crosses were retained since Inca-No-Mezame has dark nodal bands. Information on the early development of MSEE815-06 was scarce and 37 with nodal bands were retained as diploid controls.

3.4.3 *Guard Cell Counts*

The number of plastids within guard cell pairs of potential monoploids were evaluated to determine if they were above or below the threshold expected of monoploids. Eighty-four plants from maternal crosses had guard cell counts of or under 11 per pair (5.5 per guard cell). Monoploid progeny of adapted diploids were identified with counts under 5.5 per guard cell (Table 6.) All categories had some progeny with guard cell counts under the threshold except for one. No progeny from *S.microdontum* had guard cell counts of or under 11 per pair (Table 7.)

Table 6. Average (n=10) number of plastids per guard cell with standard deviation of identified monoploids and their maternal parent. Family ME1x0032 is derived from BD1250-1 and family ME1x0021 is derived from BD1222-1

Line	Avg # Plastids Per Guard Cell
ME1x0032-27	4.3 ± 1.33
ME1x0032-17	3.6 ± 0.70
BD1250-1	5.75 ± 1.16
ME1x0021-19	3.7 ± 1.00
ME1x0021-25	3.6 ± 1.11
BD1222-1	6.05 ± 1.17

Table 7. Average (n=10) number of plastids per guard cell with standard deviation from *S.microdontum* failed induction crosses.

Line	Avg # Plastids Per Guard Cell
ME1x0031-01	5.75 ± 0.78
ME1x0031-02	5.6 ± 0.73

A total of 96 screened plants including four of the diploid parents had guard cell pair counts lower than the threshold of 11 (5.5 per cell). Three aneuploids, identified using SolCal SNP data, were excluded. A Welch's T test was performed comparing the population of monoploids (n=5) and the population of diploids (n=88). The 95% confidence interval for the difference in means between the two groups ranged from 1.85 to 4.21. The average guard cell count for the diploid group was 10.29, while the average guard cell count for the monoploid group was 7.26. The diploid and monoploid populations were significantly different ($t(4.21) = 6.99$, $P < .002$). Monoploids ranged from 5.9 to 8.6 plastids per guard cell pair, only one diploid fell within this range with 8.4 plastids per guard cell pair.

3.4.5 *SolCAP Infinium Single Nucleotide Polymorphism Array*

Two previously documented monoploids were obtained from the USDA-GRIN collection to provide a reference for monoploid identification; the expected values of 0 and 4 were observed as well as fewer than 100 calls of 1 and 3 (fig 5.) Using this reference four monoploids were identified from the adapted diploid category that had values within the expected range. No monoploids were identified from any other category. The monoploids identified were from two families of the adapted diploid ME1x0021

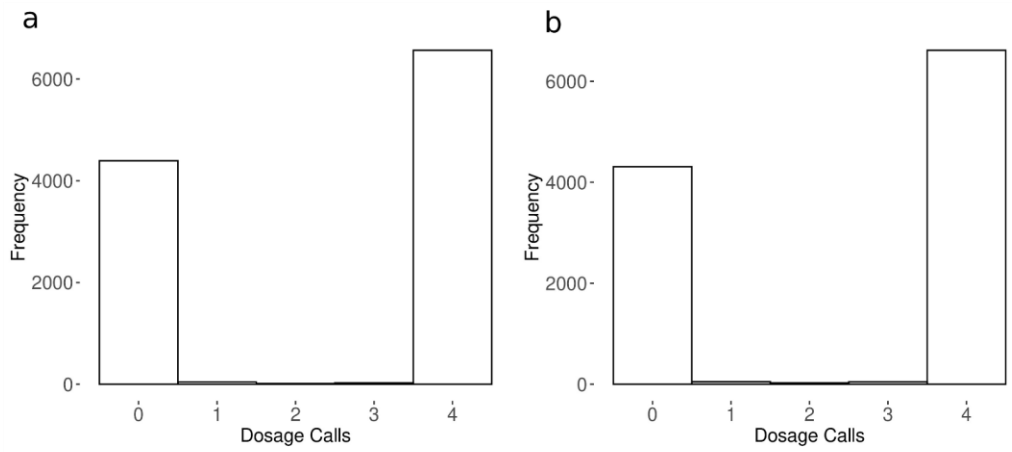

Figure 5. Histograms of tetrasomic dosage call frequencies from previously documented monoploid potato lines derived from the SolCAP Infinium array. 5a. 08-1 (GS 222) displaying frequencies of 0 and 4 5b. T5-268 (GS 221) displaying frequencies of 0 and 4.

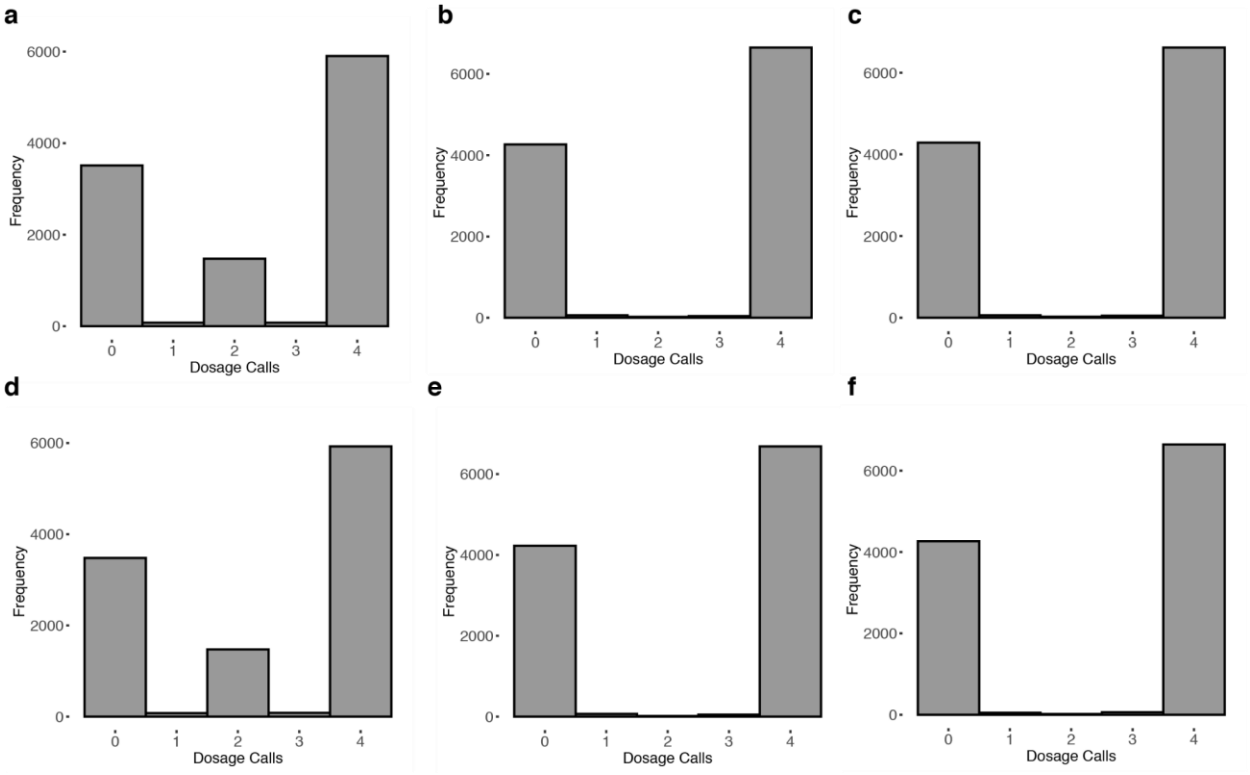

Figure 6. Histograms of tetrasomic dosage call frequencies from adapted diploid parents and their corresponding monoploids. (a) Histogram plot from BD1250-1, the maternal parent of ME1x0032 family displays dosage calls of 0, 2 and 4 as expected of a diploid. (b) ME1x0032-17 and (c) ME1x0032-27 monoploids only display dosage calls of 0 and 4. (d) Histogram plot from BD1222-1, the maternal parent of ME1x0021 family displays dosage calls of 0, 2 and 4 as expected of a diploid. (e) ME1x0021-19 and (f) ME1x0021-25 monoploids only display dosage calls of 0 and 4.

3.4.6 *Dosage calling plots for aneuploid identification*

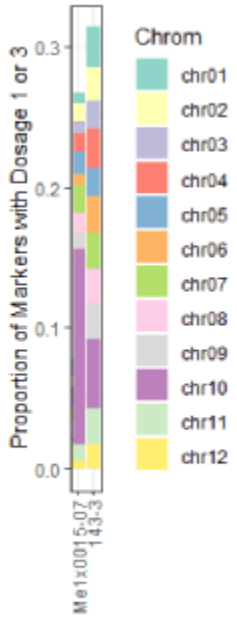

Figure 7. Dosage markers of 1 or 3 of aneuploid ME1x0015-07 and reference diploid 143-3. Chromosome 10 in ME1x0015-07 has a greater proportion of markers of 1 or 3 in chromosome 10 than across the other eleven chromosomes.

Using the PolyBreedR Geno4x data, chromosomes with higher proportions of markers of 1 or 3 were visualized. Three aneuploids were identified through this method, one of which ME1x0015-07 survived. Aneuploid ME1x0015-07 shows an increased proportion of markers with dosages of 1 or 3 in chromosome 10 (fig 7).

3.4.7 *Metaphase chromosome counting from root squash*

Root squashes were performed on ME1x0032-27 and ME1x0015-07 for cytogenetic characterization of mitotic metaphase chromosomes. This method confirmed that ME1x0032-27 has 12 chromosomes. Although only one metaphase cell was observed from the root squash of ME1x0015-07, between 10 and 13 chromosomes were visible, suggesting that this line is most likely an aneuploid of a monoploid ($2n + 1 = 13$).

3.4.8 *Monoploid Fertility*

Figure 8. ME1x0032-27 flower at anthesis with petals removed. The anther size is reduced.

ME1x0032-27 had no detectable pollen shed (n=6) and displayed shortened anthers associated with male infertility (Fig 8.).

3.4.9 *Doubled monoploid production*

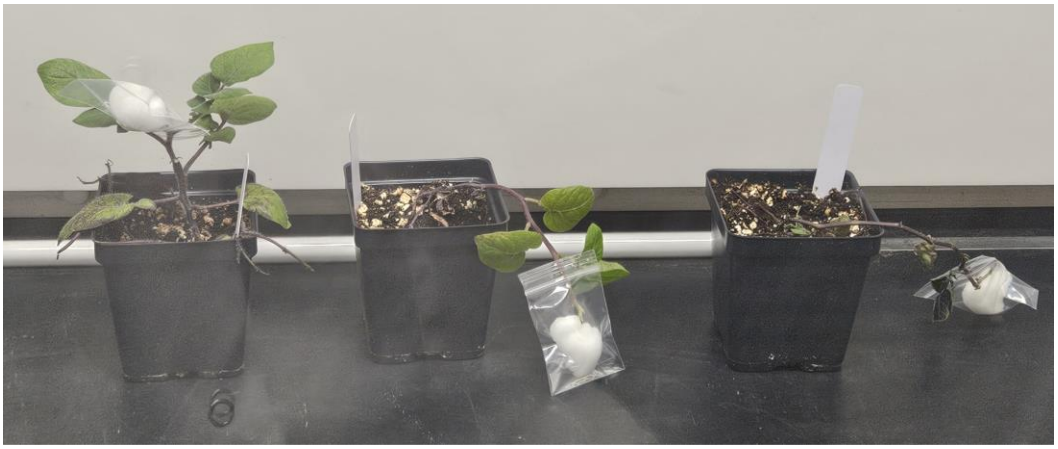

Figure 9. Three diploid potato plants undergoing colchicine treatment. Cotton balls saturated in 0.25% colchicine have been placed over decapitated axillary buds.

Figure 10. ME1x0032-27 undergoing revised colchicine treatment. Cotton balls saturated with a 0.25% aqueous colchicine solution are taped over three nodes

As a reference point, a small subset of diploid plants derived from failed haploid induction crosses underwent treatment prior to and in addition to the monoploids vigorous enough to undergo treatment (fig 9). Using the revised method shown in figure 10, the breakdown of colchicine treatment by genotype was as follows: ME1x0032-27 3 plants, 3 apical tips and 27 nodes, ME1x0036-20 2 plants, 3 apical tips and 6 nodes, diploid, 1 apical tip and 7 nodes. None of the three diploid axillary buds from the initial trial regenerated. Two shriveled, and one failed to regenerate. Regeneration has not yet occurred from the revised in vivo method. 22 days post planting initial regeneration can be seen in the in vitro trials in figure 11.

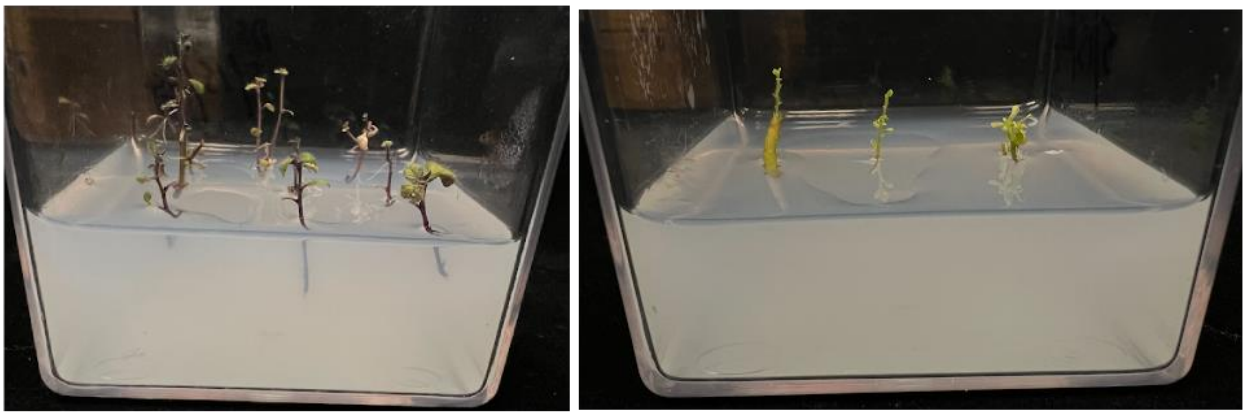

Figure 11. Monoploids 22 days post planting in colchicine media. On the left, nine cuttings of ME1x0036-22, new nodal growth and aerial roots are visible but there has been no regeneration

from below the media level. On the right two cuttings and one miniature tuber of ME1x0032-27, 22 days post planting in colchicine media.

3.4.10 *Evaluation of cytoplasm types from diploid parents*

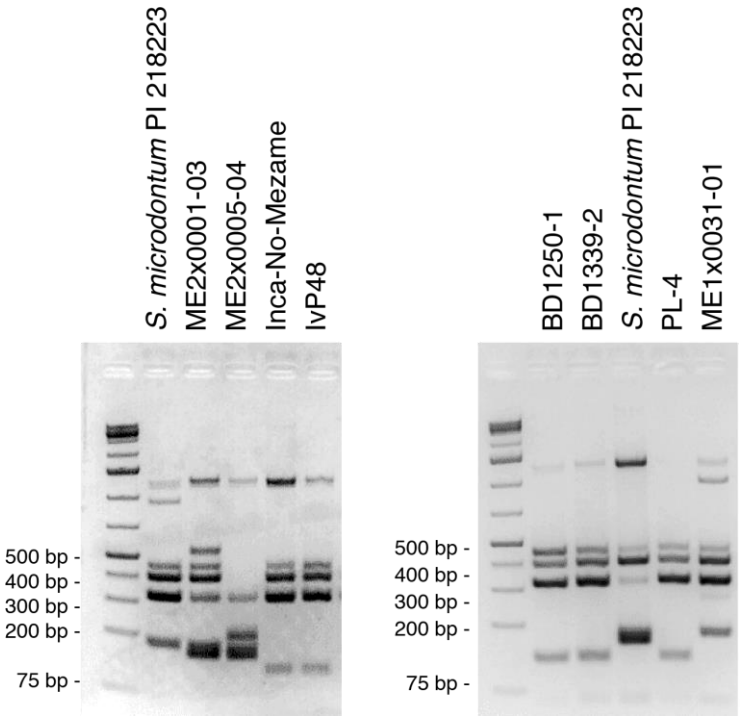

Figure 12**.** Multiplex PCR for the identification of W-, T-, M-, A-, and P-type cytoplasm using S, A, D, T and SAC markers on a 3% agarose gel in 1X TAE buffer. The far left lane on each panel shows the GeneRuler 1kb Plus DNA ladder followed by DNA from *S. microdontum* PI 128223, ME2x0001-03, ME2x0005-04, Inca-No-Mezame, IvP48, BD1250-1, BD1339-2, PL-4, and ME1x0031-01. Bandings indicative of A-type cytoplasm can be seen with *S.microdontum* (PI 128223) and ME1x0031-01. Banding indicative of D-type cytoplasm can be seen with ME1x0001-03 and T-type cytoplasm indications can be seen with ME1x0005-04. Bandings indicative of P-type cytoplasm are displayed on Inca-No-Mezame, IvP48, BD1250-1, BD1339-2 and PL-4.

Multiplex PCR was used to identify the cytoplasm type of the parents. Based on data obtained from multiplex PCR for potato cytoplasm, the banding patterns of Inca-No-Mezame, Ivp48, BD1250-1, BD1339-2 and PL-4 are consistent with P-type cytoplasm. ME1x0001-03 displays the band associated with D-type cytoplasm, and ME1x0005-04 displays the band associated with T-type cytoplasm. *S.microdontum* in the first replicate and its daughter Me1x0031-01 display both bands indicative of A-type cytoplasm, the second replicate of *S.microdontum* only displays one of the bands indicative of A-type cytoplasm (figure 12)

3.5 *Discussion and Conclusion*

Haploid induction of diploid potato to generate monoploids offers breeders a tool for the production of inbred lines in a single generation. This work provides a feasible workflow that tests the most recent diploid germplasm, using current technologies and techniques to produce monoploids within a single crossing season. Coupled with established root squash chromosome counting methods, we show that an efficient phenotyping pipeline to identify monoploids was achievable in the span of a few months. In addition, our work suggests that it might be prudent to first screen for cytoplasm type of diploid lines before inducing monoploids from breeding lines.

Haploid induction was only successful in one of the populations that were screened. We were not able to recover monoploids from the first generation and selected clones from *S. tuberosum* Group *Tuberosum* and other diploids such as *S. microdontum* and Inca-No-Mezame. HI crosses from most lines resulted in fruit set and unspotted seeds were identified and grown out. Earlier emasculation may be useful for male fertile lines with T-type cytoplasm. Minor risks

of contamination from pollinators and insufficiently cleaned tools may have also played a part in false negatives with unspotted seeds but the expected incidences of spotted and shriveled seeds. The sample size was insufficient for the first generation *S. tuberosum* Group *Tuberosum* with only 172 pollinations performed, further subdivided by the two families having different cytoplasm types. A larger sampling of genotypes across both present cytoplasm types, D-type and T-type would allow for more confidence before discarding this group from future induction cross trials.

The adapted diploid population had a greater number of pollinations across a greater number of genotypes, all within the same cytoplasm type, P-type. Four monoploids were identified from this population and all had P-type cytoplasm. Phureja-type cytoplasm was the best represented cytoplasm in the diversity of varieties trialed and absolute number of pollinations. Adapted diploids seem to represent the best pool for developing monoploids. Introgression of other diploid stock of interest with adapted diploids seems to be a route forward. The heritability of haploid induction rates is unknown. Introgressions of other trialed varieties with adapted diploid lines may be a route for creating germplasm better suited for haploid induction. Currently undergoing single hill trials are 250 Inca-No-Mezame by BD1339-2 progeny and 100 Inca-No-Mezame by BD1250-1 progeny. Inca-No-Mezame progeny appeared to have restored anther size and pollen shed. No detailed analysis of fertility was performed. BD1339-2 was crossed with both of the fertile *S.microdontum* producing 250 offspring from the and 10 from the other. It was also successfully crossed with ME2x0005-4 producing 20

offspring. These progenies would have P-type cytoplasm and may have improved fertility traits.

Low sample size limited all other trialed populations. Haploid induction crosses failed to produce monoploids in *S. microdontum*; however, a factor to be considered when growing out a crossing population of PI 218223 is the incidence of crazy sepal, a mutation that prevents normal flower development. One of three plants grown out displayed this mutation reducing the trial size. With a reported incident rate in PI 473166 of 16% growing out an additional plant per five plants may be warranted (Bamberg, 2006). General fertility was not a concern, both individuals without the mutation readily hybridized with the USDA adapted diploid line BD1339-2. F1's from this population are undergoing single hill trials at the USDA farm in Chapman, Maine for the 2024 season.

The effective use of embryo rescue for monoploid production in cucurbits and *Arabidopsis thaliana* provides precedent in other crop systems (Hooghvorst & Nogués, 2020, Ravi & Chan, 2010). Embryo rescue did not result in increased monoploid production from the genotypes trialed for maternal haploid induction crosses. Losses may have been higher than was inherent to the condition of the seeds or non-viable genotypes. Shriveled seeds faced higher rates of damage, loss or entanglement than unspotted seeds requiring greater care when handling. Seeds were also exposed to hot humid conditions and stagnant air for a period of several days due to HVAC failures. Contamination of germination plates occurred several times appearing in some cases to be deleterious to germination. The sample size was also very low, only 39 plants

derived from shriveled seeds survived to be assessed for guard cell counts. Embryo rescues across maternal and paternal crosses only netted one additional monoploid at the expense of increased labor and material cost. The sample size is insufficient to draw conclusions on whether on a larger scale in better conditions embryo rescue could increase monoploid yield. The route forward with embryo rescues is unclear.

It may be possible to raise the threshold when screening for monoploids to streamline the process. Diploids and monoploids guard cell counts do overlap, and there is no monoploid data for predominantly *S.tuberosum* Group *tuberosum* genotypes. Also the 95% confidence interval would include all diploid and monoploids analyzed. There is not enough information to raise the threshold and increase the intensity of selection without risking the loss of monoploids, however if a threshold of 9 chloroplasts per pair of guard cells was set for this experiment there would have been no losses.

Another area for streamlining the process is using PolyBreedR for identifying monoploids. Using the methods described here PolyBreedR can be used to quickly sort out monoploids and aneuploids with minimal training. Root squashes represent a more significant investment of labor and are a necessity for sorting monoploid and diploid aneuploids, but the reliability of PolyBreedR means perhaps they could be eschewed with clear monoploids.

Haploid induction to the monoploid stage holds the potential to expedite Potato 2.0 producing inbred lines in a single generation (Bamberg, 2022 ; Jansky, 2016). This study demonstrates existing technologies and methodologies make monoploid production feasible in

one crossing season and manageable within the career of a single masters student. P-type cytoplasm and the adapted diploid population represent the most promising cytoplasm type and population for future haploid induction trials. Challenges of low sample size and tissue culture contamination need to be overcome for future production. Embryo rescues produced one additional monoploid supporting their use in monoploid production, but with caveats around labor and material costs. Future trials may be able to optimize selection thresholds with guard cell counts and streamline the identification process with PolyBreedR.

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