Chapter 1

The Use and Impact of Biotechnology in Potato Breeding: Experience of the Potato Breeding Program at INIA, Chile

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http://dx.doi.org/10.5772/intechopen.72961

Abstract

The potato breeding program of Instituto de Investigaciones Agropecuarias (INIA) Chile has developed and released 11 commercial varieties of potato. It is estimated that these varieties have 50% of the Chilean potato market and are being evaluated in seven foreign countries. The aim of this work is to summarize the current importance and scope of biotechnology in breeding in Chile, by presenting a program that has generated widespread material among farmers and consumers. The germplasm bank is the source of genetic diversity for controlled crosses. Techniques to introduce the material to in vitro conditions and thermotherapy to obtain pathogen-free in vitro plants are applied. The material is characterized by SSR markers. There is a flow of material from gene bank to the annual scheme of controlled crosses and selection in the plant breeding program. In the selection plots, molecular markers associated with one or few genes that have a large and heritable effect in important traits are used: golden nematode resistance, virus resistance, and late blight resistance. Then, in the early stages of seed production, all the material of the new varieties is checked by fingerprint and molecular and ELISA test for pathogen, to assure the identity and pathogen-free status of the starting seed material.

Keywords: breeding, germplasm bank, molecular markers, varietal fingerprinting, varietal development, seed production

1. Introduction

The potato (*Solanum tuberosum* L.) is one of the three most commonly consumed crops along with wheat and rice. The annual worldwide potato production is approximately 330 million tons [1],

and the annual Chilean production is 1 million tons including 50,000 ha with 60,000 farmers. Therefore, potato has a strong economic and social importance. For this reason, Instituto de Investigaciones Agropecuarias or the Agricultural Research Institute (INIA) established a potato breeding program. In Chile, official statistics indicate that among the eight potato varieties mostly sold in wholesale markets, three correspond to varieties developed by INIA [2].

Potato varieties must fulfill the requirements of the market and consumer preferences, as well as to show good agronomic performance in several environments and wide adaptation to productive systems. Traits as high yield, tuber conformation, early maturity, and resistance to biotic and abiotic stresses are the most important goals for potato breeding in Chile.

The breeding of potatoes needs to deal with some complicated issues that make potato breeding a special case in genetic improvement of crops:

- Most of the cultivated potatoes are tetraploid and show tetrasomic inheritance.
- Tetraploidy, together with severe inbreeding depression upon repeated selfing, renders the generation of pure lines, recombinant inbred lines (RILs), or near-isogenic lines (NILs) impractical.
- Tetraploid potato genotypes are therefore highly heterozygous. The heterozygous genotypes are fixed and maintained by vegetative propagation via tubers.
- Current breeding of marketable varieties comprises the generation of genetic variation by crossing elite tetraploid parents, usually varieties and advanced breeding clones.
- Evaluation and selection of approximately 13 main characters of plant and tuber in the recombinant F1 generation via multiyear and location trials. The selection cycle from crossing to variety release requires approximately 10–12 years.
- As potato is clonally propagated, diseases are accumulated and transmitted to descendant tubers; therefore, a system for cleaning and maintaining virus-free stocks of seed is essential.

These issues make potato breeding to concentrate a large effort in developing a system of controlled crosses that generate seeds from several families producing a large F1 population that will be the source of new breeding lines with potential to become varieties. Additionally, during the process, a big expense of resources is destined to rouging and maintaining of clean seed stocks of seeds.

Under optimized agricultural practices, potato production can yield more than 40 tons per hectare within 4 months from planting to harvest. To achieve this yield, it is essential to have high-quality seeds and improved cultivars as well as good agronomic practices and pest and disease control. With low technology, average yields are much lower ranging from 5 to 20 tons per hectare.

It is expected that through applications of biotechnology such as tissue and cell culture, genetic engineering, marker-assisted technologies, genome-assisted technologies, or a combination of

all the technologies for the improvement, potato has the potential to provide an increased proportion of the food intake required for the anticipated population expansion over the coming decades. Access to these biotechnological techniques is of vital importance for developing countries. However, the highly heterozygous genotypes produce a strong segregation in the progeny from controlled crosses; therefore to obtain a precise combination of characters or the improvement of some specific traits without losing other relevant genetic controlled traits is a difficult task. Genetic engineering could be the key to reach some specific gain in a particular trait preserving good genetic background to address better development of varieties. Nevertheless, GMOs are questioned for public opinion and even forbidden in numerous countries, as the case of Chile. In this way, the role of biotechnology is an assistant for the processes of classical breeding to make them more effective and to know in a better way the plant material at the genetic level.

So how is the experience of potato breeding program in Chile by using biotechnology to assist the development of Chilean varieties, specially adapted to local environments and productive systems?

Presently, 11 varieties have been released and inscribed in official system of seed certification. With the 11 varieties, it is possible to obtain 40 tons per hectare of yield in dryland conditions and 80 tons with irrigation, a good yield for Chilean conditions.

2. Breeding schemes of potato at INIA Chile

The mission of the potato breeding program in Chile is to develop potato varieties for different uses and productive systems to meet the Chilean market demand, with international projection.

The INIA uses a breeding scheme that is similar to classical potato breeding programs [3–5] with modifications according to local requirements. The potato breeding program begins with the selection of a large number of genotypes to be used as crossing parents. In the early steps of selection, around 100 crosses are made and 30,000 genotypes are evaluated. Selection of F1 progeny at early breeding stages (i.e., the primary individual selection of seedlings and the secondary individual clonal selection) is based on characteristics with high heritability and little annual variation, such as skin color, flesh color, and tuber shape, according to Chilean consumer preferences. The elimination of progeny with severe defects that can devastate potato production (e.g., hollow heart, growth cracks, and brown spots) also occurs at these stages. Progeny that is extremely susceptible to diseases as PVY, PVX, common scab, and late blight is discarded based on visual inspections of the field, although molecular markers are also available for genotype analysis. In later stages (i.e., line selection and the performance yield test), selection is carried out based on quantitative characteristics, such as yield, maturity, cooking qualities, and aptitude for chips or French fries. Molecular markers are applied in all the advanced breeding lines in order to confirm combinations of several resistance genes for diseases. A fixation process is unnecessary as they are clonally propagated.

The main objectives of the program are:

- Good performance for different end uses (fresh market and processing) for national or international demand
- Conformation and appearance of tuber
- Good agronomic characteristics: high yield and wide adaptation to agro-climatic zones
- Industrial uses
- Specific objectives:
    - Late blight resistance
    - Golden nematode resistance
    - PVY resistance

To achieve these objectives, the activities of the program involve controlled crosses every year (around 100), with 30,000 novel genotypes that are evaluated in multiyear and locations in field conditions.

3. Participation of biotechnology to support and improve the breeding process in the INIA potato program

Figure 1 shows an organization chart about the role of biotechnology in the potato breeding program in Chile. In first place, the germplasm bank is the source of genetic diversity for controlled crosses. This in vitro gene banks hold the varieties developed, advanced breeding lines, and imported breeding material that can be used of donor of some characters and native landraces. With this system, the material is preserved free from pathogens and suitable to be transferred to foreign countries in case of any need of varieties and breeding lines.

Techniques to introduce the material to in vitro conditions and thermotherapy to obtain pathogen-free in vitro plants are applied. Thermotherapy in combination with previous chemotherapy can be employed successfully, but efficiency is variable depending on virus types to remove. Results of DAS ELISA test before and after thermotherapy of a group of potato accessions from the field strongly infected by different viruses are shown in **Table 1**, indicating that in the case of PVY, 51% of the materials could be cleaned after two rounds of thermotherapy.

The materials stored in germplasm bank are analyzed by SSR markers to characterize them by molecular fingerprint. Currently, eight SSR markers are used. These markers are employed as a routine test for varietal identification since all the varieties are released with a described molecular profile that allows tracking of the varieties in the markets after available to farmers and to solve problems as mixture of varieties. These markers have been used in an overview of the genetic diversity and genotype numbers in germplasm bank of Chilean collection,

http://dx.doi.org/10.5772/intechopen.72961

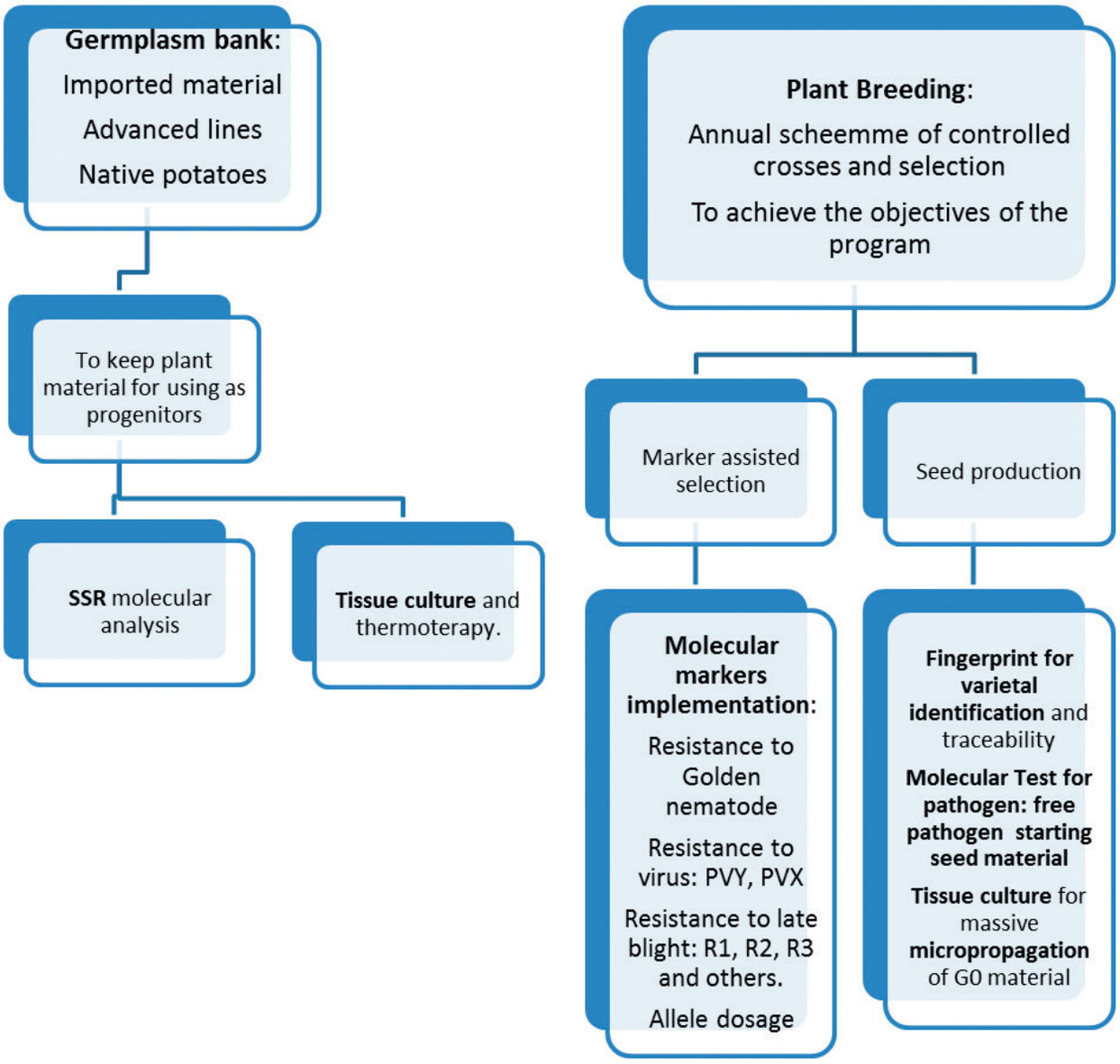

Figure 1. Organization chart about the role of biotechnology in the Chilean potato breeding program.

including native potatoes, commercial varieties, and valuable breeding lines. It is important to notice that SSR markers must be polymorphic enough to distinguish between the different varieties that it is necessary to discriminate. In the case of using molecular markers in the process of plant propagation of potato, as a tool to assure the identity of the commercial varieties that are being propagated in vitro, it is convenient to determine the set of markers that allow to produce different allele phenotypes (band patterns) for all the commercial varieties that are multiplied by the seed program, in order to differentiate them. In the case of wild material collected and kept in germplasm bank, or breeding lines with unknown pedigree, the SSR markers could discriminate different allele phenotypes, but it depends on the numbers of markers and polymorphism detected. Always it is possible that different genotypes could not be differentiated because no polymorphism in the regions of the genomes are being analyzed, but once a different band pattern is found between some plant materials, it is proven that they

Situation prior to thermotherapy treatment	Number or %	Situation after in vitro thermotherapy treatments	Number or %	Efficiency of thermotherapy (INI-FIN)/ FIN × 100
Number of accessions subjected to in vitro thermotherapy	157	Number of accessions cleaned after two rounds of thermotherapy	66	42%
Number of accessions infected with at least one virus	157	Number of accessions infected with at least one virus	91	
% incidence of PVX	26	% incidence of PVX	3.2	88%
% incidence of PVY	69	% incidence of PVY	33	51%
% incidence of PVS	78	% incidence of PVS	7	91%
% incidence of PLRV	86	% incidence of PLRV	21.7	75%
% incidence of PVA	8.9	% incidence of PVA	0.6	93%
% incidence of PVM	1.2	% incidence of PVM	0	100%
Average number of viruses present per infected accession	2.6	Average number of viruses present per infected accession	0.63	

Table 1. Efficiency of thermotherapy treatments for virus cleaning in a group of potato accessions from the field strongly infected by different viruses.

are different genotypes. This is very useful when studying collections of material with similar morphological features or when not all the descriptors are available to be examined (collections of tubers, in vitro plants or others where no flowers or leaves are available, or material affected by virus that affects the phenotype). In the case of Chilean collection of native potatoes, we have found 320 different allelic phenotypes using four SSR markers, indicating that there are at least 320 different genotypes in the collections. Of these, 158 belonging to the INIA collection were not found in another collection belonging to other Chilean institutions. As expected, different genotypes were known under the same popular name by the farmers. The molecular information is useful to know the genetic structure of the material preserved or used for breeding. For more details of our results, please see [6].

There is a flow of material from the gene bank to the annual scheme of controlled crosses and selection in the plant breeding program. Some genotypes are selected in order to combine characteristics in the progeny through controlled crosses and grown to obtain flowers and used as donor of valuable traits. On the other hand, promissory breeding lines from the field are introduced to in vitro culture and kept in the bank.

During the phases of selecting/discarding clones in field plots, molecular markers are implemented. Molecular markers associated with one or few genes that have a large and heritable effect in important traits are used (e.g., disease resistance in gene per gene model). Molecular markers for golden nematode resistance, virus resistance, late blight resistance, and some markers for flesh color are involved in the battery of markers to assist the selection and verify the combination of several resistance genes (**Table 2**). We investigate the allele dosage in some

	Gene	Marker	Resistance to	Reference
Routine markers for resistance genes implemented in PMGP-INIA	*H1*	57R	*G. rostochiensis*	[7] Finkers-Tomczak et al.
		TG689		De Jong, W. Cornell University, (unpublished); [8] Galek et al.
	Gro1–4	Gro1–4		[9] Paal et al.
	GroV1	U14		[10] Jacobs et al.
	Ry_{adg}	Ry3.3.3S/RyADG23R	PVY	[11] Kasai et al.
	Rx2	AC15	PVX	[12] Bendahmane et al.
	Rx1 y Rx2	Ask		[13] Bendahmane et al.
	R1	R1	*P. infestans*	[14] Ballvora et al.
	R2	R2		[15] Kim et al.
	R3a	R3a		[16] Huang et al.
	R3b	R3b		[17] Rietman
N°	10	11		

Table 2. Molecular markers used for routine assays to select breeding lines with resistance genes to potato diseases.

parents by means of study segregation of the marker in an F1 population in order to know the frequency of progeny that can hold the desired character and recognize most efficient parents for controlling crosses.

Once the new variety is ready to enter to the market, it is necessary to produce the stock of seed to support the entrance in the seed certification system.

Then, in the early stages of seed production in certification system, all the mother plant materials for the new varieties are checked by molecular fingerprint, PCR, and ELISA test for pathogen diagnosis, to assure the identity and pathogen-free status of the starting seed material and then micropropagated before entering to the certification system in the field. PVY and *Pectobacterium* are tested by PCR (**Table 3**), and PVX, PVM, PVS, PVA, PLRV, and PVY are diagnosed by DAS ELISA test.

In this stage, tissue culture for massive micropropagation of new varieties is still a pivotal biotechnological technique:

- Today, in the official certification system, all the varieties are propagated via in vitro culture before being multiplied in the field.
- Introduction of new varieties to in vitro condition is essential to produce certificated seed and makes possible that the varieties be distributed in the market.

Test	Marker	Gene target	Reference
Pectobacterium spp.	Y1/Y2	*Pel*	[18] Darrasse et al., modification of protocol
PVY	PVYF/PVYR	Capsid protein, strain: N	[19] Du et al., modification of protocol

Table 3. Procedures used for molecular diagnosis for ***Pectobacterium*** spp. and PVY in potato plants.

Currently our program keeps in vitro the 11 INIA varieties, 134 advanced breeding lines from INIA program, and 32 foreign varieties and breeding lines with research purposes.

Molecular fingerprints have been done to characterize 61 varieties, 25 advanced breeding lines, and 823 native landraces. We use the CIP identity kit for molecular profiling of the most valuable material for reliable identification and traceability during breeding process and seed production and in the future to track the presence in the market.

We use 11 molecular markers for marker-assisted selection, and at the date, we have analyzed 461 breeding lines and 33 varieties. The most important advantage of applying these markers is to allow more precision to choose parents for crossing in order to combine or pyramiding genes.

In **Table 4**, we can see the markers associated with resistance genes and light yellow flesh color present in the released varieties. It is possible to see that many varieties hold markers associated to golden nematode resistance, a quarantine pest in Chile but present in some

Variety	Golden nematode resistance	PVX resistance	Late blight resistance	PVY resistance	Light yellow flesh/ white flesh
Karú-INIA	*H1*; *Gro VI*				*Allele 3 BCH 2*
Patagonia-INIA	*Gro VI*				*Allele 3 BCH 2*
Pukará-INIA	*Gro VI*; *Gro 1–4*	*RX2*			*Allele 3 BCH 2*
Puyehue-INIA	*H1*; *Gro VI*; *Gro 1–4*		*R3a*		*Allele 3 BCH 2*
Yagana-INIA	*H1*; *Gro VI*	*RX2*			*Allele 3 BCH 2*
Fueguina-INIA	—		*R3a–R3b*		—
Ona-INIA	—		*R1*		*Allele 3 BCH 2*
Pehuenche-INIA	*Gro VI*; *Gro 1–4*		*R3a*		*Allele 3 BCH 2*
Purén-INIA	*Gro VI*		*R1*; *R3a*; *R3b*		—
Kuyén-INIA	—	*RX2*	—		
Rayún-INIA	*H1*; *Gro VI*		*R3b*		
R87009–28				Ry_{adg}	

Table 4. Molecular markers associated with resistance genes and light yellow flesh color present in the released varieties.

areas with potato cultivation in northern part of the country. For this pest, it is not possible to conduct field trials in south of Chile, so molecular marker implementation is crucial to track resistance in the progeny from controlled crosses with appropriate donor parents able to produce offspring with different resistance genes.

4. Conclusions about the role of biotechnology in Chilean potato breeding program

The program has implemented six biotechnological techniques; these are applied in the stages of characterization of the gene bank, selection of parents, marker-assisted selection, characterization of varieties, and propagation of material for seed production. One hundred percent of the varieties have been released involving biotechnology, especially by the use of in vitro culture techniques to produce pathogen-free material for initial stages of seed production of advanced lines. Biotechnological techniques have participated in the improvement of 2 of the 13 main characteristics associated with the program objectives. Two of the 11 varieties were characterized by molecular fingerprint at the time of their release. Biotechnological techniques such as in vitro culture, molecular fingerprint, and molecular diagnosis of diseases are used to produce primary multiplication of reproductive material for 100% of the varieties released by INIA currently present on the market.

Some important facts about the use of biotechnology in breeding and development of varieties are:

- Tissue culture is essential in the maintenance of varieties with the same genotype and initial steps of seed production system.
- Molecular fingerprint is important for varietal identification: vital in traceability of stock plants during micropropagation, and it has possibilities to be used to track the presence of varieties in the market.
- Molecular markers associated with one or few genes that have a large and heritable effect in important traits are used (e.g., disease resistance in gene per gene model).
- Molecular markers allow to have more precision to choose parents for crossing in order to combine or pyramiding genes.
- Markers for multigenic traits such as stress tolerance or cold tolerance have not been developed yet and remain as a big challenge to develop molecular genetic tools for multigenic traits.

In potato breeding, the selection of desirable phenotypes from a large breeding population will remain essential.

- Automatic, low-cost, and high-throughput phenomic technologies would be a valuable tool for massive screening of phenotypes.

- Screening methods based on next-generation sequencing technologies promise to revolutionize screening for desired genotypes, but it is necessary to solve the problem of distinguish between three different heterozygous genotypes (AAAB, AABB, and ABBB) in traits where plex number affects the character under selection.
- In order to make more precise the addressing of breeding procedures to improve specific traits (i.e., compounds with nutritional value or to eliminate undesired characters), methods as the new biotechnological techniques (NBTs) could be promising in countries where GMOs are not allowed. These new technologies as CRIPSR/Cas can be used to develop a genetic engineering with no transgenic status of the final product.

5. Biotechnology techniques to support clone selection procedures to pyramiding resistance genes to late blight

Biotechnology can be easily combined with classical breeding methods with the objective to pyramiding resistance genes (R genes) to avoid the breakdown of resistance in the case of fast evolving pathogens. We will describe below our experience in developing a strategy to pyramiding R genes for late blight resistance in breeding lines.

For this purpose, we are using the Ma*R8* and Ma*R9* genotypes as sources of resistance to late blight in the Chilean potato breeding program.

5.1. Late blight as a major threat for potato production and food security

The potato late blight caused by *Phytophthora infestans* (Mont.) de Bary is a major challenge to potato production worldwide [20]. Reliance on susceptible potato cultivars in commercial agriculture has meant that fungicides are widely used to control late blight. However, such materials have significant monetary and environmental costs to society [21–23]. To control this disease, up to 14 applications of fungicides may be needed for a crop season.

P. infestans can infect the entire plant, including the stems, leaves, and tubers. When left unchecked, it can quickly destroy a potato crop within a few days. The success of this pathogen is not only due to its elevated virulence but also to its remarkable capacity of rapidly adapt to resistant plants. Therefore, new resistant potato varieties with multiple resistance genes must be produced, as the use of varieties with genetic resistance to late blight is essential for growing low-cost, healthy, and environmentally sustainable potatoes.

However, only three of the 25 potato varieties used in Chile have some intermediate level of resistance to late blight. Notably, while *S. tuberosum* lacks significant resistance, wild potato species are rich sources of late blight resistance genes. *Solanum demissum*, a hexaploid Mexican wild *Solanum* species, is an important source of resistance to late blight. The major resistance (R) genes from *S. demissum* have late blight race specificity.

Eleven R gene differentials containing R genes introgressed into *S. tuberosum* from *S. demissum* were collected by Mastenbroek [24] and are referred to as the Mastenbroek differential set: Ma*R1*

to Ma*R11*. In Ma*R8* and Ma*R9*, at least four (*R3a*, *R3b*, *R4*, and *R8*) and seven (*R1*, *Rpi-abpt1*, *R3a*, *R3b*, *R4*, *R8*, and *R9*) R genes were present, respectively [15, 25]. This set can be used to simultaneously introduce multiple R genes. However, since this set has a low agronomic value, crosses must be made with elite breeding material to obtain breeding lines suitable for propagation.

Significantly, the resistance provided by the R genes is background dependent as genes that suppress R genes can also be segregated into F1 offspring plants [26]. Thus, more knowledge is needed about how R genes perform in different genetic backgrounds. Research has also focused on creating GM organisms carrying constructions with the described R genes [27]. However, this class of plant material is not allowed in many parts of the world for human or animal consumption including Europe and Chile. Furthermore, society is suspicious about its sustainable utilization because of the health, environmental, and social implications of GMOs. For this reason, it is necessary to investigate the applications of the natural stacking of several R genes to overcome *P. infestans.*

The objectives of our work are:

a. To combine multiple R genes that confer resistance to late blight in new lines of the potato breeding program of INIA Remehue, Chile

b. To evaluate the level of resistance to *P. infestans* in genotypes carrying different R genes inherited from hybridization between Ma*R8* and Ma*R9* with elite breeding material

5.2. Materials and methods

Controlled crosses were performed between five commercial varieties and the genotypes Ma*R8* and Ma*R9* that hold four and seven genes of resistance, respectively.

Ten progenies from each cross were randomly selected for molecular analysis and phenotypic evaluations in order to assay for the presence of R genes. The performance of these genotypes was monitored under pathogenic pressure in field conditions.

During the 2013–2014 season, 90 randomly selected progenies were evaluated for pathogen resistance. We calculated the area under disease progress curve (AUDPC) in individual plants under natural infections in the field. To promote infections, the progeny was watered by a spray system twice a week although the natural inoculum was high in Osorno, Chile.

For the molecular analysis, DNA extractions and PCR amplification were performed using genetic markers as described in **Table 2** for *P. infestans* to track the presence or absence of R genes in the Ma*R8* or Ma*R9* crossed with elite material progeny.

Progeny was also phenotypically evaluated. We determined the percentage of leaves affected by late blight during plant development by visually estimating the green and non-green portions of the leaves. The estimations were integrated into the AUDPC or area under the disease progress curve. AUDPC is obtained by the repeated visual inspections and estimation of the percentage of the leaf affected in a set of plants. The value is calculated by the formula used by Jo et al. [28]. Percentages of damaged foliage are plotted through a period of time. AUDPC was calculated for the 10 randomly selected individuals from each cross in the field.

For the following 2014–2015 season, the tubers of 71 genotypes that were not damaged by late blight were harvested. The experiment was designed with three replicates in randomized blocks. We planted three plants of each genotype that hold R genes in front of three plants of the susceptible Atlantic cultivar (susceptible control) per each replicate.

Rows of the susceptible Atlantic cultivar were also planted in the border and interspersed in the complete area of the assay. AUDPC values were calculated for all genotypes and susceptible control plots. A pairwise comparison was performed between each genotype and the respective control plot. We calculated the AUDPC from the visual estimation of the percentage of infected foliage.

5.3. Results

In the 2013–2014 season, an evaluation of randomly selected individual plants from the progeny of Ma*R8* and Ma*R9* crossed with the commercial varieties indicated that progeny carrying the *R2* gene had less foliage damage represented by lower AUDPC values. Furthermore, higher AUDPC values were found in plants that did not contain R genes. We were not able to perform statistical analysis as there were different numbers of clones holding R genes.

In the second season, we utilized undamaged plants from the first season. Most of the plants carrying R genes again had lower AUDPC values than the control plots. Interestingly, plants carrying the *R3* gene did not have different AUDPC values compared to the susceptible control plots.

In conclusion, we found that plants carrying R genes were only slightly affected by late blight in conditions of high pathogenic pressure, with the exception of the *R3a* and *R3b* genes (**Table 5**). Out of the 11 genotypes that did not show differences compared to the susceptible control, nine were holders of genes *R3a* and *R3b*, suggesting that these R genes are less resistant to late blight and, therefore, should not be used in breeding programs in Chile.

	Number of genotypes tested	**% genotypes significantly different to susceptible**
Genotypes carrying 1 R genes	5	60
Genotypes carrying 2 R genes	21	71
Genotypes carrying 3 R genes	18	94
Genotypes carrying 4 R genes	13	92
Any genotype carrying R1	27	96
Any genotype carrying R2	17	88
Genotypes carrying only R3a or R3b	20	55

Table 5. Genotypes harboring different numbers of R genes and % showing an AUDPC value significantly lower than the susceptible control plots.

5.4. Conclusions about pyramiding R genes for resistance to late blight

The Ma*R8* and Ma*R9* crosses were successful and generated hybrid genotypes harboring at least four different R genes that are now available for breeding. The progeny carrying R genes has been selected as parents for backcrosses or early clonal selection step and entered to the scheme of the potato breeding program.

A major challenge remains to develop an efficient and reliable system of phenotyping for late blight damage and for large-scale screening of breeding lines.

Acknowledgements

Financial support: Subsecretary of the Chilean Ministry of Agriculture through Potato Breeding Program project and Conservation of Genetic Resources program (501453-70). CORFO (Corporación de Fomento a la Producción) through INNOVA-Public Goods 14BPC4-28525.

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