THESIS

CHARACTERIZING THE LINK BETWEEN CYTOPLASMIC MALE STERILITY AND PVY RESISTANCE IN POTATO

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ABSTRACT

CHARACTERIZING THE LINK BETWEEN CYTOPLASMIC MALE STERILITY AND PVY RESISTANCE IN POTATO

Cytoplasmic male sterility (CMS) in potatoes is a common reproductive issue in *Potato Virus Y* (PVY) breeding programs, as resistant sources typically have a wild cytoplasmic back ground (W, D, or T). In the San Luis Valley breeding program, the cytoplasmic types and PVY resistant clones' density are unknown. Similarly, the effects of these cytoplasmic types and resistance genes on reproductive traits have not been determined.

This study aimed to characterize the cytoplasm types and investigate cytoplasm types and resistance genes' effects on pollen viability, pollen quantity, and seed set. The presence of T and W cytoplasm types was determined among the seven clones, with W type being more prevalent. This finding indicates that PVY resistant genes are concentrated between only these two cytoplasmic types. While the analysis revealed an impact of the cytoplasm types and genes on pollen viability, no significant differences were found in pollen quantity or seed set.

All clones exhibited absent or low pollen viability and produced either absent or minimal amounts of pollen, suggesting low male fertility levels. The observed differences in pollen viability and seed set are thought to stem from difficulties in crossing and the influence of the female clones used. Notably, no berries were obtained from clones carrying the Ry_{sto} gene with W type cytoplasm, indicating their complete male sterility.

The characterization of these cytoplasm types and the male sterility status will influence breeders in designing efficient mating combinations. They will contribute to sustainable seed production, which requires a substantial quantity of seeds per cross.

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TABLE OF CONTENTS

CHAPTER 1: LITERATURE REVIEW

Introduction

The potato (*Solanum tuberosum L.*) originates from South America (Kumar Chakrabarti et al., 2017). Potatoes were first cultivated around 200 B.C. in the Andes Mountains of South America by the Inca civilization (How the Potato Changed the World’s History, 2024).The Andes Mountains are home to nearly 4,000 genetic varieties (How the Potato Changed the World’s History, 2024). Europeans were introduced to potatoes in the 16th century. Introduced to their countries by the Spanish in 1533, potatoes subsequently spread to France in 1540, England, Ireland, and Scotland, and later to other European countries and North America (Hawkes & Francisco-Ortega, 1993).

Potato Production in the World

The potato has had a profound impact on global society (De Jong, 2016). Today ranked as the world's third-largest food crop, following wheat and rice, so it plays a vital role in global food security by feeding the world (FAO, 2021). According to FAO's data in 2022, the global potato production was 375 million tonnes. In 2022, the total harvested area worldwide was 17.8 million hectares, down 3 percent from the 2021 crop (FAO, 2022). China and India were the top producers, with China contributing 95.5 million tonnes and India 56 million tonnes (FAO, 2022). The U.S. produced 17.8 million tonnes of potatoes in 2022, making it the fifth-largest producer globally (FAO, 2022).

Potato Production in the USA

Potatoes remain the leading vegetable crop in the United States, with commercial cultivation occurring in 30 states (USDA, 2023). Idaho is the top producer with almost 5.5 million metric tons of potatoes in 2022. Washington ranks as the second-largest producer with about 4 million metric tons(USDA, 2023). Wisconsin, Oregon, North Dakota follow them in that order almost 1.5 million metric tons, 1.1 million metric tons and 1 million metric tons. In 2022, Colorado had nearly the same potato production as North Dakota, with a production of 990.00 metric tonnes, and was ranked 6th in production (USDA, 2023).

The Potato as a Global Food

Potatoes are the most important food crops worldwide, owing to their high yield and nutritional value (Zaheer & Akhtar, 2016).This versatile and adaptable tuber originated in the high Andes of South America and has since spread to various elevation zones in temperate regions across all continents (Zaheer & Akhtar, 2016).Advances in breeding and the development of pigmented varieties, which are rich in antioxidants and phytochemicals, have further solidified the potato's status as a significant and diverse crop in terms of both production and consumption (Zaheer & Akhtar, 2016).The economic factors contributing to the popularity of potatoes are noteworthy. High food prices have been influenced by increasing rising costs of biofuels and fossil fuels (Beals, 2019 and Avendano, 2012).Their affordability, compared to other staple foods, makes them a crucial dietary resource in economically disadvantaged regions (Beals, 2019 and Avendano, 2012).Therefore, potatoes play a crucial role in global nutrition and health. They are used not only as a source of livelihood but also as a fundamental food item that provides essential nutrients (Zaheer & Akhtar, 2016). Compared to other grains, potatoes offer higher levels of carbohydrates, protein, and minerals, making them a vital component of the diet in many regions (Zaheer & Akhtar, 2016). Therefore, potatoes play an important role in addressing global food security challenges (Zaheer & Akhtar, 2016).

Potato Nutritional Value

Potatoes are considered starchy vegetables, which leads to a focus on their contribution as a source of carbohydrates (Beals, 2019). Moreover, potatoes are an excellent source of essential vitamins and minerals which are potassium, magnesium, and iron (Wijesinha-Bettoni & Mouillé, 2019).They are particularly rich in vitamin C, which plays a crucial role in supporting immune function, promoting healthy skin, and enhancing iron absorption (Potato USA,2024). Potatoes offer 27 mg of vitamin C, which constitutes 45 percent of the daily recommended value (Potato USA,2024).Vitamin C is a water soluble vitamin that plays an important role in protein metabolism and acts as an antioxidant by reducing cellular damage and limiting the unwanted effects of free radicals (National Institutes of Health, n.d.). Additionally, it supports collagen synthesis, which is essential for maintaining healthy gums and facilitating wound healing (Brown, 1999). Reseach has shown that potatoes are among the top five dietary sources of vitamin C for Americans (Beals,2019). This highlights their significant role in providing this essential nutrient in the American diet.

Potatoes are a source of vitamin B6 which is important for energy metabolism and the formation of red blood cells. (Wijesinha-Bettoni & Mouillé, 2019). Potatoes are crucial for various metabolic processes, particularly in carbohydrate and protein metabolism by providing 10% of the daily value of B6 (Potato USA,2024). Potatoes are a superior source of potassium compared to other foods often considered high in potassium, such as bananas and oranges (Beals,2019). When including the skin, potatoes provide 15% of the daily recommended value of potassium (Potato USA,2024). Potassium helps regulate blood pressure, thus alleviating the effects of high sodium consumption that can lead to high blood pressure (Centers for Disease Control and Prevention, n.d.). It also supports heart health (Potatoes USA, 2024).

Magnesium is one of the nutrients that is commonly under-consumed by Americans. A medium-sized potato (5.3 oz), including its skin, provides approximately 48 mg of magnesium, contributing roughly 5% to the total magnesium intake in the American diet(Beals, 2019). his contribution is significant, as magnesium plays a crucial role in supporting muscle and nerve function, among other physiological processes (Beals, 2019).A medium-sized potato contains 2 grams of dietary fiber, which constitutes approximately 8% of the recommended daily value for fiber (Potatoes USA,2024). Dietary fiber not only promotes a sense of satiety, aiding in weight management but also functions as a carbohydrate that helps regulate blood glucose levels (Potatoes USA, 2024).

In addition to vitamins and minerals, potatoes also contain a variety of phytochemicals with antioxidant properties, most notably carotenoids and anthocyanins (Brown, 1999).The total carotenoid content of potatoes varies significantly, ranging from 35 µg to 795 µg per 100 g of fresh weight (Brown,1999). Dark yellow cultivars contain approximately ten times more total carotenoids than white-flesh cultivars (Beals, 2019).For instance, Reddivari et al. (2007) examined the effects of a purple potato anthocyanin fraction on the proliferation of prostate cancer cells. The results indicated that the anthocyanin fraction resulted in a decrease in cancer cell proliferation Reddivari et al. (2007).

Objectives in Potato Breeding Program

With more than 10,000 cultivated types, potatoes are among the most diverse crops in the world (Dobnik et al., 2021). Over the past 45 years, the International Potato Centre (CIP) in Latin America has collected a remarkable 17,326 accessions of local potato varieties(International Potato Center, n.d.). By using selective breeding techniques that capitalize on natural diversity, these varied potato cultivars have been created over centuries (Dobnik et al., 2021).

It is essential to establish precise breeding goals that emphasize both qualitative and quantitative aspects is crucial when planning a potato breeding program in order to create new varieties with desired qualities (Zhang et al.,2022).The following characteristics are taken into account in potato breeding programs: One of the main objectives of potato breeding is yield. To meet the increasing demand for potatoes as a staple food worldwide, high-yielding varieties are crucial (Zhang et al.,2022).

The other one is Tubers' quality for processing and cooking which are glycoalkaloid content, damage resistance, hollow heart and tuber cracking, and sensitivity to greening (Wayumba et al.,2019).Abiotic stress-related characteristics include resistance to heat stress or high temperatures, resilience to drought or water stress, and resistance to cold stress like sweetening of tubers during cold storage, or tolerance to frost (Dobnik et al., 2021).Biotic stress focuses on resistance to pests and diseases. Sustainable potato production requires breeding for resistance to nematodes, viral infections, late blight, and other bacterial and fungal diseases (Dobnik et al., 2021).

Breeding with R genes for Resistance to *Potato Virus Y*

With its global production reaching 375 million tons (FAO,2022), it holds great importance in the food institute and international trade, providing livelihoods for farmers and contributing to the economies of many countries (FAO, 2022 and 2023). However, despite the numerous advantages of potatoes, they also face challenges, including pests and disease threats that can affect their production and sustainability (Zhang et al., 2022).

Among these threats, PVY is recognized as a significant viral pathogen for potatoes, causing decreases in both yield and quality(Lacomme et al., 2017). And this virus has spread rapidly through infected seeds, tubers, and vector aphids (Lacomme et al., 2017). Some methods to control PVY include rotation practices, selecting certified virus-free seeds, implementing pest management techniques, and using resistant varieties(Lacomme et al., 2017).However, developing resistant varieties is one of the effective long-term solutions because it reduces the use of pesticides and other chemicals, helps prevent the spread of the disease through tubers, and aids in preserving the quality of potatoes (Lacomme et al., 2017).

In potato breeding, the identification and utilization of dominant resistance genes, commonly referred to as R genes, are essential for developing cultivars that can withstand important diseases, particularly PVY (Dobnik et al., 2021), which can cause quality deterioration and yield reductions (Solomon-Blackburn & Barker, 2001; Singh et al., 2008). And this virus has spread rapidly through infected seeds, tubers, and vector aphids (Lacomme et al., 2017). To control PVY, some methods include rotation practices, selecting certified virus-free seeds, implementing pest management techniques, and using resistant varieties. However, the generation of resistant cultivars is regarded as the most cost-effective and environmentally friendly approach to managing viral diseases in potato crops (Solomon-Blackburn & Barker, 2001).

Potato germplasm has identified four *R* genes, namely Ry_{adg}, Ry_{sto}, Ry_{chc} and $Ry\text{-}f_{sto}$ conferring extreme resistance to all known strains of PVY, derived from *Solanum tuberosum* ssp. *andigena*, *Solanum stoloniferum*, *Solanum chacoense* and *Solanum stoloniferum,* respectively (Nie et al., 2004), and molecular markers associated with these genes have been developed and are accessible for use. The molecular markers for detecting the Ry_{adg} gene, derived from *Solanum tuberosum* ssp. *andigena* and located on chromosome XI, encompass RYSC3, and RYSC4 (Kasai et al., 2000). Likewise, for identifying the Ry_{chc} gene from *Solanum chacoense*, positioned on chromosome IX, the marker 38–530 are employed (Hosaka et al., 2001). Additionally, for the Ry_{sto} gene originating from *Solanum stoloniferum*, found on chromosome XI, the markers GP122, STM003, and YES3-3B are utilized (Kasai et al.,2000; Song et al., 2005; Valkonen et al.,2008), and and for *Ry-f_{sto}* derived from *Solanum stoloniferum*, located on chromosome XII, the marker GP122718 is employed (Flis et al., 2005). The success of a potato breeding program is not solely dependent on the use of resistance genes (*R* genes). It also relies on factors such as the expression of various resistance sources and other agronomic and morphological traits in progeny under specific breeding conditions, as well as the suitability of intra-species crossbreeding, including considerations such as male sterility (Dobnik et al., 2021).

The Role of Cytoplasmic Male Sterility in Potato Breeding Programs

Cytoplasmic male sterility (CMS) is observed in various plant species. This condition is thought to arise from disruptions in the cell's interactions between nuclear and cytoplasmic elements (Rena et al., 2021).The mitochondrial genome is crucial in this process, acting as a cytoplasmic component contributing to the observed male sterility (Sanetomo & Gebhardt, 2015).Consequently, male sterility trait is transmitted to progeny via maternal inheritance when they inherit the mitochondria responsible for the condition (Sanetomo & Gebhardt, 2015).

The issue of male sterility poses challenges that hinder the effectiveness of breeding strategies aimed at improving characteristics such as yield and disease resistance, particularly in the context of true seed production in potatoes (Santayana et al., 2022).Cross-bred true potato seed (TPS) is acknowledged for enhancing genetic diversity and is distinguished by its improved seed quality, increased seedling vigour, and higher tuber yield (Gopal, 1993).

Male sterility, which adversely affects pollen viability and the overall reproductive cycle of the potato plant, represents a significant issue in breeding programs focused on true potato seed production (Santayana et al., 2022). The issue of male sterility significantly restricts the selection of male parents, as many varieties cannot be used as male parents because they are unable to produce viable pollen (Rena et al., 2021). This limitation diminishes genetic diversity (Kemble & Shepard, 1984).

Additionally, wild potato species have been incorporated into potato breeding programs due to their beneficial traits, such as resistance to pests and diseases (Rena et al., 2021). However, despite their many advantageous features, these species often possess cytoplasmic backgrounds that can cause male sterility (Rena et al., 2021).Nonetheless, with resistance breeding, there has been an increase in the prevalence of cytoplasmic factors that induce male sterility within the cultivated potato gene pool (Rena et al., 2021).

Another problem caused by male sterility is the resulting genetic uniformity (Hosaka & Sanetomo, 2012).Mitochondrial and chloroplast-specific DNAs are present in the cytoplasm and are inherited exclusively from the female parent in most plant species (Sanetomo & Gebhardt, 2015). Consequently, many varieties are used as females due to male sterility. As a result, certain types of cytoplasm have become more prevalent in the potato gene pool (Kemble & Shepard, 1984).This limitation leads to genetic uniformity within the heritable cytoplasmic elements (Kemble & Shepard, 1984).

The most comprehensively documented example of issues related to cytoplasmic gene function has been observed in southern corn leaf blight (Kemble & Shepard, 1984).Texas (T) male-sterile corn cytoplasm increases susceptibility to toxins produced by the fungal pathogen *Helminthosporium maydis* race T (Kemble & Shepard, 1984).This susceptibility has been shown to be significantly influenced by mitochondrial membranes and genes encoded in mitochondrial DNA (mtDNA) (Sanetomo & Gebhardt, 2015). By the 1970s, 85% of hybrid corn in the United States contained T cytoplasm, which led to one of the most severe disease epidemics in corn history (Kemble & Shepard, 1984). Ullstrup (1972) concluded from this experience that "a major cultivated species should never be bred to such uniformity that it becomes universally vulnerable to pathogen attack, insect infestation, or environmental stress. It is essential to maintain diversity in both the genetic and cytoplasmic makeup of all significant crop species."

Diverse Types of Cytoplasmic Male Sterility in Potatoes

Breeders encounter a significant challenge when dealing with hybrids and varieties resistant to PVY and this is cytoplasmic male sterility (Hosaka & Sanetomo, 2012).In plant cells, chloroplast and mitochondrial DNA exist in multiple copies, alongside a single copy of nuclear DNA and this unique genomic arrangement implies that nuclear gene expression and function can be influenced in various ways by the genes present in chloroplasts and mitochondria (Sanetomo & Gebhardt, 2015). A notable example of such an interaction is cytoplasmic male sterility, a condition frequently observed in crop species, which arises from the interplay between nuclear and mitochondrial genes (Rena et al., 2021).This phenomenon underscores the complex interdependence among different genomic compartments and their collective impact on plant fertility and development (Sanetomo & Gebhardt, 2015). Potato (*Solanum tuberosum L.*) is a crop that exhibits various types of cytoplasmic genomes within a cultivar group and cytoplasmic genome types in potatoes can be classified into several distinct categories, including A, M, P, T, W, and D types (Goryunova et al., 2023). Three of these types (T, W, and D) are linked to male sterility in specific hybrid combinations (Goryunova et al., 2023).

Each of these cytoplasmic is associated with specific traits that which can influence breeding strategies and performance. The A-type cytoplasm is most common type found in *Solanum tuberosum* ssp. *andigena* (*S. tuberosum* group *Andigena*). P-type cytoplasm was derived from *Solanum phureja* (*S. tuberosum* group *Phureja*) (Goryunova et al., 2023). The M-type cytoplasm represents an ancestral form from which the P- and A-type cytoplasms are derived; both

P- and A-type cytoplasms are considered subtypes of the M-type cytoplasm. This M-type cytoplasm has been identified in various wild and cultivated potato species. Currently, M-type cytoplasm appears to be the rarest form within the modern gene pool (Goryunova et al., 2023).

The predominant cytoplasmic type found in contemporary potato varieties is known as the "Chilean" or T-type and this type is characteristic of *Solanum tuberosum* subsp. *tuberosum* (including *S. tuberosum* cultivars from the Tuberosum group, 2n=4x=48) and their ancestral Chilean landrace varieties from the Chilotanum group (Goryunova et al., 2023). The reason for the dominance of this type of cytoplasmic genome in the potato gene pool, supported by selection, is its economically beneficial traits(Goryunova et al., 2023). In classical breeding, the cytoplasmic genome of *S. tuberosum* subsp. *tuberosum* (primarily T-type cytoplasm) has been reported to be associated with higher tuberization rates, increased tuber yield, greater tuber numbers, and earlier vine maturity (Goryunova et al., 2023).

However, it is characterized by T-type chloroplast DNA and β-type mitochondrial DNA and this T/β cytoplasm is predominant in this potato species, and male sterility is an expected problem (Hosaka & Sanetomo, 2012). T-type cytoplasm is associated with various abnormalities in the development of reproductive organs, such as reduced pollen viability, withering, non-opening anthers, shriveled or indehiscence anthers and poor flowering, which contribute to the plant's fruiting insufficiency (Goryunova et al., 2023). It has been noted that one-third of varieties with T-type cytoplasm were unable to develop berries (Gavrilenko et al., 2019). Through the study of the genetic control of fertility restoration, a nuclear *Rf* gene that can lead to the recovery of male fertility in T-type cytoplasm carriers has been identified ; however, the *Rf* gene has not been studied at the molecular level (Santayana et al., 2022).The D-type cytoplasm was introduced into the potato gene pool through crosses with the hexaploid wild Mexican species *Solanum demissum* (Sanetomo

& Hosaka, 2011).This wild species has been extensively utilized in breeding programs as a source of resistance to late blight, caused by the pathogen *Phytophthora infestans*, which is one of the most devastating diseases affecting potato crops globally (Dufková et al.,2023). Potato samples possessing the D-type cytoplasm exhibit a unique characteristic: functional sterility of pollen (Hosaka & Sanetomo, 2012).In these plants, while the pollen grains appear morphologically normal and can be well-stained, they are non-functional, meaning they are incapable of fertilization(Hosaka & Sanetomo, 2012).The W/γ-type cytoplasm is derived from the wild species *Solanum stoloniferum* which is one of the most common species of wild potatoes in North and Central America (Rena et al., 2021).

Solanum stoloniferum carries the nuclear Ry_{sto} gene which is known for providing extreme resistance to multiple strains of PVY(Hosaka & Sanetomo, 2012). But the W/γ-type cytoplasm exhibits the most pronounced sterilizing effect caused by associaition with the characteristic mtDNA derived from *S. Stoloniferum*, characterized is called "tetrad sterility" or "lobed sterility" (Hosaka & Sanetomo, 2012).Because sterile pollen grains are grouped together in tetrads, they have a four-lobed appearance and tetrads do not disintegrate in the end of microsporogenesis (Sanetomo & Gebhardt, 2015) (Gavrilenko et al., 2019).

The use of metabolic profiling methods in anthers has been demonstrated that tetrad sterility is associated with a sudden disorder in carbonic exchange in anthers, particularly in the polysaccharide spectrum and amino acid and fatty acid metabolism (Gavrilenko et al., 2019). In T/β and W/γ cytoplasms, male sterility is characterized by visible abnormalities, including various deformities of pollen and anthers and these include a complete absence or significant reduction in pollen shedding and deformities in pollen and anthers (Hosaka & Sanetomo, 2012).

Reproductive Traits in Potatoes: Flowering Behavior, Pollen Production, and Breeding Challenges

Flowering

Potatoes have various flowering characteristics, and their inflorescences are usually composed of 7 to 15 flowers, although some genotypes can bear up to 30 flowers (Muthoni et al., 2012). Each flower is characterized by five sepals and five petals that form a crown that can take a star-like or round shape depending on the particular genotype flowers (Muthoni et al., 2012). The color of the flowers, resulting from genetic factors, can vary from white to blue and purple (International Potato Center, 2017).In the potato flower, five stamens are attached to the corolla tube and in some species, the anthers responsible for producing pollen exhibit bright yellow or purple pigmentation (International Potato Center, 2017).

However, pale yellow or yellow-green anthers are an indication of male sterility, which affects the plant's ability to produce viable pollen(Ordoñez, 2014). Flowering behavior in potatoes is determined by genetics and environmental factors such as photoperiod, light intensity, and temperature, and these factors affect the success of flowering and tuber development (Bethke & Jansky, 2021). There are early-maturing and late-maturing genotypes in potatoes. For early-maturing genotypes, flowering usually begins 39 to 45 days after planting (DAP), with peak flowering occurring at approximately 60 DAP (Potato International Center, 2021). In contrast, late-maturing genotypes can reach their peak flowering at 90 to 100 DAP. Photoperiod greatly affects flowering in potatoes. The best conditions for healthy flower production are a 16-hour photoperiod and night temperatures of 15–20 °C and short days can lead to flower drop (Potato International Center, 2021).

Pollen production and 2n gametes

In potatoes, pollen production is the product of plant microsporogenesis and carries the genetic information needed for the next generation. Potatoes have bilobed stigma emerging from between the anthers. Viable and vigorous pollen successfully germinates on the stigma. It travels through the style to the ovary, fertilising the egg cell (n) to produce a diploid embryo (2n), which will develop into a new plant (Ordoñez et al., 2014; Potato International Center, 2021).

Keeping the pollen alive is one of the essential points for genetic analysis and healthy reproduction of the plant, and for this, pollen should be collected from mature anthers. Anther dehiscence occurs 3 -5 days after anthesis. Anthers with bright yellow flesh and brown tips are indicators of mature anther and good pollen sources. Pollen should be stored in cool and dry areas to maintain its vitality. Pollen can maintain its viability for approximately one month when stored at 2.5°C and up to a year at –24°C (Ordoñez et al., 2014; Potato International Center, 2021).

Reproductive barriers

There are obstacles in the potato plant that affect its ability to reproduce effectively. One of these is that unlike wild potato relatives or specific landraces, advanced tetraploid clones do not flower sufficiently. When they do flower, they usually show a problem of male sterility and these plants typically have low or non-viable flower production or low or non-viable pollen production. Therefore, they have an issue of not being able to produce seeds. Any genetic blockage from bud initiation to seed set can cause sterility. In addition, self-incompatibility is another obstacle to effective reproduction. In order to effectively identify reproductive barriers and develop strategies, pollen viability, flower production and compatibility issues should be evaluated (Potato International Center, 2021).

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CHAPTER 2: CHARACTERIZING CYTOPLASM

Introduction

With thousands of years of diversification, the genetic components of a crop have become complex and difficult to trace back (Hosaka, 2003). However, chloroplast DNA is inherited maternally in most plants, so tracing the maternal ancestry of crops is considered a reliable source (Hosaka, 2003). In plant cells, chloroplast and mitochondrial DNA exist in multiple copies, alongside a single copy of nuclear DNA and this unique genomic arrangement implies that nuclear gene expression and function can be influenced in various ways by the genes present in chloroplasts and mitochondria (Sanetomo & Gebhardt, 2015). A notable example of such an interaction is cytoplasmic male sterility, a condition frequently observed in crop species arising from the interplay between nuclear and mitochondrial genes (Sanetomo & Gebhardt, 2015).

In potato breeding, cytoplasmic differences have been determined for various agronomic traits by reciprocal crossbreeding methods (Hosaka & Sanetomo, 2012). For example, the cytoplasm of the most commonly used potato (*Solanum tuberosum* L. ssp. *tuberosum,* $2n = 4x = 48$) was associated with high tuber formation, high tuber yield, many tubers, poor flowering, early vine maturity, low pollen stainability, and poor pollen shedding (Hosaka & Sanetomo, 2012). In other words, most potato genotypes with desired traits are subject to sterility problems (Hosaka & Sanetomo, 2012). The common potato, grown worldwide, is a highly heterozygous tetraploid crop. Its cytoplasmic genome is characterized by the presence of T-type chloroplast DNA and β-type mtDNA (Hosaka & Sanetomo, 2012). Although the cytoplasmic sterility factor usually resides on mtDNA, β-type mtDNA has shown complete association with T-type ctDNA, and therefore, T/β cytoplasm is common in potatoes (Hosaka & Sanetomo, 2012). The T-type chloroplast DNA was identified by a deletion of 241 base pairs (bp), which set it apart from other chloroplast DNA found in Andean potatoes (Hosaka, 2003). In addition to the T/β type of intrinsic sterility, specific male sterility is associated with cytoplasmic genomes (Hosaka & Sanetomo, 2012). Cultivars carrying the gene for resistance to the *Potato Virus Y* (Ry_{sto}), mostly released in Germany, exhibit male sterility caused by the combination with the characteristic mtDNA derived from *S. stoloniferum* Schlechtd (Hosaka & Sanetomo, 2012). This cytoplasm is known as W/γ-type and is called tetrad sterility or lobed sterility (Hosaka & Sanetomo, 2012).This is so called because the anthers are often clustered in tetrads and have a four-lobed appearance (Hosaka & Sanetomo, 2012). In T/β and W/γ cytoplasms, male sterility is characterized by visible abnormalities, including various deformities of pollen and anthers (Hosaka & Sanetomo, 2012). These include a complete absence or significant reduction in pollen shedding and deformities in pollen and anthers (Hosaka & Sanetomo, 2012).

The D-type cytoplasm was introduced into the potato gene pool through crosses with the hexaploid wild Mexican species Solanum demissum Lindl (2n = 6x = 72) (Sanetomo & Hosaka, 2011). This wild species has been extensively utilized in breeding programs as a source of resistance to late blight, caused by the pathogen *Phytophthora infestans*, which is one of the most devastating diseases affecting potato crops globally (Dufková et al.,2023). Potato samples possessing the D-type cytoplasm exhibit a unique characteristic: functional sterility of pollen (Hosaka & Sanetomo, 2012). In these plants, while the pollen grains appear morphologically normal and can be well-stained, they are non-functional, meaning they are incapable of fertilization (Hosaka & Sanetomo, 2012). *S. demissum* is unilateral incompatible with common potato (*S. tuberosum L.*, 2n = 4x = 48) and can be easily crossed with *S. tuberosum* pollen and produces backcross offspring with pentaploid hybrids using only female parents (Sanetomo & Hosaka, 2011). Therefore, the cytoplasm of *S. demissum* has been transferred to bred varieties and in this way, the cytoplasm of *S. demissum* (D type) is transferred to cultivated cultivars (Hosaka & Sanetomo, 2012). Band 1 is a DNA marker developed to determine *S. demissum* cytoplasm and this DNA sequence is completely specific to *S. demissum* (Hosaka & Sanetomo, 2012). Thus, it is an important indicator distinguishing *S. demissum* cytoplasm (Hosaka & Sanetomo, 2012).

This study was conducted based on information provided by Hosaka and Sanetomo, 2012. We used two markers to identfy potato cytoplasm, T and D marker. The primary objective of the SAC Marker was to separate the samples into two distinct groups. The Type 1 band indicates the presence of the S-, A-, or C-type ctDNA, while the Type 2 band indicates the T-, W-, or D-type cytoplasm groups. In this context, T-type cytoplasm was distinguished from W-type cytoplasm by a diagnostic Type 3 marker band. This indicates that the Type 3 band was found in T-type ctDNA carriers characterized by a 241-bp deletion. Furthermore, D- type cytoplasm was distinguished from W-type cytoplasm by the presence of a specific D marker band, which produced a single band only in the *S. demissium* accession (Hosaka & Sanetomo, 2012).

In the Colorado State University potato breeding program, potato clones that produce low pollen levels, indicating male sterility, are utilized as female parents. Clones carrying the PVY resistance gene are particularly preferred, leading to the prevalence of certain cytoplasm types within the program. However, the cytoplasm types of none of the clones used in this program have been determined thus far. In this study, we aimed to identify the cytoplasm types of our samples containing the PVY resistance gene to prevent potential future genetic uniformity.

Materials and Methods

Plant Material and Tissue Collection

This research was conducted at the San Luis Valley Research Center and at the main campus of Colorado State University in Fort Collins. Nine clones were selected based on their resistance to PVY and low pollen production. CO01198-2Radg (*Ry*$_{adg}$), A12305-2Radg (*Ry*$_{adg}$), CO17140-2Ychc (*Ry*$_{chc}$), ORC19007-1W (*Ry*$_{chc}$), CO15016-1Rusto (*Ry*$_{sto}$), CO17135-4Rusto (*Ry*$_{sto}$), ORC19205-1RU (*Ry-f*$_{sto}$) and MN13142-32 (non resistant to PVY but with low pollen production).

Additionally, two clones used as pollinators at the San Luis Valley Research Center were included, but they were utilized as female clones in the test study: CO15211-1R and CO14371-3RU.

Plant materials were collected randomly from potato leaves. To ensure high DNA quality, samples were specifically taken from young leaves. The collected leaf samples were labeled, and DNA extraction and PCR were performed.

DNA Extraction

For DNA extraction, leaf tissue was collected and weighted (100 mg per extraction) into a 2 mL Eppendorf tube labeled with sample identification and replication number. The DNA extraction protocol was based on QIAGEN DNeasy Plant Kit. 400 µl Buffer AP1 and 4 µl RNase A stock solution was added to the disrupted plant and was vortexed vigorously until not to any tissue clumps.

Plant tissue was lysed to release DNA using buffer AP1, while RNase A enhanced the purity of the DNA by degrading RNA present in the lysate. The mixture was incubated for 10 min at 65°C and mixed 3-4 times by inverting the tube during incubation. High heat enhances the effectiveness of the lysis buffer and breaks down cellular components so that this step lyses the cells. Plant materials stuck inside the tube lid were moved back down using a mini centrifuge.

30 µl Buffer P3 was added to the lysate. The samples were mixed and incubated for 5 minutes on ice to precipitate detergent, proteins, and polysaccharides. Then, the lysate was centrifuged for 5 min at 20,000 x g (14,000 rpm) to remove very viscous lysates and large amounts of precipitates.

The lysate was pipetted into the QIAshredder Mini spin column, placed in a 2 ml collection tube, and centrifuged for 2 min at 20,000 x g (14,000 rpm). The QIA shredder Mini spin column removes most precipitates and cell debris.

The flow-through fraction was transferred into a new tube without disturbing the pellet. Typically, 450 µl lysate was recovered, 1.5 volumes of Buffer AW1 were added to the cleared lysate, and the mixture was mixed by pipetting.

650 µl of the mixture was pipetted into the DNeasy Mini spin column and placed in a 2 ml collection tube. It was centrifuged for 1 min at 6000 x g, and discarded the flow-through. This step was repeated with the remaining samples.

The DNeasy Mini spin column was placed into a new 2 ml collection tube and was added 500 µl Buffer AW2, and centrifuge for 1 min at 6000 x g. The flow-through was discarded.

500 µl Buffer AW2 was added to the DNeasy Mini spin column and centrifuged for 2 min at 20,000 x g (14,000 rpm) to dry the membrane and prevent interference with the residual ethanol's subsequent reactions. The DNeasy Mini spin column was carefully transferred to a 2 ml microcentrifuge tube to prevent contact with the column flow-through. Then, 100 µl Buffer AE was pipetted directly on the DNeasy membrane. It was incubated for 5 min at room temperature and then centrifuged for 1 min at ≥6000 x g (≥8000 rpm) to elute. The previous step was repeated.

Selection of Markers

There are six types of cytoplasmic genome in potatoes: A, M, P, T, W, and D and three of these types (T, W, and D) are associated with male sterility in specific hybrid combinations (Goryunova et al., 2023). Five PCR markers have been developed to determine all cytoplasm types. Because the samples used in the study were thought to be associated with male infertility, the focus was placed on three PCR markers (SAC, T, and D). Marker selection and PCR conditions was made according to the information provided by Hosaka and Sanetomo, 2012.

The main function of the SAC marker among these markers is to determine the S-, A-, and C- type ctDNAs; that is, it is used to distinguish them from the W-, T-, and D-type ctDNAs. However, since our samples were thought to belong to the W, T, and D cytoplasmic types, we did not use this marker in our study. The T-type cytoplasm was distinguished from the W-type cytoplasm using a T-marker. The D-type cytoplasm was distinguished from the W-type by the presence of a specific D-marker band. So we focused on the markers to determine T- and D- type ctDNAs, while the remaining sample was considered to belong to W-type ctDNAs.

PCR Analysis

After DNA extraction from the leaves, DNA samples were used in PCR. The table shows the PCR markers used to identify cytoplasmic types in potatoes (Table 2.1). 2 µl DNA sample were used for total 50 µl PCR mix (Table 2.2). The amplification condition for PCR was given in Table 2.3.The primers and PCR conditions used to determine the potato cytoplasm type were performed as described by Hosaka and Sanetomo (2012).

Table 2.1 PCR markers

Marker	Primer (5′–3′ sequence)
T	GGAGGGGTTTTTCTTGGTTG AAGTTTACTCACGGCAATCG
D	CGGGAGGTGGTGTACTTTCT ACGGCTGACTGTGTGTTTGA

Table 2.2 PCR Reaction ingredients

Component	Quantity for 1 reaction (µl)
d2H2O	39.41
10X buffer (+MgCl)	5
10 mM dNTPs	1
100 µM T-f	0.12
100 µM T-r	0.12
20 mg/mL RA	3.75
3 Units Taq	0.6
DNA sample	2
Total	50

Table 2.3 PCR amplification conditions

Temperature	Time	Number of Cycles
94 °C	3 min	1
94 °C	30 sec	35
60°C	30 sec	35
72°C	1 min	35
72°C	10 min	1

After the reaction, 1.5% agarose gel electrophoresis was performed to see the result of the PCR products. Electrophoresis was performed in 1XTAE. For gel electrophoresis, samples with a volume of 10 µl were mixed with 3 µl of loading dye I, and 10 µl sample volumes were loaded on agarose gel. Loaded products were electrophoresed under 80 volts for 50 minutes.

Photographic images were captured using a UV lamp.

Results

The T marker generated three banding types. Type 1 exhibited a slightly shorter band from Type 2. Type 3 was found only in the T-type ctDNA carrier, characterized by a 241-bp. According to these results, two out of 7 samples, ORC19205-1RU from $Ry\text{-}f_{sto}$ and ORC19007-1W from Ry_{chc}, had T cytoplasm type (Figure 2.1).

Following identifying T-type cytoplasm using a diagnostic T-marker, which produced a Type 3 band, the D marker was used to distinguish between W and D-type cytoplasmic types. The D marker is a presence/absence indicator specific to the *S. demissum* accession. While samples with D-type cytoplasm were expected to exhibit a strong D-marker band, none of the analyzed samples displayed such a band. This absence indicates that the remaining samples are representative of W-type cytoplasm.

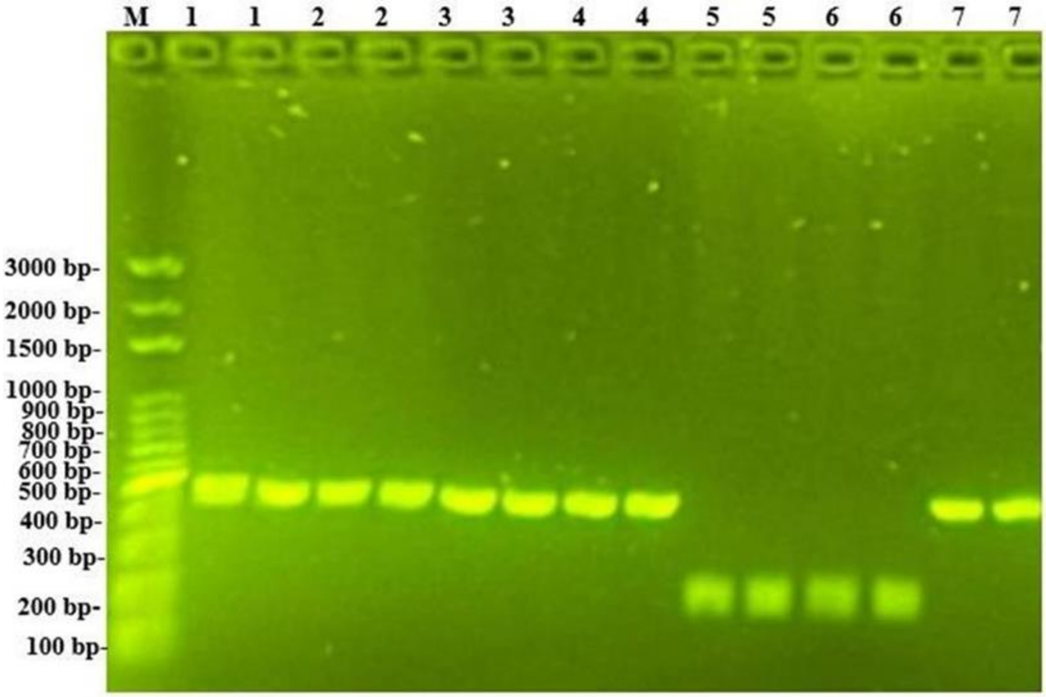

Figure 2.1 T marker was detected on a 1.5% agarose gel in 1× TBE buffer. M: 3000 bp DNA marker. The samples 1 and 2 from Ry_{adg}, 3 and 4 from Ry_{sto}, 5 from $Ry\text{-}f_{sto}$, 6 and 7 from, and Ry_{chc}.

Results of the Evaluation of Our Clones

According to the PCR results, two of the seven samples exhibited T-type cytoplasm, while the remaining five were classified as W-type (Figure 2.2). Examination of the cytoplasmic distribution according to the genes, we observed T-type cytoplasm in one of the two clones carrying the Ry_{chc} gene, ORC19007-1W, as well as in the clone with the $Ry\text{-}f_{sto}$ gene, ORC19205-1RU. All samples carrying the Ry_{adg} and Ry_{sto} genes had W-type cytoplasm (Figure 2.3).

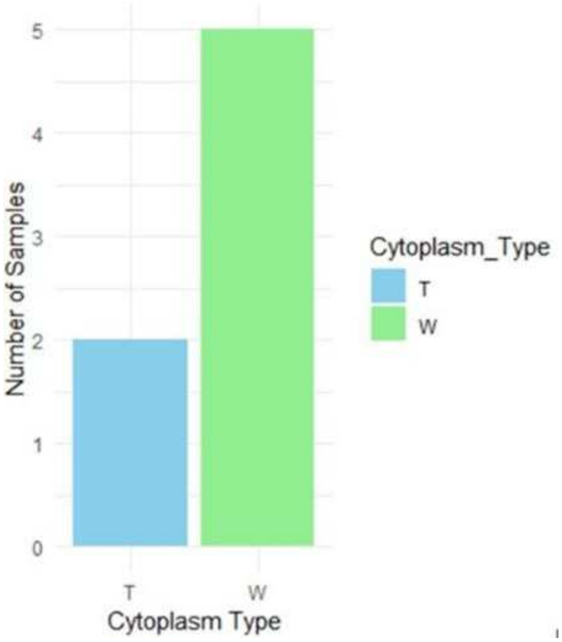

Figure 2.2 Number of cytoplasm type distribution among PVY resistance clones.

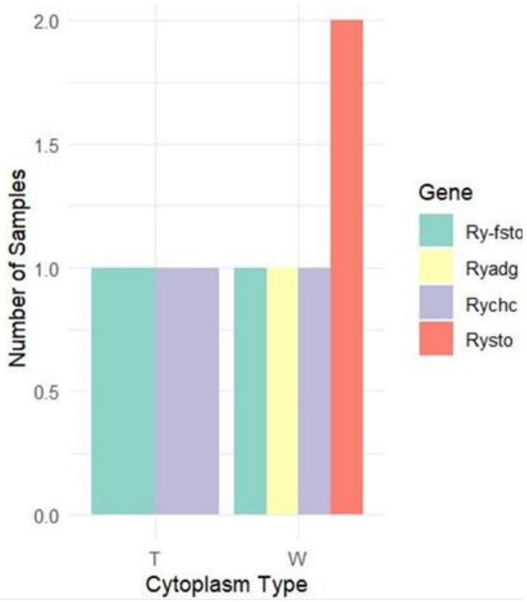

Figure 2.3 Distribution of samples with genes across cytoplasm types.

Discussion

Among the analyzed samples, two different cytoplasm were identified, W and T, with W being more dominant. This is an expected result of the gradual increase in these two cytoplasm types in many breeding programs. This increase is because the common potato (*Solanum tuberosum L.* ssp. *tuberosum*) has the T type and is associated with desirable traits such as high tuber yield and early maturity. Similarly, W-type cytoplasm is frequently used in breeding programs because it is usually associated with resistance to PVY.

Conclusion

Characterization of cytoplasm types in potato clones carrying resistance genes for PVY is important to address potential genetic uniformity issues in breeding programs. Identifying cytoplasm types, W and T, showed that the W type was dominant among them. This finding suggests that there may be concerns regarding genetic diversity among PVY-resistant lines. Breeders must consider cytoplasm types to reduce this risk when selecting parents for crossbreeding. Therefore, it is recommended that cytoplasm types be evaluated for all PVY-resistant lines within the CSU, which will help maintain genetic diversity.

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CHAPTER 3: REPRODUCTIVE TRAIT EVOLUTION

Introduction

Potato is the third most important food product in production in the world (FAO, 2021). They are an important crop because they yield high nutritional value per unit of time and space (Rizza et al., 2006). However, potato diseases, especially viruses, severely decrease potato production. *Potato virus Y* is one of the most important viruses that infect potatoes and causes serious economic problems by causing yield losses of up to 80% (Li et al., 2022). Viruses pose a significant problem, bringing costly breeding, crop inspection, and diagnostics, including the increasing use of postharvest tuber testing associated with formal seed potato production and certification processes (Rizza et al., 2006). This virus has spread rapidly through infected seeds, tubers, and vector aphids (Lacomme et al., 2017).Some methods to control PVY include rotation practices, selecting certified virus-free seeds, implementing pest management techniques, and using resistant varieties (Lacomme et al., 2017). Insecticides cannot control PVY because aphids can rapidly transmit the virus via the stylets before the insecticides can act (Rizza et al., 2006). Chemical control of aphids is also expensive, causing populations of resistant aphids to increase, and pesticide residues in food are a concern (Rizza et al., 2006).

Among these methods, developing resistant varieties is one of the effective long-term solutions because it reduces the use of pesticides and other chemicals, helps prevent the spread of the disease through tubers, and aids in preserving the quality of potatoes (Lacomme et al., 2017). Also, breeding cultivars resistant to PVY provides the most straightforward and economical solution to preventing the damage caused by PVY (Rizza et al., 2006). Various breeding programs in Latin America have been concerned with incorporating sources of disease resistance into improved cultivars, so genotypes from many breeding programs today carry resistance to PVY (Li et al., 2022). There are two types of single dominant resistance genes for PVY: *Ny* genes, hypersensitive response, which are effective against individual strains of PVY, and *Ry* genes, which are extreme resistance genes and are effective against all strains (Slater et al., 2020). Hypersensitivity prevents the spread of the virus from cell to cell and through the vascular system, while extreme resistance strongly suppresses the replication of the virus (Slater et al., 2020). Therefore, Ry genes are primarily used in potato breeding programs to develop cultivars resistant to PVY. Several *Ry* genes, Ry_{adg}, Ry_{sto}, $Ry\text{-}f_{sto}$, and Ry_{chc} have been introgressed from wild potato species *Solanum tuberosum* ssp. *andigena*, *Solanum stoloniferum*, and *Solanum chacoense* respectively (Nie et al., 2004).

Potato breeding programs in the US report predominately using Ry_{adg} and Ry_{sto}. Molecular markers for these genes have been developed and are available for use, offering a more cost-effective and earlier screening methodology than traditional screening (Solomon-Blackburn & Barker, 2001). The molecular markers for detecting the Ry_{adg}, gene, derived from *Solanum tuberosum* ssp. *andigena* and located on chromosome XI, encompass RYSC3 and RYSC4 (Kasai et al., 2000). Likewise, for identifying the Ry_{chc} gene from *Solanum chacoense*, positioned on chromosome IX, the marker 38–530 are employed (Hosaka et al., 2001). Additionally, for the Ry_{sto} gene originating from *Solanum stoloniferum*, found on chromosome XII, the markers GP122, STM003, and YES3- 3B are utilized (Slater et al., 2020), and and for $Ry\text{-}f_{sto}$ derived from *Solanum stoloniferum*, located on chromosome XII, the marker GP122718 is employed (Flis et al., 2005).

Table 3.1 The origins of the genes and their chromosomal positions

Gene	Origin	Chromosome Position
Ry_{adg}	*Solanum tuberosum ssp. andigena*	XI
Ry_{chc}	*Solanum chacoense*	IX
Ry_{sto}	*Solanum stoloniferum*	XII
$Ry\text{-}f_{sto}$	*Solanum stoloniferum*	XII

However, breeders encounter a significant challenge when dealing with hybrids and varieties resistant to PVY. This is cytoplasmic male sterility arising from the interplay between nuclear and mitochondrial genes. *S. stoloniferum* carrying the nuclear Ry_{sto} gene, which confers resistance to the most destructive PVY strain, exhibits the most pronounced sterilizing effect, characterized by tetrads (Hosaka & Sanetomo, 2012). This pressing issue is male sterility, which adversely affects reproductive processes and is notably prevalent in cultured tetraploid varieties. Research indicates a complex interaction between cytoplasmic and nuclear genetic factors, suggesting that nuclear genes play a crucial role in the genetic control of pollen viability (Hosaka & Sanetomo, 2012). The prevalence of male sterility in potatoes negatively impacts the management of breeding programs.

Specifically, the Colorado State University (CSU) breeding program requires a large quantity of seed per cross for sustainable seed production. Since male sterile plants cannot produce viable pollen, they can only serve as female parents, restricting parent selection and reducing genetic diversity. Therefore, male fertility is crucial when selecting parents in a breeding program, as it helps prevent crossing failures and low seed sets. Therefore, assessing male fertility and its indicators, specifically pollen viability and quantity, and testing the pollen's ability to pollinate and produce seeds are crucial for the sustainability of potato production.

Extreme resistance to PVY has been incorporated into the Colorado Potato Breeding Program; however, the relationship between these resistant genes and male sterility, which leads to reproductive failures, low seed sets, and limitations in parent selection, has not yet been established within the San Luis Valley breeding program. Therefore, this study aims to determine the relationship between clones carrying PVY resistant genes and male sterility.

The project seeks to facilitate the improvement of potato germplasm by identifying male fertile varieties that are resistant to PVY. In doing so, it aims to enhance crop yield, quality, and resistance to PVY, thereby contributing to the sustainable development of improved potato varieties.

Materials and Methods

This research was conducted at the San Luis Valley Research Center (SLVRC) in Colorado and at the main campus of Colorado State University in Fort Collins. The reproductive traits related to male fertility/sterility were phenotyped in potato clones resistant to PVY (methods described below). Plants grown in the greenhouse were evaluated for reproductive traits and male fertility/sterility by performing test crosses with female plants.

Plant Material

Seven clones were selected from the breeding program based on which resistance genes they carry: CO01198-2Radg (Ry_{adg}), A12305-2Radg (Ry_{adg}), CO17140-2Ychc (Ry_{chc}), ORC19007-1W (Ry_{chc}), C015016-1Rusto (Ry_{sto}), CO17135-4Rusto (Ry_{sto}), ORC19205-1RU(Ry-$_{fsto}$).

These clones were used as pollinators to evaluate fruit and seed development in clones carrying PVY resistance genes. Three clones were selected to be used as females based on PVY susceptibility and relevance to the breeding program: Silverton russet, CO14371-3RU, and CO15211-1R.

The experimental unit was defined as a single plant per pot. Each clone was sown in medium- sized pots three repetitions in the greenhouse and a complete randomized design (CRD) was used. Fertilization and pruning were performed to promote flowering and pollen production.

Additionally, excess lateral branches at the lower parts of the plant were pruned to direct the plant's energy towards flowering. Regular watering was maintained to ensure that the soil remained consistently moist.

Reproductive Trait Evaluation

Pollen Viability Test

Assessing male fertility and its indicators, specifically pollen viability, quantity, and testing the ability to pollinate and produce seeds, is crucial for the sustainability of potato production (Ordoñez et al., 2017).The assessment of pollen viability relies on a limited number of methods, including dye tests and in vitro germination evaluations (Ordoñez et al., 2017).The Acetocarmine glycerol jelly test is among the most commonly employed staining techniques for analyzing pollen viability in potatoes (Ordoñez, 2014). This staining method provides a rapid evaluation of enzymatic activity within the pollen grain membrane and the integrity and coloration of the cytoplasm (Ordoñez et al., 2017).

A vital advantage of this procedure is that unaffected by environmental conditions (Potato International Center, 2021).The underlying principle of the staining technique is that cytoplasmic staining agents effectively penetrate only intact cells with functional membranes, thereby allowing visualization of compounds associated with pollen viability (Potato International Center, 2021).Notable reagents such as potassium iodide, aniline blue, and acetocarmine selectively stain starch, callose, and chromatin structures (Potato International Center, 2021). Viable pollen grains exhibit a well-defined, rounded structure and show staining, whereas non- viable grains are typically unstained and exhibit deformities (Ordoñez, 2014). The presence of abnormalities, such as tetrads, four cores with pollen grains, indicates infertility within the sample (Ordoñez B. 2014).

Acetocarmine Glycerol Jelly Stain

Mature anthers were collected, and the pollen was extracted using a pollen vibrator. Extracted pollen from one or two anthers was placed on a microscope slide. A drop of 2% Acetocarmine Glycerol Jelly Stain was placed on the slide, and the pollen was spread in the dye with light circular movements using a toothpick. To allow the pollen grains to absorb the stain, it was left for approximately 3 minutes, and the coverslip was covered. It was left for one day to ensure thorough absorption of the stain. This test was conducted as described by Ordoñez (2014).

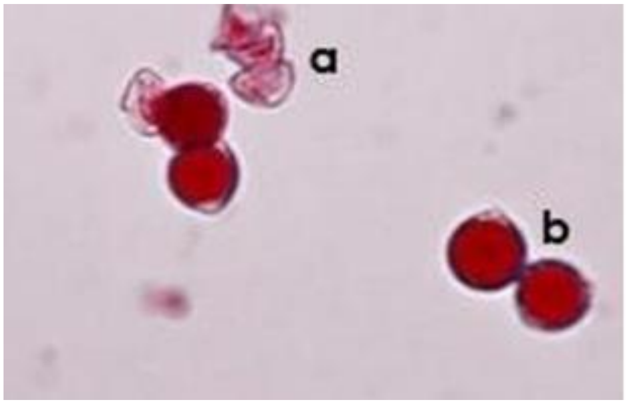
Observation

Stained pollen grains were examined under a light microscope at magnifications of 400X. The bright red staining of the cytoplasm of a pollen grain indicates viability (Figure 3.1a), whereas cytoplasm that is not red or pink shows non-viable or sterile pollen (Figure 3.1b). Compared to viable pollen grains, sterile ones exhibit deformation, lack a regular shape and appear wrinkled. Observing abnormalities, such as tetrads or four-core pollen grains, indicates the pollen sample is infertile (Figure 3.2).

250 pollen grains across different regions of the slide were counted to determine the viability percentage. The obtained pollen samples were systematically categorized into distinct groups based on their stainability percentages (Table 3.2)(Ordoñez, 2014).

Table 3.2 Ranges of pollen viability in potatoes (PolV)

Category	Viability Range (%)	Description
1	0	Sterile
3	<50	Low
5	>50-80	Moderate
7	>80-100	High



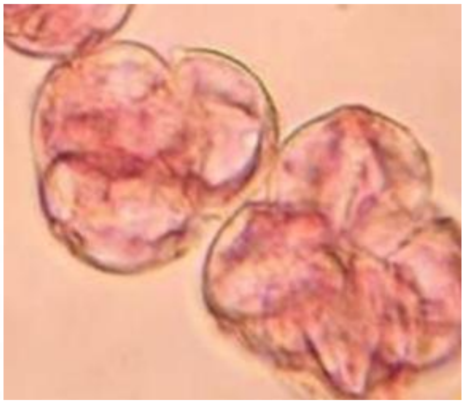


Figure 3.1 a) Unviable pollen b) Viable pollen Figure 3.2 Sterile tetrads
Source: Ordoñez B. 2014. Brochure: Pollen Viability Assessment

Pollen Quantity Measurement

After the flowers were fully opened, mature anthers that were either near dehiscence or completely dehiscent were collected from 10 flowers per experimental unit (plant) using tweezers. The collected anthers were placed in a clean and dry petri dish and left to dry for 24 hours at room temperature (25 °C). At the end of the 24 hours, pollen was extracted from anthers using a vibrator designed for pollen release. The collected pollen was then transferred to a 500 µl Eppedorf® tube. The pollen volume was categorized according to the rates shown in Table 3.3 and Figure 3.3. The pollen volume was categorized according to the rates shown in Table 3.3 and Figure 3.3 and was done as described by Santayana et al (2022).

Table 3.3 Categories and Descriptions for Visual Estimation of Pollen Quantity (QPol)

Qpol category	Volume	Description
0	$Qpol = 0$	Absence
1	$0 < QPol \leq 50$	Little
3	$50 < QPol \leq 250$	Moderate
5	$250 < QPol$	Abundant

Figure 3.3. Reference guidelines for QPol rating utilizing a 500 µl eppendorf tube
Source: International Potato Center (CIP), 2021

Seed Set Evaluation

The collected pollen was carefully cross-pollinated with the female clone. Approximately 60 days after pollination, the fruits were harvested from the greenhouse, and the number of berries was recorded for further analysis. After harvesting, the berries were kept at room temperature until they softened, which is crucial for the subsequent extraction process. Seeds were manually extracted from the softened berries by gently squeezing them into a water- filled container. Subsequently, the seeds were rinsed under a softly flowing tap to ensure cleanliness. The seed set calculation was determined by dividing the total number of seeds by the total number of fruits within a cross. According to Santayana et al. (2022), seed set equal to or higher than 20 seeds/berry indicates male fertility. This metric provided information on the fertility and reproductive success of clones.

Statistical Analysis

Shapiro-Wilk was utilized to determine the normality, and Levene's test was conducted to assess the homogeneity of the data for each variable. While pollen viability and seed set showed a slightly normal distribution. The homogeneity of variances across groups was heterogeneity based on Levene's test. So, the Analysis of Variance (ANOVA) test was chosen to assess the differences in pollen viability among different potato clones. Then the Harmonic Distribution (HDS) test was conducted for a more detailed evaluation of the results. Pollen quantity was analyzed using the Kruskal-Wallis non-parametric test.

Results

Pollen Quantity for Genes

Pollen production was measured to test the prediction that there is a significant variation in pollen quantity among the clones with PVY resistance genes. Results were shown in Table 3.4.There was no statistically significant difference in pollen quantity between the gene groups (p=0.4724). One possible reason for this lack of statistical difference could be the greenhouse conditions in which the experiments were conducted. Additionally, pollen viability and seed set were assessed simultaneously throughout the year (in 2023 and in the plant growth facilities at Colorado State University) and in the same greenhouse environments. However, pollen abundance was assessed in the San Luis Valley greenhouse in 2024. Therefore, this change in conditions may have contributed to the differences in the observed results.

Table 3.4 Result of pollen quantities by gene and clone

Gene	Clone	1.plant	2.plant	3.plant
Ry_{adg}	CO01198-2Radg	0	0	1
Ry_{adg}	A12305-2adg	1	1	1
Ry_{sto}	CO15016-1RUsto	1	1	1
$Ry\text{-}f_{sto}$	ORC19205-1RU	0	1	0
Ry_{chc}	ORC19007-1W	1	0	0

In general, all clones produced pollen at little and absence levels.The same distribution was observed in the clones with Ry_{chc}, $Ry\text{-}f_{sto}$ and one of the clones from Ry_{adg} genes CO01198-2Radg Out of the three sample plants for each clone, two did not produce any pollen,while just one plant produced less than 50 µL of pollen.The another clones from Ry_{adg}, A12305-2adg, and clone from Ry_{sto} produced less than 50 µL of pollen for all plant. Although this indicates low pollen production, they had the highest pollen production values among the clone groups.

Pollen Quantity for Cytoplasm Types

Pollen production was measured to test the prediction that there was a significant variation in pollen quantity among the clones with T and W cytoplasmThere was no statistically significant difference in pollen quantity between the cytoplasm type ($p > 0.05$). Results were shown in Table 3.5. However, this is thought to be related to the samples being grown under different dates and conditions in different greenhouses.

Table 3.5 Result of pollen quantities by cytoplasm and gene

Cytoplasm Type	Clone	Gene	1.plant	2.plant	3.plant
W	CO01198-2Radg	Ry_{adg}	0	0	1
W	A12305-2adg	Ry_{adg}	1	1	1
W	CO15016-1RUsto	Ry_{sto}	1	1	1
T	ORC19205-1RU	$Ry\text{-}f_{sto}$	0	1	0
W	ORC19007-1W	Ry_{chc}	1	0	0

W cytoplasm type shows more variety in pollen production than T cytoplasm type. However, in general both cytoplasm types produced pollen at little and absence levels. Some clones of W type (A12305-2adg and CO15016-1RUsto) produced pollen at little level, while others produced pollen at absence level. In T type, only one clone, ORC19205-1RU, produced pollen at little level.

Pollen Viability Associated with Genes

It was predicted that pollen viability varied among genes and was correlated with male sterility in clones carrying PVY resistance genes. The percentage distribution of pollen viability among four genes related to PVY resistance was observed under a light microscope at magnifications of 400X (Figure 3.4 and Figure 3.5).

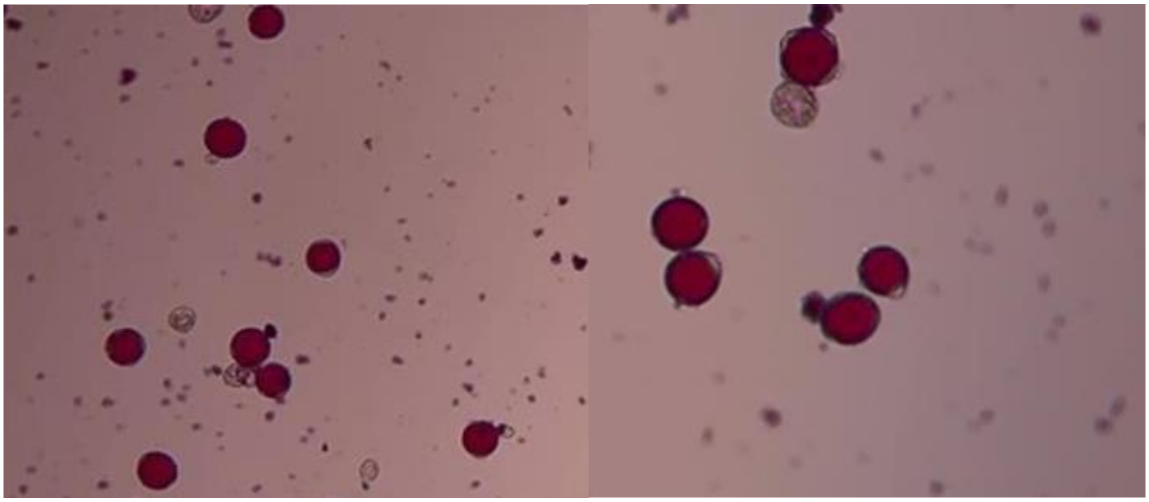


Figure 3.4 Pollen stained with acetocarmine showing viable pollen in CO17140-2Ychc (a)

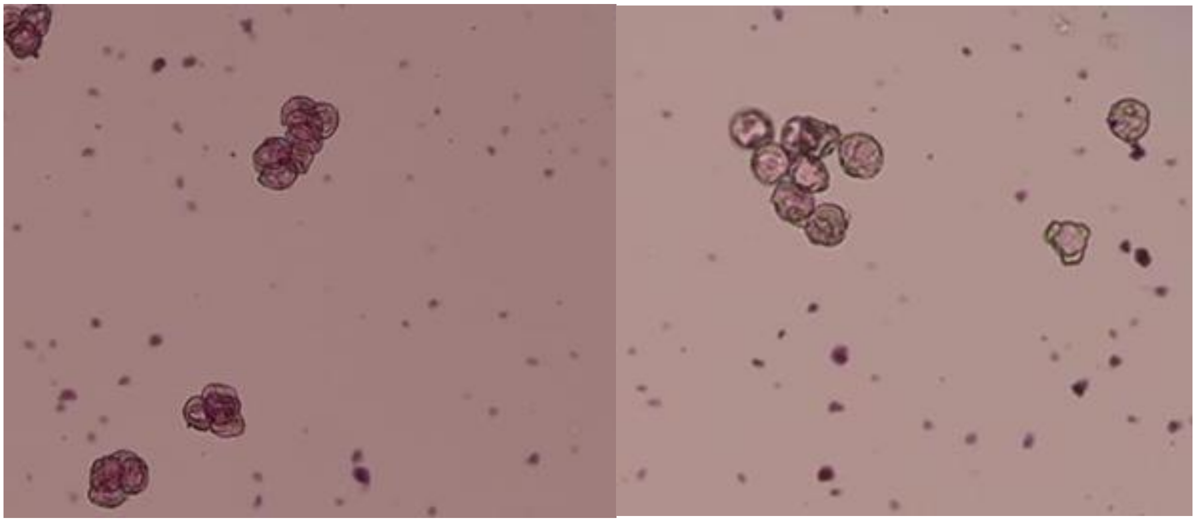


Figure 3.5 Pollen stained with acetocarmine showing unviable pollen in CO15016- 1Rusto

The pollen viability of each gene was evaluated by calculating the average of 3 replicates. Result showed statistically significant difference between the mean pollen viability of gene groups ($p < 0.01$). Results were shown in table 3.6. Ry-f_{sto}, Ry_{chc}, and Ry_{sto} gene groups show minimal variability, while *Ryadg* gene has more significant variability.

However, all clones carrying *Ry*-$_{fsto}$, *Ry*$_{sto}$, and *Ry*$_{chc}$ genes showed low pollen viability levels by staying under 50% viable pollen. However, among the clones *Ry*$_{chc}$, CO17140-2Ychc and ORC19007-1W, had the highest pollen viability with 44.9%. CO01198-2Radg, carrying the Ryadg gene had the lowest pollen viability value with 0.4%, indicating male sterile. The other sample, A12305-2adg, carrying the Ryadg gene had a low pollen viability of 40.6% and was the second-highest pollen percentage among all evaluated genes. Clones, ORC19205-1RU, carrying the *Ry-f*$_{sto}$, and clones, CO15016-1RUsto and CO17135-4Rusto, carriying the *Ry*$_{sto}$ genes exhibited similar pollen viability levels with 29.0% and 27.2%, respectively (Figure 3.6).

Table 3.5 Result of different genes on pollen viability

Gene	Clone	PolV/plant 1	PolV/plant 2	PolV/plant 3
Ry$_{adg}$	CO01198-2Radg	0.4	0.4	0.4
Ry$_{adg}$	A12305-2adg	40.7	41.6	39.6
Ry$_{sto}$	CO15016-1RUsto	25	32.4	25
Ry$_{sto}$	CO17135-4RUsto	24.4	30.4	26
Ry-f$_{sto}$	ORC19205-1RU	27.2	34.8	25
Ry$_{chc}$	CO17140-2Ychc	47.7	38.4	50
Ry$_{chc}$	ORC19007-1W	46.8	48.4	38

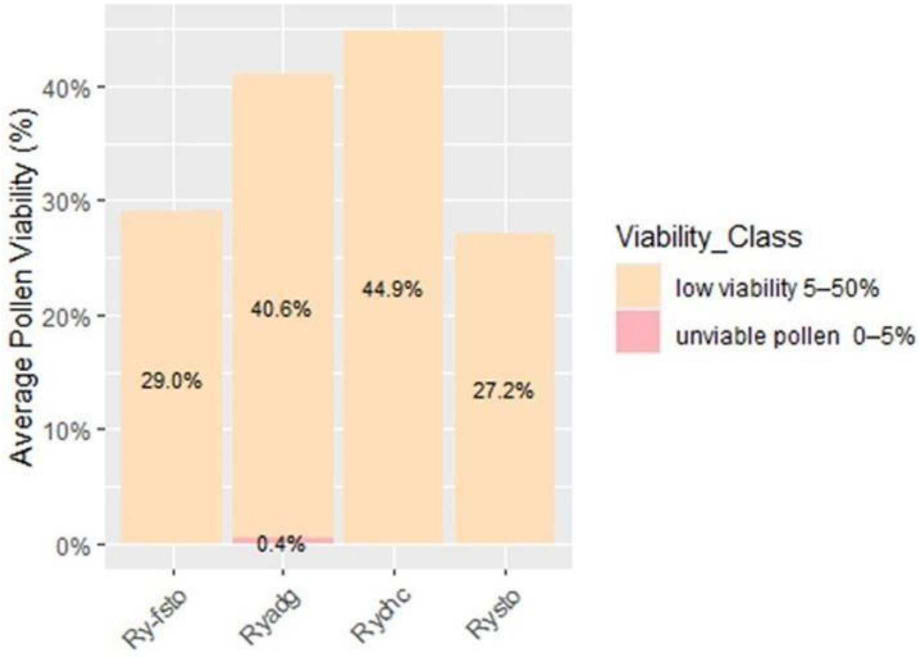


Figure 3.6 Distribution of pollen viability among four genes related to PVY resistance

Pollen Viability Associated with Cytoplasm Types

It was predicted that pollen viability varied among clones carrying PVY resistance genes and that this variation was associated with the cytoplasm type. Result showed a statistically significant difference between the mean pollen viability of cytoplasm groups ($p < 0.01$).

Results are shown in Table 3.6. The data show that both W and T cytoplasm types do not show medium or high levels of pollen viability (Figure 3.7). Clones with W cytoplasm generally have low pollen viability. In particular, clone CO01198-2Radg showed no viability in pollen production. However, there is a significant difference between other clones carrying the Ry_{adg} gene with the same cytoplasm type. The average pollen viability of this clone is 0.4 percent, while the other is 40.6 percent. CO17140-2Ychc clone with W cytoplasm has the highest pollen viability among the Ry_{chc} gene clones with 45%. ORC19007-1W clone with T cytoplasm reaches the highest pollen viability among T cytoplasms, showing a value of 44.4.

Table 3.6 Result of different cytoplasm types on pollen viability

Gene	Cytoplasm Type	Clone	PolV/plant 1	PolV/plant 2	PolV/plant 3
Ry_{adg}	W	CO01198-2Radg	0.4	0.4	0.4
Ry_{adg}	W	A12305-2adg	40.7	41.6	39.6
Ry^{sto}	W	CO15016-1RUsto	25	32.4	25
Ry_{sto}	W	CO17135-4RUsto	24.4	30.4	26
$Ry\text{-}f_{sto}$	T	ORC19205-1RU	27.2	34.8	25
Ry_{chc}	W	CO17140-2Ychc	47.7	38.4	50
Ry_{chc}	T	ORC19007-1W	46.8	48.4	38

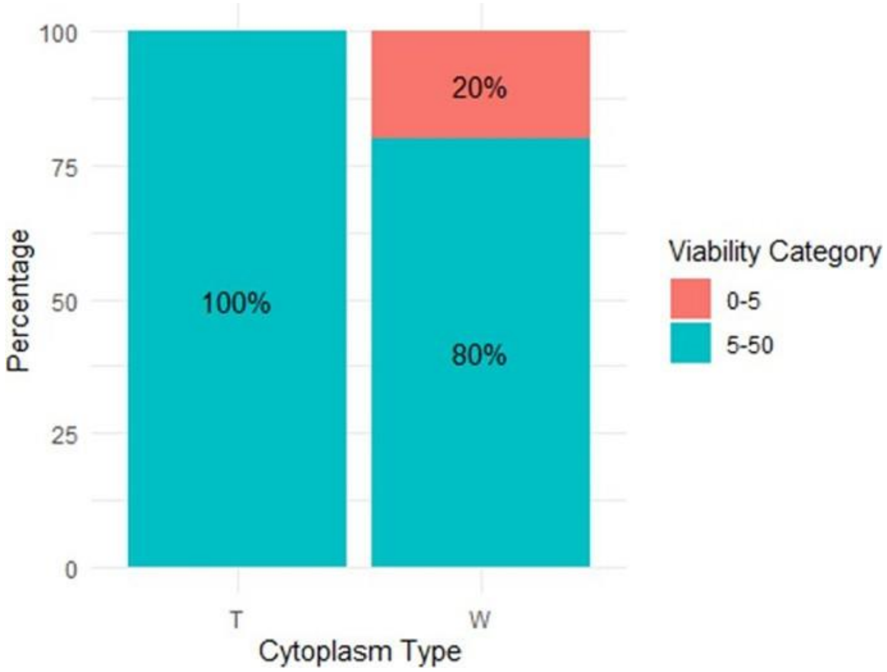


Figure 3.7 Mean pollen viability for different cytoplasm types

Seed Set Associated with Genes

We hypothesized that specific PVY resistance genes significantly influenced seed set in clones, with varying levels of seed production observed across different gene types. To test the hypothesis, we looked at the seed set produced by clones carrying the PVY resistance gene for each gene. To better understand how PVY resistance is associated with male sterility, clones carrying PVY resistance were used as male plants in crosses, and the seed set was evaluated. Due to difficulty in crossing, only two plants per clone were used for crossing, and there was high variation between the two plants (Figure 3.8), which lead to no statistical difference on seed set (p=0.4724). However, seed sets equal to or higher than 20 seeds/berry were considered indicative of male fertility. In this case, all clones except CO15016-1Rusto and CO17135-4RUsto carrying the Ry_{sto} gene were considered male-fertile. Among the clones thr Rychc gene, CO17140Ychc showed the highest seed set value,287, while ORC19007-1W clone had the lowest seed set value, 85.3. A12305-2Radg belonging to Ryadg had the second highest seed set , 194.4,while CO01198-2Radg had the second lowest value,85.5, which was shown the tetrad sterility (Figure 3.8).ORC19205-1RU with the $Ry\text{-}f_{sto}$ gene had a seed set value of 135, making it the sample with the 3rd highest seed set production.

The most striking value from this study was seen in the clones belonging to the Ry_{sto} gene. The seed set value of the clones CO15016-1RUsto and CO17135-4RUstowith the Ry_{sto} gene was zero, meaning these clones did not produce any berries (Figure 3.9).

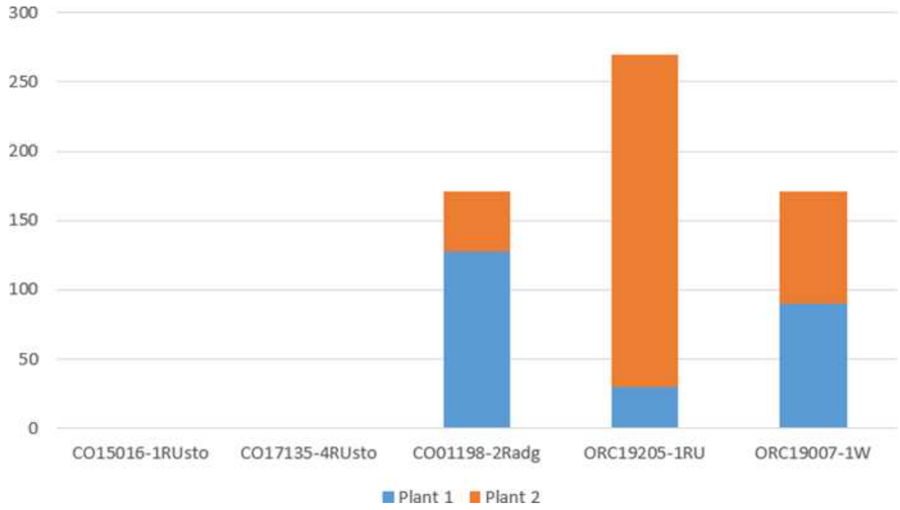


Figure 3.8 Seed set variation observed between the two plants used for crossing within each clone

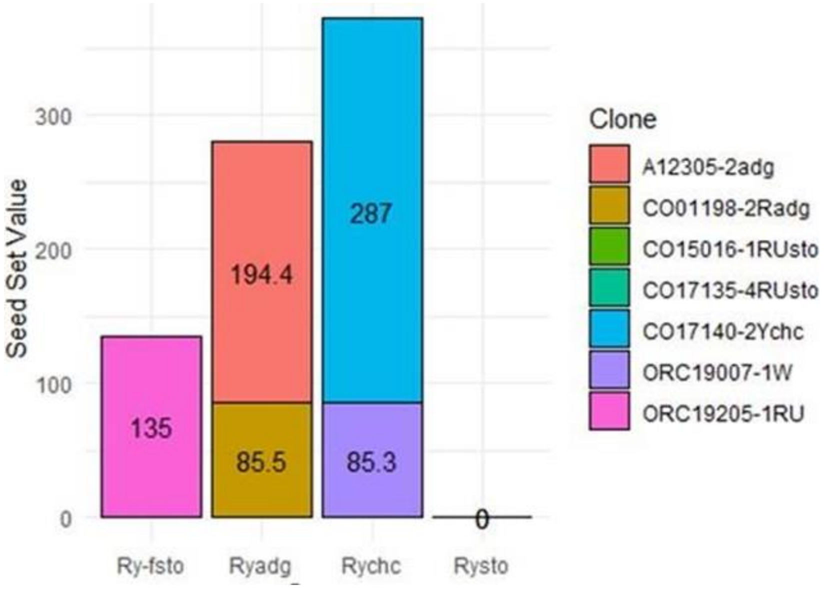


Figure 3.9 Seed set values among different genes

Seed Set Associated With Cytoplasm Type

To test the hypothesis that there is a difference in seed set production among cytoplasm types, the seed set production of clones with W and T types was compared. Result showed there is no statistically significant difference between the seed set and cytoplasm groups ($p > 0.05$). However, clones with W cytoplasm produced a total of 566.9 seed sets from 5 clones, while clones with T cytoplasm produced 220.3 seed sets from 2 clones (Figure 3.10). The clone with W type and Ry_{chc} gene has the highest seed set of 287. However, another clone carrying Ry_{chc} gene has a different cytoplasm and has the lowest seed set. Similarly, cloneA12305-2Radg with W-type cytoplasm shows the second-highest seed set production.

On the other hand, the clone with W-type cytoplasm and Ry_{adg} gene has a low seed production of 85.5. The clone with T-type cytoplasm and carrying the $Ry\text{-}f_{sto}$ gene produced 135 seed sets. Both clones carrying the Ry_{sto} gene did not produce any berry and, therefore, had no seed set (Figure 3.11).

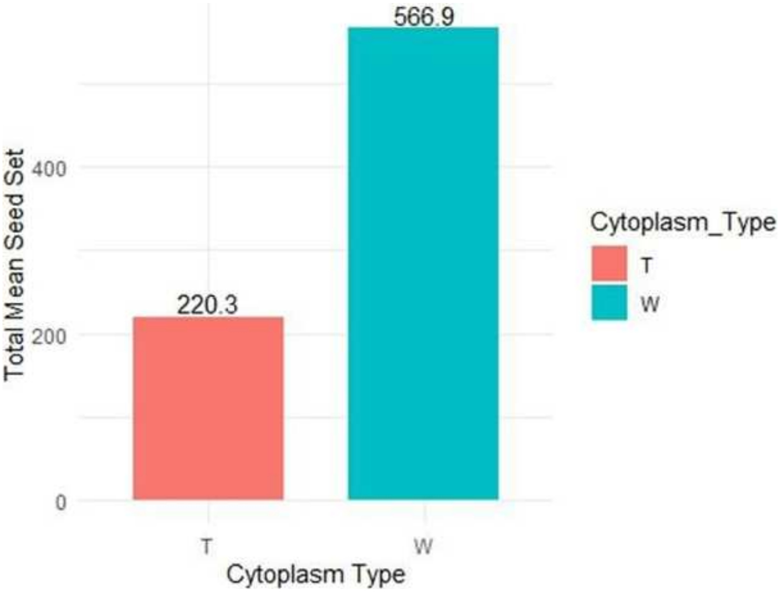


Figure 3.10 Total seed sets of clones according to cytoplasm type

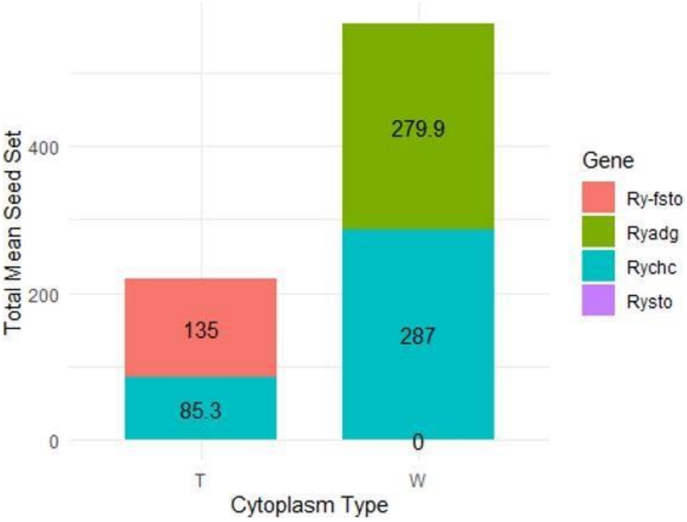


Figure 3.11 The number of seed sets produced by each clone

Discussion

This study evaluated the effects of gene and cytoplasm type on male fertility. There is a statistically significant difference between the gene groups and cytoplasm types for pollen viability. However, no significant difference was found between pollen amount and seed set.

The reason for the lack of significant difference in pollen amount is thought to be due to different greenhouse conditions. Pollen viability and seed set were evaluated in the same greenhouse environment at the plant growth facilities at Colorado State University in 2023 throughout the year. However, pollen quantity was assessed in the San Luis Valley greenhouse in 2024. Therefore, it is thought that the changes in these conditions may have contributed to the differences in the observed results. The lack of a significant difference between the seed sets was thought to be related to the difficulty of crossing and the female parents' effect. Many crossings were performed to obtain berries from our samples. However, since each clone was not crossed with the same female clone, reason suggests that the genetic structure and pollen accepting ability of the female plant used may affect seed retention. Clones carrying the Ry_{chc} genes exhibited high pollen viability among all clones. However, those with T-type cytoplasm produced lower seed sets, while those with W-type cytoplasm had higher ones. This situation may arise from genetic and cytoplasmic interactions. This indicated that the clone with W-type cytoplasm is likely to better results in test crosses. According to Santayana et al. (2022), seed production is important in determining whether a clone is male fertile or sterile. In particular, 20 or more seed sets per fruit are considered an indicator of male fertility. The results revealed that only the Ry_{sto} gene with W-type cytoplasm clones showed complete male sterility, as they did not produce berry in the test crosses. This is one indication that the effect of the cytoplasm or the gene on seed set production remains unclear. In addition, tetrad sterility was observed under the microscope in two of our Ry_{sto} gene clones, which was considered an indicator of strong male sterility. It is consistent with studies supporting the conclusion that all those showing tetrad sterility are male sterile (Santayana et al., 2022). However, our samples showed little pollen viability despite being tetrad sterile and not producing seeds. This indicates the pollen was stainable but did not produce functionally viable pollen.

On the other hand, we had another clone showing tetrad sterility, one of the clones carrying the Ry_{adg} gene with W-type cytoplasm. Differences were observed between this clone and the other clone carrying the Ry_{adg} gene in terms of pollen viability and seed set. In this case, it is thought that these two samples may have different accessions. However, it is noteworthy that our sample carrying the Ry_{adg} gene shows tetrad sterility but produces seeds. The fact that a tetrad sterile clone can produce seeds is an important finding, and this suggests that the ability of a tetrad sterile clone to produce seeds may be due to a fertility-enhancing gene. Although seed production is an important criterion for determining whether the seed is male fertile or sterile, fruit set percentage (FSP) values should also be considered in future studies. FSP is calculated by dividing the number of fruits formed by the number of flowers fertilized. Low FSP may indicate low pollination rates and may be another indicator of male sterility.Therefore, evaluating fruit set percentages may provide more information about the reproductive success of these clones.

In addition, we cannot separate possible clonal effects from the effects of genes; there may be background genetic effects independent of the resistance gene within each clone, and there is no way to evaluate this. However, to reduce the variation found in our samples, especially in the Ry_{adg} gene, more clones containing the Ry_{adg}, Ry_{chc}, Ry_{sto}, *and* $Ry\text{-}f_{sto}$ genes should be evaluated for reproductive traits, and the same female plant should be used to understand the difference in seed set.

Conclusion

In conclusion, this study highlights the significant effects of gene and cytoplasm type on male fertility in potato clones. While a statistically significant difference was observed in pollen viability across gene groups and cytoplasm types, no such difference was found in pollen quantity or seed set. By identifying male-fertile varieties resistant to PVY, we can enhance crop yield and sustainability by reducing reproductive failures, low seed sets, and limitations in parent selection. Thus, we can help the potato industry face the ongoing challenges posed by viral infections.

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