DIPLOID POTATO BREEDING: PLOIDY DETERMINATION IN 2X × 2X *Solanum tuberosum* DIHAPLOIDS CROSSES BY SELF-COMPATILE DONORS WITH SCREENING AND IDENTIFICATION OF SNP MARKERS ASSOCIATED WITH *Ralstonia solanacearum* RESISTANCE IN THE WILD SPECIES *Solanum commersonii*

By

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ABSTRACT

DIPLOID POTATO BREEDING: PLOIDY DETERMINATION IN 2X × 2X *Solanum tuberosum* DIHAPLOIDS BY SELF-COMPATIBLE DONORS WITH SCREENING AND IDENTIFICATION OF *Ralstonia solanacearum* RESISTANCE SNP MARKERS IN WILD SPECIES OF *Solanum commersonii*

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Solanum (*2n = 2x* = 24) species (*Solanum chacoense*) when used as male self-compatible donor can be easily hybridized with other wild species (*2n = 2x* = 24) and dihaploids of *Solanum tuberosum* (*2n = 4x* = 48). Previous work carried out using diploid breeding techniques has had success and this has culminated into potato breeding platforms that exploit the *2n* gamete breeding scheme with a focus on creating genetic diversity. In a diploid breeding program, the spontaneous occurrence of tetraploid progeny complicates the breeding process. Thirty-five 2x × 2x crosses were made to examine spontaneous incidences of tetraploid progeny in the F_1 hybrid progeny of 2x × 2x *S. tuberosum* dihaploids by self-compatible donors *S. chacoense* (M6) and DRH S6-10-4P17. In these 2x × 2x crosses we expect both diploid and tetraploid progeny. Results showed that by using chloroplast counting and SNP genotyping, the frequency of tetraploid progeny ranged from 0 to 40% in the 35 crosses between Atlantic or Superior dihaploids to self-compatibility donors. This result is attributed to bilateral sexual polyploidization (BSP) which is the result of *2n* egg and pollen formation. The results of this study will inform breeders developing diploid germplasm the potential consequences of 2x × 2x crosses between Solanum species and dihaploids of *S. tuberosum*.

Bacterial wilt, caused by *Ralstonia solanacearum* (Rs), is a major disease in most tropical, subtropical and temperate potato producing regions of the world. Breeding for bacterial wilt disease resistance in potatoes is challenging due to the pathogen's aggressive nature and persistence in the environment. Accessions of *S. commersonii* are known to harbor resistance, therefore, breeding for genetic resistance to bacterial wilt may be an effective strategy to control bacterial disease. The genetic variation among S_1 selfed progeny of *S. commersonii* line MSEE912-08 was characterized by artificially inoculating them with an isolate of Rs. One hundred and twenty individual S_1 selfed progeny were screened for Rs resistance by artificial inoculation with Rs isolate NAK66 in a glasshouse replicating each line three times in the experiment. Percent

disease reaction was calculated as the relative area under disease progress curve (RAUDPC). RAUDPC was fitted to a random effects mixed model and the means were transformed as a Best linear unbiased prediction (BLUP) score for each clone. BLUP values ranged from -19.25 for the most resistant to16.83 for the most susceptible clones. Genotyping S_1 individuals using the V3 Illumina Infinium Array generated 117 polymorphic single nucleotide polymorphic (SNP) markers from the selfed population. Using 98 of the 120 progeny the significance of the markers was confirmed by single marker analysis from WindowsQTLCartographer 2.5, as well as a single marker ANOVA test using JMP®, Pro13. Significant markers were identified on Chromosome 4 which explained 9.8% to 13% of the phenotypic variation. SNP markers identified resistance associated with a homozygous genotype. Specifically, the genotypic value prediction from BLUP scores in single marker ANOVA showed that the homozygous genotype of the SNP markers in solcap_snp_c2_35970, PotVar0015326, PotVar0075537, solcap_snp_c1_10181, solcap_snp_c1_4109 and solcap_snp_c2_12904 were significantly associated with Rs resistance. This study was able to discover self-compatibility in *S. commersonii*. This study provides potential germplasm that can be used in marker assisted breeding in potato. Secondly, self-compatibility was identified in *S. commersonii* and should be further evaluated for use in a diploid breeding program.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1. DIPLOID POTATO BREEDING PROSPECTS AND OPPORTUNITIES

1.1 GENETICS AND BIOLOGICAL ASPECTS OF POTATO PLOIDY

The cultivated potato *Solanum tuberosum* is a polysomic tetraploid (*2n = 4x =* 48) that is propagated vegetatively (Peloquin et al., 1999). Previous research has utilized dihaploid plants (*2n = 2x =* 24) obtained from tetraploids to capture their genetic diversity (Peloquin et al., 1999). The use of dihaploids presents the advantage of a diploid potato breeding scheme. Previous studies demonstrated that *2n* gametes facilitates efficient transfer of allelic diversity of both quantitative and qualitative traits. Additionally, geneticists and breeders can exploit sexual polyploidization by rigorously identifying genotypes that have high *2n* gamete production for potato polyploid production (Lim et al., 2004).

2n gametes are the result of modified meiosis affecting specific stages of micro- and megasporogenesis, otherwise referred to as meiotic anomalies. *2n* gametes possess an unreduced chromosome number. *2n* gametes are essential in genetic expansion of cultivated tetraploid genotypes, allowing for spread of allelic diversity, transmission of high parental heterozygosity, epistasis and useful genes from crops wild relatives gene pool through sexual polyploidization breeding scheme (Mendiburu and Peloquin, 1977; Camadro and Masuelli, 1995; Carputo et al., 1999; Peloquin et al., 1999). There is integration of genetic diversity of multiple diploid species ensured through *2n* gamete formation. The phenomenon consequently allows for progressive introgression of polyploids which contributes significantly to the heterozygosity or new allelic diversity in the progenitors, compared to somatic doubling which does not change the genotype except for allele redundancy (Watanabe and Peloquin, 1989; Camadro and Masuelli, 1995; Carputo et al., 1999; Peloquin et al., 1999; Carputo et al., 2000; Mason and Pires, 2015). Additionally, *2n* gametes play a fundamental role in capturing a relatively high reservoir of untapped diversity. *2n* gametes present a potential breeding value with genetic gain that can arise from qualities like disease resistance in wild cultivars coupled with environmental adaptation (Ehlenfeldt and Hanneman, 1984).

1.1.1 Causes of *2n* gamete formation

There are myriad of mechanisms behind *2n* gamete formation such as parallel spindles, synaptic mutation, abnormal meiotic aberrations like premeiotic doubling, chromosome pairing anomalies and abnormal premature cytokinesis (Douches and Quiros, 1988; Dewitte et al., 2012; Younis et

al., 2014). Many authors attribute most of *2n* gamete mechanisms to either first division restitution (FDR) or second division restitution (SDR) type gametes. The utilization of *2n* gamete mechanisms has allowed for interspecific hybridization for the transfer of desirable and novel traits to new varieties (Younis et al., 2014). In meiotic polyploidization, *2n* gametes have been used extensively to enhance intergenomic recombination between alien chromosomes leading to higher levels of diversity resulting in enhanced broad selection for development (Ramanna and Jacobsen, 2003; Younis et al., 2014).

1.1.2 Mechanism of *2n* gamete formation

Mok and Peloquin (1975) attribute heterosis of yield in most plants to the mode of *2n* pollen formation in the 2x parent. The parallel spindle (*ps*) gene results in parallel orientation of the second meiotic division spindle and is genetically equivalent to FDR. In this phenomenon, all loci between the centromere and beginning of the first cross over that are heterozygous in the 2x parent will be heterozygous in the gamete and half of the loci between the first and second cross-over will be homozygous (Hermundstad and Peloquin, 1987). 80% of total heterozygosity of the 2x parent is channeled to 4x progeny via the parallel spindle mechanism. The SDR is believed to play a key role in the mechanism of *2n* egg formation which contrasts from FDR because only about 40% of parental heterozygosity is channeled from parent to the offspring (Johnston and Hanneman, 1980; Carputo et al., 2003). If a SDR *2n* egg combines with FDR *2n* pollen it can establish highly heterozygous 4x progeny (Peloquin et al., 1989).

Some previous work has also highlighted the utilization of dihaploid crossing with another diploid species hybrid combining *2n* gametes which results in tetraploid progeny which is referred to as bilateral sexual polyploidization (BSP) (Hermundstad and Peloquin, 1985). This scheme maximizes heterozygosity due to the fact that there is both inter- and intra-locus interactions from both parents that is transmitted through the *2n* gamete mechanisms to the offspring (Hermundstad and Peloquin, 1985). Other schemes have focused on unilateral sexual polyploidization (USP), which involves 4x cultivar crossing with 2x with the capability to produce *2n* gametes (Peloquin et al., 1989). One benefit of this system allows for the utilization of an already adapted cultivar as one of the parents (Peloquin et al., 1989). Contrary to the first breeding scheme USP has transmission of *2n* gametes from only one parent therefore having lower levels of interactions

which is less effective as in bilateral sexual polyploidization scheme (Hermundstad and Peloquin, 1985).

1.1.3 Tuber formation capability of dihaploid-species hybrids

Research conducted over the last 40 years, showed the high potential for the utilization of dihaploids in the breeding program. Some of the parents used in the production of dihaploid possessed good tuberization ability. Furthermore, dihaploid evaluation via crosses to wild species showed high variability in their ability to tuberize (Hermundstad and Peloquin, 1985).

Hermundstad and Peloquin (1985) noted that "the reason for the variability of tuberization within dihaploid species is attributed to a two-locus model for maturity". At one locus, the maturity locus *m*, that causes late maturity is dominant. The differences in the *Tuberosum* germplasm ability to tuberize may be due to differences at the maturity locus. This is also evident among dihaploids derived from the same tetraploids. Therefore, *MM*, *Mm* and *mm* dihaploids could be derived from a duplex (*MMmm*) tetraploid (Hermundstad and Peloquin, 1985). The *MM* dihaploid if produced, would be late and hence produce late hybrids that are unable to tuberize in the particular growth environments. Alternatively, though, early hybrids would result from crosses between either *Mm* or *mm* dihaploids and diploid species. Therefore, the dihaploid maternal gametes derived from a tetraploid parent allows for selecting gametic genotypes that possess desired traits from the tetraploids. This allows for selection of dihaploids that are either homozygous recessive or heterozygous. Most diploid wild Solanum species on the other hand have been documented as highly diverse and self-incompatible. This self-incompatibility is observed when they are crossed with dihaploids with a high level of within-family variability as was noted by Hermundstad and Peloquin (1985).

1.1.4 Intra-locus interactions for tetra allelic and dihaploids derivatives of tetraploids

Previous heritability studies have showed low heritability for tuber weight and total yield. The low heritability is attributed to occurrence of homozygosity coupled by loss of inter-locus interactions (Peloquin et al., 1991) which has been hypothesized to play a key role in explaining the yield reduction in tetraploids (Peloquin et al., 1991). There are three types of segregation that has been identified in the polyploids including, chromosome segregation, random chromatid segregation and maximum equational segregation (Carputo et al., 2005). Additionally, at each biallelic locus

there are five genotypes: aaaa, Aaaa, AAaa, AAAa and AAAA. Under multi-allelic models there are tetrallelic (e.g. A1A2A3A4) and triallelic (e.g. A1A2A3A3) loci that provides opportunity for a high number of intra-locus interactions (Watanabe et al., 1995; Carputo et al., 2005). The use of sexual reproduction would create an abundance of diversity through the recombination of allelic variants of gene which arose from interspecific hybridization. The tubers that grow from the genetically unique seedlings can be planted as seed tubers, and consequently as distinct clones that can be established and maintained through the asexual vegetative reproduction (Carputo et al., 2005; Siri et al., 2008). Tetraploids have six possible first order interactions (Table 1.1) which have been hypothesized to contribute to maximum heterozygosity as compared to dihaploids which has only one possible first order interaction, hence the sharp yield reduction experienced (Peloquin et al., 1991).

Table 1.1 Comparison of dihaploids and tetraploid intra-locus allelic interactions

Genotype	**1st order interactions**	**2nd order interactions**	**3rd order interactions**
4 x parent	A1 A2 A3 A4 (A1 A2, A1 A3, A1 A4, A2 A3, A2 A4, A3 A4)	A1 A2, A1 A3, A1 A4, A2 A3, A2 A4, A3 A4	A1 A2 A3 A4
Dihaploid parent	A1 A2	-	-

Source: From Peloquin et al. (1991)

1.1.5 Determining ploidy levels

The chloroplast count technique has been used as the rapid and cost-effective method of determining ploidy levels at any stage of plant growth. The technique counts the chloroplast number in the stomatal guard cell under magnification. Chloroplast counting has been applied to many plant species (mulberry, red clover and sugar beet) to hasten disposal of undesirable higher ploidy levels and early identification of monoploids and diploids (Chaudhari and Barrow, 1975). Alsahlany et al. (2019) cites several examples where chloroplast counts have been given priority in applied breeding programs to ensure quality control in terms of ploidy level preservation in early generation of breeding programs to save on resources, workload and time duration. Additionally, studies comparing the correlation of chloroplast count technique to flow cytometry and Single Nucleotide Polymorphism (SNP) Array method demonstrated the efficiency of

chloroplast count to determine ploidy (Veilleux et al., 1985). Moreover, Ellis et al. (2018) used the potato SNP array in interrogating potato ploidy level at the International Potato Center (CIP) gene bank. Their study revealed that SNP genotyping is efficient and precise even though it is time consuming and expensive compared to chloroplast count. Other studies where chloroplast counting has been employed to determine ploidy levels for both in-vitro and in vivo plants (Veilleux et al., 1985; Gebhardt et al., 2006) demonstrated that the chloroplast counting was the fastest, most accurate, efficient and cost effective technique for identification of ploidy (Veilleux et al., 1985). At CIP, chloroplast counting was determined to be more precise, simple and economical due to the high correlation existing between the number of chloroplast and the number of chromosomes (Ordoñez, 2014).

Table 1.2. The ploidy level determination scale

Stomatal guard cell chloroplast number	**Ploidy level**
6-8	Diploid
9-11	Triploid
12-14	Tetraploid

Ploidy level determination scale of potato genotypes adopted from (Ordonez, 2014)

1.2 *Ralstonia solanacearum* RESISTANCE IN *Solanum commersonii*

1.2.1 Virulence factors of *Ralstonia solanacearum*

Ralstonia solanacearum (Rs) is a soil borne pathogen classified as belonging to the subdivision of Proteobacteria that causes lethal wilting disease to over 200 plants species (Poueymiro and Genin, 2009). The signature characteristic of the disease is its fitness ability to cause swift and effective bacterial colonization in xylem tissue of the affected plant. Additionally, Rs has the ability to persist in the environment (Hayward, 2000; Milling et al., 2009; Poueymiro and Genin, 2009). Rs have Type III effector genes (T3SS) also called Type III protein secretion system (T3SS), which is highly conserved in the species. The pathogen can maneuver the primary defense mechanisms against microbes' effects also known as pathogen-associated molecular pattern (PAMP). The mechanism has ability of the T3SS basal pathogenicity to restrain this PAMP triggered immunity

in host plants. This is coupled by possessing substrates that suppresses hypersensitive response (HR) which occurs in some plants like tomato (Poueymiro and Genin, 2009).

The pathogen's ability to cause disease to the host plant is caused by the well characterized virulence factors. There is a myriad of factors leading to increased pathogenicity of bacterial wilt in plants (Genin and Denny, 2012). The increased pathogenicity is caused by occurrence of high levels of extracellular polymeric substances (EPS) molecular mass. The EPS is attributed to enhancing speedy systemic colonization. Whereas, even if the wilt is introduced to the mutant EPS there will be no wilt occurrence despite being injected straight into the xylem (Genin and Denny, 2012). This EPS accumulation has been reported to cause vascular dysfunction that results into susceptible plants wilting. Additionally, EPS causes increased presence of ethylene coupled with salicylic defense response pathway as was experienced in the tomato breeding cultivar Hawaii7996 compared to the non-resistant cultivar (Milling et al., 2011; Genin and Denny, 2012).

The pathogen motility is another factor that increases virulence of Rs. Motility helps the pathogen to locate and invades host plant roots (Genin and Denny, 2012). Additionally, there are myriad of stressful environmental cues and compounds encountered by Rs such as reactive oxygen species (ROS) that are normally emitted by plants after infection as the first line of defense against the pathogen. This causes the Rs to develop a corresponding redundant mechanism to detoxify ROS or conversely tolerate this oxidative environments that are prevalent in the plants (Genin and Denny, 2012). The Rs has assisted pathogen to detoxify phenolic compounds even though their biological significance has not been unraveled. There is also information on Rs producing phytohormones that are believed to cause increased virulence (Genin and Denny, 2012).

1.2.2 The innate immune receptor regulation by MicroRNA

Some of the mechanisms that have been employed in introducing plant innate immune receptors. Therefore, enabling employment of a RNAi method hence, controlling gene activity by short double stranded RNA (dsRNA) and specific protein complexes. This leads to the selective degradation of specific mRNA or inhibition of translation of many mRNAs in the cell (Zuluaga et al., 2015). Potato is amenable to many genetic improvement techniques but one reason that the development of highly resistant lines to Rs has not occurred may be due to inconsistency that occurs in the sequevar (a group of two or more identical gene-sequence variants). Usually, this is

determined based on the *egl* (Endoglucanase gene sequence) thus limiting the determination in some of them using the available genetic techniques (Albuquerque et al., 2015). Albuquerque et al. (2015) notes that "use of fingerprinting techniques (e.g. repetitive sequence-based polymerase chain reaction (PCR)) has permitted apportioning of isolates to clonal groups".

Using the strategy of the utilization of a R-gene that encodes cell surface innate immune receptors with the intercellular leucine rich repeat (LRR) domain and transmembrane domain, many plants have managed to circumvent some diseases (Foolad and Panthee, 2012). A vast percentage of R-genes encode intercellular innate protein possessing nucleotide binding (NB) and LRR domain. This domain possess the active R-gene that could have arisen through a combination of positive selection and duplication mechanism in the course of evolution in plant pathogen interaction (Foolad and Panthee, 2012). Some expression profiles documented that small RNA (sRNA)-mediated transcriptional gene silencing are profoundly critical in ensuring host plant protection from pathogens (Foolad and Panthee, 2012). Rs resistance have been challenging due to scarce resources in terms of molecular basis of resistance breeding in plants (Liu et al., 2016). Fortunately, in tobacco, tomato and eggplant, progress has been made in the identification of quantitative traits loci (QTL) in Rs resistance breeding (Tran et al., 2016).

1.2.3 Resistance mechanism to *Ralstonia solanacearum* (Rs)

Rs resistance has been characterized in *Arabidopsis thaliana*. The process is mediated by Toll and Interleukin-1 receptors that mediate innate immunity in plants (TIR-NB-LRR), whereas others encode proteins with a coiled-coil domain at the N terminus (Foolad and Panthee, 2012). This therefore allows for monitoring the expression differential occurring in plants when challenged with different pathogens. The plants equally produce different immune responses to counter the pathogen. Another very important technique effectively employed in measuring the resistance levels between different lines is through proteome analysis. This method shows the different levels of up regulation of different proteins among the infected plants as opposed to the healthy plants which has in effect been used in differential protein expression under diseases scenarios (Park et al., 2016).

1.3 MOLECULAR MARKERS TO ENHANCE IDENTIFICATION OF USEFUL TRAITS IN CROPS

The utilization of traits from wild germplasm as well as uncultivated crop-wild relatives has enabled breeding of potatoes for disease resistance and important agronomic traits (Bradshaw et al., 2006; Ramakrishnan et al., 2015). Resistance to late blight is an example of introgression of wild germplasm *S. demissum* into the cultivated species (Vossen et al., 2016). Additionally, molecular markers have been used by breeders to identify by a predictive approach the best plants available in large populations (Bernardo, 2008). Molecular markers have been advantageous during generations in which phenotypic selections is impractical or ineffective thereby allowing for genomewide prediction of the performance of each population generated from clonal selections, self-pollinated species, or test cross selection in a hybrid crop (Bernardo and Yu, 2007).

Plant breeding has been conducted for several years through conventionally crossing best parents and thereafter selection and recovery of superior or individuals that out perform their parents (Collard and Mackill, 2007; Moose and Mumm, 2008). Plant breeding has been useful in increasing genetic diversity of crops and thus genetic gain has been accelerated through molecular plant breeding in hybrids crops like maize and now being extended to other crops (Moose and Mumm, 2008). High throughput genotyping coupled with molecular markers have allowed efficient characterization of genetic diversity in germplasm pool for nearly all crop species. In potato, molecular markers have provided additional information that would have otherwise not been possible through conventional breeding. Molecular information enriches investigation of plant evolution structure. Molecular markers allow for maintaining and characterizing genetic diversity reservoir for future mining of beneficial alleles (Horst and Wenzel, 2007; Collard and Mackill, 2008; Hamilton et al., 2011).

1.3.1 Major QTL identification

Thousands of SNPs can be used in the construction of a genetic maps. This density is useful in the identification of genes associated with important agronomic traits (Manrique-Carpintero et al., 2015). Several QTLs across the genome have been responsible in controlling tuber yield and specific gravity which are located on Chromosomes 1, 2, 3, 4, and 7 when different diploid

populations were evaluated (Bonierbale et al., 1993; Freyre and Douches, 1994; Schäfer-Pregl et al., 1998; Manrique-Carpintero et al., 2015).

Extensive work has led to the identification of candidate genes coupled with association mapping in the tetraploid populations. Candidate genes discovery has enabled identification of markers for superior alleles associated with tuber starch, sugar content and yield (Gebhardt et al., 2006; Draffehn et al., 2010; Li et al., 2013; Manrique-Carpintero et al., 2015). In potato, the Infinium Potato SNP array has played an important role in high throughput genotyping of SNPs (Felcher et al., 2012; Manrique-Carpintero et al., 2015). Occurrence of high density genetic maps in diploid populations of potato coupled with the ability to offer genome wide coverage and close mapping of traits of interest that are potential reference maps possessing transferrable markers (Manrique-Carpintero et al., 2015). Gebhardt et al. (2011) has proposed that to identify complex traits, it would be prudent to use a combined strategy of association mapping and candidate gene analysis having dense genetic maps exhibited with SNPs markers anchored to the genome sequence. Wang et al. (2013) suggests that bacterial wilt resistance in tomato is location-specific and strain specific (Lopes et al., 1994; Hanson et al., 1996; Li et al., 2005). Some of the stable resistance has been achieved in tomato with a resistant QTL on Chromosome 6 from the variety Hawaii 7996 (Wang et al., 2013).

1.3.2 Quantitative trait loci (QTL) mapping for crop improvement

Genetic mapping

Plant genome sequencing has played a major role in mapping gene functions, gene regulations and expression. Furthermore, high resolution SNP maps have been used to tag and identify desired genes (Mohan et al., 1997). Several research groups have deployed map-based cloning and transposon tagging to isolate genes corresponding to desirable traits. Genetic mapping allows one to obtain a fine scale linkage map using DNA markers as well as a genomic DNA library of large-sized fragments which are equally screened with linked markers of the anticipated trait (Mohan et al., 1997). Additionally, to resolve complex quantitative traits occurring in a single Mendelian complex, efforts have been geared toward construction of near isogenic lines which carry one or multiple chromosomal segments of one of the parental genetic stock required. Therefore, by using the alternative lines or the near isogenic lines thus enables the handling of a given QTL as a single Mendelian factor (Mohan et al., 1997).

Traditional establishment of linkage maps entails a segregating plant population developed through sexual reproduction which possess parents differing in one or more traits of interest. A mapping population size for preliminary genetic studies ranges from 50 to 250 individuals (Collard et al., 2005) with higher resolution mapping requiring higher population sizes. In QTL analysis the mapping population must be phenotypically evaluated by collecting trait data and subsequently QTL mapping is performed (Collard et al., 2005).There are various populations that have been previously used in mapping (Collard et al., 2005). Typically, F_2 populations and backcross (BC) populations are the simplest type of mapping population having an advantage of ease to produce the population due to short time requirement. Other mapping populations are recombinant inbred (RI) and double dihaploids which generate homozygous lines which can be multiplied and sexually reproduced (Collard et al., 2005).

Identification of polymorphism is fundamental in the construction of a linkage map through identifying DNA markers that reveal differences between the progenies. This requires sufficient amounts of polymorphism between the parents to enable linkage map construction. Therefore, there is a need to have parents that are distantly related/having adequate polymorphism to provide genetic diversity and ultimately screened across the entire population being genotyped (Collard et al., 2005).

1.3.3 Linkage analysis

Collard et al. (2005) points out in the review on construction of a linkage map, that the final step entails coding data for each of the DNA markers within the individuals in the working population facilitated by mapping software (Join Map 4.1, MapQTL6, MapChart, WinQTL Cartographer version 2.5). Where the linkage between markers are analyzed and used to construct maps, the linkages are calculated using odds ratio (Van Ooijen and Kyazma, 2009; Wang et al., 2012) and therefore accuracy is highly derived from the number of studied individuals. Some authors endorse a minimum of 50 individuals for a linkage map construction (Young, 1994).

1.3.4 QTL analysis

The principle behind QTL analysis is dependent upon detecting an association between the phenotype and the genotype markers. Therefore, these markers essentially partition the mapping population into different genotypic groups based on presence and absence of a certain marker

allelic state. Consequently, the closer a marker is to a QTL peak, the lower the chances of recombination occurring between the marker and the QTL, therefore, will be inherited together in subsequent progenies. There are several methods documented for QTL detection: single marker analysis, composite interval mapping and multiple interval mapping with each detecting technique having its pros and cons (Collard et al., 2005). Conditional probabilities are used for each QTL genotype which is then calculated at specific centimorgan (cM) intervals. The phenotypic data are ultimately regressed on the QTL genotype at each position, with the regression coefficient weighted by the restrictive probabilities of the QTL genotypes. A permutation test then is used to set the LOD threshold to identify a QTL as significant hence confirms the presence of QTL (McCord et al., 2011).

QTL analysis is useful in identifying markers linked to a trait of interest and enables identification of markers that control a given trait of interest (Collard et al., 2005; Collard and Mackill, 2007). QTL mapping is conducted with a structured population where parents are selected based on divergence in phenotypes to ensure the capturing maximum phenotypic variation to get clear correlation between genotype and trait phenotype. To identify genes responsible for a trait of interest, QTL mapping is the first step. QTL mapping is normally followed by positional cloning or through candidate gene analysis (Menéndez et al., 2002) to confirm the results of mapping. For example, many corroborative studies have been found for foliage maturity QTL on Chromosomes 3 and 5, in potato (McCord et al., 2011; Manrique-Carpintero et al., 2015; Massa et al., 2015). QTL for specific gravity on Chromosomes 2, 5, 7, 9 and 11; and QTL for yield on Chromosomes 2, 5, 6 and 8 (Freyre and Douches, 1994).

During QTL analysis, there are genetic predictors which can be calculated epistatic effects, additive and dominance. As described by Van Eeuwijk et al. (2010). The total number of base parental alleles contribution in a population plays a major role in the additive genetic predictor. Therefore, the detection of a subset of genetic predictors responsible for the observed phenotypic differences aggregates to QTL mapping. When a significant part of phenotypic variation is described by a genetic predictor a QTL is detected (Van Eeuwijk et al., 2010).

Consequently, QTL effects are normally fixed parameters unless there are effects greater than 10. In cases of multi-trait QTL analysis, the aim is to illustrate genetic variation surrounded by traits and the genetic associations among traits in terms of signs and magnitude of QTL effects (Van

Eeuwijk et al., 2010). A mixed model has been used for QTL analysis via modification of variance and correlations governing the genotypic effects. The variance-covariance matrix for genetic effects during multi environmental QTL analysis allows for partitioning of the genotypic main effects and the genotypic by environment interactions into parts due to regression on a predictor and residual as described by Van Eeuwijk et al. (2010).

1.3.5 Classes of disease resistance genes

Plant disease resistance can be clustered into two distinct groups: qualitative and quantitative resistance. The former is known to be instigated by single resistance (*R*) gene whereas quantitative resistance mediated multiple genes otherwise called QTL characterized by partial and durable resistance which is pathogen species-nonspecific but species-specific (Wisser et al., 2005; Kliebenstein and Rowe, 2009; Poland et al., 2009). Plants have, over time, defined their disease evasion routes through plant-pathogen recognition patterns. This occurs through signal transduction pathways that interact with each other to form complex network leading to defense responses. In this mechanism plants develop innate immunity by quickly discriminating self from non-self, hence pathogen/microbe associated molecular patterns (PAMPS), or MAPS (Panstruga et al., 2009; Kou and Wang, 2010).

Major QTLs have been grouped as those QTLs that explain >10% of phenotypic variation whereas those that explain <10% of phenotypic variation as minor QTLs (Poland et al., 2009; Van Eeuwijk et al., 2010). Hence these genes are associated with minor QTLs largely seen as playing a fundamental role in quantitative broad spectrum resistance and durable resistance (Kou and Wang, 2010). There are three major QTLs that have been isolated for fungal diseases resistance through map–based cloning in different crops. For most part, resistance QTLs exhibited small effects on disease resistance. Kou and Wang (2010) suggest that there are several host pattern recognition receptor (HPRR) genes and defense responsive/defense related genes that have been identified to play a key role in quantitative resistance.

The identification of QTLs governing variability of traits involved in stress tolerance is of great benefit. Additionally, the identification of such genes will help scaling up crop performance in the field at different stress scenarios. The markers identification will allows for a clearer understanding of their role in agronomic value and allelic functions in crop improvement (Tardieu and Tuberosa, 2010). Tolerance can be loosely defined as “capability of plants to withstand harsh stresses and

still manage to accomplish the growing cycle and get reasonable yields" (Tardieu and Tuberosa, 2010). Bonierbale et al. (1994) clarifies that superimposing QTL data for a wide range of phenotypic data like biochemical assays, direct screening and correlative resistance to insects as well as its adaptation to the target environment on genetic map enabled them to identify and understand the genes controlling resistance mechanisms. In my thesis research, the study focused in identifying Rs resistance progeny and detected some significant SNP markers responsible for Rs resistance which can be used in the management and control of Rs in potato.

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CHAPTER 2. PLOIDY DETERMINATION IN 2X × 2X CROSSES OF *Solanum tuberosum* DIHAPLOIDS BY SELF-COMPATIBLE DONORS

2.1 ABSTRACT

Solanum chacoense (*2n = 2x* = 24) when used as male self-compatible donor can be easily hybridized with many other wild species (*2n = 2x* = 24) and dihaploids of *Solanum tuberosum* (*2n = 4x* = 48). In a diploid breeding program, the spontaneous occurrence of tetraploid progeny complicates the breeding process. Thirty-five 2x - 2x crosses were made to examine spontaneous incidences of tetraploid progeny in the progeny of 2x - 2x *S. tuberosum* dihaploids crossed with self-compatible donors *S. chacoense* (M6) and DRH S6-10-4P17. In these 2x - 2x crosses we expect to find both diploid and tetraploid progeny. Results showed that by using chloroplast counting and SNP genotyping, the frequency of tetraploid progeny ranged from 0 to 40% in the 35 crosses between female dihaploids and male self-compatibility donors. This result is attributed to bilateral sexual polyploidization (BSP) resulting from *2n* egg and pollen formation. This study identified diploids that produce lower frequency of *2n* eggs. The results of this study suggest that dihaploids with lower frequencies of 2n eggs should be used in 2x - 2x crosses for diploid breeding schemes.

2.2 INTRODUCTION

Potato breeders have traditionally utilized the diverse germplasm of the diploid Solanum species and cultivated relatives of tetraploid cultivated potato *Solanum tuberosum* Grp. Tuberosum L. (*2n = 4x* = 48). Employing conventional breeding scheme has ensured accessibility of genes for disease resistance, tuber yield and stress tolerance to generate vigorous interspecific hybrids (Jansky and Peloquin, 2006). Notably, cultivated tetraploid potato crosses to diploids (*2n = 2x* = 24) from the South American relatives (Jansky and Peloquin, 2006; Alsahlany et al., 2019) are possible due to *2n* gamete formation in the diploid parents (Ramanna, 1983). The mechanisms of *2n* gamete formation in some clones exhibits considerable variation in the pattern of meiotic abnormalities depending upon the influence of environmental factors such as temperature, day length and age of plant (Ramanna, 1983).

The potato diploid germplasm pool has two major groups: the cultivated relatives (cultivar-group *S. tuberosum*) and the wild *Solanum* species (Jansky and Peloquin, 2006). Carputo et al. (2005)

noted that the boundary between “cultivated” and “wild” as well as the putative “wild” is unclear. Therefore, the landrace is considered to encompass the diploids formerly recognized as a distinct species which are members of *S. tuberosum* cultivar-groups such as, S. *tuberosum* and Groups Ajanjuiri, Phureja and Stenotomum (Jansky and Peloquin, 2006; Spooner, 2002). The potential offered through the utilization of diploid *Solanum* species hybrids results from the wide germplasm with higher number of male fertile plants, high tuber yield, set and size, tuber dormancy among other characteristics is enormous. These characteristics can be used in the breeding program to genetically improve the potato (Jansky and Peloquin, 2006).

The use of self-compatible diploid germplasm presents greater opportunities for genetic resource utilization of germplasm, but also make those resources available to researcher’s who can now access them more easily (Jansky et al., 2014). Additionally, diploid breeding can swiftly respond to targeted needs by releasing inbred lines or F1 hybrids as cultivars (Lindhout et al., 2011; Jansky et al., 2014). Some researchers have used potato clone M6 (previously designated *S. chc* 523-3) which is from a self-pollinated wild diploid *S. chacoense* species accession (Jansky et al., 2014). The hybrid between dihaploids crossed with M6 acts as a bridge to developing inbred potato lines (Jansky et al., 2014). Recovering tetraploids (which are naturally self-compatible (SC) in 2x - 2x crosses complicates the selection of progeny in diploid crosses. Therefore, finding 4x progeny in the diploid breeding program crosses require us to assess the frequency of its occurrence this will therefore form part of the quality control measure in diploid breeding programs.

Tetraploid progeny can result from bilateral sexual polyploidization (Mendiburu and Peloquin, 1977; Peloquin et al., 1999; Carputo et al., 2000; Carputo et al., 2003; Peloquin et al., 2008; Brownfield and Köhler, 2010). In scenarios where breeding is conducted with the dihaploids or diploid populations, there will be a need for ascertainment of the ploidy of the offspring prior to use in a crossing. The purpose of this study was to quantify and characterize the proportion of tetraploids from crossing *S. tuberosum* dihaploids with diploid SC donors. SNP genotyping and chloroplast counting were used to distinguish tetraploid and diploid in 2x - 2x crosses between *S tuberosum* dihaploids and self-compatible donors. The specific objectives were to make a series of 2x-2x crosses and to quantify the frequency of tetraploid and diploid progeny using chloroplast counting and SNP genotyping to determine ploidy level in the progeny. Employing ploidy

determination will help create effectiveness and applicability of genetic gains that can be realized in diploid potato breeding and genetics studies.

2.3 MATERIALS AND METHODS

2.3.1 Background of plant material

A total of 35 dihaploids from *S. tuberosum* cvs. Atlantic and Superior were used as female parents and were planted in 5L pots in the Winter/Spring of 2016 and 2017 at Michigan State University (Table 2.1). The female parents were from *S. tuberosum* Grp. Tuberosum. The male parents are M6 (*S. chacoense*) an inbred line derived by self-pollinating the diploid wild *S. chacoense* for seven generations (Jansky et al., 2014) and DRH S6-10-4P17, from Dr. Richard Veilleux of Virginia Tech University possessing self-compatibility traits. The plants were grown in the greenhouse under 20 - 25º C and 16 h photoperiod. Standard horticultural practices were applied to ensure optimum growth and nutrition is achieved from the plants. Seeds from 2x-2x crosses were extracted, dried and later germinated with each family progeny samples having 50 seedlings for evaluation. Two mature leaves were collected from each seedling after one month for chloroplast counting.

Table 2.1. Pedigrees of 2x - 2x crosses

No.	Code of F_1 progeny	Female parents	Male parents
1	MSEE900	R127H1	M6
2	MSEE901	R127H2	M6
3	MSEE902	ATL_V_23	M6
4	MSEE903	ATL_V_23	DRH S6-10-4P17
5	MSEE904	ATL-M-403	M6
6	MSEE905	ATL-M-170	M6
7	MSEE919	VT-SUP-96	M6
8	MSEE920	ATL-M-404	DRHS6-10-4P17
9	MSEE921	ATL-M-120	DRHS6-10-4P17
10	MSEE922	VT-SUP-96	DRHS6-10-4P17
11	MSEE923	ATL-M-427	DRHS6-10-4P17
12	MSEE924	VT-SUP-79	M6
13	MSEE925	VT-SUP-70	M6
14	MSEE926	VT-SUP-19	M6
15	MSEE927	VT-SUP-08	M6
16	MSEE928	ATL-M-120	M6
17	MSEE929	ATL-M-404	M6
18	MSEE930	ATL-M-405	M6
19	MSEE931	ATL-M-406	M6
20	MSEE932	ATL-M-409	M6
21	MSEE933	ATL-M-402	M6
22	MSEE934	ATL-M-401	M6
23	MSEE935	ATL-M-186	M6
24	MSEE936	ATL-M-188	M6
25	MSEE938	ATL-M-198	M6
26	MSEE939	ATL-M-429	M6
27	MSEE940	ATL-V-033	M6
28	MSEE941	VT-SUP-40	M6
29	MSEE942	VT-SUP-12	M6
30	MSEE943	ATL-V-006	M6
31	MSEE944	ATL-M-133	M6
32	MSEE945	ATL-M-153	M6
33	MSEE946	ATL-M-169	M6
34	MSEE947	ATL-M-187	M6
35	MSEE948	ATL-M-418	M6

2.3.2 Leaf sample collection and guard cell chloroplast counting procedure

One-month-old leaflets were collected from the apical part of the plants and placed in a moist Petri dish to prevent wilting. A strip of lower leaf epidermis was peeled off from the leaflet abaxial side near the vein structure using a pair of fine tweezers and immediately placed on a glass microscope slide (Figure 2.1). A total of 1750 progeny were counted for number of chloroplast per guard cell following the procedure by (Ordoñez, 2014).

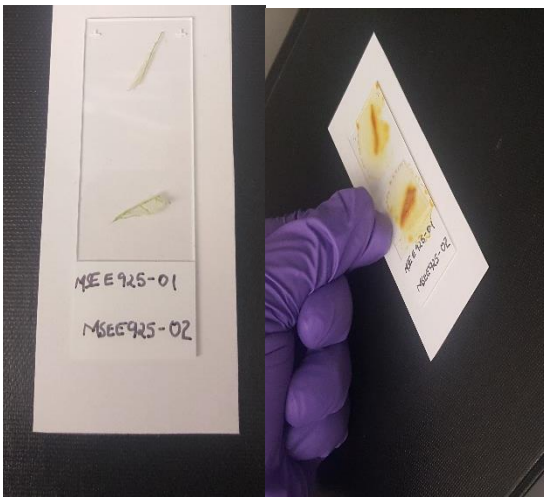

Figure 2.1. Staining of the lower epidermis on a drop of 1:1 iodine – potassium iodide to enable chloroplast visualization.

The peeled epidermis strip was then mounted on a drop of 1:1 propidium iodine- potassium iodide (PIPI). The PIPI solution was dissolved as described by (Alsahlany et al., 2019). Briefly, 500 mg of propidium iodide is dissolved together with 500 mg of potassium iodide in 50 ml of 70 % ethanol. After two minutes a cover slip was mounted, and observed under a light microscope (OLYMPUS BX60) at 400x magnification as described by Alsahlany et al. (2019) while adjusting the magnification to the level to obtain a clear view of the guard cell that appears or is seen as dark brown – black in color. A picture processing software called SPOT basic image capturing software was used to capture chloroplast image magnification which is transmitted to the computer (Fig. 2.2). The image captured by SPOT is designed for microscopy imaging having interphase preconfigured setting where image previewing is enhanced with the magnifier and focus gauges in the SPOT live window interphase into the computer (Fig. 2.2) (Spotsoftware, 2019). Chloroplasts were counted from 20 random guard cells per leaf to calculate an average.

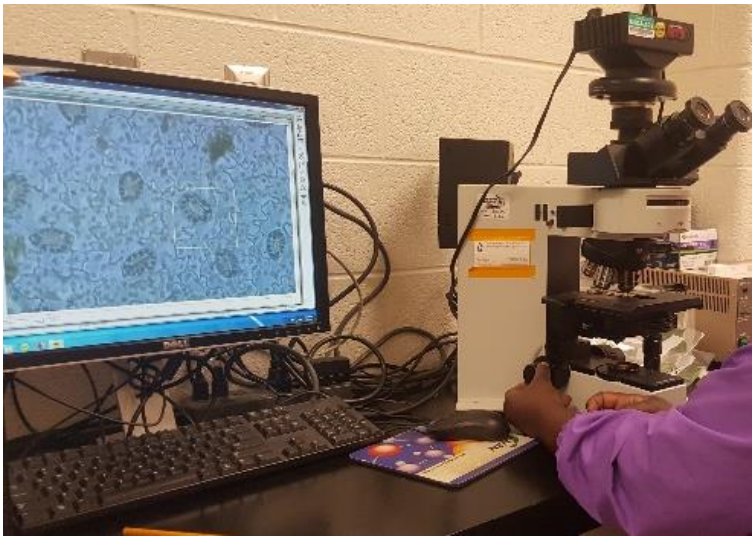

Figure 2.2. Chloroplast number counted under the microscope at 400X magnification.

2.3.3 Statistical data analysis for chloroplast count

Chloroplast count means from 20 stomatal guard cells were calculated for all 1,750 progeny. The data was analyzed with Microsoft Excel 2016 and JMP Pro 13 software (SAS Institute, Cary, NC) was used to calculate the means for classifying ploidy. The SNP calls and chloroplast count were analyzed using one-way ANOVA ($\alpha = 0.05$). Mean comparison was conducted with Tukey-Kramer honest significant difference (HSD). There were 14 reference samples for validation of ploidy: three tetraploid cultivars and 11 diploid samples derived from a recurrent selection breeding pool combining *S. tuberosum* Grp. Tuberosum and wild *Solanum* species.

2.3.3.1 Validating the chloroplast count with SNP genotyping

To compare chloroplast counting versus SNP genotyping for ploidy determination, Spearman's *p* correlation analysis was run. This study compared 137 potato progeny lines from a subset of the 35 2x - 2x crosses in this study. To validate the chloroplast count results, emerging leaf tissue was collected for DNA isolation from MSEE905, MSEE925, MSEE927, MSEE928, MSEE929, MSEE933 and MSEE939 that exhibited high level of tetraploid progeny. Additionally, 14 reference samples described above were sampled for SNP genotyping. Qiagen DNA extraction kit was used (DNeasy Plant Mini Kit (cat. Nos. 69104 and 69106, QIAGEN kit). SNP genotyping

was conducted using the 22K Infinium potato SNP array (Douches et al., 2014; Alsahlany et al., 2019).

2.3.3.2 Determination of ploidy

To analyze the SNP data, the auto-call genotypes were normalized and clustered (Illumina, San Diego, CA) using the GenomeStudio 2.0.4 Illumina software. SNP data filtering generated 11,581 SNPs. In order to identify the tetraploid SNP genotypes frequencies (AAAA, AAAB, AABB, ABBB, and BBBB) each SNP are summed across all SNPs in every sample as described by Alsahlany et al. (2019). The frequency of auto-called simplex and triplex SNPs (AAAB and ABBB, respectively) from a sample can be used to determine ploidy. The SNP genotype frequencies of each of the 137 progeny were compared to the mean simplex (AAAB) and triplex (ABBB) call frequency for the three tetraploid reference samples. To validate the sample ploidy, if the SNP auto-call simplex frequency is close to zero (<0.01), the sample is classified as diploid and when the simplex frequency is >0.20, the sample is classified as tetraploid. These simplex frequencies were based on the known reference samples (Supplemental Table 5).

2.4 RESULTS

2.4.1 Ploidy determination in 2x - 2x progeny

Based upon 11 diploids and three tetraploids reference samples, the diploid chloroplast count means ranged from 6.9 – 8.0 and tetraploids count means ranged from 12.0 -13.5 (Fig. 2.5). The individual progeny sorted into these two chloroplast count groupings. The average of chloroplast count for the diploid and tetraploid progeny was significantly different ($p < 0.0001$) (Table 2.2).

Table 2.2. Mean comparison of chloroplast count in progenies of 2x × 2x crosses.

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Ploidy	1	1.4560744	1.45607	571.6260	<.0001*
Error	150	0.3769930	0.00255		
Total	151	1.8330673			

The chloroplast counts revealed both diploid and tetraploid progeny occurring in the 35 2x × 2x crosses (Table 2.3). As expected, majority of the progeny in the 2x × 2x crosses were diploid but tetraploid progeny was observed at varying levels from 0 – 40% as shown in Table 2.3.

Table 2.3. Ploidy determination of 35 2x × 2x family based on chloroplast counts

Family	Crosses	2x Diploid (%)	4x Tetraploid (%)
MSEE929	ATL-M-404 × M6	60	40
MSEE923	ATL-M-427 × DRHS6-10-4P17	66	34
MSEE925	VT-SUP-70 × M6	66	34
MSEE920	ATL-M-404 × DRHS6-10-4P17	76	24
MSEE939	ATL-M-429 × M6	78	22
MSEE927	VT-SUP-08 × M6	82	18
MSEE928	ATL-M-120 × M6	82	18
MSEE933	ATL-M-402 × M6	86	14
MSEE905	ATL-M-170 × M6	86	14
MSEE926	VT-SUP-19 × M6	86	14
MSEE940	ATL-V-033 × M6	88	12
MSEE932	ATL-M-409 × M6	88	12
MSEE946	ATL-M-169 × M6	90	10
MSEE948	ATL-M-418 × M6	90	10
MSEE935	ATL-M-186 × M6	92	8
MSEE901	R127H2 × M6	92	8
MSEE922	VT-SUP-96 × DRHS6-10-4P17	92	8
MSEE930	ATL-M-405 × M6	94	6
MSEE903	ATL_V_23 × DRH S6-10-4P17	94	6
MSEE904	ATL-M-403 × M6	94	6
MSEE921	ATL-M-120 × DRHS6-10-4P17	96	4
MSEE902	ATL_V_23 × M6	96	4
MSEE900	R127H1 × M6	100	0
MSEE919	VT-SUP-96 × M6	100	0
MSEE924	VT-SUP-79 × M6	100	0
MSEE931	ATL-M-406 × M6	100	0
MSEE934	ATL-M-401 × M6	100	0
MSEE936	ATL-M-188 × M6	100	0
MSEE938	ATL-M-198 × M6	100	0
MSEE941	VT-SUP-40 × M6	100	0
MSEE942	VT-SUP-12 × M6	100	0
MSEE943	ATL-V-006 × M6	100	0
MSEE944	ATL-M-133 × M6	100	0
MSEE945	ATL-M-153 × M6	100	0
MSEE947	ATL-M-187 × M6	100	0

The diploid F_1 chloroplast count was significantly different from the tetraploid chloroplast count, Table 2.2 (ANOVA, $\alpha = 0.05$). The ploidy results of the 35 families found that ATL-M-404 as a female had tetraploid frequencies of 24% and 40% (Table 2.3). Thirteen families had 100% 2x progeny. Eight families had a tetraploid frequency between 4 - 8%. There were 14 families that

had a tetraploid frequency of 10 - 40%. Almost half of the Atlantic dihaploids and 11% of the Superior dihaploids used as female parents produced tetraploid progeny. R127H1 produced 2x progeny, while R127H2 has 8% tetraploid progeny (Table 2.3). This high occurrence of 4x progeny is attributed to bilateral sexual polyploidization (BSP) where both parents produce *2n* gametes thus favoring formation of tetraploid offspring (Mendiburu and Peloquin, 1976; Peloquin et al., 1989; Peloquin et al., 1999).

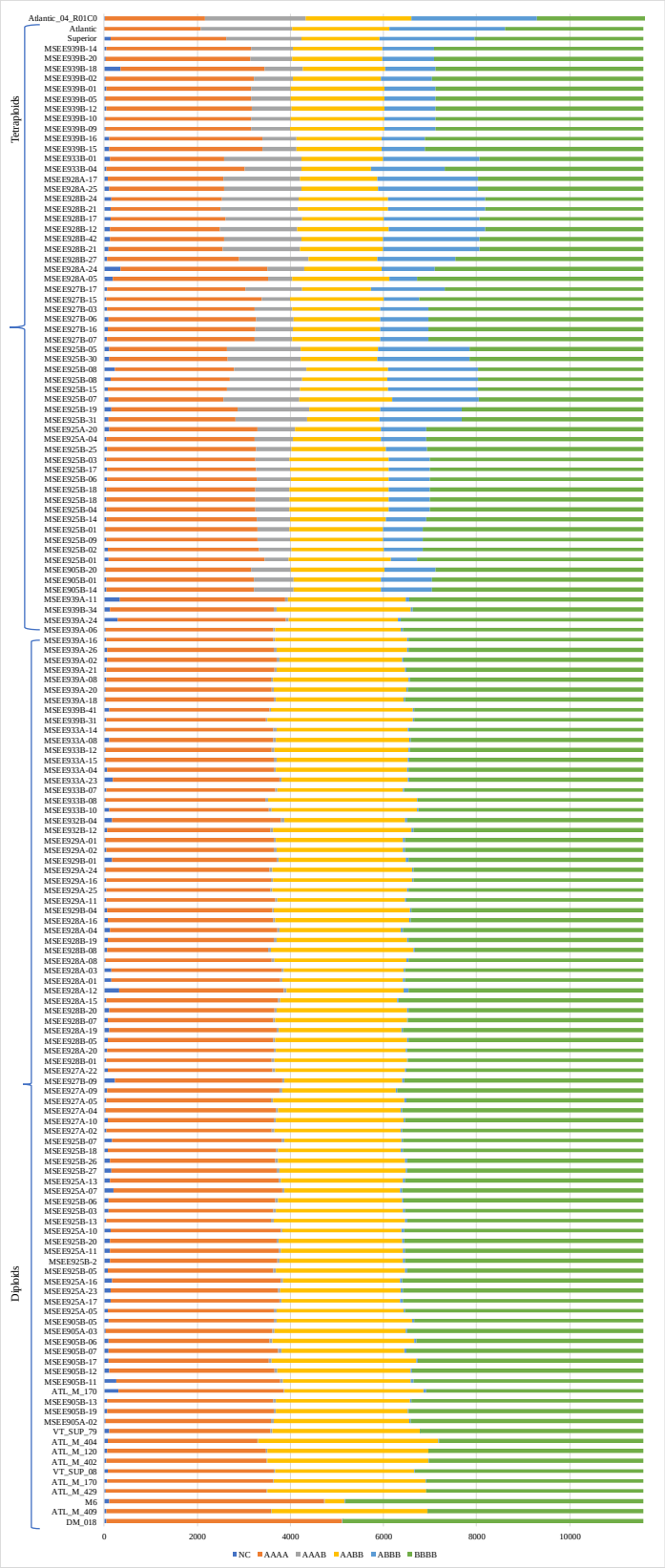

Figure 2.3. Genotypes SNP summary for over 137 individuals with 14 reference samples.

Figure 2.3 (cont'd)

* NC=No calls. Reference samples included are three rows are tetraploid (top) and bottom 11 rows are diploid.

2.4.2 SNP Genotype frequency identification

When SNP genotype frequency was auto-called, there were a total of 82 individuals that classified as diploid and 55 as tetraploid (Fig. 2.4). Based upon the 11 diploid and three tetraploid reference samples, the mean SNP genotyping standard deviation was 0.01 for diploids and more than 0.14 for tetraploids (Fig. 2.5). There were more SNP calls for the AAAA and BBBB followed by the duplex AABB. The study observed lower SNP calls frequency for the triplex (ABBB) and simplex (AAAB) as was reflected by more diploids than tetraploid counts (Fig. 2.4).

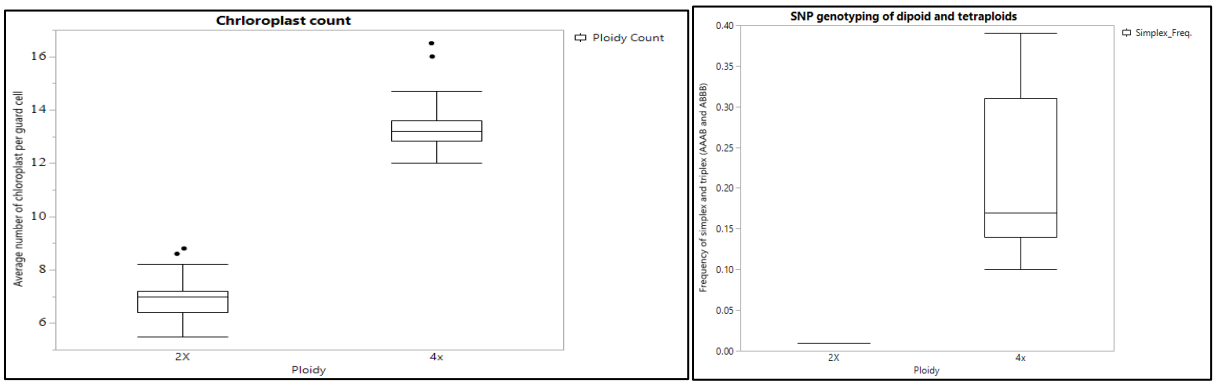

Figure 2.4. Comparison of SNP frequency and chloroplast count.

Table 2.4. SNP genotype frequency and chloroplast count correlation analysis.

Ploidy	**SNP Genotype 4x**	**SNP Genotype 2x**
Chloroplast count (4x)	48	11
Chloroplast count (2x)	6	86

Out of the 137 progenies sampled for SNP genotyping and chloroplast counting comparison, there were 48 tetraploid calls that matched and 86 diploid calls that matched (Table. 2.4). The SNP genotype and chloroplast count correlation were high, but there were 11 samples were called diploid by SNP genotype, but were called tetraploid by chloroplast count (Table 2.4). There were six samples that were SNP genotyped and called tetraploids, but the chloroplast count determined

them to be identified as diploids. The error rate could be attributed to either sampling error or biologically due to chromosome doubling.

2.5 DISCUSSION

Tetraploid occurrence was observed in *S. tuberosum* female dihaploids by self-compatible male donors' crosses. The tetraploid progeny occurrence in 2x - 2x crosses are normally attributed to bilateral sexual polyploidization (BSP) which is the result of *2n* egg being fertilized by *2n* pollen (Carputo et al., 2000). In this study, the 2x - 2x crosses were expected to possess a high number of diploid progeny since it is speculated that *2n* pollen is very rare in the self-compatibility donors used (Jansky et al., 2014). Results showed the frequency of tetraploid progeny ranging from 0 to 40% in the 35 crosses using three different dihaploid sources (Atlantic, Superior and MSR127-2) to the two self-compatibility donors (M6 and DRHS6-10-4P17). These results are consistent with reported work from Carputo et al. (2003), Peloquin et al. (1999), Werner and Peloquin (1990) and (Younis et al., 2014) who have previously observed 2x - 2x bilateral polyploidization. Carputo et al. (2003) reported that the frequency of *2n* gametes varied with diploids having *2n* pollen frequency from 1.9 to 36.3%, while *2n* egg frequency in Solanum species range from 4.9 to 22.6%. The presence of *2n* gamete in many plants shows that *2n* gamete has been occurring in many other plant species hence not just limited to the potato crop. Notably, other crops such as, Medicago, Manihot, *Dactylis glomerata*, and *Trifolium nigrescens* showed similarity in the *2n* frequency (Britognolle and Thompson, 1997).

Carputo et al., (2003) observed the range of *2n* gamete to be between 1% and 40%. This ratio of 1n anf 2n gametes could be beneficial in that a 2x-2x cross can preserve the diploid ploidy level while permitting polyploidization. This study yielded comparable research findings with Carputo et al. (2000). Tetraploid progeny ranged from 0 to 40% indicating that in many of the 2x-2x crosses both parents were producing *2n* gametes. The occurrence of a high frequency of *2n* gametes due to *2n* pollen and *2n* egg production was common in wild diploid species (Peloquin et al., 1991; Carputo et al., 2000; Carputo et al., 2003). The 4x frequency may reflect *2n* egg frequency in dihaploids and cytological examination of the megaspore would confirm this hypothesis (Peloquin et al., 1989; Watanabe and Peloquin, 1993).

Out of the 35 families evaluated, we focused on seven families that had highest tetraploid incidence to compare chloroplast counting to SNP genotyping. The ploidy results attained from SNP

genotyping and chloroplast counts methods did not agree completely for the analyzed samples showed SNP call and chloroplast count data comparison to establish tetraploid occurrence in the 2x - 2x dihaploid crosses. This discrepancy could have been because both chloroplast count and SNP genotyping were done at different times. Hence, there could have been a sampling error that caused the discrepancy in the ploidy determination. It is also possible that the lack of complete correlation may be that the tetraploids were the result of chromosome doubling. SNP genotyping would not detect tetraploids formed in this way. Most of the SNP data when compared with the chloroplast data, demonstrated high similarity confirming that the chloroplast data can be adopted as a cheaper and quicker technique for breeders to identify diploids in the breeding programs (Alsahlany et al., 2019). For practical purposes the chloroplast count can be employed for quality control in applied diploid breeding program to ensure the proper ploidy level is maintained. Additionally, early ploidy screening can be used to enhance breeding efficiency in early generation selection stages of breeding programs (Alsahlany et al., 2019).

For diploid breeding programs the occurrence of 4x progeny must be minimized. Hence, breeders need to select parental lines that produce no *2n* eggs or a low frequency of *2n* eggs (Carputo et al., 2000). *2n* egg formation could be used for in the future to study the individualized *n* and *2n* gamete combining ability. The heterotic response of those lines will play a major role on how they behave in predicting performance of the crosses in cases where bottleneck hypothesis is favored (Mendiburu and Peloquin, 1977). Occurrences of both 4x and 2x progeny was previously important to allow for testing the value of polyploidy for vigor plus yield. For diploid breeding, the interest is to maintain dihaploids that have lower frequency of *2n* eggs to enable maintenance of potato breeding specifically at the diploid level. This study was able to identify dihaploids without *2n* gamete occurrence that can be used in the diploid breeding program.

2.6 CONCLUSION

Both SNP genotype call and chloroplast count methods confirmed the occurrence of the tetraploid in the progeny. The occurrences confirm that the possible cause of the phenomenon could have been resulted from *2n* gamete resulting from meiotic aberrations. Additionally, the high level of diploids in the offspring also confirms that plants tend to hold a higher frequency of *n* gametes. This points to the need of ploidy confirmation when one carries out crosses in the breeding programs to ensure diploid breeding of potatoes is achieved. Breeders need to ensure that the

hybrids produced from 2x - 2x crosses are the true intent or objective of program since there is possibility of tetraploid progeny occurrence. The study identified progeny with low level of tetraploid occurrence which could be used effectively in diploid breeding. The experiment ultimately validated the chloroplast counts findings using SNP genotyping via the 22K Infinium potato SNP arrays. The results of this study support the use of the chloroplast count technique to distinguish diploid from tetraploid progeny in 2x-2x crosses.

APPENDIX

Table 2.5. SNP genotyping and chloroplast count data comparison to establish ploidy in the 2x-2x dihaploid crosses.

Sample ID	SNP genotype simplex and triplex frequency	Ploidy of individuals	Means of chloroplast count	Sample background
Diploid reference Samples				
DM_018	0.01 b	2x	7.0 d	*S. tuberosum* Grp. Tuberosum
ATL_M_409	0.01 b	2x	7.2 d	*S. tuberosum* Grp. Tuberosum
M6	0.01 b	2x	6.2 d	Wild Solanum species
ATL_M_429	0.01 b	2x	7.6 d	*S. tuberosum* Grp. Tuberosum
ATL_M_170	0.01 b	2x	7.0 d	*S. tuberosum* Grp. Tuberosum
VT_SUP_08	0.01 b	2x	7.2 d	*S. tuberosum* Grp. Tuberosum
ATL_M_402	0.01 b	2x	6.3 d	*S. tuberosum* Grp. Tuberosum
ATL_M_120	0.01 b	2x	6.9 d	*S. tuberosum* Grp. Tuberosum
ATL_M_404	0.01 b	2x	6.2 d	*S. tuberosum* Grp. Tuberosum
VT_SUP_79	0.01 b	2x	7.0 d	*S. tuberosum* Grp. Tuberosum
ATL_M_170	0.01 b	2x	7.1 d	*S. tuberosum* Grp. Tuberosum
2x-2x, diploid cross samples				**Female X male cross**
MSEE905A-02	0.01 b	2x	6.3 d	ATL-M-170 × M6
MSEE905B-19	0.01 b	2x	7.2 d	ATL-M-170 × M6
MSEE905B-13	0.01 b	2x	6.5 d	ATL-M-170 × M6
MSEE905B-11	0.01 b	2x	6.9 d	ATL-M-170 × M6
MSEE905B-12	0.01 b	2x	7.3 d	ATL-M-170 × M6
MSEE905B-17	0.01 b	2x	8.1 d	ATL-M-170 × M6
MSEE905B-07	0.01 b	2x	7.7 d	ATL-M-170 × M6
MSEE905B-06	0.01 b	2x	7.0 d	ATL-M-170 × M6
MSEE905A-03	0.01 b	2x	8.8 d	ATL-M-170 × M6
MSEE905B-05	0.01 b	2x	6.6 d	ATL-M-170 × M6
MSEE925A-05	0.01 b	2x	8.0 d	VT-SUP-70 × M6
MSEE925A-17	0.01 b	2x	6.2 d	VT-SUP-70 × M6
MSEE925A-23	0.01 b	2x	6.5 d	VT-SUP-70 × M6
MSEE925A-16	0.01 b	2x	6.7 d	VT-SUP-70 × M6
MSEE925B-05	0.01 b	2x	7.6 d	VT-SUP-70 × M6
MSEE925B-2	0.01 b	2x	6.5 d	VT-SUP-70 × M6
MSEE925A-11	0.01 b	2x	5.9 d	VT-SUP-70 × M6
MSEE925B-20	0.01 b	2x	7.5 d	VT-SUP-70 ×M6
MSEE925A-10	0.01 b	2x	6.8 d	VT-SUP-70 × M6
MSEE925B-13	0.01 b	2x	7.8 d	VT-SUP-70 × M6
MSEE925B-03	0.01 b	2x	7.0 d	VT-SUP-70 × M6

Table 2.5 (cont'd)

MSEE925B-06	0.01 b	2x	6.7 d	VT-SUP-70 × M6
MSEE925A-07	0.01 b	2x	7.0 d	VT-SUP-70 × M6
MSEE925A-13	0.01 b	2x	6.7 d	VT-SUP-70 × M6
MSEE925B-27	0.01 b	2x	6.9 d	VT-SUP-70 × M6
MSEE925B-26	0.01 b	2x	6.9 d	VT-SUP-70 × M6
MSEE925B-18	0.01 b	2x	6.0 d	VT-SUP-70 × M6
MSEE925B-07	0.01 b	2x	7.0 d	VT-SUP-70 × M6
MSEE927A-02	0.01 b	2x	8.2 d	VT-SUP-08 × M6
MSEE927A-10	0.01 b	2x	6.7 d	VT-SUP-08 × M6
MSEE927A-04	0.01 b	2x	5.5 d	VT-SUP-08 × M6
MSEE927A-05	0.01 b	2x	6.9 d	VT-SUP-08 × M6
MSEE927A-09	0.01 b	2x	6.3 d	VT-SUP-08 × M6
MSEE927B-09	0.01 b	2x	7.6 d	VT-SUP-08 × M6
MSEE927A-22	0.01 b	2x	6.3 d	VT-SUP-08 × M6
MSEE928B-01	0.01 b	2x	6.7 d	ATL-M-120 × M6
MSEE928A-20	0.01 b	2x	7.2 d	ATL-M-120 × M6
MSEE928B-05	0.01 b	2x	7.2 d	ATL-M-120 × M6
MSEE928A-19	0.01 b	2x	5.5 d	ATL-M-120 × M6
MSEE928B-07	0.01 b	2x	7.0 d	ATL-M-120 × M6
MSEE928B-20	0.01 b	2x	7.0 d	ATL-M-120 × M6
MSEE928A-15	0.01 b	2x	6.1 d	ATL-M-120 × M6
MSEE928A-12	0.01 b	2x	6.2 d	ATL-M-120 × M6
MSEE928A-01	0.01 b	2x	7.5 d	ATL-M-120 × M6
MSEE928A-03	0.01 b	2x	6.0 d	ATL-M-120 × M6
MSEE928A-08	0.01b	2x	7.8 d	ATL-M-120 × M6
MSEE928B-08	0.01b	2x	7.0 d	ATL-M-120 × M6
MSEE928B-19	0.01 b	2x	7.6 d	ATL-M-120 × M6
MSEE928A-04	0.01 b	2x	7.0 d	ATL-M-120 × M6
MSEE928A-16	0.01 b	2x	6.5 d	ATL-M-120 × M6
MSEE929B-04	0.01 b	2x	6.9 d	ATL-M-404 × M6
MSEE929A-11	0.01 b	2x	6.3 d	ATL-M-404 × M6
MSEE929A-25	0.01 b	2x	6.2 d	ATL-M-404 × M6
MSEE929A-16	0.01 b	2x	7.2 d	ATL-M-404 × M6
MSEE929A-24	0.01 b	2x	7.0 d	ATL-M-404 × M6
MSEE929B-01	0.01 b	2x	6.5 d	ATL-M-404 × M6
MSEE929A-02	0.01 b	2x	6.2 d	ATL-M-404 × M6
MSEE929A-01	0.01 b	2x	7.2 d	ATL-M-404 × M6
MSEE932B-12	0.01 b	2x	8.2 d	ATL-M-409 × M6
MSEE932B-04	0.01 b	2x	7.2 d	ATL-M-409 × M6

Table 2.5 (cont'd)

MSEE933B-10	0.01 b	2x	7.8 d	ATL-M-402 × M6
MSEE933B-08	0.01 b	2x	7.0 d	ATL-M-402 × M6
MSEE933B-07	0.01 b	2x	7.2 d	ATL-M-402 × M6
MSEE933A-23	0.01 b	2x	7.0 d	ATL-M-402 × M6
MSEE933A-04	0.01 b	2x	7.0 d	ATL-M-402 × M6
MSEE933A-15	0.01 b	2x	6.7 d	ATL-M-402 × M6
MSEE933B-12	0.01 b	2x	7.3 d	ATL-M-402 × M6
MSEE933A-08	0.01 b	2x	5.7 d	ATL-M-402 × M6
MSEE933A-14	0.01 b	2x	6.6 d	ATL-M-402 × M6
MSEE939B-31	0.01 b	2x	7.1 d	ATL-M-429 × M6
MSEE939B-41	0.01 b	2x	7.2 d	ATL-M-429 × M6
MSEE939A-18	0.01 b	2x	6.4 d	ATL-M-429 × M6
MSEE939A-20	0.01 b	2x	6.4 d	ATL-M-429 × M6
MSEE939A-08	0.01 b	2x	6.4 d	ATL-M-429 × M6
MSEE939A-21	0.01 b	2x	8.1 d	ATL-M-429 × M6
MSEE939A-02	0.01 b	2x	6. 0 d	ATL-M-429 × M6
MSEE939A-26	0.01 b	2x	7.3 d	ATL-M-429 × M6
MSEE939A-16	0.01 b	2x	6.4 d	ATL-M-429 × M6
MSEE939A-06	0.01 b	2x	7.1 d	ATL-M-429 × M6
MSEE939A-24	0.01 b	2x	7.5 d	ATL-M-429 × M6
MSEE939B-34	0.01 b	2x	6.1 d	ATL-M-429 × M6
MSEE939A-11	0.01 b	2x	8.6 d	ATL-M-429 × M6
2x-2x tetraploid, samples				
MSEE905B-14	0.17 a	4x	13.6 b	ATL-M-170 × M6
MSEE905B-01	0.17 a	4x	13.1 bc	ATL-M-170 × M6
MSEE905B-20	0.17 a	4x	14.4 b	ATL-M-170 × M6
MSEE925B-01	0.17 a	4x	13.2 bc	VT-SUP-70 × M6
MSEE925B-02	0.27 a	4x	16.5 a	VT-SUP-70 × M6
MSEE925B-09	0.16 a	4x	13.9 b	VT-SUP-70 × M6
MSEE925B-01	0.23 a	4x	14.0 b	VT-SUP-70 × M6
MSEE925B-14	0.16 a	4x	13.6 b	VT-SUP-70 × M6
MSEE925B-04	0.16 a	4x	12.7 bc	VT-SUP-70 × M6
MSEE925B-18	0.15 a	4x	12.7 bc	VT-SUP-70 × M6
MSEE925B-18	0.15 a	4x	12.7 bc	VT-SUP-70 × M6
MSEE925B-06	0.15 a	4x	13.1 bc	VT-SUP-70 × M6
MSEE925B-17	0.15 a	4x	12.5 bc	VT-SUP-70 × M6
MSEE925B-03	0.16 a	4x	13.1 bc	VT-SUP-70 × M6
MSEE925B-25	0.16 a	4x	14.7 b	VT-SUP-70 × M6
MSEE925A-04	0.16 a	4x	13.2 bc	VT-SUP-70 × M6

Table 2.5 (cont'd)

MSEE925A-20	0.16 a	4x	13.3 bc	VT-SUP-70 × M6
MSEE925B-31	0.29 a	4x	13.7 bc	VT-SUP-70 × M6
MSEE925B-19	0.29 a	4x	13.5 bc	VT-SUP-70 × M6
MSEE925B-07	0.30 a	4x	14.3 b	VT-SUP-70 × M6
MSEE925B-15	0.30 a	4x	13.0 bc	VT-SUP-70 × M6
MSEE925B-08	0.31 a	4x	13.4 bc	VT-SUP-70 × M6
MSEE925B-08	0.31 a	4x	13.4 bc	VT-SUP-70 × M6
MSEE925B-30	0.31 a	4x	13.8 bc	VT-SUP-70 × M6
MSEE925B-05	0.31 a	4x	13.1 bc	VT-SUP-70 × M6
MSEE927B-07	0.16 a	4x	13.7 b	VT-SUP-08 × M6
MSEE927B-16	0.16 a	4x	13.3 bc	VT-SUP-08 × M6
MSEE927B-06	0.16 a	4x	13.5 bc	VT-SUP-08 × M6
MSEE927B-03	0.16 a	4x	13.0 bc	VT-SUP-08 × M6
MSEE927B-15	0.17 a	4x	13.3 bc	VT-SUP-08 × M6
MSEE927B-17	0.24 a	4x	13.7 bc	VT-SUP-08 × M6
MSEE928A-05	0.14 a	4x	13.6 bc	ATL-M-120 × M6
MSEE928A-24	0.17 a	4x	13.2 bc	ATL-M-120 × M6
MSEE928B-27	0.27 a	4x	12.8 bc	ATL-M-120 × M6
MSEE928B-21	0.32 a	4x	12.8 bc	ATL-M-120 × M6
MSEE928B-42	0.32 a	4x	13.3 bc	ATL-M-120 × M6
MSEE928B-12	0.33 a	4x	16.0 a	ATL-M-120 × M6
MSEE928B-17	0.33 a	4x	12.6 bc	ATL-M-120 × M6
MSEE928B-21	0.33 a	4x	12.8 bc	ATL-M-120 × M6
MSEE928B-24	0.33 a	4x	13.4 bc	ATL-M-120 × M6
MSEE928A-25	0.33 a	4x	13.5 bc	ATL-M-120 × M6
MSEE928A-17	0.33 a	4x	13.3 bc	ATL-M-120 × M6
MSEE933B-04	0.24 a	4x	12.9 bc	ATL-M-402 × M6
MSEE933B-01	0.33 a	4x	13.4 bc	ATL-M-402 × M6
MSEE939B-15	0.14 a	4x	12.4 c	ATL-M-429 × M6
MSEE939B-16	0.15 a	4x	12.8 bc	ATL-M-429 × M6
MSEE939B-09	0.17 a	4x	12.9 bc	ATL-M-429 × M6
MSEE939B-10	0.17 a	4x	12.6 bc	ATL-M-429 × M6
MSEE939B-12	0.17 a	4x	13.2 bc	ATL-M-429 × M6
MSEE939B-05	0.17 a	4x	13.1 bc	ATL-M-429 × M6
MSEE939B-01	0.17 a	4x	12.2 c	ATL-M-429 × M6
MSEE939B-02	0.17 a	4x	12.9 bc	ATL-M-429 × M6
MSEE939B-18	0.17 a	4x	12.8 bc	ATL-M-429 × M6
MSEE939B-20	0.18 a	4x	13.5 bc	ATL-M-429 × M6

Table 2.5 (cont'd)

MSEE939B-14	0.18 a	4x	13 bc	ATL-M-429 × M6
Tetraploid reference samples				
Atlantic	0.39 a	4x	12.0 c	*S. tuberosum* Grp. Tuberosum
Superior	0.32 a	4x	13.6 bc	*S. tuberosum* Grp. Tuberosum
Atlantic_040_R01C0	0.32 a	4x	0.32 a	*S. tuberosum* Grp. Tuberosum

* Means followed by same letters are not significantly different from each other $P = 0.001$ level of probability.

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CHAPTER 3. SCREENING FOR *Ralstonia solanacearum* RESISTANCE IN *Solanum commersonii*

3.1 ABSTRACT

Worldwide, potato (*Solanum tuberosum L*) is rated as one of the most important food crops after wheat, rice and maize. Potato contributes to the total annual production output of root and tuber crops. Most of the available commercial cultivars are highly susceptible to a myriad of plant pathogens, namely, bacterial, viral and fungal diseases. Bacterial wilt caused by *Ralstonia solanacearum* (Rs), is rated as one of the most economically important diseases affecting potato. Currently, new diploid breeding strategies coupled with epidemiological disease surveillance available to the potato breeding community has created an opportunity to breed for Rs resistant cultivars. The purpose of this study was to identify diploid germplasm with resistance to bacterial wilt. The disease incidence was assessed by analyzing the wilt percentage and severity on the *Solanum commersonii* clones which were artificially inoculated with Rs. Two different Rs strains (CMR15 and UW551) were used to inoculate the clones and revealed promising clones with impressive resistance levels under growth chamber conditions. One clone was identified as highly resistant (MSEE912-08). It exhibited a disease score of 0.4 (less than 25% wilted leaves) when inoculated with UW551 Rs strain and 1.3 with the CMR15 strain, hence was considered the most resistant to the pathogen infection. The resistance observed was characterized by decline in disease, rather than lack of disease. The most resistant clones from this study were used to create a mapping population to identify single nucleotide polymorphic markers associated with Rs resistance.

3.2 INTRODUCTION

Potato wild relative *Solanum commersonii* has been reported to carry resistance to bacterial wilt caused by *Ralstonia solanacearum* (Rs) (Laferriere et al., 1999; Fock et al., 2000; Aversano et al., 2015). This presents an opportunity to study the pathogen's interaction with Rs resistant germplasm and thus develop and test progeny from a breeding population of *Solanum commersonii*. The causal agent for bacterial wilt is Rs, which is considered a quarantine organism worldwide as documented in A2 list from European and Mediterranean Plant Protection Organization. It is a *β*-proteobacterium that infects the plant by colonizing xylem, hence blocking the flow of water, soluble nutrients, including organic ions from the roots to the rest of the plant,

thus causing wilting and subsequent death of the plant leading to high economic yield loss (Champoiseau et al., 2009; Albuquerque et al., 2015; Clarke et al., 2015). Bacterial wilt is also commonly called brown rot. The transmission mechanism of the pathogen is through soil, water or infected plant material and enters the plant through root invasion (Zuluaga et al., 2015). Bacterial wilt has been reported specifically in tropical and Mediterranean climatic regions. However, this disease has also been recorded in temperate regions of the world, with specific reference to Europe. In the United States, bacterial wilt R3bv2 is classified as a Select Agent since they pose severe threat to plant health and so has zero-tolerance reinforcement quarantine regulations and sanitation protocols to avert the introduction of the pathogen (Champoiseau et al., 2009).

Due to the aggressiveness of the pathogen, it has been listed under stringent biosecurity plant regulation in 2002 to promote guidelines for management and prevent pathogen spread (Champoiseau et al., 2009). The Rs strain four main characteristic strains with reported genomic instability since the genomes harbor many transposable elements that take part in active acquisition, alteration of genetic material thus, contributing to their genomic variation (Álvarez et al., 2010). The variation within Rs groups during classification has warranted it to be referred as "species complex" (Fegan and Prior, 2005; Champoiseau et al., 2009). This variation contributes to the Rs species complex, giving it the power to effectively colonize plant tissues and cause bacterial wilt (Álvarez et al., 2010; Clarke et al., 2015).

Four comprehensive phylotypes of Rs, corresponding to geographical and genomic diversity groups have been described. The Phylotype can be identified with DNA primers specific to the four species complex (Carputo et al., 2009; Albuquerque et al., 2015). The phylotypes are further subdivided into sequevars based on the sequence of the endoglucanase (*egl*) gene (Champoiseau et al., 2009). Rs strains have been described as Phylotype I which include lowland tropical strains and possesses a wide host range. Other strains belong to Phylotype II, which is subdivided into phylotype IIA and IIB-1 (Cellier and Prior, 2010). Phylotype II is known to mostly infect highlands potato growing regions and cold tolerant potatoes. It is also known as brown rot strain which belongs to the phylotype IIB – 1 (Franc et al., 2001; Cellier and Prior, 2010). Recently, phylotype IIA documented to have a broad host range was predominantly isolated from tropical regions (Cellier and Prior, 2010). The Phylotype III strains originated from Africa and Phylotype IV strains are from Indonesia (Champoiseau et al., 2009).

Potato breeders have used diploid wild relatives of potato (*Solanum, tuberosum* L.) to access genes for disease resistance, tuber yield and stress tolerance to generate vigorous interspecific hybrids (Jansky and Peloquin, 2006). Notably, there has been success in introgression of useful traits from diploid potato into cultivated tetraploid potato (2n = 4x = 48). The diploid species cross easily to South American diploid relatives via utilization of *2n* gametes. Diploids can be crossed with tetraploids and produce tetraploid offspring due to formation of *2n* gametes (Jansky and Peloquin, 2006). Using conventional breeding methods associated with self-compatible diploid potatoes, coupled with epidemiological disease surveillance available to the potato breeding community, has the potential to create resistant cultivars against bacterial wilt. Ideally, epidemiological assessment of the pathogen colonization patterns of the strain will enable pathologists and breeders to better predict the biological properties of the strain on the breeding lines and help in the development of effective disease management strategies. A survey carried out by CIP (International Potato Center) points out that Rs is among the key challenges facing seed potato production in tropical, subtropical and warm temperate regions of the world and goes on to emphasize the impact of Rs in the production levels for the above regions (Champoiseau et al., 2009).

The current research was conducted using self-fertilized, wild potato clones of *S. commersonii*. This research assessed the disease incidence by artificially inoculating the clones derived from *S. commersonii*. The total disease incidence was defined as the number of plant units infected expressed as a percentage of wilted leaves and hence quantified by wilting degree (WD) in leaves ranging from 0 to 4 (Fock et al., 2001; Carputo et al., 2009). The WD is also known as disease incidence scores. The disease incidence scores was depicted by diseased plant parts for the different Rs strains on inoculated plants. Disease assessment revealed the most promising clones to be used to study the genetics of Rs resistance. The Rs resistant clones in the study will continue to be evaluated as a source of germplasm for Rs resistance in potato breeding research and variety development.

3.2.1 Objectives

Our main goal was to assess *S. commersonii* progenies for *R. solanacearum* resistance.

3.2.2 Specific objectives

1. To obtain progeny from *S. commersonii*.

2. To conduct Rs bioassays to identify resistant clones for future breeding and genetics research.

3.3 MATERIALS AND METHODS

3.3.1 Developing the germplasm

S. commersonii is a diploid wild relative of the cultivated potato. *S. commersonii* is native to Uruguay, Argentina and Brazil with reported resistance to Rs, verticillium wilt *Verticilium dahliae*, nematode *Ditylenchus destructor*, *Alternaria solani* fungus and tolerance to low temperature (Laferriere et al., 1999; Caruso et al., 2008). The planting material *S. commersonii* (2n = 2x =24) seeds and seedling from true potato seeds was provided by Dr. John Bamberg of NRSP-6 Potato Gene Bank, USDA-ARS. Self-pollination was carried out in the greenhouse using CEC03-07, also known as PI320266 which is bacterial wilt resistant *S. commersonii* Dun. (2n = 2x =24) line with 1 EBN (Endosperm Balance Number). This selfing of the different CEC03 individuals generated a population against which two strains of Rs pathogen was exposed to identify the most resistant individuals. The original plan of this study was to create, a hybrid (F_2 population) between *S. commersonii* (CEC03-and *S. chacoense* (M6). M6 was selected to introduce self-compatibility. M6 is highly self-compatible, a trait that allows it to be crossed to many potato clones in order to introgress self-compatibility (Jansky et al., 2014). Consequently, the self-compatibility trait is fundamental in generating F_2 populations and other filial generations (Jansky et al., 2014). *S. commersonii* × M6 crosses were made, fruits developed, seeds were harvested from these fruits, and were germinated to generate F_1 progeny. Based on SNP genotyping, the progenies were determined to only have alleles from *S. commersonii*. This was discovered by SNP genotyping which showed absence of M6 alleles in the putative F_1s, leading to the determination that the progeny were self-fertilized individuals *S. commersonii*. This absence of hybrid progeny may be explained by the uneven EBN in the progeny, as *S. chacoense* has an EBN = 2 and *S. commersonii* has an EBN = 1 (Hawkes., 1990). Despite the setback, the study proceeded since phenotypic evaluation had already been conducted, and segregation for resistance level was observed.

The seeds from the self-fertilized *S. commersonii* line were planted in the tissue culture laboratory using Murashige and Skoog (MS) medium containing 3% sucrose 0.8 % agar and pH of medium was adjusted to 5.8 (Murashige and Skoog, 1962). The plantlets were kept in the growth rooms at 20 ± 2°C under 16 h light/8 h dark photoperiod. The true potato seeds (TPS) from the self-fertilized *S. commersonii* progeny were then germinated in the tissue culture lab using the above-mentioned

growth conditions. The seedlings were transplanted in the greenhouse for rapid multiplication. One-and-half month old plant material were later transferred to growth chambers for a bacterial wilt resistance bioassay.

3.3.2 Bioassay for Rs resistance

Bacterial strain inoculum preparation

In order to screen the plant material for resistance, Rs inoculum was prepared and diluted to1×10^7 cfu/ml. This screening was done at the University of Wisconsin, Madison in the laboratory of Dr. Caitlyn Allen. Two strains were used in the study. The Rs strain UW551 which is equivalent R3bv2 or phylotype IIB sequevar 1 and another strain belonging to phylotype I, CMR15 were used to rate bacterial wilt disease development. The *Rs* strain UW551 is equivalent of NAK66 wild type background race 3 biovar (R3bv2 or phylotype IIB sequevar 1), a Kenyan strain from the Nakuru region (NAK66). The bacterial colony was grown for 48 h at 28 ºC on Kelman's growth media having 2,3,5 triphenyl tetrazolium chloride (TTC) as previously described by Siri et al. (2009).

3.3.3 Conditions in growth chambers for virulence assay

Greenhouse plants that were six-weeks-old were transferred to the growth chambers. The growth chamber temperature was 24 ºC to 28 ºC, at 16 h photoperiod and a relative humidity of 80%. A suspension having 0.9% saline solution was prepared and Rs inoculum was adjusted to a concentration of 1×10^7 cfu/ml (colony forming units). The purpose of wounding roots is to ensure entry of the pathogen, thus ensuring the infection of the plants. Root inoculation of wounded plants was done by pouring 50 ml of bacterial suspension into the soil.

Disease scoring: Plants were monitored daily for Rs disease progression and wilting symptoms were rated. Symptoms were scored using wilting degree in leaves according to Carputo et al. (2009) and Fock et al. (2001) where 0 = no wilted leaves, 1 =1 - 25% total wilted leaves, 2 = 26 - 50% wilted leaves, 3 = 51 - 75% wilted leaves and 4 = 76 - 100% of leaves entirely wilted. The Rs scores were conducted 20 days post inoculation. The percentage of total wilting was recorded after every five days for a period of 20 days.

3.3.4 Experimental design and statistical data analysis

The *S. commersonii* plant material was tested in a complete block design (CBD) with an experimental unit consisting of eight plants per clone. One data point represented each plant the average of the eight plants in a unit (one plant per replication, hence 8 replications). Hence the 8 reps per clone were averaged. The plants were artificially inoculated with two different strains of Rs namely, UW551 (Phylotype IIB) and CMR15 (Phylotype I), in plant growth chambers. Two parental clones CEC03-03 and CEC03-08 and four *S. commersonii* self-fertilized progenies (MSEE908-03, MSEE912-08, MSEE914-06, MSEE915-02 and M6) was used as a susceptible check) were screened. Disease WD was tested for significant differences between clones using JMP pro 13 software (SAS Institute, Cary, NC). The wilting percentage was used for a one-way analysis of variance considering different clones and Rs strains as factors. The analysis of variance (ANOVA) was used to determine the significant difference between each clone. Additionally, Tukey's HSD (honestly significant difference) was used to test all pairwise differences for all the clones to declare clones significantly different.

3.4 RESULTS

3.4.1 Rs evaluation

From the assay, the most bacterial wilt resistant clone was MSEE912-08. It had disease incidence score of 0.4 against UW551 strain (Figure 3.1). The most susceptible parental clone was CEC03-08, (4.0 disease incidence score) when inoculated by CMR15 and UW551 Rs strain. The progeny, MSEE914-06 and MSEE915-02 were the most susceptible to bacterial wilt to both strains. They had a mean disease incidence score against strain UW551 of 4.0 and 2.3, respectively (Figure 3.1 and 3.2). Score is equivalent to 50% of the leaves of MSEE915-02 and over 76% wilted leaves for clone MSEE914-06 were wilted 20 days post inoculation. Data indicate the progeny were much more susceptible to the disease.

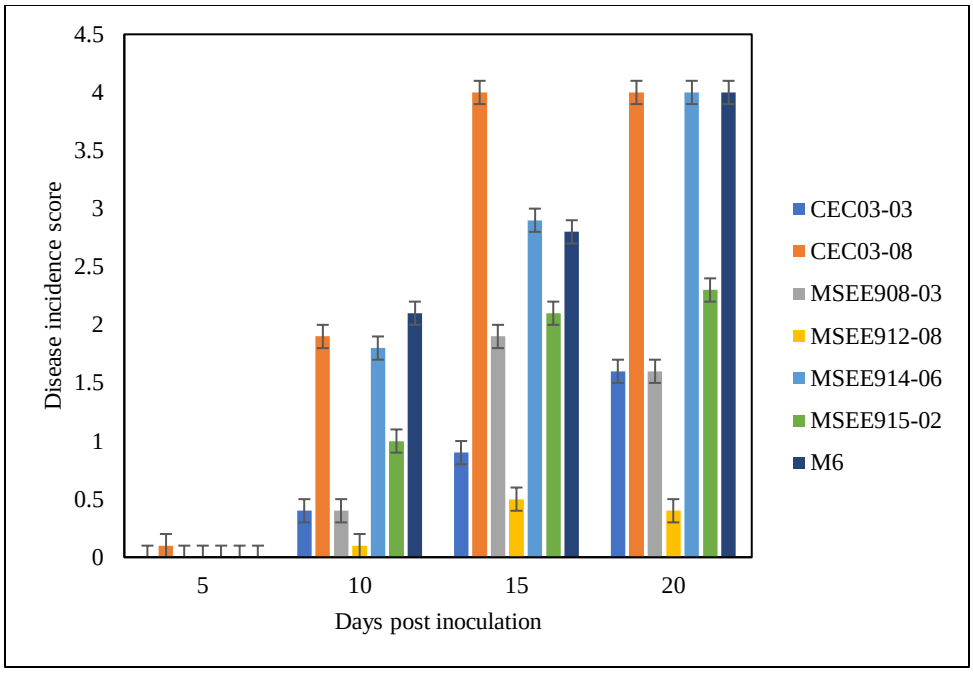

Figure 3.1. *Ralstonia solanacearum* strain UW551 disease incidence progress curve on *S. commersonii* clones, self-fertilized progenies and M6. Error bars indicate standard error.

Most of the clones were not infected by the Rs pathogen at 5 days post inoculation. All the clones showed infection from day 10 post inoculation according to the disease incidence score (Figure 3.1). This trend was further discussed in Figure 3.2.

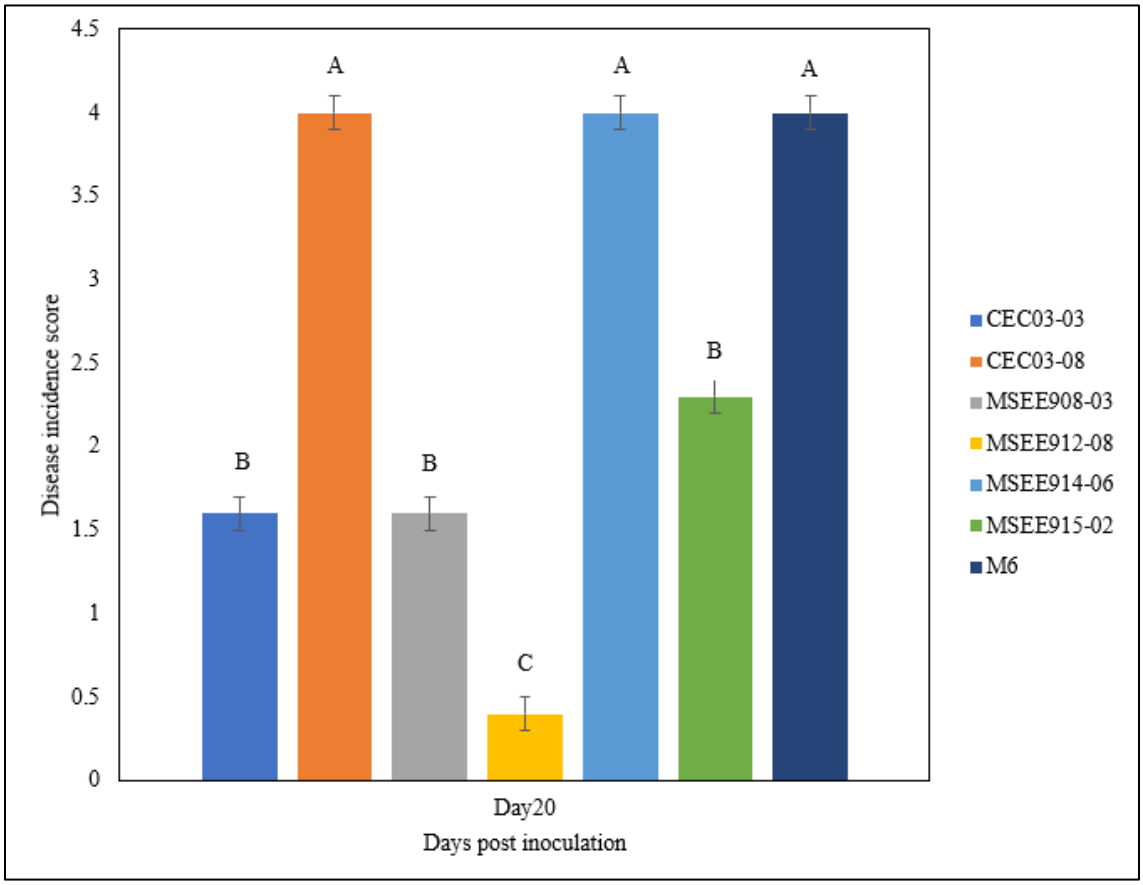

Figure 3.2. *Ralstonia solanacearum* strain UW551 disease incidence progress curve at 20 days post inoculation on *S. commersonii* clones, self-fertilized progenies and M6. Bars with different letters above them were significantly different according to Tukey test (P = 0.05).

When the Tukey HSD all pairwise comparison for significant difference between the clones was performed, different Rs strains showed varied significance levels for the different strains as shown in Table. 3.2 and 3.3 (supplementary Tables 3.3). The graphical disease progression 20 days post inoculation shows that MSEE912-08 was significantly more wilt resistant to UW551 compared to all the other clones evaluated. This clone was chosen for further selfing and evaluation for Rs resistance markers. In contrast, MSEE915-02 and MSEE914-06 as well as CEC03-08 were much more susceptible to both strains of the disease as about half of the inoculated plants wilted (Figure 3.2). CEC03-03, MSEE908-03 and MSEE915-02 were not significantly different from each other as shown above (Figure 3.2) on the Rs strain UW551.

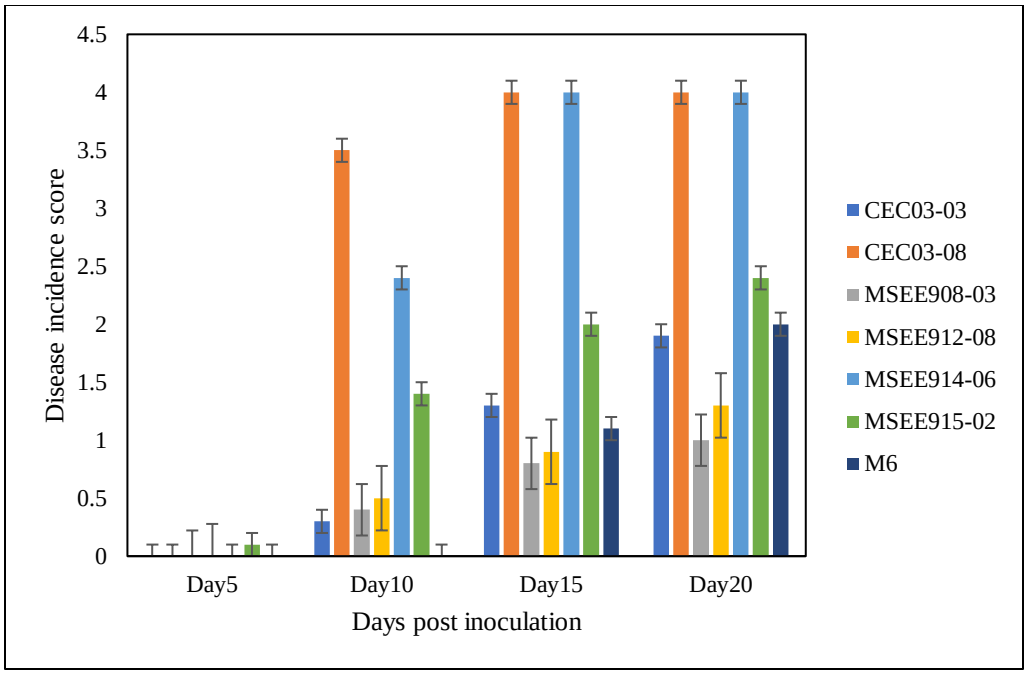

Figure 3.3. *Ralstonia solanacearum* strain CMR15 disease incidence progress curve at 5, 10, 15 and 20 days post inoculation on *S. commersonii* clones, self-fertilized progenies and M6. Error bars indicate standard error.

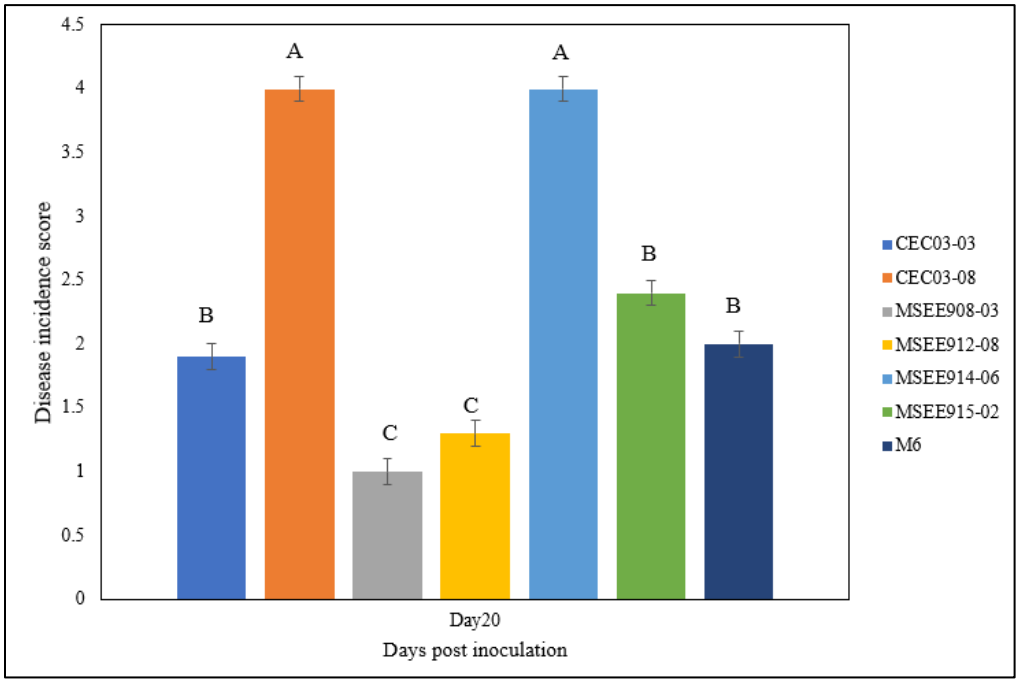

Figure 3.4. *Ralstonia solanacearum* strain CMR15 disease incidence progress curve at 20 days post inoculation on *S. commersonii* clones, self-fertilized progenies and M6. Bars with different letters above them were significantly different according to Tukey test (P = 0.05).

The disease incidence score indicated that MSEE908-03 and MSEE912-08 were the most tolerant clones to bacterial wilt infection CMR15 (Figure 3.3 and 3.4). This led to the overall selection of MSEE912-08 which was significantly different from all the other clones as shown by the different mean disease incidence scores (Table 3.1 and 3.2).

Table 3.1. Comparison of CMR15 and UW551disease incidence score differentiation among clones.

	UW551 disease incidence score (%)			**CMR15 disease incidence score (%)**		
Plant name	D10	D15	D20	D10	D15	D20
CEC03-03	0.4±0.084	1.3±0.28	1.6±0.36	0.3±0.32	1±0.42	1.9±0.21
CEC03-08	1.9±0.42	4±0.89	4±0.89	3.5± 0.21	4±0.21	4.0±0.22
M6	2.1±0.48	2.8±0.61	4±0.89	0.6±0.21	2±0.42	2.0±0.42
MSEE908-03	0.4±0.08	1±0.22	1.6±0.36	0.4±0.20	1±0.32	1.0±0.27
MSEE912-08	0.125±0.03	0.3±0.06	0.45±0.08	0.5±0.32	1±0.27	1.3±0.42
MSEE914-06	1.75±0.40	2.9±0.48	4±0.50	2.4±0.43	4±0.21	4.0±0.41
MSEE915-02	1±0.29	2.1±0.52	2.3±0.64	1.4±0.31	2±0.21	2.4±0.42

Disease incidence score is weighted average disease incidence range from 0 to 4: 0 = no wilted leaves, 1 = 1 - 25% total wilted leaves, 2 = 26 - 50 % wilted leaves, 3 = 51 - 75% wilted leaves and 4 = 76 - 100% = plant entirely wilted. Values in the same column with the same letter are not significantly different as determined by the Tukey test (P =0.05); D; day; +/- values are the standard error.

The bacterial wilt inoculation results shown in Table 3.1 displays the mean disease incidence score based on wilting degree values. The clonal parent CEC03-08 appeared to be highly susceptible when inoculated with bacterial strain UW551 (Table 3.1). The CEC03-08 was highly infected and progressed steadily until the last day of scoring. Whereas, the progenies MSEE915-02 and MSEE914-06 were the most susceptible over the days as shown in both Figure 3.1, 3.3 and Table 3.1. MSEE908-03 was moderately resistant to UW551 and significantly resistant to Rs using strain CMR15 (Table 3.1 and Figure 3.4). Comparison of ranking between different strains identified MSEE912-08 as the most resistant clones from the bioassay (Table 3.1). The different days were highly significant ($P = 0.0001$) between the 10^{th}, 15^{th} and 20^{th} day post inoculation using Tukey $P = 0.05$. Day 10 was significantly different from day 15 and day 20. Whereas, day 15 and day 20 were not significantly different from each other $P = 0.4604$. We discovered self-compatibility in the *S. commersonii* population even though this was not the core intention of the study. Therefore, it would be important to pursue further study of this germplasm for self-compatibility.

3.5 DISCUSSION

The results from this study suggest that the selfed progeny for *S. commersonii* can be useful as a source of bacterial wilt resistance in potatoes through diploid breeding. The study showed that the *S. commersonii* selfed progenies were significantly more wilt resistant than the parental clone. One of the source materials, CEC03-08, was highly susceptible to both bacterial wilt strains. We were able to identify MSEE912-08 as the most wilt resistant clone for further wilt resistance study. The original plan was to develop a population by crossing self-compatible *S. chacoense* to *S. commersonii*. After SNP genotyping the population (discussed in Chapter 4), we discovered that there were no hybrids, hence the progeny was all self-fertilized (or possibly the result of sib-mating). This study encountered crossing barrier that has been recorded by other groups who had reported it as being likely due to differences in endosperm balance number between the parents (Fock et al., 2000). Due to a lack of *S. chacoense* alleles in the progeny, the population was treated as self-fertilized population of *S. commersonii*.

These results indicated a quantitative disease resistance that is expressed as a reduction in disease rather than a total absence of disease symptoms (Poland et al., 2009). This resulted in the identification of a wilt resistant clone among the progenies of *S. commersonii*, MSEE912-08 which had a disease score of 1 for two different strains. Our experiment showed the first symptoms of disease wilting three days post inoculation, which is consistent to previous work done by Ishihara et al. (2012) in which they examined transcriptomic information on the early response of tomato against Rs at three to seven days post inoculation. The occurrence of segregating resistance is believed to be under polygenic control for the attributes showing that this durability for resistance can be prolonged when properly managed (Zuluaga et al., 2015). There was no significant difference within some of the progeny of self-fertilized *S. commersonii* when assayed together with *S. chacoense* as the susceptible check. The evaluation of the population was done using one of the most aggressive strains of Rs, UW551. Screening was conducted under optimum conditions that favored the development of disease symptoms. It would be interesting to test if the clones that had higher levels of resistance to UW551 are also resistant to other strains of bacterial wilt, ensuring the correct discrimination of the so-called resistant genotypes. It would be likewise remarkable to identify whether the genes segregating in this *S. commersonii* population coincide with those that Zuluaga et al. (2015) identified and could be used to breed for bacterial wilt

resistance. Resistant progeny clones from this experiment are seen as those that had wilted disease score of ≤ 1 , this was seen to be in concurrence with the study described by Carputo et al. (2009). Our results showed resistant clones exhibited wilt to a degree < 1 which can be categorized as resistant. There should be further work to study the genes underlying the resistance loci of these tolerant clones. Once the resistance genes have been identified, they may be employed through marker assisted selection to support the development of improved cultivars with durable resistance.

A study by Habe et al. (2019) analyzed the inoculation age and determined the resistance and susceptibility occurrence in genotypes showed comparison to our study. Even though we did not particularly concentrate in studying the optimal age for inoculation, we draw comparisons with their bioassay since they used the same phylotype as was used in this study. The group analyzed nine different genotypes which were subjected to the same incubation temperature and the same strain (Phylotype I) of Rs. Another study which used different potato clones and a different Rs strain as was done by Carmeille et al. (2006), and suggests that strain specificity of markers ought to be translated in terms of phylotype-specificity. Siri et al. (2011) explained that the Rs resistance has proved to be unstable across different geographical regions. Therefore, this calls for an emphasis in targeted breeding against the prevalent regionally specific strain to achieve meaningful progress in bacterial wilt breeding as a whole. *S. commersonii* , *S. brevidens* and *S cardiophylum* are believed to be among diploid species that have been utilized in Rs resistance and studies (Johnston and Hanneman, 1980; Carputo et al., 1999). Previous research work used sexual hybrids of *S. multidissectum*, *S. sparsipillum*, and *S. chacoense* to transferred Rs resistance into a cultivated background but proved challenging. The resistance conferred was moderate resistance with undesirable traits like high glycoalkaloid content (Siri et al., 2011). Additionally, Fock et al. (2000) reports on bacterial wilt resistance derived from *S. phureja* that was considered dominant and was discovered to be controlled by three unlinked genes. Furthermore, four major loci have equally been reported to play a key role in bacterial wilt in potato (Fock et al., 2001). There are reports that different sources of bacterial wilt resistance in solanaceous crops have different biological mechanisms, as different Rs strains have extensive genetic diversity (Huet, 2014). Therefore, the best approach to look for sources of resistance is through employing appropriate screening methods. This can be achieved through focusing on breeding germplasm adapted only to a given ecosystem, coupled by using an effectoromics approach which aims at understanding mechanisms fundamental to plant immune responses (Huet, 2014).

Diploid potato breeding will be based on the generation of inbred lines that will be used to make F_1 hybrid potato cultivars (Jansky et al., 2016).In order to achieve progress in diploid breeding, self-compatibility is critical. In this aspect, the approach of selfing requires self-compatibility to produce inbred lines. This is achieved when a series of homozygous sets of additive loci recombination's follows (Jansky and Peloquin, 2006; Jansky et al., 2014). This combinations of additive loci results in genetic gain through exploiting inbreeding (Fasoula and Fasoula, 2002; Jansky et al., 2016). The discovery of a dominant S-locus inhibitor (*Sli*) gene was critical for incorporating self-compatibility in diploid potato (Hosaka and Hanneman, 1998). Additionally, breeding at the diploid level encounters high genetic load of deleterious recessives. A deleterious recessive mutation is typically 'masked' in the autotetraploid but 'exposed' in the diploids (Herman et al., 1994; Dekkers and Hospital, 2002; Jansky and Peloquin, 2006; Jansky et al., 2016). Deleterious recessives are purged by inbreeding, which is only possible if the line has self-compatibility. The strategy is to purge deleterious recessives alleles from potato during inbreeding, by taking advantage of the *Sli* gene. There is documented progress of self- compatibility introgression from *S. chacoense* or M6 (Jansky et al., 2014).

This study serendipitously, allowed us to demonstrate the occurrence of self-compatibility in a *S. commersonii* population. This will provide an effective breeding strategy for bacterial wilt resistance. By moving away from the old paradigm, which was characterized by looking for identifiable phenotypes that uses molecular linkage. Self-compatibility enables the individuals possessing the trait or region of interest to be repeatedly backcrossed to the recurrent parent (Tanksley and McCouch, 1997). Individuals with residual heterozygosity in the region or trait of interest are self-pollinated to select for individuals homozygous for the trait or region of interest or desired characteristics (DeVicente and Tanksley, 1993; Tanksley et al., 1996; Tanksley and McCouch, 1997). Consequently, utilization of selfing can be postulated to result in series of inbred lines with bacterial wilt resistance available for introgression in diploid cultivated potato breeding programs.

Proper screening and quantification of latent infection of Rs will enable tracking bacterial colonization. This will ensure success in Rs prevention and production of resistant cultivars. These resistant clones can be used to develop markers for selection in the breeding programs and can therefore be added into the cultivated diploid breeding species using marker assisted selection.

They can also be used for improving some of the high yielding but vulnerable cultivars that are already present in the market. This will allow for an analysis of the genetic mechanism of resistance with continued crossings and detailed molecular studies.

As reported by Huet (2014), resistance breeding remains the most sustainable strategy to prevent Rs infection. We hope that challenges confronting bacterial wilt breeding like genetic variability of the pathogen, desirable agronomic traits. When linkage drag occurs, the unfavorable alleles are transferred along with the target gene or QTL allele (Bernardo, 2014). This therefore reduces genetic gain progression. Additionally, durable resistance and adaptation to different agro-ecological zones will hopefully, be considered in breeding efforts (Boschi et al., 2017). With commitment towards characterization of bacterial wilt resistance, there is optimism that a comprehensive resistance to the pathogen might be achieved. Hence, continued research with wild relatives of potato like *S. commersonii* will facilitate the introgression of bacterial wilt resistance and other desirable agronomic traits into diploid potato breeding programs.

APPENDICES

APPENDIX A

UW551 and CMR15 combined disease incidence at 20 days post inoculation on *S. commersonii* clones

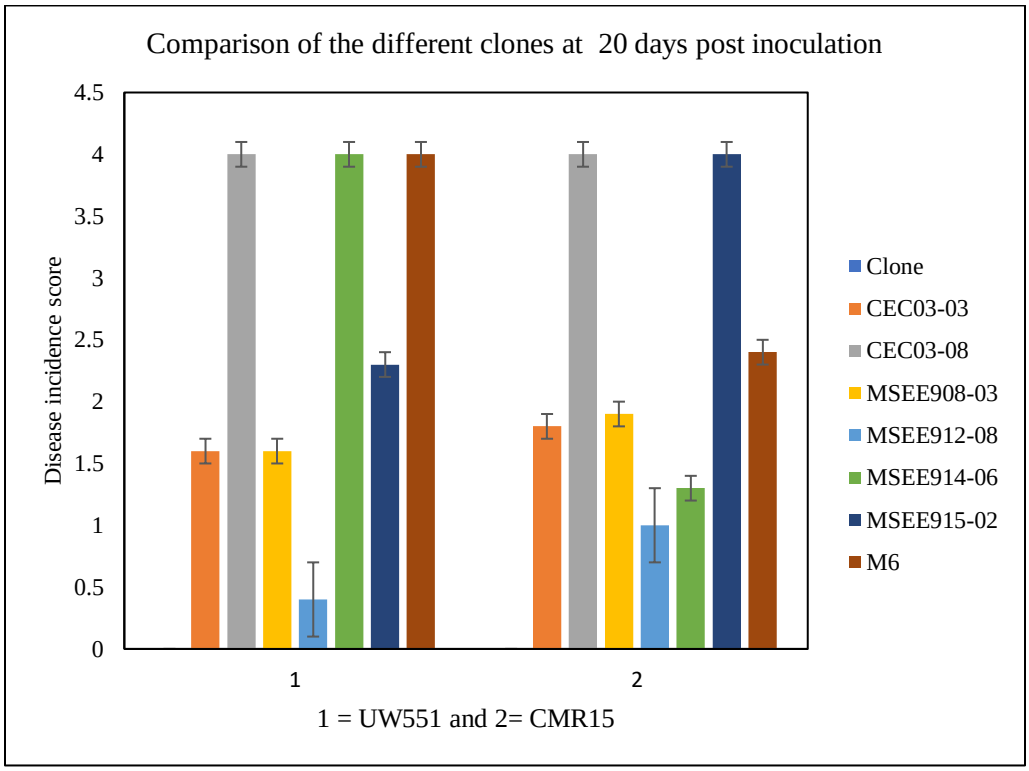

Figure 3.5. *Ralstonia solanacearum* strains UW551 and CMR15 combined disease incidence at 20 days post inoculation on *S. commersonii* clones, self-fertilized progenies and M6. Error bars indicate standard error.

APPENDIX B

Pairwise comparison for all the clones when evaluated with CMR15.

Table 3.2. Tukey HSD all pairwise comparison for all the clones when evaluated with CMR15.

Plant name	Plant name	Difference	t Ratio	Prob>|t|
CEC03-03	CEC03-08	-1.67857	-8.15	<.0001***
CEC03-03	EE908-03	0.20238	0.98	0.9571
CEC03-03	EE912-08	0.10119	-3.41	0.0146*
CEC03-03	EE914-06	-1.47619	-7.17	<.0001***
CEC03-03	EE915-02	-0.50000	-2.43	0.1953
CEC03-03	M6	-0.10119	-0.49	0.9989
CEC03-08	EE908-03	1.88095	9.13	<.0001***
CEC03-08	EE912-08	1.77976	8.64	<.0001***
CEC03-08	EE914-06	0.20238	0.98	0.9571
CEC03-08	EE915-02	1.17857	5.72	<.0001***
CEC03-08	M6	1.57738	7.66	<.0001***
EE908-03	EE912-08	-0.10119	-0.49	0.9989
EE908-03	EE914-06	-1.67857	-8.15	<.0001***
EE908-03	EE915-02	-0.70238	-3.41	0.0146*
EE908-03	M6	-0.30357	-1.47	0.7600
EE912-08	EE914-06	-1.57738	-7.66	<.0001***
EE912-08	EE915-02	-0.60119	-2.92	0.0610
EE912-08	M6	-0.20238	-0.98	0.0089*
EE914-06	EE915-02	0.97619	4.74	0.0001***
EE914-06	M6	1.37500	6.67	<.0001***
EE914-06	M6	0.20238	0.98	0.9571

*Significant at 0.05 probability level, **Significant at 0.01 probability level, *** Significant at 0.001 probability level.

The different clones are compared from each other when the clones were evaluated with CMR15 strain as shown in Table 3.2.

APPENDIX C

Tukey HSD all pairwise comparison for all the clones when evaluated with UW551 strain.

Table 3.3. Tukey HSD all pairwise comparison for all the clones when evaluated with UW551 strain.

Plant name	Plant name	Difference	t Ratio	Prob>|t|
CEC03-03	CEC03-08	-1.30357	-6.41	<.0001**
CEC03-03	EE908-03	0.05357	0.26	1.0000
CEC03-03	EE912-08	0.54762	2.69	0.01074*
CEC03-03	EE914-06	-1.00000	-4.92	<.0001**
CEC03-03	EE915-02	-0.37500	-1.84	0.5203
CEC03-03	M6	-0.92857	-4.57	0.0002**
CEC03-08	EE908-03	1.35714	6.68	<.0001**
CEC03-08	EE912-08	1.85119	9.11	<.0001**
CEC03-08	EE914-06	0.30357	1.49	0.7483
CEC03-08	EE915-02	0.92857	4.57	0.0002**
CEC03-08	M6	0.37500	1.84	0.5203
EE908-03	EE912-08	0.49405	2.43	0.0194*
EE908-03	EE914-06	-1.05357	-5.18	<.0001**
EE908-03	EE915-02	-0.42857	-2.11	0.3535
EE908-03	M6	-0.98214	-4.83	<.0001**
EE912-08	EE914-06	-1.54762	-7.61	<.0001**
EE912-08	EE915-02	-0.92262	-4.54	0.0002**
EE912-08	M6	-1.47619	-7.26	<.0001**
EE914-06	EE915-02	0.62500	3.07	0.0397*
EE914-06	M6	0.07143	0.35	0.9998
EE914-06	M6	-0.55357	-2.72	0.1001

*Significant at 0.05 probability level, **Significant at 0.01 probability level, *** Significant at 0.001 probability level.

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LITERATURE CITED

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CHAPTER 4. IDENTIFICATION OF SNP MARKERS ASSOCIATED WITH *Ralstonia solanacearum* RESISTANCE IN THE WILD POTATO SPECIES *Solanum commersonii*

4.1 ABSTRACT

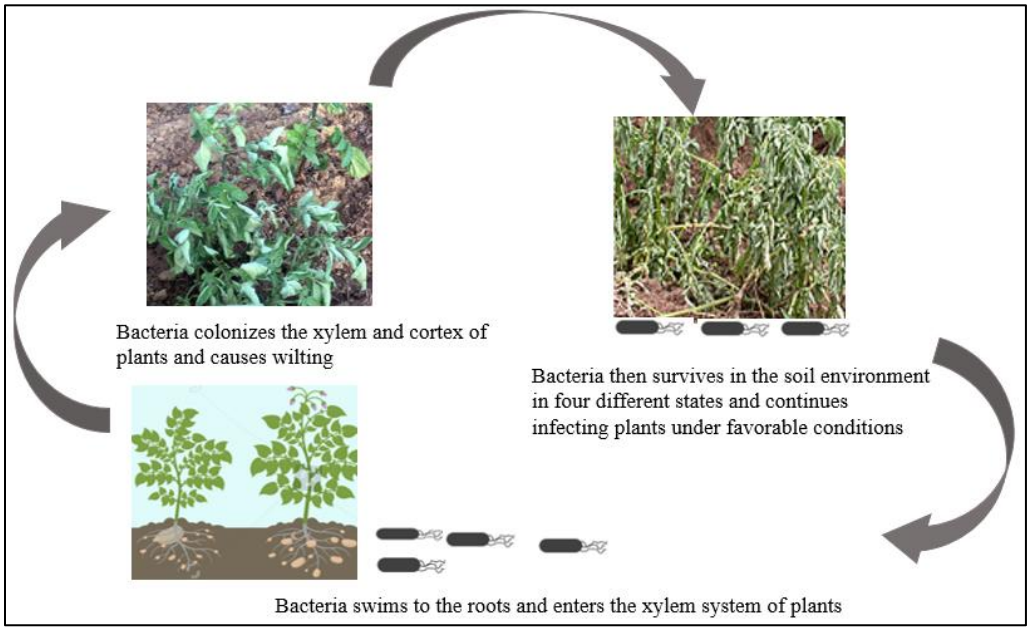
Bacterial wilt, caused by *Ralstonia solanacearum* (Rs), is a major disease in most tropical, subtropical and temperate potato producing regions of the world. Breeding for bacterial wilt resistance in potatoes is challenging due to the pathogen's aggressive nature and persistence in the environment. Accessions of *S. commersonii* are known to harbor resistance, therefore, breeding for genetic resistance to bacterial wilt may be an effective strategy towards controlling bacterial resistance. Genetic variation among S_1 progeny of *S. commersonii* line MSEE912-08 was characterized for Rs resistance. Ninety-eight individual S_1 selfed progeny were screened by artificial inoculation of Rs isolate NAK66 in a glasshouse with each line having three replications in the experiment. Percent disease reaction was calculated as the relative area under disease progress curve (RAUDPC). RAUDPC was fitted to a random effects mixed model and the means were transformed as a Best linear unbiased prediction (BLUP) score for each clone. BLUP values ranged from -19.25 for the most resistant and 16.83 for the most susceptible clones. Genotyping S_1 individuals using the Illumina 22K Potato SNP array generated 117 polymorphic single nucleotide polymorphic (SNP) markers from the selfed population. The significance of the marker linkage to Rs resistance was confirmed by single marker analysis from WindowsQTLCartographer 2.5, as well as a single marker ANOVA test using JMP®, Pro13. Significant markers were identified on Chromosome 4 which explained 9.8% to 13% of the phenotypic variation. Resistance was associated with a homozygous genotype. We interrogated Chromosome 4 from the Spud DB Genome browser to investigate if there was an annotated gene mapped disease related candidate gene. The genotypic value prediction from BLUP scores in single marker ANOVA showed that the homozygous genotype for solcap_snp_c2_35970, PotVar0015326, PotVar0075537, solcap_snp_c1_10181, solcap_snp_c1_4109 and solcap_snp_c2_12904 were significantly associated with Rs resistance. Additionally, the study identified WRKY transcription factor that could have been contributing to the Rs disease resistance in this study. This study provides potential germplasm that can be used in Rs resistance breeding in potato.

4.2 INTRODUCTION

Bacterial wilt is caused by the bacteria *Ralstonia solanacearum* (Rs) and is listed as one of the most economically important bacterial diseases of plants (Álvarez et al., 2010). The pathogen was first discovered in eggplant (Smith, 1896) then in tobacco (Johnson, 1921), tomato and potato and other crops (Álvarez et al., 2010). The bacterial disease is now known to infect more than 200 plant species and over 50 botanical families (Hayward and Hartman, 1994; Elphinstone, 2005; Champoiseau et al., 2009; Álvarez et al., 2010). Previous genetic studies have identified major genes that confer resistance to Rs in tomato (Wang et al., 2013). Nonetheless, little work has been done to identify Rs resistance genes in potato (Habe et al., 2019). To date, several wild potato species have been used in breeding for resistance to Rs. *S. commersonii* holds several resistance traits not commonly observed in cultivated potato, including resistance to bacterial wilt, verticillium wilt, potato virus X, root knot nematode, tobacco etch virus, soft rot, blackleg, common scab, and late blight (Bamberg and Martin, 1993; Micheletto et al., 2000; Aversano et al., 2015). Additionally, *S. commersonii* has been reported to be freezing tolerant and have the capacity to cold acclimate which are not found in cultivated potato (Bamberg, 1994). Rs resistance is heritable as sexual hybridization of *S. commersonii* and *S. tuberosum* yielded offspring with partial Rs resistance (Carputo et al., 2009).

Bacterial wilt is mostly prevalent in the lowlands of tropical and sub-tropical regions of potato production areas because bacterial wilt cannot survive in temperatures below 4º C for long durations (Champoiseau et al., 2009). Notably, there is one subgroup of *R. solanacearum,* Race 3 biovar 2 (R3bv2) that predominantly infects plants at high altitudes or in some warm temperate zones (Champoiseau et al., 2009). The pathogen causes brown rot of potato in the highland area of Asia and Latin America, as well as in Africa. Additionally, the pathogen is considered a species complex due to its significant variation of clusters of closely related isolates whose individual members may represent more than one species (Fegan and Prior, 2005). The pathogen causes wilting, stunting and yellowing of foliage (Yao and Allen, 2007). Other symptoms include leaf epinasty coupled with internal symptoms of progressive discoloration of vascular tissues in the xylem that can exude viscous ooze which is a signature symptoms for bacterial wilt in plants (Hayward, 1991). Additionally, dried leaves maintain green coloration even after wilting (Fegan and Prior, 2005). The bacteria are believed to infect the roots of the plants, followed by rapid

colonization of the plant's vascular system (Milling et al., 2009). The Rs can release large amounts of exopolysaccharide, thereby preventing water flow within the xylem vessel characterized by wilting and plant death. (Denny, 2007; Champoiseau et al., 2009; Álvarez et al., 2010). Global damage of bacterial wilt in potato is estimated to cause economic losses up to $950M per annum (Champoiseau et al., 2009).



Source: Adapted from Álvarez et al. (2010)

Figure 4.1. The life cycle of *Ralstonia solanacearum* outside the plant into the soil and environment.

The life cycle of *Ralstonia solanacearum* (Figure 4.1): The bacterium moves to the plant roots, penetrating the epidermis, flowing through the cortex into the xylem and colonizing the xylem vessel leading to wilting of the plant. Once the plant is dead, the bacterium is released to the environment where it can survive in four different states namely; viable but non-culturable form (VBNC), starved cells, biofilm, and phenotypic conversion until it comes into contact with another host (Álvarez et al., 2010).

Phylogenetic classification of Rs system has been developed to reflect the strains' phylogeographical origins. Phylotype I strain originated from Asia, Phylotype II from America, Phylotype III originated from Africa and Phylotype IV originated from Indonesia. Further subdivision of phylotypes have been based on the sequence of the endoglucanase (*egl*) gene (Fegan and Prior, 2005; Champoiseau et al., 2009; Álvarez et al., 2010; Ferreira et al., 2017). This is a hydrolytic cell wall degrading enzyme that promote entry of the pathogen into host tissue, hence contributing to bacterial virulence (Álvarez et al., 2010). Phylotype II (R3bv2) of Rs is becoming

prevalent in the highland potato growing areas of Kenya where it is causing economic losses for small holder farmers in the region (Muthoni et al., 2012). Without a feasible control strategy, its management has been difficult since chemical control measures, such as application of bleaching powders coupled with resistance inducers (e.g. acibenzolar-S-methyl) or phosphorous acid (Champoiseau et al., 2009) are not feasible in the field. Reports indicated a lack of feasibility for chemical application on a large scale, since it can cause environmental damage, or have high costs and labour inputs (Laferriere et al., 1999; Champoiseau et al., 2009; Milling et al., 2009). Therefore, some of the strategies used for bacterial wilt control in endemic regions have included, use of certified seeds, cultural sanitation, use of pathogen free soil and water coupled with crop rotation (Champoiseau et al., 2009; Boschi et al., 2017). Rs resistance breeding in potato has been achieved through utilization of protoplast fusion of *S. stenotomum* and *S. tuberosum,* the resulting hybrids exhibiting partial Rs resistance (Fock et al., 2001). Additionally, somatic hybrids of *S. commersonii* and *S. tuberosum* were studied and reported to result into Rs resistance (Laferriere et al., 1999; Fock et al., 2000). Most studies are indicate that Rs resistance is quantitative resistance, as was identified in tomato (Mangin et al., 1999; Wang et al., 2000; Carmeille et al., 2006; Boschi et al., 2017), eggplant (Lebeau et al., 2013), tobacco (Qian et al., 2012) and in potato (Habe et al., 2019). Currently, there are no commercial potato varieties resistant to Rs available (Huet, 2014; Boschi et al., 2017).

In order to introduce genetic resistance as a sustainable solution for managing Rs, the current research sought to identify genetic regions or SNP markers associated with Rs resistance from *S. commersonii.* Previously, *S. commersonii* had been identified as a potential source of Rs resistance (Laferriere et al., 1999; Caruso et al., 2008; Zuluaga et al., 2015). Using single marker analysis, we were able identify SNP markers associated with Rs resistance. This study used a selfed population from a *S. commersonii* clone to identify loci associated with resistance to Rs. Genetic variation for Rs among the progeny of selfed *S. commersonii* clone was identified by artificially inoculating the plants with the isolate NAK66 (R3bv2), rating the clones for wilting degree and conducting a single marker association analysis. The phenotype data was associated with SNP data from the Illumina 22K Potato SNP array platform to perform significant SNP marker identification analysis. The selfed population from *S. commersonii* is understood to possess a complement of resistance genes. The significant SNPs associated with Rs resistance identified in this study may serve as a candidate for marker assisted breeding for this economically important trait.

4.3 MATERIAL AND METHODS

4.3.1 Plant Material

The *S. commersonii* PI line 320266 (CEC03-07) was used in the study. The accession was kindly provided by USDA/ARS potato gene bank at Wisconsin Madison (Dr. John Bamberg). This PI has been previously identified to have cold tolerance (Stone et al., 1993) and resistance to Rs (Laferriere et al., 1999; Zuluaga et al., 2015). CEC03-07 was self-fertilized to generate MSEE912-08 family. The clone MSEE912-08 was selected among selfed progeny that had exhibited high resistance to Rs from preliminary Rs bioassay previously carried out at the University of Wisconsin, Madison (Dr. Caitilyn Allen's lab) in the fall of 2017. The clone MSEE912-08 was selfed to generate seed for in Spring 2018. Plant material was then taken to Kenya for establishment and screening against Rs in the Spring and the Summer of 2019. The S_1 population consisted of 120 individual progeny (herein coded as MSEE912-08:1-120). All the progeny were germinated on Murashige and Skoog (MS) salts/media containing 3% sucrose 0.8 % agar with the pH was adjusted to 5.8 (Murashige and Skoog, 1962). The plantlets were kept in the growth rooms at 20 ± 2°C under 16 h light/8 h dark photoperiod. The mapping population was confirmed to be a *S. commersonii* selfed population based on SNP genotyping. Shangi, Tigoni and Unica were used as susceptible check lines in the study. The clone MSEE912-08 and M6 were also included.

4.3.2 Bacterial culture and phenotypic data collection

4.3.2.1 Rs Strain preparation and maintenance

The Rs strain was maintained in a sterile distilled water stock culture as described by Carputo et al. (2009). Briefly, two days prior to inoculation with the pathogen, the pots were not watered to ensure that the plants would uptake the inoculum during the assay. Rs strain, NAK66 was cultured on solid agar media containing 2,3,5 -tri-phenyl tetrazolium chloride (TZC) at 28º C for 48 hrs. Subsequently, the virulent, white-colored, irregular-shaped, pink center was picked and transferred to casamino acid peptone (CPG) medium for further culturing overnight (Kelman, 1954; Carputo et al., 2009; Champoiseau et al., 2009; Milling et al., 2009; Huerta et al., 2015). The bacterial suspension concentration was suspended in sterile distilled water and standardized to an optimal

density of 600 nm (OD_{600}) and further adjusted/diluted to final cell density of approximately 5×10^7 cfu mL^{-1} for use in the inoculation of the population (Huerta et al., 2015).

4.3.2.2 Inoculation of plant material

Uniformly grown plant material was established through apical cutting of the 120 S_1 progeny in a 40 mm diameter pot in the greenhouse. One-and-a-half-month-old plant material was transferred to a glasshouse where they were acclimatized for one week before the bioassay was done. (Note: to establish the plant material, they were grown in a greenhouse to carry out the disease bioassay the plants were transferred to a glasshouse in a plant quarantine station). Optimum greenhouse conditions were maintained to promote plant establishment. The Rs strain NAK66 (R3bv2) was kindly provided by Dr. Kalpana Sharma of CIP Kenya, for use in the resistance bioassay. Briefly, using the aggressive strain NAK66, a total of 1890 clonally propagated plants were evaluated. (120 progeny, MSEE912-08, M6 and the three susceptible check varieties consisting of three replicates of 5 plants). The population was artificially inoculated with Rs using the soil soak method. Wilting degree was estimated using a standardized scale from Carputo et al. (2009) to evaluate the individual plants (Fock et al., 2000). The soil soak method is as follows: a suspension of inoculum was prepared and adjusted to a concentration of 1×10^7 cfu (colony forming unit). Six-week-old plants were inoculated with the Rs strain NAK66 by root inoculation (root stabbing) 5 cm away from the stem of the plants. 25 ml of the bacterial suspension was poured into the pot (Huerta et al., 2015).

Bacterial wilt screening of all the plant material was conducted at a designated pathology laboratory (and glasshouses) mandated to handle the aggressive pathogens strains at the Kenya Plant Health Inspectorate Service (KEPHIS), Plant Quarantine Station, Muguga, Kenya. Additionally, fifteen clonal plants of three local elite Rs susceptible varieties (Shangi, Tigoni and Unica) in Kenya were also evaluated. The plants were acclimatized to glasshouse growth conditions for one week and watered and fertilized as needed. Appropriate agronomic requirements were applied before inoculation with Rs strains NAK66. The plants were distributed in the glasshouse under 28 ºC ±2 ºC for 12 hr during the day, while the night temperature was 23 ºC ±2 ºC for 12 hr. with 80% relative humidity. After inoculation the plants were covered with plastic bags for 48 hr to accelerate pathogen infection. The wilting of the population was monitored from the 2nd day after inoculation up till 30 days post inoculation according to Siri et al. (2009).

4.3.3 SNP Marker genotyping analysis

Tissue was harvested from young leaves of the 120 S_1 progeny and the parent clone (MSEE912-08) and then freeze dried. Tissue was also collected for Tigoni, Shangi, Unica, CEC03-03, M6, and MSEE912-08. DNA was extracted from leaf sample using DNeasy plant mini kit (QIAGEN) as enumerated by Manrique-Carpintero et al. (2015). Briefly, DNA was quantified using the Quant-iT^{TM} PicoGreen$^{(R)}$ dsDNA assay kit and adjusted to a concentration of 30 ng/ml in dH2O and arranged in 96-well microtiter plates for genotyping. SNP genotyping was conducted on the Illumina Infinium 22K Potato SNP array. Processing of DNA quantification and SNP genotyping was performed for the samples as described by Hamilton et al. (2011), Stich et al. (2013), Douches et al. (2014), Alsahlany et al. (2019). To analyze SNP data, GenomeStudio 2011.1 (Illumina, San Diego, CA) software was used (Antanaviciute et al., 2012). GenomeStudio$^{(R)}$ 2.0 data analysis software was used to normalize clusters, and call genotype for data quality monitoring and quality control. A total of 123 diploid samples were genotyped for each SNP locus with one of three probable genotypes automatically assigned to the individuals using GenomeStudio. The SNP markers clustered according to diploid genotypes AA, AB, or BB as a reference as enumerated by Stich et al. (2013). The SNPs that were of low- quality or monomorphic, and those that mapped to more than one site were eliminated from the data set. Visual assessment of clusters in the genome studio graphs was also conducted to remove SNPs confounded in paralogues, smaller sub clusters of individual genotypes call that did not cluster tightly or that did not cluster in expected regions and either shifted from 0 or 1 axis as reported by Manrique-Carpintero et al. (2015) and Antanaviciute et al. (2012). Ninety-eight progeny were used for the SNP single marker analysis. Single marker analysis (SMA) was calculated from BLUP scores data for the Rs resistance trait with all the marker classes and potential relationship between the SMA were related for significance for all the genotype trait. Experimental design and data analysis

4.3.3.1 Phenotyping of Rs resistance.

The 120 progeny and six reference clones were tested in a Randomized Complete Block Design (RCBD) by artificially inoculating them with the NAK66 strain of Rs. The percentage of wilted leaves were scored for 30 days post inoculation (DPI) with readings taken every day. The Rs resistance scale was as described by Carputo et al. (2009); Briefly, disease wilting was recorded as 0, 25, 50, 75 or 100% on individual plants. Using percent disease wilting from 1st day to the 30th

day relative area under disease progress curve (RAUDPC) was calculated (Simko and Piepho, 2012). An ANOVA of RAUDPC values was used with random effects mixed model, and best linear unbiased predictions (BLUP) scores were calculated for each clone (Piepho et al., 2007). Clones were considered to be resistant if the clone exhibited a wilting percentage average of ≤ 25% (Carputo et al. 2009).

$$RAUDPC = \sum_{i=1}^{n} \frac{(yi+yi+1)}{2}(t_{i+1} - t_{i+1}) \quad (1)$$

in which n = total number of observations, *yi* = injury intensity (usually incidence in crop health data) at the *i*th observation, and *t* = time at the *i*th observation. Since the unit for *y* in the sample data is % and the unit for *t* is development stage, the unit of the AUDPC is % development stage unit. The first term in the equation is the height of the rectangle (estimated as the midpoint y_i and y_{i+1}) and the second term is the width of the rectangle (Simko and Piepho, 2012).

4.3.3.2 Statistical analysis

Percentage of wilting leaves was used for a one-way analysis of variance (ANOVA) considering one treatment (Rs bioassay) factor with several different clones. ANOVA (SAS 9.4) was used to test the means among the clones and to quantify the differences to determine the significant differences in the overall means of disease severity of the different clones and their response R3bv2 (NAK66). R statistical package was used for principal component analysis of the 98 individuals BLUPs wilting scores to estimate marker effects. Statistical analyses of the wilting percent data were performed using JMP®, Pro13 (SAS Institute Inc., Cary, NC, 1989-2019). For all clones tested, the mean was estimated for each individual clone by Proc LS mean. Data normality and residual error were estimated using a W-test (Shapiro and Wilk, 1965). The marker selection via BLUP in the plant was used to predict genotypic value (total genetic variance) from the phenotypic value in the population. The mean separations were done using Tukey's multiple comparison procedure test P = 0.05 to determine significant differences. R software (https://uc-r.github.io/hc_clustering) was used for computing hierarchical clustering by computing dissimilarities values and generating a dendrogram (RCore, 2019) for the S_1 population and for principal component analysis for the 7, 14, 21 and 28 DAI on wilting degree of the Rs infected plants. Marker genotypes were tested using a single marker t-test or ANOVA (David et al., 2019). The model is expressed as;

$$Y = \mu + f(marker) + error \quad (2)$$

Where Y is equal to trait value, μ is equal to population mean, f (marker) is a function of the molecular markers and error represents the residual error in the model. For a simple regression model; y = b0 + b1 + e (error) which test whether the marker is linked to a QTL. The F statistics compares the hypothesis H_0: b1= 0 to an alternative H_1: b1 not 0. The pr (F) was used as a much support there is for H_0. Additionally, non- parametric statistics such as Kruskal-Wallis statistics was used in single marker ANOVA analysis (SMANOVA). SMANOVA was done for all chromosomes to see if there were any other markers not identified through the single marker analysis. This allowed for clustering of clones based on disease score similarities for their resistance to wilting.

4.3.4 SNP Marker analysis

Eliminating individuals that had high SNP no call rates and those with unexpected segregation patterns based on the parental genotypes resulted in a population of 98 individuals for further analysis. A total of 117 polymorphic markers were used for linkage map construction. A linkage map was constructed using JoinMap ® 4.1. SNP markers were filtered to remove markers with a high number of missing genotypes. Additionally, SNPs were filtered according to similarity of loci by excluding markers with similarity value of 1.000. This filtering of the 98 individual clones resulted in 93 SNPs markers positioned on nine linkage group and this data set used for a marker analysis (Van Ooijen, 2006; Wang et al., 2013). JoinMap ® 4.1 was used to generate the linkage map (Meijer et al., 2018) using the regression mapping as the mapping algorithm. An independence test with a logarithm of odds (LOD) score 4.0 was used to group markers into linkage groups. The marker order and genetic distance were calculated using regression mapping algorithm with the Kosambi map function as described by Wang et al. (2013) and Van Ooijen (2006). This allowed for determination of order and distance among markers within each linkage groups. This study used WinQTL Cartographer version 2.5. for SNP marker detection (Wang et al., 2012). The Single Marker Analysis (SMA) procedure was used to identify significant markers. Single marker analysis calculated the phenotypic values for the different genotypes for a given molecular marker as described by David et al. (2019). The proportion of phenotypic variation of each clone for bacterial wilt resistance represented as BLUPs estimated the phenotypic variance (R^2) value and additive gene effects of the markers. The SMA was performed using a variety of

statistical analyses, including t-tests, ANOVA, regression, maximum likelihood estimations, and log likelihood ratios as described on the statistical analysis section above to determine the association between phenotype for Rs resistance and genotype of the S_1 population.

Additionally, a nucleotide sequence homology search was conducted by using SNP marker sequence data into the Nucleotide BLAST program (http://www.ncbi.nlm.nih.gov) and conducting a search of nucleotide by the Basic Local Alignment Search Tool (BLAST program against the GenBank and dbEST database) for annotation. Lastly, the significant SNPs on Chromosome 4 were further interrogation in Spud DB Genome Browser to view and analyze the genome for the occurrence of positionally mapped disease gene candidates.

4.4 RESULTS

4.4.1 Rs bioassay

The S1 population showed a highly variable response to Rs inoculation depicting segregation for resistance and susceptibility after the inoculation.The disease score incidence for the S_1 population, parental and local elite lines inoculated with NAK66 of *R. solanacearum* BLUP scores ranged from -19.25 for the most resistant and 16.83 for the most susceptible clones (Table 4.6 supplementary). Progeny from MSEE912-08 S1 population with bioassay RAUDPCx100 scores of ≤ 25 were as follows: MSEE912-08:63, MSEE912-08:19, MSEE912-08:88, MSEE912-08:14, MSEE912-08:29, MSEE912-08:6, MSEE912-08:118. The clones with the highest level of susceptibility (≥ 50 RAUDPCx100) were MSEE912-08:25, MSEE912-08:52, MSEE912-08:55, MSEE912-08:57, MSEE912-08:99, MSEE912-08:98 which were equivalent to the susceptible checks Unica Tigoni and Shangi (Fig. 4.4 and Table 4.6). As the assay progressed, most of the susceptible clones were infected by the pathogen (Table 4.1). The clones were highly significantly different from each other with clone and DAI significant sources of variation (Table 4.2) ($P = 0.05$).

Table 4.1. Covariance parameter showing the relationship between variable after the population is inoculated with NAK66 of *Ralstonia solanacearum.*

Covariance Parameter Estimates				
Covariance Parameter	**Estimate**	**Standard Error**	**F Value**	**P-value**
Clone number	0.1918	0.03152	6.09	<.0001
Day*Clone number	0.05780	0.01755	3.29	0.0005
Residual	0.4777	0.02153	22.19	<.0001

Table 4.2. ANOVA showing repeated measure for wilting disease incidence of the S_1 population parental and local elite lines inoculated with NAK66 strain of *Ralstonia solanacearum.*

Source of variation	**d.f.**	**Variance**	**P value**
Replication	2	13.95	
Clone	125	3.54	<.001***
Residual	252	26.65	
Day	29	2707.47	<.001***
Clone*Day	3625	2.46	<.001***
Residual	7366		

Tukey's multiple comparison test, $P = 0.05$, for mean disease incidence score 30 DAI,

*** denoted as highly significant $P = 0.001$.

Among the selfed progeny of the *S. commersonii* line there were some S_1 clones that had high Rs resistant phenotypes, as compared to the local elite cultivars and parental clone (CEC03 clones). Using Rs strain NAK66, the from Kenyan isolate used, there was no significant difference between the local cultivars and some of the susceptible S_1 progenies (Supplementary Table 4.6). MSEE912-08 had lower disease resistance compared to the S_1. Initial screening of the genotypes showed the clone as amongst the most tolerant line in comparison to other S_1 clones tested earlier.

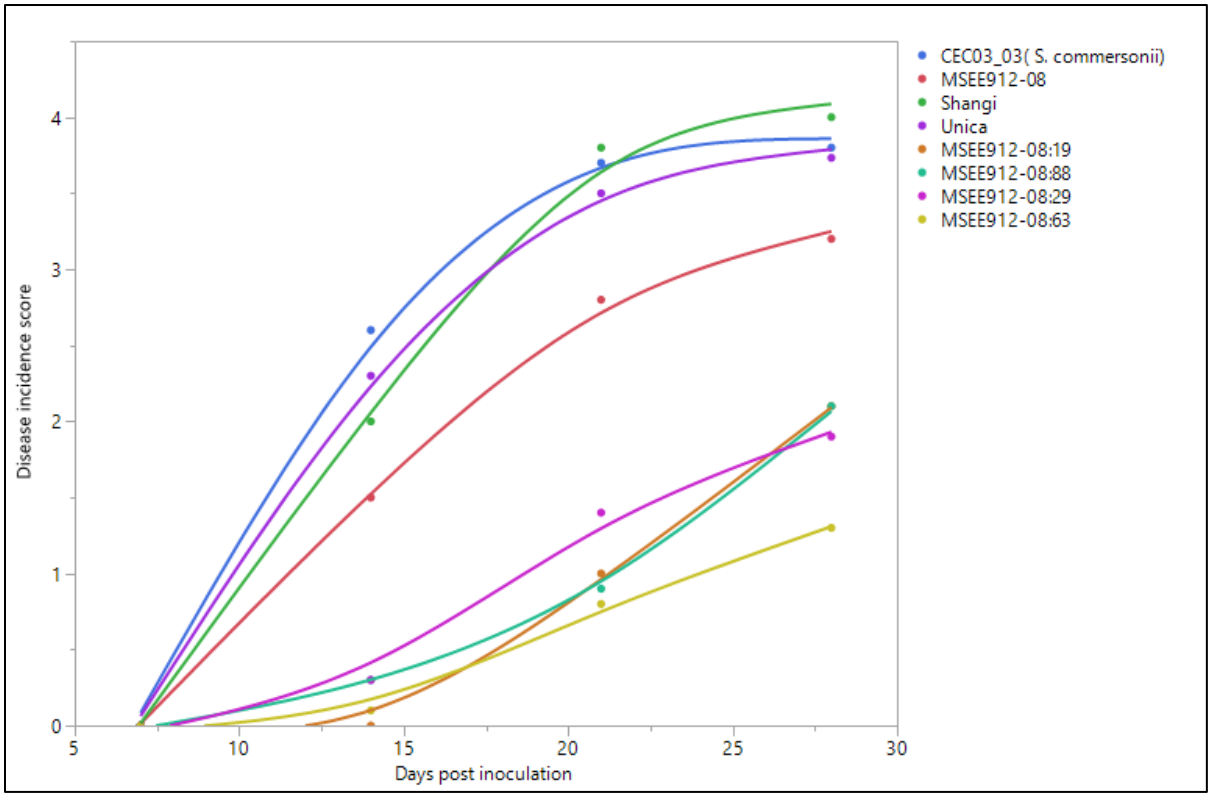


Figure 4.2. Disease incidence showing disease progression amongst tolerant progeny, CEC03-03, MSEE912-08 and susceptible controls and the local elite genotypes that were inoculated with *R. solanacearum.*

Based upon the ANOVA, genotype and DPI were significant. The S_1 progenies displayed a highly variable response segregating from resistant to a susceptible reaction. Thus, MSEE912-08:63, MSEE912-08:19, MSEE912-08:88, MSEE912-08:14, MSEE912-08:29, MSEE912-08:6, MSEE912-08:118 progeny classified as resistant (Table 4.6. and Figure 4.2). Most of the local elite varieties were highly susceptible possessing overall disease score above 50% wilted leaves. Compared to local elite varieties the S_1 population exhibited resistance to Rs.

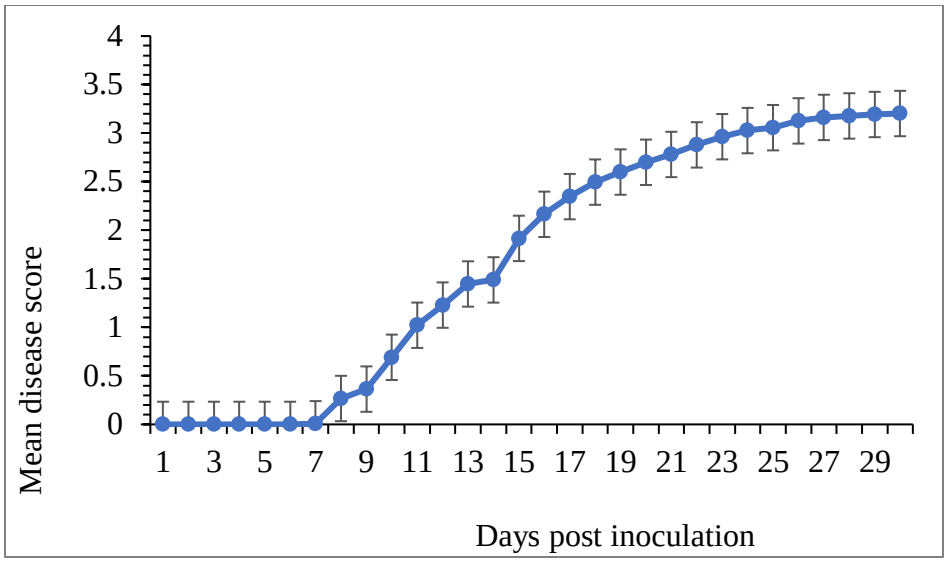


Figure 4.3. The disease score progression for all the plant material 30 days post inoculation calculated from daily mean score of 15 plants per clone.

The bacterial wilt disease in the S_1 population over time shows the level of disease DPI. There were some clones that exhibited significantly lower wilt disease score hence should be further evaluated to confirm their resistance level as shown in Figure 4.4. that used BLUP data.

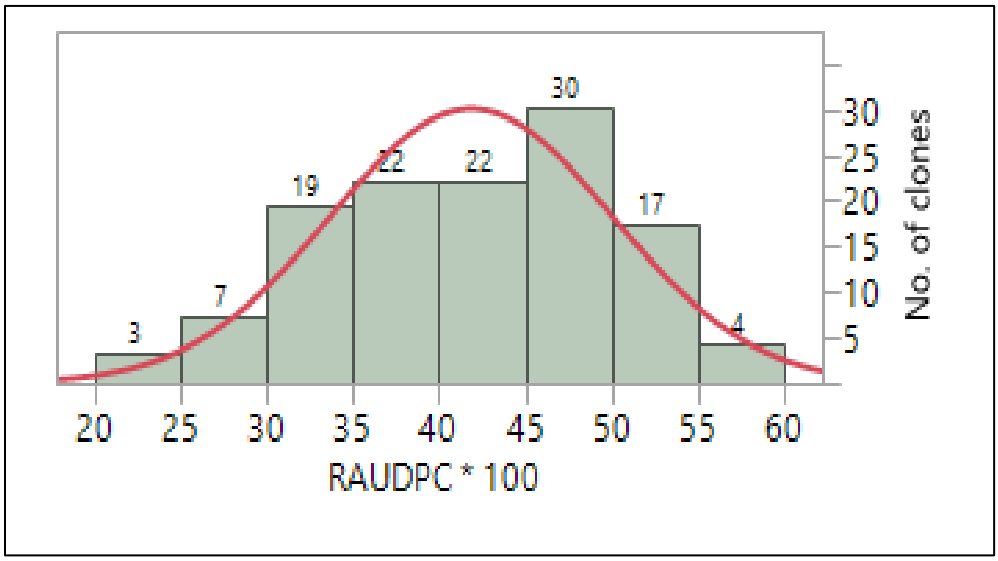


Figure 4.4. The Rs RAUDPC distribution between different clones.

The graph in Figure 4.4 shows the distribution of the RAUDPC scores for the different S_1 population. The graph also shows that about 10 individuals had significantly lower RAUDPC of below ≤ 25 hence more resistant to Rs. There were more than 51 individuals that were highly susceptible. Additionally, this included the local susceptible checks as well as the MSEE912-08

since their BLUP score were 7.5 to 14.9 (Supplementary Table 4.6). Among the progeny tested, 8 % showed RAUDPC of less than ≤ 25 shown on Figure 4.4. There were 75 % of individuals ≥ 50 while 17 % of the progeny showed RAUDPC of above ≥ 55 (Figure 4.4).

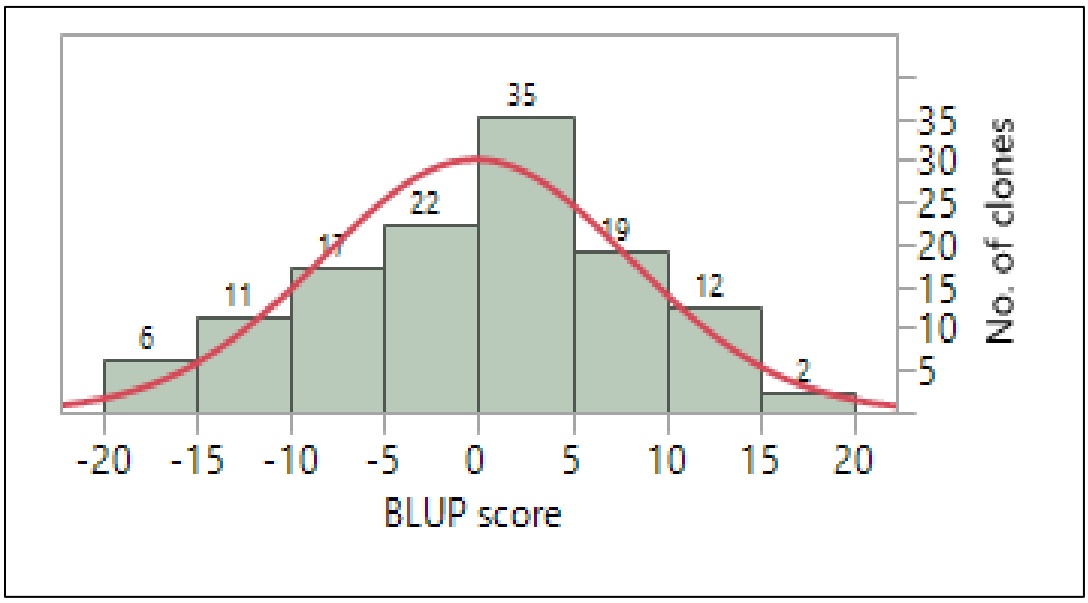


Figure 4.5. The BLUP distribution between different clones.

The graphical of BLUP score distribution among the different clones (Figure 4.5). The graph also shows that about 17 individuals had significantly lower BLUP score (BLUP score of -10 to – 19.2, supplementary Table 4.6).

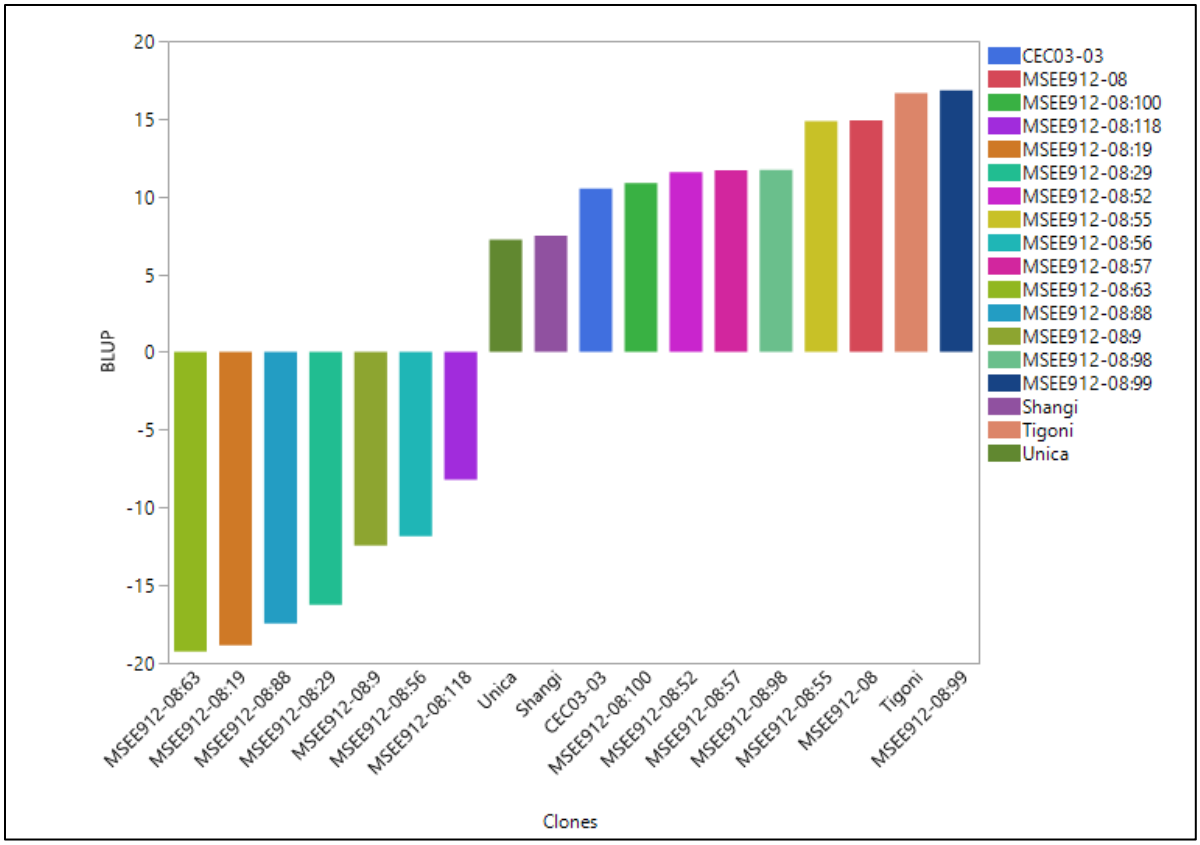


Figure 4.6. The BLUP score for 7 most resistant clones and 6 susceptible clones, MSEE912-08, CEC03-03 and susceptible check varieties.

The graphical discrimination of most resistant and susceptible clones is shown in Figure 4.6.

4.4.2 Markers and individual filtering

There was a total of 20,670 SNP markers from the Illumina Infinium 22K Potato SNP array. Based on questionable SNPs, with multiple hits to pseudomolecules and monomorphic SNPs, 8,682 were filtered out. After filtering the SNPs that did not cluster appropriately according to the validation standard set, low-quality SNPs, SNPs that did not segregate in the S_1 and SNPs with high rates of no calls, 117 SNPs remained. The SNPs were then used to calculate heterozygosity in the population. For the population filtering, after removing duplicates, and individuals with a high no-call rate, a total 98 progeny remained for single marker linkage analysis.

4.4.3 Genetic linkage and physical positions of SNPs on DM V4.03 Pseudomolecule

In the S_1 population, the 117 SNPs were distributed across eight of the twelve potato chromosomes Figure 4.7 shows the mapping of the distinct position of the SNP markers. Figure 4.7 shows the correlation between the genetic map and the physical map position of the SNP markers. Due to low marker density in Chromosomes 3, 6, 8, and 11 no linkage groups were not constructed (Figure 4.7)

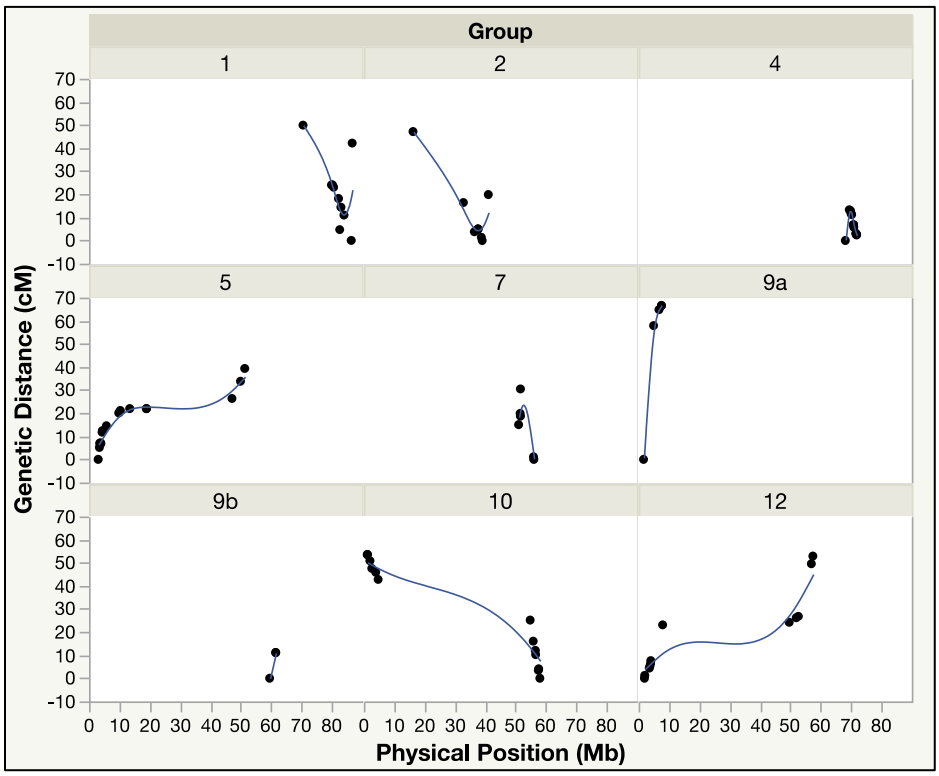


Figure 4.7. Concordance between the physical (Mb) and genetic (cM) of the eight chromosomes in S_1 progeny mapping population.

Figure 4.7 shows concordance between the genetic and physical maps linkage groups corresponded to physical pseudomolecules and groups 9a and 9b represent the arms of Chromosome 9. However, markers were not evenly distributed across Chromosomes 1, 4 and 7 and there are troubling concordance issues on Chromosomes 1, 2, 4 and 7 which could have been due to genetic differences (Figure 4.7) between the wild *S. commersonii* and reference genome sequence DM1-3 516/R44 (*Solanum phureja*) (Hardigan et al., 2017).

4.4.4 Single marker analysis

The segregating 93 SNPs used in the single marker analysis resulted into the detection of several SNPs associated with Rs resistance on Chromosome 4. Analysis of SNP markers positions detected in the segregating S_1 mapping population of *S. commersonii* revealed Chromosome 4 as having six significant markers as is shown in Table 4.4 The phenotypic variation explained by those markers was 9.8 % to 13.0 %, (analyzed by WinQTL Cartographer version 2.5) as shown in Table 4.3. Consequently, when single marker ANOVA analysis (SMANOVA) was performed on each of the SNP markers. The significance of the markers was corroborated with both single marker analysis and single marker ANOVA as significant (Table 4.3).

Table 4.3. Summary of single marker analysis for markers with linkage to bacterial wilt resistance in the population as calculated by WindowsQTLCartographer.

Chromosome	**SNP marker**	**LOD**	**cM**	**Mb**	**pr (F)**	**% R^2**
4	PotVar0075537	3.02	0.0	68.1	0.000231	13***
4	solcap_snp_c2_35970	2.50	6.807	70.7	0.00286	9.8**
4	solcap_snp_c1_10181	2.85	11.220	70.0	0.000347	12***
4	PotVar0015326	2.98	11.629	70.0	0.000255	13***
4	solcap_snp_c1_4109	2.95	12.897	69.8	0.000275	12***
4	solcap_snp_c2_12904	3.03	13.307	69.4	0.000226	13***

Significant at 0.01 probability level, * Significant at 0.001 probability level; LOD, logarithm of odds; %R^2, percentage of explained variance of traits being analyzed; cM, centimorgan; Mb, megabase pair.

When SMANOVA analysis was carried out on the six SNP markers, it was determined that the homozygous state of the SNP markers was significantly associated with Rs resistance (Table 4.4). This was seen across the six markers in Chromosome 4 (Supplementary Figure 4.8).

Table 4.4. Single marker Analysis of Variance (SMANOVA) for the SNPs associated with Rs resistance calculated using JMP.

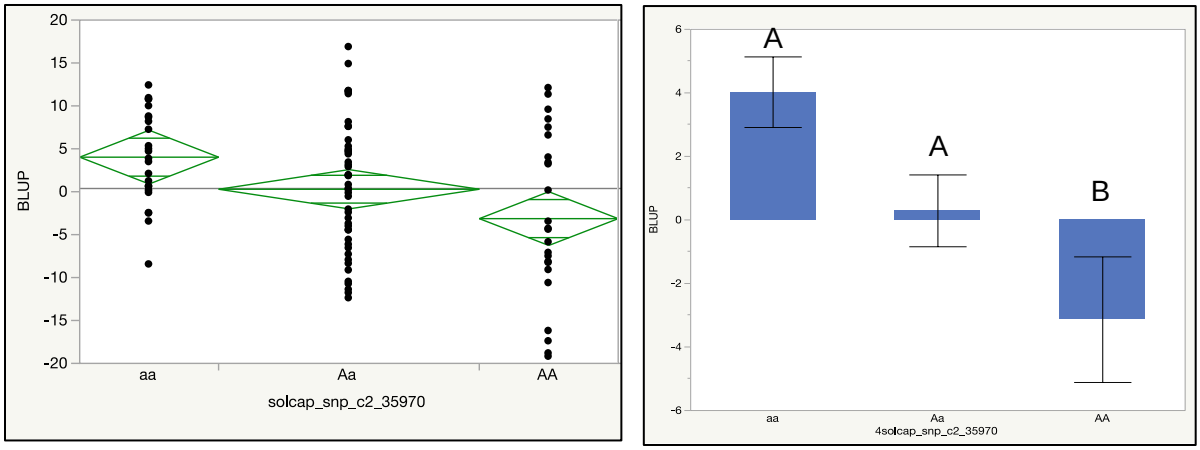
Source (SNP markers)	Chr	cM	Mb	AA	Aa	Aa	Prob > F
PotVar0075537 ±SE	4	0.0	68.1	-4.3 c	0.33 b	4.30 a	0.0086**
solcap_snp_c2_35970 ±SE	4	6.807	70.7	-2.85 b	0.29 ab	3.73 a	0.0139*
solcap_snp_c1_10181 ±SE	4	11.220	70.0	-4.6 b	1.26 ab	3.60 a	0.0065**
PotVar0015326 ±SE	4	11.629	70.0	-4.06 c	0.50 b	4.19 a	0.0091**
solcap_snp_c1_4109 ±SE	4	12.897	69.8	-4.02 b	0.50 a	4.02a	0.0093**
solcap_snp_c2_12904 ±SE	4	13.307	69.4	-4.31 b	0.67 a	4.10 a	0.0077**

Mean followed by same letters are not significantly different at 0.05α level using Student's t mean separation analysis, *Significant at 0.05 probability level, **Significant at 0.01 probability level; Chr, chromosome; cM, centimorgan; Mb, megabase pair.

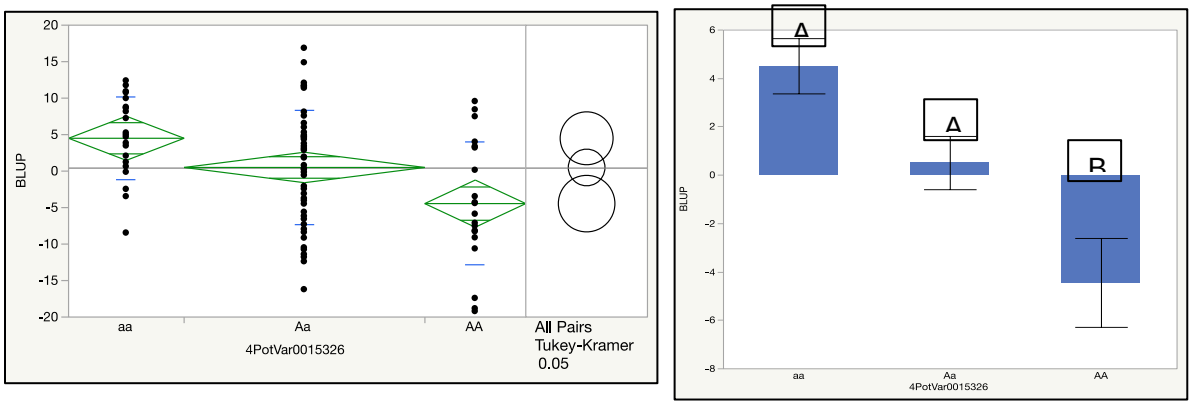
Some of the significant SNPs are in annotated genesso to. A BLAST search of the nucleotide sequence of SNP marker solcap_snp_c2_35970 is in senescence associated protein SPA15, which is believed to be senescence associated gene induced during leaf senescence in plants (Yap et al., 2003) (Table 5). Based on the SNP marker position, it was evident the markers are physically close together, therefore the other four SNPs PotVar0075537, solcap_snp_c2_35970, solcap_snp_c1_4109 and solcap_snp_c2_12904 did not show any unique specific biological reference to disease resistance but they may play other biological roles in the plant (Table 4.5).

Table 4.5. Annotation of SNP markers from Chromosome 4 associated with bacterial wilt resistance.

Trait/marker	Chromo-some	Protein	Function
PotVar0075537	4	protein DCL (Dicer-Like protein) in plants for antiviral defense and small RNA during virus infection	Chloroplast development and palisade cells morphogenesis in leaves (Keddie et al., 1996; Bellaoui and Gruissem, 2004)
solcap_snp_c2_35970	4	Senescence-associated protein SPA15	Senescence associated gene induced during leaf senescence in plants (Yap et al., 2003)
solcap_snp_c1_10181	4	BEL1-like (BEL1-like) homeodomain protein 3	Heterodimer in both floral, apical shoot meristem and vegetative development (Rutjens et al., 2009; Sharma et al., 2014)
PotVar0015326	4	-	-
solcap_snp_c1_4109	4	3-ketoacyl-CoA synthase 4	Protein involved in fatty acid biosynthesis, which is part of lipid metabolism (Blacklock and Jaworski, 2006)
solcap_snp_c2_12904	4	J domain-containing protein required for chloroplast accumulation response 1	Plays role in protein translation translocation and act in ATPase activity



SNP marker solcap_snp_c2_35970



SNP marker PotVar0015326

Figure 4.8. Two different examples of significant SNP marker showing SMANOVA analysis carried. (a) SNP marker solcap_snp_c2_35970, (b) SNP marker PotVar0015326 on Chromosome 4.

There was an association between reduced disease index score as showed by higher negative BLUP scores that showed homozygous alleles to be associated with resistance (Figs. 4.8 and 4.9). Showing that the SNP markers identified resistance associated with a homozygous genotype. This trend was observed for most of the SNP markers. SMANOVA SNP analysis identified four other SNP markers: PotVar0075537, solcap_snp_c2_35970, solcap_snp_c1_4109 and solcap_snp_c2_12904 (Supplementary Figure 4.9).

4.4.5 Comparison of SMANOVA with SMA from WinQTL cartographer

Both analyses identified the same six SNPs associated with Rs resistance. The single marker ANOVA (SMANOVA) using JMP software was able to show which genotype was correlated with Rs resistance. Out of the six SNPs, two SNPs (solcap_snp_c2_35970 and PotVar0015326) may be in candidate genes. We interrogated Chromosome 4 from the Spud DB Genome browser to investigate if there was an annotated gene mapped disease related gene candidate. The occurence the WRKY transcription factor (Supplementary Figure. 4.14) could have been contributing to the Rs disease resistance in this study.

4.5 DISCUSSION

In this study, MSEE912-08 selfed progeny had high levels of Rs resistance from *S. commersonii*. The results from this study suggests that *S. commersonii* can be useful as source of bacterial wilt resistance for cultivated potato. Additionally, when the S_1 progenies were assayed together with the local cultivated, they expressed significantly less disease comparable to local elite cultivars in Kenya. The resistant individuals from this study will be a useful resource in advancing Rs resistance breeding. These individuals can also be tested with other Rs isolates to compare their performance and ensure they possess stable resistance.

Bacterial wilt resistance has been previously achieved through protoplast fusion from *S. stenotomum* into cultivated potato (Fock et al., 2001). Kim-Lee et al. (2005), Laferriere et al. (1999) and Fock et al. (2000). Bacterial wilt resistance was discovered through somatic hybridization of *S. commersonii* and *S. tuberosum*. Additionally, *S. phureja* has been reported to offer resistance to bacterial wilt (Fock et al., 2000). Other bacterial wilt resistance sources identified in wild and cultivated tuber bearing *Solanum* species include, *S. acaule, S. bulbocastanum* and *S. clarum* (Machida-Hirano, 2015; Ferreira et al., 2017).

Some of the individuals analyzed had difference in performance towards the bioassay. The *S. commersonii* accession are speculated to have heterozygous alleles thereby having the resistance that may be recessive. The S_1 progeny displayed phenotypes that were resistant after selfing in relation to the parental lines (Wenzel and Uhrig, 1981). This was seen since the progenies displayed some phenotypes exceeding the parental line with literature reporting the incident happening more frequently in intraspecific than it occurs in interspecific selfing (Rieseberg et al.,

2003) . The population depicting correlation with resistance that was seen to deviating from gene to gene systems but rather showing polygenic resistance (Bonierbale et al., 1994; Leonards-Schippers et al., 1994; Caruso et al., 2008) as was reported by Thoquet et al. (1996). Additionally, there are more likely possibility is that UW551 and NAK66 had different pathogenicity on MSEE912-08, despite being the same phylotype and biovar.

Our research was able to identify SNP markers on Chromosome 4 associated with Rs resistance. Chen et al. (2013) investigated over 109 specific SSR (single sequence repeat) markers on *S. chacoense* and identified three markers that were associated with a quantitative resistance to bacterial wilt (Kliebenstein and Rowe, 2009; Poland et al., 2009). They discovered plants that possessed a quantitative resistance mediated by multiple genes. Additionally, quantitative resistance was characterized by a partial and durable resistance which is pathogen species-non-specific but species-specific (Wisser et al., 2005; Kliebenstein and Rowe, 2009; Poland et al., 2009; Kou and Wang, 2010). Findings examining the tomato cultivar Hawaii7996, known to possess resistance, revealed QTLs on Chromosomes 3, 6, 8 and 10. Other work on resistant cultivar L285 revealed QTLs in Chromosomes 6, 7 and 10 showing that bacterial wilt resistance in tomato is under polygenic control (Thoquet et al., 1996; Denny, 2007; Ishihara et al., 2012). In this study, we report the use of an S_1 mapping population of *S. commersonii* to identify SNP markers associated with Rs resistance. These markers were all detected on Chromosome 4 and explained from 9.8% to 13.0 % of the phenotypic variance observed. Habe et al. (2019) discovered five QTL for resistance in diploid hybrid potato on Chromosomes 1, 3, 7, 10 and 11 (qBWR-1, qBWR-2, qBWR-3, qBWR-4, qBWR-5) which explained 9.3 – 18.4 % of the phenotypic variance that they observed. Other research groups identified bacterial wilt resistance QTL on Chromosomes 2 and 9 (Chen et al., 2013; Habe et al., 2019). This could be due to the differences in the strains of pathogen used in the various experiments, as well as the species from which the resistance was previously identified. There is need to validate the resistance with the already identified clones with other strains.

Carputo et al. (2009) studied Rs resistance. They were able to estimate the degree of dominance and reported that the phenotypic Rs resistance was mostly derived from *S. commersonii* rather than susceptible *S. tuberosum* group that they tested. Our study demonstrates the possibility to use an S_1 population to identify bacterial wilt resistance lines which can potentially be utilized in marker

assisted selection. Because our marker density in this study was low we did not carry out QTL mapping since this would not be appropriate as it can lead to over estimation of the QTL effects (Dekkers and Hospital, 2002; Holland, 2007; Bernardo, 2008; Desta and Ortiz, 2014; Iqbal and Rahman, 2017).

Marker assisted selection is seen as a tool that will accelerate genetic gain in a breeding cycle due to reduction in time and costs associated with phenotyping (Desta and Ortiz, 2014). Currently, to increase the mean performance of any population, cheap and abundant molecular markers have been utilized in genomic selection. Rather than identifying sets of markers associated with the trait of interest, genomic selection exploits all markers without first identifying subset of markers with significant effects (Bernardo and Yu, 2007; Bernardo, 2008). Advances in molecular breeding have enabled an increase in favorable gene action, increased knowledge base and ability to characterize most crop germplasm hence accelerate genetic gain which is every breeders goal (Moose and Mumm, 2008; Liu et al., 2017; Varshney et al., 2018). From a quantitative genetics perspective, the hypothetical concept of heritability quantifies the proportion of the phenotypic variation that is controlled by the genotype (Moose and Mumm, 2008). The gene action allows for breeding value to be characterized by progeny testing where genomic segments can be identified when they exhibit statistically significant association with quantitative traits (Moose and Mumm, 2008). Marker assisted selection has enabled increased knowledge gaps on the ability to characterize genetic diversity available in any genetic pool which can be mined effectively to maximize genetic gain by expanding the speed that generations can be completed to achieve breeding goals (Moose and Mumm, 2008).

Plant breeders have previously employed single marker analysis for detecting association between molecular markers and traits of interest (David et al., 2019). To identify molecular markers associated with Rs resistance, we employed two different single marker analyses. The significant genomic regions for Rs resistance were identified based on P value <0.001 and the SNP marker solcap_snp_c2_35970 was found to have been functionally annotated protein known as senescence associated protein SPA15. These genes reported to be involved in defense proteins and protein degradation hence are correlated gene induced during leaf senescence in plants (Yap et al., 2003; Sarwat et al., 2013). The marker is located on Chromosome 4.

This gene model is annotated to code for protein which is reported to be associated with the family of proteins which plays a key role in defense protein signaling that leads to senescence of leaves (Sarwat et al., 2013). Additionally, the SPA15 gene model possesses a putative domain related to C-terminal region of potato leaf protein remorin which is postulated to be involved in signal transduction events responsible for plant defense response due to SP15 immunolocalization on the plant cell wall (Yap et al., 2003). An overlap in plant defense response and leaf senescence has been reported by Yap et al. (2003) in *Arabidopsis thaliana.*

The other SNP marker, solcap_snp_c1_10181 is associated with a BEL1- like protein which plays a role in meristem and floral development and enhancing tuberization and root growth in potato as reported by Sharma et al. (2014) and Campbell et al. (2008). This protein was not reported for any disease related genes. The biological explanation for the observation should be treated as speculative, as they might arise due to common resistance evolution response mechanisms as reported by (Mangin et al., 1999).

There is a need to look for other genes on the Chromosome 4 region identified by this study not only the genes that have SNPs. Focusing on Chromosome 4 from the Spud DB Genome browser to understand the gene density enabled this study to investigate if there was an annotated gene mapped disease related candidate gene. The search identified a candidate gene, e WRKY transcription factor. WRKY transcription factors are members of the complex family of genes that have been implicated in the regulation of transcriptional reprogramming associated with plant immune responses (Eulgem and Somssich, 2007; Pandey and Somssich, 2009). WRKY factors have been additionally fundamental in activation of defense programs (Rushton et al., 2010).

This study, by a fortunate stroke of serendipity, led to the discovery that *S. commersonii* possesses self-compatibility. *S. commersonii* can be used as additional source of self-compatibility which is infrequent among the diploid species. The *S. commersonii* clones in this study were selfed to the first generation. Self-compatibility was identified by Jansky et al. (2014) who identified self-compatibility in *S. chacoense.* This germplasm was repeatedly self-pollinated to the seventh generation. The line is homozygous for the dominant self-incompatibility inhibitor gene *Sli* hence, has been exploited for diploid breeding. It would be worthwhile, to characterize this *S. commersonii* as a new source of self-compatibility. Utilization of *S. commersonii* will be of great value since it will also increase the genetic pool for other traits of interest that have been previously reported. These traits include, resistance to bacterial and verticillium wilt, potato virus X, root knot

nematode, tobacco etch virus, soft rot and blackleg, common scab, and late blight (Bamberg and Martin, 1993; Micheletto et al., 2000; Aversano et al., 2015).

As the exploration of diploid potato breeding continues, our research is contributing toward the development of a Rs resistant diploid crop. The reinvention of potato crop at a diploid breeding level will enhance improvement towards understanding the genetics of both yield and quality traits (Jansky et al., 2016). Tetraploid potato breeding has been hindered in large part by low recombination rates. Additionally, some of the factors that have led to less remarkable gains in the tetraploid level have been due to inbreeding depression, lack of adequate disease resistance, polyploidy and long generation cycles (Jansky et al., 2014; Jansky et al., 2016). Additionally, some unfavorable alleles remain hidden in the tetraploid potato genome and can only be spotted in proceeding breeding cycles adding to the reduction in potato genetic gain as was reported by Lindhout et al. (2011). Therefore, utilization of diploid breeding is seen as reduction in ploidy that will result in the development of inbred lines that can exploit heterosis (Jansky et al., 2016). To achieve these inbred lines, self-compatibility is believed to be the driving force that will propel the process forward. The approach of selfing allows the production of inbreds which will create a series of homozygous sets of additive loci, as well as fixing resistance alleles. When recombination events occur will be fundamental in genetic gain upon combinations of genes and hence exploits inbreeding (Fasoula and Fasoula, 2002; Jansky et al., 2016). Self-compatibility, coupled with allelic variation and the *Sli* gene is among the fundamental elements in inbreeding tolerance and consequently hasten production of F_1 hybrid seed potato breeding (Lindhout et al., 2011). Self-pollination normally allows for binding together including favorable interacting alleles at different loci located on different chromosomes (Allard, 1999; Jansky et al., 2016). Therefore, this could have been one of the reasons why the S_1 population had higher bacterial wilt resistance due to having advantage of being organized into "multiple favorable interacting and stable epistatic systems" (Jansky et al., 2016).

The wild species *S. commersonii* has been previously reported to have bacterial wilt resistance when compared to other potato cultivars (Laferriere et al., 1999; Kim-Lee et al., 2005; Carputo et al., 2009). Generation of homozygous inbred lines will allow production of recombinant inbred lines, introgression libraries, or near isogenic lines which can be considered functional genomic tools useful in forward genetic studies. These will enable isolation, identification and mapping of

genes responsible for Rs resistance and therefore, advance potato genetic improvement (Lindhout et al., 2011; Jansky et al., 2014).

There is a need to design a breeding strategy to combine resistance from wild species which can be achieved through the already established breeding schemes. To obtain homozygosity, repeated selfing of the population will generate homozygous lines (Meijer et al., 2018). Marker assisted recurrent selection exists as one of the markers based recurrent selection criterion for studying complex traits (Meuwissen et al., 2001; Lorenzana and Bernardo, 2009). The abundance of molecular markers have enabled prediction of genotypic values from molecular markers hence increasing genetic gain and precision to breeding (Lorenzana and Bernardo, 2009). Jansky et al. (2016) describes a diploid potato breeding system as having genetic diversity for favorable alleles highly maintained while diversity for deleterious alleles or allelic combinations is greatly diminished via an inbred line hybrid being approached.

Additionally, results from this study will provide potential germplasm for future research opportunities which can be implemented using the different breeding strategies namely; recurrent selection which is used extensively in the improvement of gene frequencies coupled with combining ability in cross pollinated crops (Fasoula and Fasoula, 2002). Recurrent selection operates on the principle of changing gene frequencies in a breeding population using a cyclic method of population improvement where the best individuals are chosen as parents for the next generation, in assuring that this generation will exceed the parental generation (Jenkins, 1940; Hull, 1945). The strategy will generate populations in which inbred lines systematically assemble desirable combinations of genes and exploits heterosis (Fasoula and Fasoula, 2002; Jansky et al., 2016). Therefore, this strategy when employed will increase the frequency of Rs resistant alleles in the breeding population. The *S. commersonii* resistant lines will be inbred and then crossed to other diploid inbred line as part of an F_1 hybrid system. It's worth noting that selection in breeding population has by and large concentrated on additive effects, while the non-additive effect has also contributed greatly to the performance of most hybrid crops. When genes are known, the genotype building strategy can be employed to merge favorable alleles at all loci to create new genotypes (Dekkers and Hospital, 2002). This is considered as a new breeding germplasm specifically when there is upgrading of existing parent line by employing repeated backcrossing that aims at recovering the genotype of the recurrent parent with the addition of a gene(s) controlling a

desirable trait provided by the donor parent carrying the target gene also known as foreground selection (Lindhout et al., 2011). Further work is required to map the loci for Rs resistance more precisely. This will help define and clarify the biological nature of Rs resistance. Finally, the study of *S. commersonii* will enable researchers to dissect the genetic variation and accelerate progress in diploid breeding with special emphasis in understanding and revealing alleles that contribute Rs resistance in the tropical and sub-tropical regions of the world where Rs is a menace to successful potato production.

APPENDICES

APPENDIX A

Segregating population mean disease score results

Table 4.6. S_1 segregating population mean disease score in comparison to the other clones

Clone	BLUP	Mean Disease score	Prob>|t|
MSEE912-08:10	-3.23181	1.56	0.5283
MSEE912-08:26	4.068426	1.96	0.4273
MSEE912-08:48	2.044686	1.84	0.6899
MSEE912-08:63	-19.251	0.64	0.0002
MSEE912-08:76	-8.49996	1.26	0.0975
MSEE912-08:89	-0.19534	1.72	0.9696
MSEE912-08:97	3.960225	1.95	0.4397
MSEE912-08:108	0.594448	1.95	0.3847
MSEE912-08:4	8.07493	2.19	0.1154
MSEE912-08:29	-16.2571	0.82	0.0016
MSEE912-08:2	-10.6704	1.23	0.0376
MSEE912-08:11	-6.61619	1.36	0.1969
MSEE912-08:27	-8.31616	1.27	0.105
MSEE912-08:41	-3.13925	1.56	0.5402
MSEE912-08:50	3.372104	1.92	0.5106
MSEE912-08:90	-7.1532	1.32	0.163
MSEE912-08:99	16.82568	2.67	0.0011
MSEE912-08:109	-2.56829	1.60	0.6163
MSEE912-08:13	1.184519	1.79	0.8172
MSEE912-08:30	8.11859	2.18	0.1135
MSEE912-08:3	1.914186	1.85	0.7087
MSEE912-08:15	-7.36394	132	0.151
MSEE912-08:28	11.29152	2.36	0.0278
MSEE912-08:42	4.576012	1.99	0.372
MSEE912-08:51	-0.61169	1.71	0.905
MSEE912-08:68	-10.5264	1.14	0.0403
MSEE912-08:78	-9.19352	1.63	0.1226
MSEE912-08:91	3.73061	1.94	0.4667
MSEE912-08:100	10.8718	2.34	0.0342
MSEE912-08:117	3.329162	1.92	0.516
MSEE912-08:17	-6.20533	1.29	0.2262
MSEE912-08:31	5.27453	2.02	0.3035
MSEE912-08:43	-2.51238	1.60	0.624
MSEE912-08:53	3.062984	1.90	0.5501
MSEE912-08:69	-7.56081	1.31	0.1404
MSEE912-08:79	-4.08174	1.51	0.4258
MSEE912-08:92	0.104746	1.74	0.9837

Table 4.6 (cont'd)

MSEE912-08:101	**11.34299**	**2.37**	**0.0271**
MSEE912-08:16	-5.91353	1.41	0.2487
MSEE912-08:19	-18.8676	0.68	0.0002
MSEE912-08:44	7.192239	2.13	0.1608
MSEE912-08:54	6.528337	2.09	0.2029
MSEE912-08:70	4.324349	1.98	0.3989
MSEE912-08:81	-0.16793	1.73	0.9739
MSEE912-08:93	-4.52401	1.48	0.3775
MSEE912-08:102	5.219116	2.02	0.3086
MSEE912-08:18	-3.77452	1.53	0.4615
MSEE912-08:35	9.93494	2.29	0.0529
MSEE912-08:45	1.894942	1.84	0.7115
MSEE912-08:59	-2.47386	1.59	0.6293
MSEE912-08:71	-3.5024	1.54	0.4944
MSEE912-08:83	3.163572	1.90	0.537
MSEE912-08:94	-11.4525	1.09	0.0257
MSEE912-08:21	4.935922	2.01	0.3356
MSEE912-08:38	7.443422	2.14	0.1467
MSEE912-08:22	-10.7964	1.14	0.0354
MSEE912-08:37	-9.15063	1.21	0.0745
MSEE912-08:46	7.535456	2.16	0.1417
MSEE912-08:61	-2.11146	1.62	0.6803
MSEE912-08:74	-3.51433	1.57	0.596
MSEE912-08:95	10.72412	2.32	0.0367
MSEE912-08:104	10.68939	2.32	0.0373
MSEE912-08:98	11.70696	2.38	0.0226
MSEE912-08:23	-7.34691	1.33	0.152
MSEE912-08:9	-12.434	1.02	0.0155
MSEE912-08:39	0.756163	1.79	0.8827
MSEE912-08:47	9.524875	2.26	0.0634
MSEE912-08:62	-5.64375	1.42	0.271
MSEE912-08:75	0.170911	1.74	0.9734
MSEE912-08:87	0.31643	1.76	0.9508
MSEE912-08:96	2.832059	1.89	0.5805
MSEE912-08:107	3.429826	1.92	0.5034
MSEE912-08:25	12.35339	2.42	0.0162
MSEE912-08:52	11.56778	2.38	0.0243
MSEE912-08:1	-8.42865	1.27	0.1004
MSEE912-08:55	14.84332	2.38	0.0039
MSEE912-08:56	-11.837	1.06	0.0212
MSEE912-08:57	11.68066	2.38	0.0229
MSEE912-08:58	7.520026	2.15	0.1426
MSEE912-08:64	4.692975	2.00	0.3599

Table 4.6 (cont'd)

MSEE912-08:66	3.810822	1.94	0.4572
MSEE912-08:73	5.954829	2.07	0.2454
MSEE912-08:80	8.733816	2.22	0.0887
MSEE912-08:86	-8.00197	1.29	0.1187
MSEE912-08:88	-17.4532	0.75	0.0007
MSEE912-08:12	-4.53084	1.44	0.3767
MSEE912-08:105	-4.45578	1.47	0.3847
MSEE912-08:113	12.03976	2.40	0.019
MSEE912-08:114	0.109331	1.78	0.9858
MSEE912-08:60	-4.34871	1.48	0.3962
MSEE912-08:72	1.782639	1.83	0.7279
MSEE912-08:110	8.393439	2.20	0.1018
MSEE912-08:118	-8.21457	1.26	0.2576
MSEE912-08:84	4.643831	2.00	0.365
MSEE912-08:111	1.859769	1.84	0.7167
MSEE912-08:119	4.866428	1.94	0.3668
MSEE912-08:112	8.591938	2.21	0.094
MSEE912-08:82	0.529272	1.76	0.9177
Shangi	7.476948	2.16	0.1449
Tigoni	16.65036	2.66	0.0012
Unica	7.235292	2.14	0.1583
CEC03-03	10.5228	2.32	0.0403
MSEE912-08(S_1)	14.90506	2.56	0.0037

APPENDIX B

The Principal component analysis results

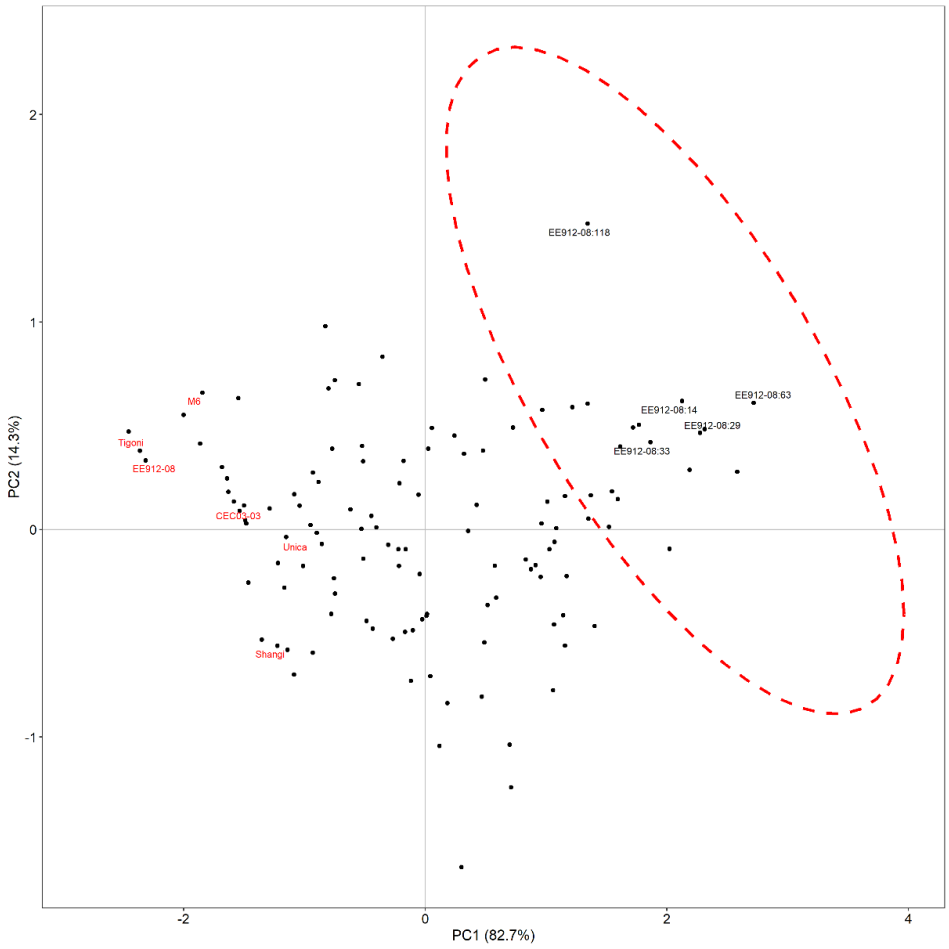


Figure 4.9. The Principal component analysis at days 7, 14, 21 and 28 on wilting degree post inoculation of the MSEE912-08 and S1 parent and parent *S. commersonii* (CEC03-03) plus local checks. Showing an ellipsis considering 95% of confidence (dashed).

Using PCA, there are some materials within the ellipsis that exhibited mean resistances lower than two. The tolerant materials were those with scores smaller than two by day 28. Checks cultivars were also included, and they are in red (Figure 4.9). The study captured 97 % of variance by using the PC1 82.7% and PC2 14.3%. therefore, allowing for clustering and separation of tolerant and susceptible clones.

APPENDIX C

Dendrogram clustering of the 98 genotypes

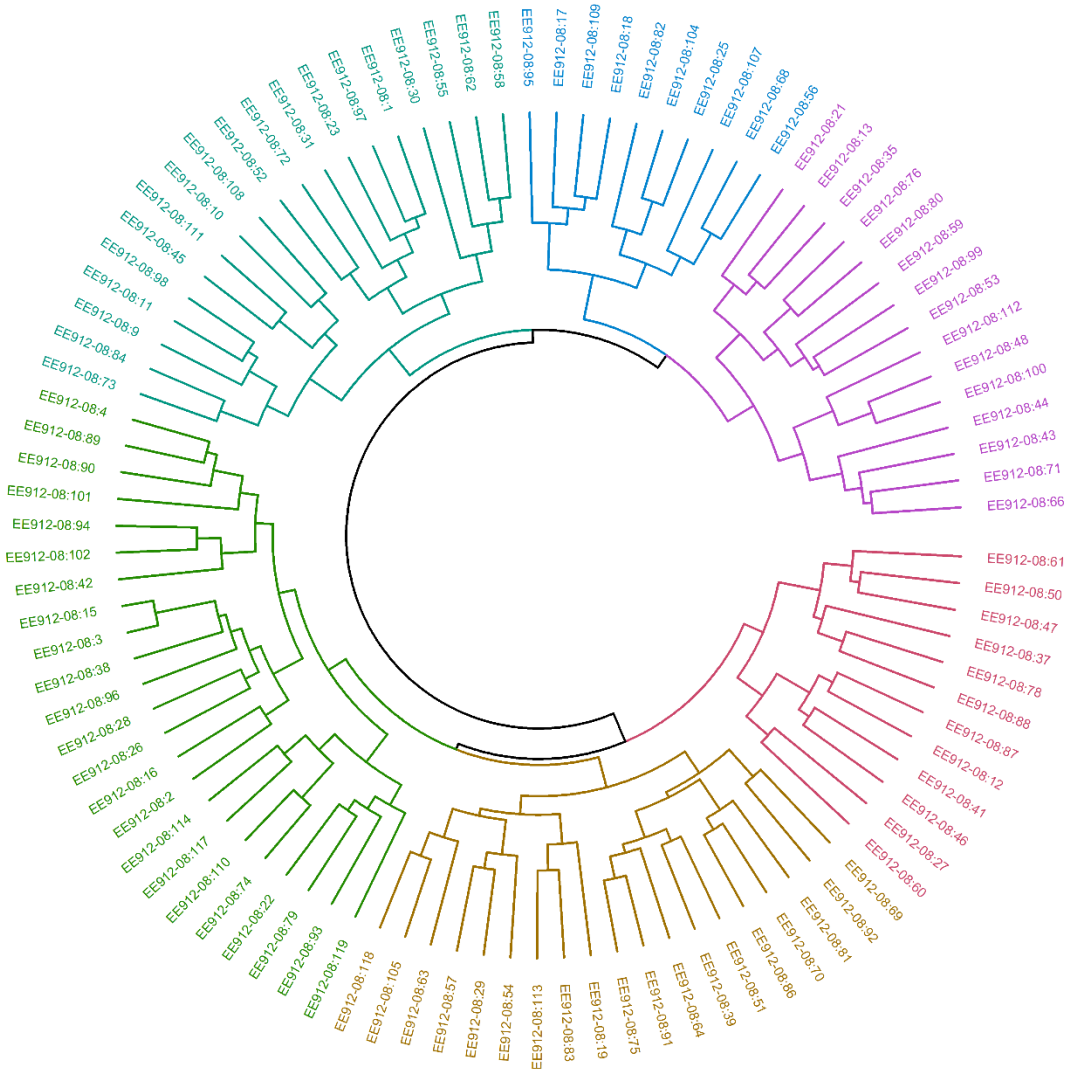


Figure 4.10. The circular dendrogram of the 98 genotypes. There similarity was compare using Jaccard similarity coefficient. It shows clustering into six main groups.

APPENDIX D

Single marker Analysis results

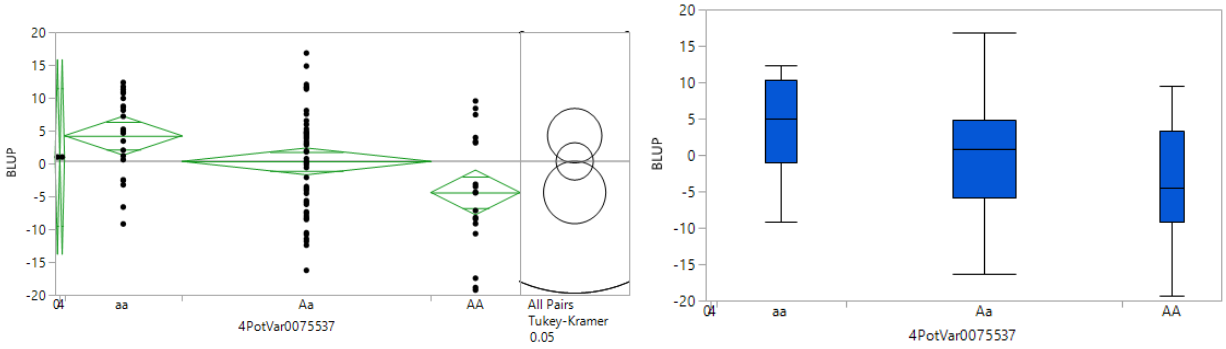
Table 4.7. Table showing all the significant marker in single marker analysis for chromosome1, 4 and 12.

Chrom.	SNP marker	b0	b1	-2ln(L0/L1)	F(1,n-2)	pr(F)	% R^2
1	solcap_snp_c1_2466	0.561	-2.186	4.159	4.161	0.044105	4.14*
4	PotVar0075537	0.075	-4.298	13.912	14.642	0.000231	13***
4	PotVar0017413	0.274	3.05	7.761	7.911	0.005958	6.8**
4	solcap_snp_c2_10616	0.276	3.049	7.775	7.926	0.005912	7.6**
4	PotVar0017188	0.308	-2.975	7.241	7.362	0.007898	7.3**
4	PotVar0017260	0.246	3.015	7.429	7.561	0.007129	7.3**
4	PotVar0015935	0.338	3.367	9.574	9.852	0.002254	9.3**
4	PotVar0015665	0.338	-3.291	9.127	9.371	0.00286	8.8**
4	PotVar0015588	0.338	3.291	9.127	9.371	0.00286	8.8**
4	solcap_snp_c2_35970	0.338	-3.291	11.317	9.371	0.00286	9.8**
4	solcap_snp_c1_10181	0.338	4.075	13.133	13.767	0.000347	12***
4	PotVar0015326	0.212	-4.119	13.725	14.432	0.000255	13***
4	solcap_snp_c1_4109	0.255	-4.053	13.577	14.266	0.000275	12***
4	solcap_snp_c2_12904	0.254	-4.106	13.957	14.694	0.000226	13***
12	PotVar0053629	0.251	-2.398	4.165	4.168	0.043947	4.46*

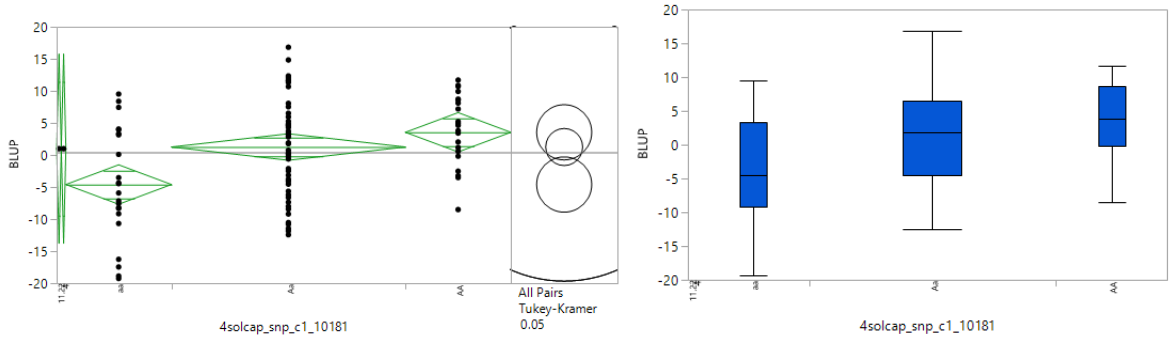
*Significant at 0.05 probability level, **Significant at 0.01 probability level, *** Significant at 0.001 Probability level; Chrom; chromosome. H0: b1= 0 to an alternative H1: b1 not 0. -2ln(L0/L1); denotes two times the negative the natural log of likelihood Ratio test statistics with likelihoods L0 and L1 respectively is the number of traits being analyzed.

APPENDIX E

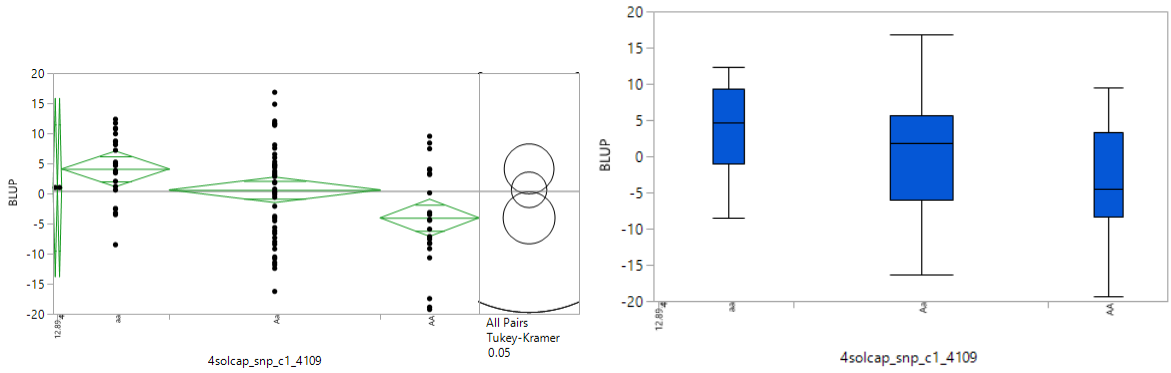
Single marker ANOVA results



(a) SNP marker 4Potvar0075537



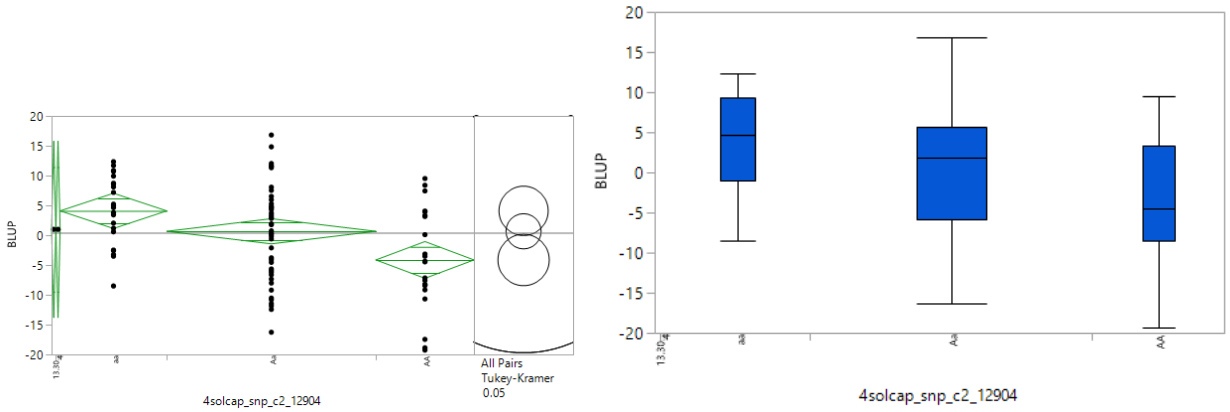
(b) SNP marker 4solcap_snp_c1_10181



(c) SNP marker 4solcap_snp_c1_4109

Figure 4.11. Showing single marker analysis and occurrence of the genotypes within the different SNPs markers a, b, and c as shown above.

Figure 4.11 (cont'd)



(d) SNP marker 4solcap_snp_c2_12904

Most of the SNP markers showed genotype AA to be significantly responsible for the Rs resistance as is shown in the Figure 4.11 (a, b, c and d).

APPENDIX F

Cluster normalizing for the different SNPs

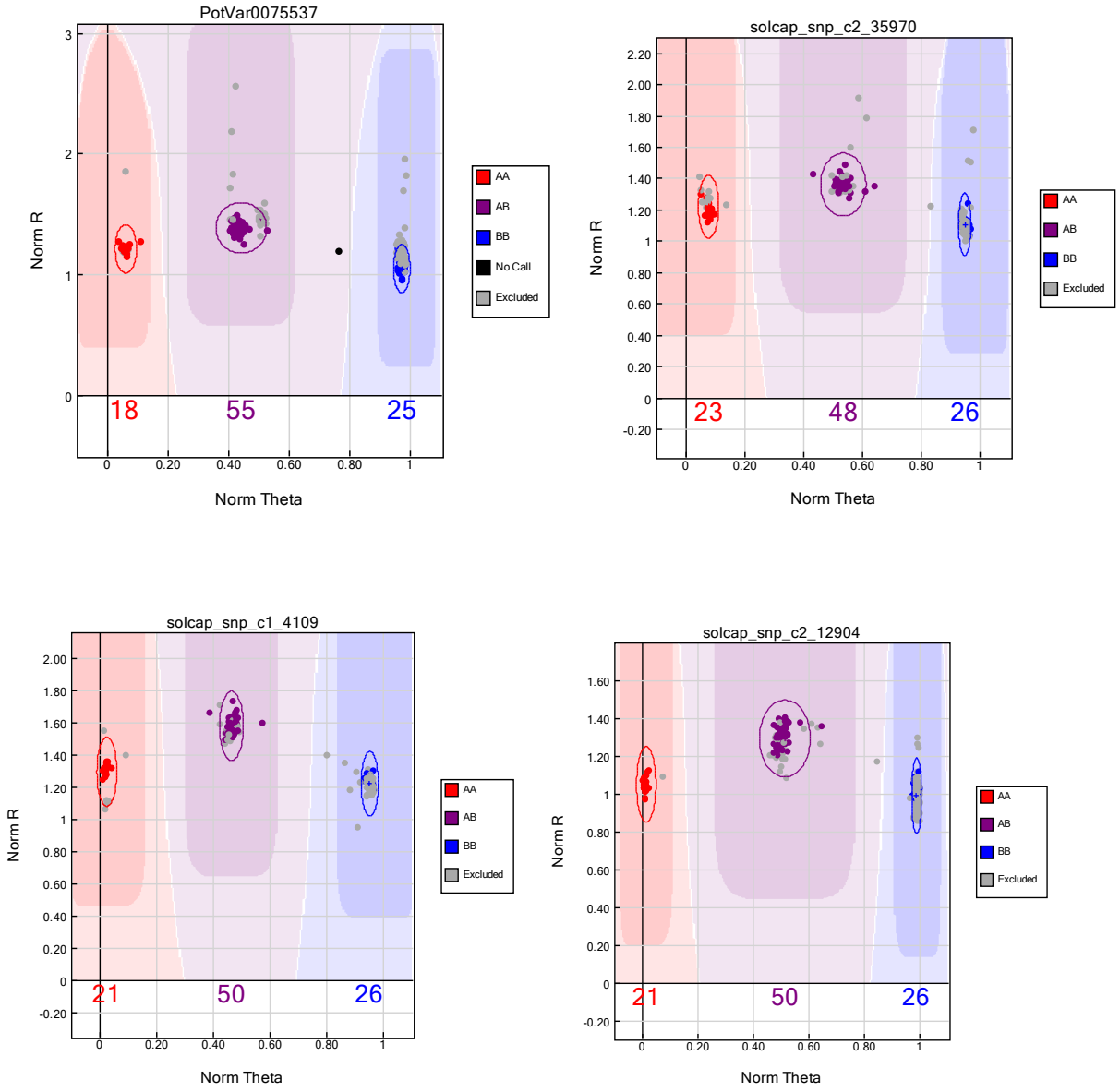


Figure 4.12. Cluster normalizing for the four different significant SNPs markers on genome studio showing expected segregation ratio for the call genotypes.

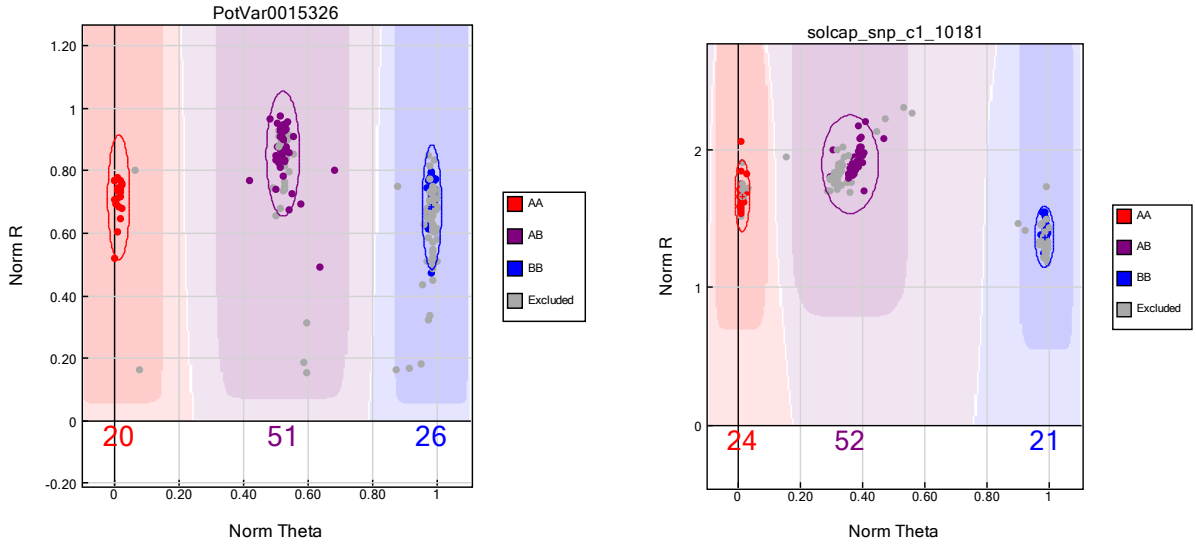


Figure 4.13. Cluster normalizing for the two different significant SNPs markers on genome studio showing expected segregation ratio for the call genotypes.

APPENDIX G

Genome Browser expression results

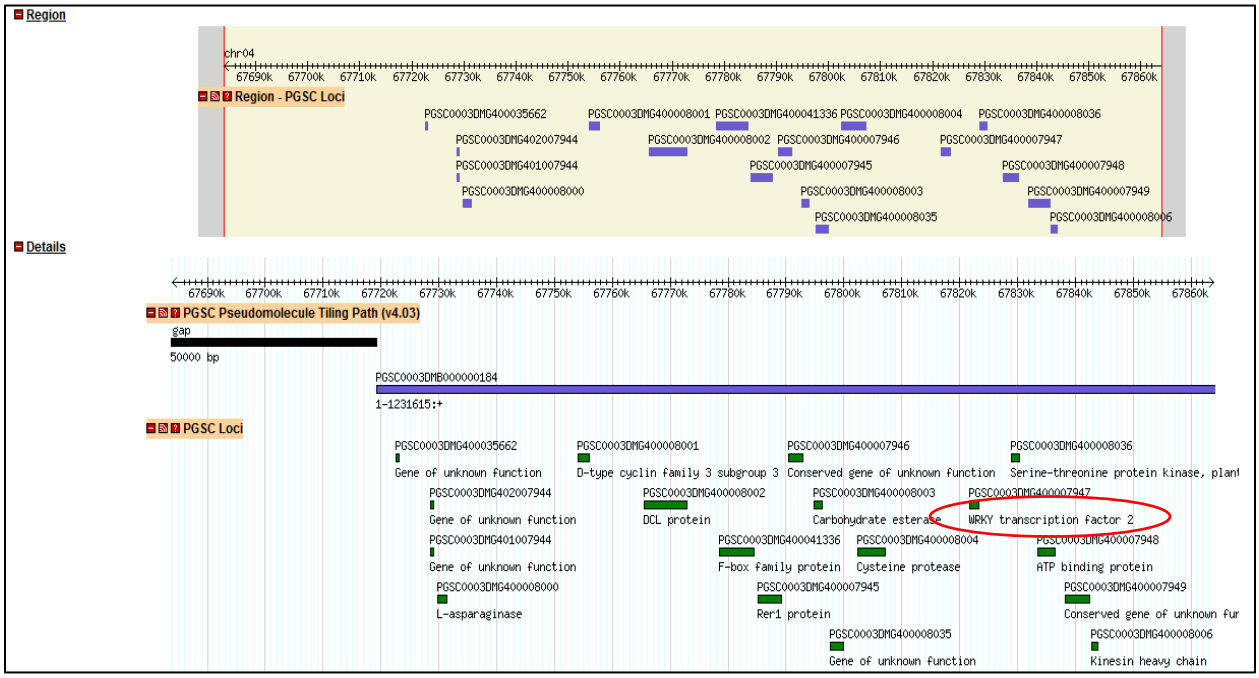


Figure 4.14. Visualization of gene prediction and protein expression showing some known genes from Genome Browser.

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LITERATURE CITED

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CHAPTER 5. GENERAL CONCLUSIONS

5.1 *2N* GAMETES IMPLICATION IN DIPLOID BREEDING

There are over 200 species of wild diploid potatoes (Watanabe, 2015). Hybridization enables the wild potatoes to introgress genetic diversity into the cultivated potatoes (Mok and Peloquin, 1975; Ehlenfeldt and Hanneman, 1984; Peloquin et al., 1989; Watanabe, 2015). *2n* gametes plays a fundamental role in potato genetic resource for both cultivated and wild species. Additionally, *2n* gamete have been utilized for ploidy manipulation in breeding for majority of higher plants (Dewitte et al., 2012; Watanabe, 2015). Dewitte et al. (2012) reports on the sources of *2n* gametes starting with interspecific/intergeneric hybrids which is in lily, wheat, roses, and clover.

2n gamete played a major role in creating new cultivars at higher ploidy levels. Therefore, creating bridges in which highly desirable genes from wild diploid species can be integrated into cultivated polyploid genetic pool (Veilleux, 1985; Watanabe and Peloquin, 1991; Watanabe and Peloquin, 1993; Carputo et al., 2000; Carputo et al., 2003; Dewitte et al., 2012). *2n* gamete additionally allowed for introduction of novel genes flow from diploid to hexaploid species. This has led to improvement of traits of interest, allelic diversity and increased heterozygosity (Mok and Peloquin, 1975; Carputo and Barone, 2005).

Meiotic disturbance or restitution are capable of producing *2n* gametes with considerable positive breeding value (Veilleux et al., 1982; Veilleux et al., 1985; Watanabe et al., 1992; Watanabe, 2015). Meiotic aberrations frequently showing abnormal to absent chromosome pairing are characterized by univalent, lagging chromosome or absence of chromosome and chromosome bridges leading to *2n* gametes (Dewitte et al., 2012). Other causes of *2n* gametes is meiotic mutations that is capable of disturbing spindle formation or cytokinesis, hence resulting in *2n* gamete (Meredith et al., 1995; Erazzú and Camadro, 2007).

Some of the features of interspecific hybrids enables both *2n* egg and *2n* pollen to be produced simultaneously. This is also known as bilateral sexual polyploidization (Carputo et al., 2000).The formation of *2n* gametes is therefore important in allowing for sexual polyploidization. *2n* gametes can be utilized effectively in unilateral sexual polyploidization breeding schemes since it involves 4x - 2x crosses or 2x - 4x crosses. The unilateral sexual polyploidization is effectively functional through the utilization of *2n* pollen or *2n* egg (Carputo et al., 2000). Alternatively, bilateral sexual

polyploidization occurrences (BSP) involves 2x - 2x crosses. BSP requires the functional of both *2n* pollen and *2n* eggs formation. Therefore, during diploid breeding, tetraploid occurrence is due to BSP (Carputo et al., 2000). BSP occurs in hybrids especially if there are superior *2n* egg and *2n* pollen producing hybrids during sexual polyploidization (Carputo et al., 2000; Dewitte et al., 2012). The proper detection of *2n* pollen has been done through studying pollen size (Veilleux et al., 1985).

Diploid breeding will enable alleviation of some of the bottlenecks that occurs in conventional tetraploid breeding schemes. Due to the spontaneous occurrence of tetraploids in the diploid breeding, there is a need to employ quality controls. By routinely checking the hybrid progeny ploidy to ensure breeding scheme is in tandem with the intended goals of every breeding program. Constant chloroplast counts can be employed as an efficient tool to ensure that ploidy is maintained at the diploid level in the breeding program.

5.2 CONTRIBUTIONS OF WILD CULTIVARS IN RS RESISTANCE BREEDING

Potato has numerous resistance R genes in which different diseases and pests have been studied and mapped in several chromosomal spots. High levels of Rs resistance was identified in *Solanum phureja* except that the resistance was unstable due to environmental difference. Temperature was reported to be responsible for disease resistance breakdown (Sequeira and Rowe, 1969). *S. tuberosum* and *S. commersonii* hybrid pentaploid and *S. stenotomum* (with somatic hybrid) have also been identified to have resistance to Rs (Fock et al., 2000; Fock et al., 2001; Kim-Lee et al., 2005; Caruso et al., 2008; Zuluaga et al., 2015; Ferreira et al., 2017). Durable resistance of potato towards Rs has been difficult to achieve (Zuluaga et al., 2015). Some reports have shown Rs resistance loci or genes for quantitative trait loci in tomato and tobacco (Nishi et al., 2003; Qian et al., 2013) and for eggplant (Lebeau et al., 2013).

The use of genetic resistance seems to be the best feasible strategy available towards Rs control. Most of the partial resistance were identified to be largely under polygenic control (Cook et al., 1989; Gillings and Fahy, 1993; Carmeille et al., 2006). QTL controlling resistance to race 1 Rs strain that was largely found in tomatoes (Carmeille et al., 2006). Notably, integrated strategies employed in Rs control are complex due to the fact that bacteria are capable of infecting most crops as soilborne, water and tuber borne organism. Therefore, its recommended to avoid dissemination of pathogen through use of latently infected tubers (Huet, 2014).

Most resistance studies of bacterial wilt have been on *Arabidopsis thaliana* and *Medicago truncatula*. The genetic analysis have used recombinant inbred lines that identify QTL involved in Rs resistance and fine mapping of those QTL (Huet, 2014). Studies have determined the recessive RRS-R (Resistance to *Ralstonia solanacearum* 1) gene in Arabidopsis. The gene encode atypical R protein harboring a C- terminal WRKY DNA binding domain hence perceived as the negative regulator of plant defense (Deslandes et al., 2002; Huet, 2014). This study was able to identify candidate gene, known as the WRKY transcription factor on the Chromosome 4 locus. This therefore calls for further validation of the study.

Resistance of solanaceous crops to Rs behave differently under various environmental conditions. Different studies report that different strains exhibit extensive genetic diversity worldwide (Huet, 2014). This study showed varying Rs population performance when inoculated with the same strain and results giving a totally different phenotypic observation during bioassays in the different regions. Huet (2014) explains that identification of resistance source can only be accelerated by exploiting *R solanacearum* effectors through an effectoromics approach (Vleeshouwers and Oliver, 2014) coupled with molecular characterization of their virulence and a virulence functions. The use of resistance breeding coupled by integrated pest management can be used to avoid selection pressure that can increase resistance breakdown towards bacterial wilt (Lopez and Biosca, 2005; Huet, 2014).

5.3 SNP MARKERS ANALYSES AND IDENTIFICATION FOR CROP IMPROVEMENT

Plant genome sequencing has played a major role in mapping gene functions, gene regulations and expression. SNP marker identification are possible through high resolution SNP marker to tag and identify desired genes (Mohan et al., 1997). Several research groups have deployed map-based cloning and transposon tagging to isolate genes corresponding to desirable traits. Genetic mapping allows one to obtain a fine scale linkage map using DNA markers. Additionally, genomic DNA library of large- sized fragments are equally screened with linked markers for anticipated trait of interest (Mohan et al., 1997). To resolve complex quantitative traits occurring in a single Mendelian complex, efforts have been geared toward construction of near isogenic lines which carry one or multiple chromosomal segments of one of the parental genetic stock required.

Therefore, by using near isogenic lines enables the handling of a given QTL as a single mendelian factor (Mohan et al., 1997).

Traditional establishment of linkage maps entails a segregating plant population developed from sexual reproduction possessing parents differing in one or more traits of interest. A mapping population size for preliminary genetic studies ranges from 50 to 250 individuals (Collard et al., 2005). To achieve higher resolution mapping the population size needs to be higher. In QTL analysis the mapping population must be phenotypically evaluated by collecting trait data and latter QTL mapping (Collard et al., 2005).

There are various populations that have been previously used in mapping (Collard et al., 2005). Typically, F_2 populations and backcross (BC) populations are the simplest type of mapping population have an advantage of ease to produce the population due to short time requirement. Other mapping populations are recombinant inbred (RI) and double di-haploids which generate homozygous lines which can be multiplied and sexually reproduced (Collard et al., 2005).

Identification of polymorphism is fundamental in the construction of a linkage map through identifying DNA markers that reveal difference between the progenies. This requires enough amounts of polymorphism between the parents to enable linkage map construction. Therefore, there is need to have parents that are distantly related or having adequate polymorphism to provide genetic diversity and ultimately screened across the entire population referred genotyping (Collard et al., 2005).

This study was to identify significant SNP markers for Rs resistance that can be used in potato breeding. This study was able to identify six significant SNP markers in chromosome 4. Another study that identified QTL in potato responsible for Rs was carried out by Habe et al. (2019) where the group found five QTL in Chromosomes 1, 3, 4, 7, 10 and 11, in hybrid between two diploid potato crosses. These QTL explained 9.3 - 18.4% of the phenotypic variance. This study showed that the SNP markers in Chromosome 4 explained 9.8 - 13.0% of the phenotypic variance. This therefore shows that molecular markers will assist in identifying molecular markers governing partial resistance. Through symptoms development surveillance and proper Rs resistance will be achieved (Mohan et al., 1997; Carmeille et al., 2006; Hackett et al., 2013).

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