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Research Paper

Identification of sources of cytoplasmic male sterility in onion (*Allium cepa* L.) germplasm using molecular markers

González-Chavira, M.M., Pons-Hernández, J.L., *González-Pérez, E., Guerrero-Aguilar, B.Z. and Villalobos-Reyes, S.
(Campo Experimental Bajío-INIFAP. Carretera Celaya-San Miguel de Allende km 6.5, C.P. 38124, Celaya, Guanajuato. México.)

ABSTRACT: *Onion is a very important crop that demands the use of hybrid seed to satisfy the need for production that currently prevails. To obtain it, it is necessary to identify in the germplasm, the types of cytoplasm that are found and the genetic condition of the Ms nuclear locus, with this the elements would be available to identify male-sterile genotypes, maintainers and restorers of fertility that would allow the formation of hybrids. With this objective, 29 populations were analyzed using the molecular markers: 5'Cob, orfA501, to identify the type of cytoplasm and AcPMS1 to establish the genotype of the Ms locus. It was found that, in the analyzed germplasm, the three types are present. Of cytoplasm S, N and T, being the normal cytoplasm (N) the most frequent being in 76% of the families, followed by the sterile cytoplasm (S) present in 41% and the T cytoplasm that was only found in the 21%. In the case of the Ms locus, the results indicate that the most common genotype is the recessive homozygote (msms) which is in 25 of the 29 families (86%), followed by the dominant homozygote (MsMs) which is found in 22 of the 29 families. families (76%), with the heterozygous genotype being the least frequent (Msms) present in 8 accessions (28%). With this information it was possible to identify androsterile (A), maintainer (B) and restorer (C/R) plants of male fertility, which allows the program to have the elements to start a breeding scheme by hybridization.*

KEYWORDS: *Types of cytoplasm, fertile male sterile plants, maintainer plants, restorer plants.*

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I. INTRODUCTION

The onion (*Allium cepa* L.) according to the value of its production is the second most important vegetable in the world, surpassed by the tomato (Valencia and Zetina, 2017). In Mexico, it is the third cultivated vegetable, with an average production in the last decade of 1.4 million tons, of which Guanajuato contributed 197,127 t in 2019, placing it as the second producing state (SIAP, 2020). Production based on the use of F1 hybrid cultivars that present better physical characteristics, which are due to genetic causes and that are reflected in a vigor that exceeds that of the parents, while the use of native materials and open-pollinated (OP) varieties is limited. The 'hybrid vigour' or 'heterosis' effect is reflected in the increase in bulb yield, as well as in the uniformity of characters of agricultural interest, in comparison with open-pollinated varieties (Evoor *et al*., 2007).

Obtaining genotypes that present this hybrid vigor is the main trend in onion genetic improvement in recent years. From a commercial point of view, the advantage of hybrid materials lies in the control of seed production that depends on the parental lines. For the formation of hybrids, it is necessary to cross between two inbred parental lines. From the crossing of inbred lines, the F1 population is obtained, which is used to produce commercial seed. Carrying out this activity manually is not practical, since the mechanical removal of maturing anthers before they shed pollen is not economically feasible, due to the structure of the umbel that contains 200 to 600 flowers that open successively (Brewster, 2008). This is why male sterility is of great importance to make the formation of hybrid material economically viable. This inability to produce fertile pollen is essential for the production of hybrid seeds, since plants that have this characteristic do not self-fertilize, therefore, any seeds that are produce in these plants must be the result of cross-pollination. .

*Corresponding Author: gonzalez.enrique@inifap.gob.mx

Campo Experimental Bajío, INIFAP, México.

It has been established that the factors inducing male sterility in onion, or cytoplasmic male sterility (CMS), are found in the mitochondrial genome, so it is a character that is transmitted only from mother to daughter offspring. For the production of hybrid onion seeds, the CMS-S (Jones and Emsweller, 1936; Jones and Clarke, 1943) and CMS-T (Berninger, 1965) nuclear-cytoplasmic sterility systems are used. In these systems, there are three types of cytoplasms (mitotype) the fertile cytoplasm ("N") and the sterile cytoplasms ("S" and "T"). The CMS-S system is the most widely used due to its stability in different environments (Havey, 2000). In this system, a single gene (*Ms*) is needed to restore fertility, while in the CMS-T system, alleles of three genes are required to restore it (Kim *et al*., 2015). For the production of hybrids in the CMS-S system, there must be male-sterile lines (lines "A" or *Smsms*), maintainer lines (lines "B" or *Nmsms*) and lines that restore fertility (lines "C/R" or *MsMs*) that are the parents of the fertile hybrid (Santos *et al*., 2008).

The identification of the type of cytoplasm was obtained from the molecular markers *5'cob* and *orfA501*. The *5'cob* is obtained using the specific oligonucleotides designed by Sato (1998), obtained from the sequencing of the mitochondrial *cob* gene, which reveals an insertion in the chloroplast DNA sequence, in the "upstream" region of this gene in the cytoplasm S, distinguishing the polymorphisms between the N and S cytoplasms allowing the identification of these cytoplasms in individual plants. The *orfA501* is a marker based on an insertion in the mitochondrial sequence of chives that forms an open reading frame (*orfA501*) (Engelke *et al*., 2003) that complements the identification of the S, N and T cytoplasms.

For the identification of the genotype of the locus (*Ms*) the gene responsible for the restoration of male fertility in onion, different types of markers closely linked to it have been developed (Gokce and Havey, 2002; Bang *et al*. 2011; Havey, 2013; Kim, 2014; Huo *et al*., 2015), which together with the molecular markers reported by Kim *et al*. (2015), including those linked to the *AcPMS1* gene are involved in the DNA replication error repair pathway, which they consider to be the best "candidate gene" to be responsible for the restoration of male fertility in the onion. These markers make possible the complete identification of the 'A' and 'B' lines, allowing marker assisted selection (MAS) of these types of lines.

Various works on the application of MAS, using PCR markers to determine the types of S/N and T cytoplasm and the *Ms* locus in onion, have been reported by several researchers (Khar and Saini, 2016; Ferreira *et al*., 2017 ; Ferreira and Santos, 2018; Ahmad et al., 2020; Manjunathagowda and Anjanappa, 2020; Dehghani et al., 2021). Based on the above, the objective of this work was to identify through PCR markers the cytoplasmic types present in 29 onion populations of the Vegetable Program of the Bajío Experimental Field of INIFAP, as well as the genotyping of the *Ms* locus, in order to reduce the time in the identification of male-sterile (A), maintainer (B) and restorer (C/R) populations, a fundamental step in the process of obtaining hybrids.

II. MATERIALS AND METHODS

The study was develop at the facilities of the Vegetable Program of the Bajío Experimental Field of the National Institute of Forestry, Agriculture and Livestock Research (INIFAP). Located in Celaya, Guanajuato, Mexico at the geographic coordinates 20º 32' 05" NL and 100º 48' 49" LO, at 1750 m, during the autumn-winter cycle of 2017-2018, under conditions of average temperature of 29.4 °C.

2.1. Onion genetic material.

Twenty-nine populations from the germplasm bank of the Bajío Experimental Field Vegetable Program were use. From each population in trays containing sterile peat, 12 seedlings were produce, which remained under greenhouse conditions until the seedling stage (12 ± 1 cm in height). Three hundred forty eight individuals were individually analyze.

2.2. Extraction of DNA.

From the 12 seedlings of each genotype, leaf tissue was collect and the individual extraction of genomic DNA was carry out, which was done using the CTAB method (Doyle and Doyle, 1990). The obtained DNA concentrations were quantify with a spectrophotometer (NanoDrop ND-1000; Thermo Fisher Scientific, Waltham, MA) and the quality was evaluate by electrophoresis in 1% agarose gels.

2.3. Analysis of the type of cytoplasm.

For the analysis of the type of cytoplasm, several independent amplifications were performed on the DNA of the 29 accessions, using different primers in the PCR reactions. The first two were with the primers and conditions recommended by Sato (1998), known as: *5'cob* (S)-specific primer (5'-GTCCAGTTCCTATAGAACCTATCACT-3'), *5'cob* (N)-specific primer (5'-TCTAGATGTCGCATCAGTGGAATCC-3') and an anti common sense primer for both (5'-CTTTTCTATGGTGACAACTCCTCTT-3') with an alignment temperature of 53 ºC. Which amplify the 180 and 414 bp fragments. In addition, the primer *orfA501* (Engelke *et al*., 2003) primer 1: (5'-

*Corresponding Author: gonzalez.enrique@inifap.gob.mx

ATGGCTCGCCTTGAAAGAGAGC-3') and primer 2: (5'-CCAAGCATTTGGCGCTGAC-3') was used in this reaction the expected fragment is 473 pb.

The reaction mixture was in a volume of 20 µL: 0.25 µM of each primer, 0.15 mM of each dNTP, 1X PCR buffer, 2.0 mM MgCl2, 2.5 U of the enzyme Taq DNA polymerase, and 50 ng of DNA. The program for the amplification of *5'cob* was: an initial cycle of 2 min at 94°C, 36 cycles of 30 s at 94°C, 1 min at 53°C and 2 min at 72°C and a final cycle of 5 min at 72°C. For the *orfA501* marker it was an initial cycle of 2 min at 94°C, 40 cycles of 30 s at 94°C, 1 min at 60°C, 2 min at 72°C and a final cycle of 5 min at 72°C. The sizes of the amplified fragments were estimated by comparison with the 1kb plus molecular marker bands in 8% acrylamide-bisacrylamide gel electrophoresis and 2.5% agarose gels.

Table 1. Differences between the -(N), -(T) and -(S) cytoplasms by combination of the *5' cob* primers and the *orfA501* marker according to Engelke *et al*. (2003) +, amplification of the expected fragment; –, no amplification of the expected fragment.

Citoplasma	***5'cob***		***orfA501***
	180pb	**414pb**	**473pb**
(N)	+	-	-
(T)	+	-	+
(S)	+	+	+

The results of these two reactions were considered as complementary in the identification of the types of cytoplasm according to what was proposed by Engelke *et al*. (2003) (Table 1). The frequencies of the different types of cytoplasms were calculated by dividing the number of individuals that presented the expected banding patterns by the total number of individuals analyzed.

2.4. Evaluation of the male fertility restoration (*Ms*) locus.

For the genetic evaluation of the *Ms* locus, the primers AcPMS1F (5'-GGTCACCAGGTGGAGAGAGAA-3') and *AcPMS1R* (5'-TCATTGAGCTGCATCCAAA-3') were used (Kim *et al*., 2015; Ferreira *et al*., 2017). The reaction mixture was at a final volume of 10.0 µL: 10 ng of DNA, 0.2 µM of each of the primers, 0.2 mM of each dNTP, 1X PCR buffer, 2.0 mM MgCl2, and 1 U of Taq DNA polymerase. The amplification program used was 95 °C for 5 min; six cycles of 95 °C for 30 s, 69 °C for 1 min with a decrease of 2 °C per cycle, and 72 °C for 45 s; seven cycles of 95 °C for 30 s, 59 °C for 1 min with a 1 °C decrease per cycle, and 72 °C for 45 s; and finally 27 cycles of 95 °C for 30 s, 52 °C for 1 min, and 72 °C for 45 s.

The *AcPMS1* marker was considered as: 1) dominant homozygous (*MsMs*) if it amplified a 242bp fragment, 2) homozygous recessive (*msms*) if the amplified fragment was 276bp, and 3) heterozygous (*Msms*) if both fragments were amplified. 242 and 276bp. The sizes of the amplified fragments were estimated by comparing them with the 1kb plus molecular marker bands, in a 2.5% agarose gel electrophoresis. The frequencies of the different genotypes were calculated by dividing the number of individuals that presented the expected banding patterns by the total number of individuals analyzed.

III. RESULTS AND DISCUSSION

3.1. Analysis of the type of cytoplasm.

As can be seen, the amplicons obtained are those expected according to what has been reported in the literature (Sato, 1998; Engelke *et al*., 2003). In 82% of the plants analyzed, the results were consistent and the type of cytoplasm could be identified. In the remaining 28% of plants, fragments of different sizes than expected were amplified or no amplicon was obtained without ruling out that this is due to methodological problems. The possibility that fragments of alleles different from those expected are also being amplified or in the case of no amplification, that the target site of the primers is modified in these individuals, but this is the subject of another investigation. The molecular profiles found with the markers used for the analysis of the cytoplasm are shown in Figure 1.

These molecular profiles were used to identify the types of cytoplasm according to what was proposed by Engelke *et al*. (2003), and the different types of cytoplasms were quantified based on their frequency in the different accessions analyzed.

*Corresponding Author: gonzalez.enrique@inifap.gob.mx

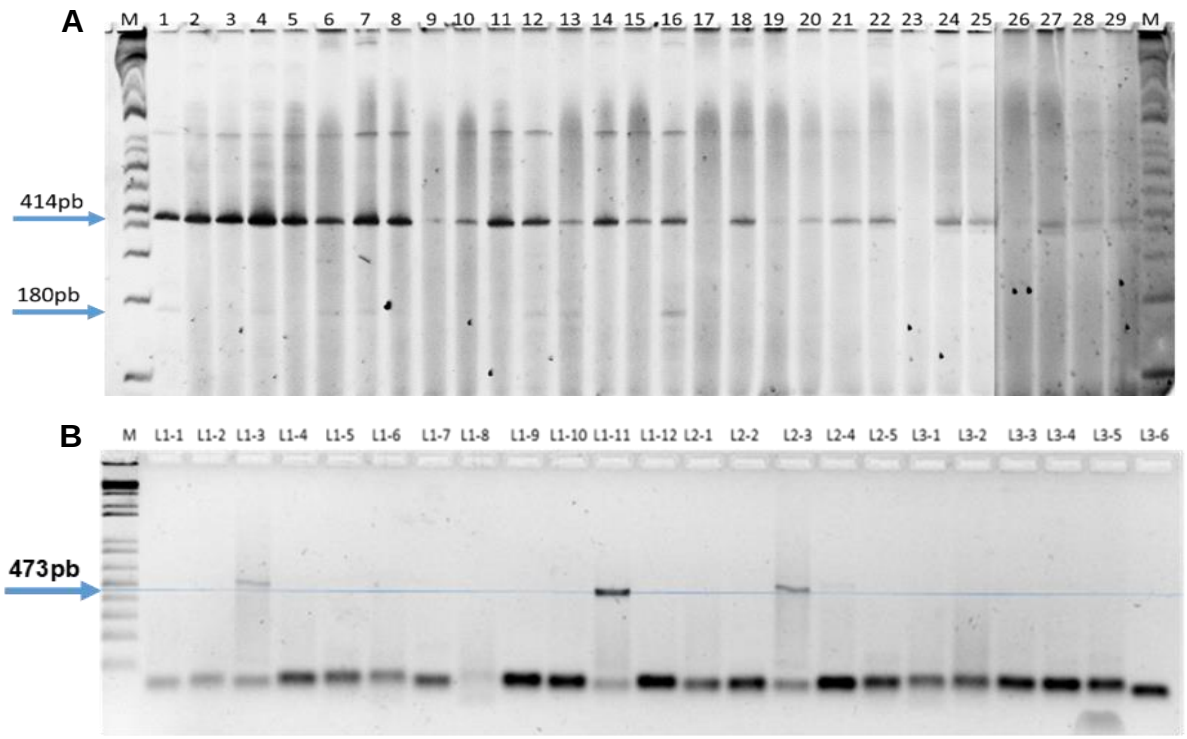

Figure1: Examples of: A) Molecular profile for the *5'cob* marker, showing the 180bp and 414bp fragments, on an 8% acrylamide-bisacrylamide gel electrophoresis in 1X TBE, M= 1Kb Plus marker. B) Molecular profile for the *orfA501* marker, showing the 473pb amplicon, in a 2.5% agarose gel electrophoresis in 1X TBE, M= 1Kb Plus marker.

This analysis indicates that the most frequently found cytoplasm is the normal one (N) which is found in 22 of the 29 families (76%), followed by the sterile cytoplasm (S) present in 12 genotypes (41%) and in contrast the T cytoplasm. it was only found in 6 accessions (21%) (Table 2). Saini *et al*. (2015) analyzed 350 plants from three Indian onion populations, concluding that the *5'cob* and *prfA501* markers could distinguish the three types of cytoplasm. Engelke *et al*. (2003) using the same markers reported the three types of cytoplasm (N, S and T) in 361 onion plants from Turkey.

As in this work, Ferreira *et al*. (2017) when analyzing with the markers *5'cob* and *orfA501*, found in the Brazilian germplasm that the most frequent cytoplasm was N, followed by S and the least frequent was T cytoplasm with frequencies of 0.47, 0.28 and 0.25, respectively. Muso *et al*. (2011), using the markers *5'cob*, *orfA501* and *orf725*, cytoplasmically characterized 20 half-sib families resulting from crosses by open pollination between the onion cultivars "INIA colorada" and "Rojo duro" and 9 commercial hybrids, and found the three types of possible cytoplasms, S, T and N, with a frequency of 0.45, 0.30 and 0.25 respectively. They concludes that their results are consistent with the cytoplasms present in the progenitors that gave rise to them, since in a previous work he found in a sample of 20 individuals of "INIA Colorada" 16% of plants with normal cytoplasm (N) and 84% of plants with type T androsterile cytoplasm.

This could be the difference in the proportions of the types of cytoplasm between different works of this nature, which had already been pointed out by Van Der Meer and Van Bennekom (1971), who point out that genetic analyzes indicate that the *ms* and *S* factors would occur in different frequencies in varieties of onions from different countries. On the other hand, when analyzing within the accessions, variable frequencies of the types of cytoplasm were also found, finding at least two types of cytoplasm in 48% of the genotypes, only one type in 42% and in the remaining 10% of the accessions no type of cytoplasm could be identified (Table 2). These mixtures of cytoplasms in a single genetic material, without ruling out that they could be contaminations, had already been found by Dehghani *et al*. (2021) who reported mixtures of N, S and T cytoplasms in the "Dorcheh Pop" population derived from a hybrid and in the "Red Hybrid", "Seria Hybrid" and "Baran Hybrid" hybrids.

Within the germplasm in which only one type of cytoplasm was identified, the populations ACeP-4, -18, -20, -21, -25, -27 and -28 stand out, where most of the plants analyzed had the cytoplasm N, which due to this condition cannot have androsterile individuals. Another important population is ACeP-15, which has only type S cytoplasm in more than half of the plants analyzed (67%), this indicates that it can only have male-sterile plants or type B maintainer plants depending on the condition of its locus. Furthermore, this may be due to

*Corresponding Author: gonzalez.enrique@inifap.gob.mx

differences in the cytoplasms of the parents from which they were derived. The genotypes with the T cytoplasm are important for the breeding program, because it allows having various sources of male sterility and this is desirable to avoid the genetic vulnerability of a single cytoplasm (Vu *et al*., 2011).

Table 2. Frequencies of fertile (N) and male-sterile (S) and (T) cytoplasms in 29 onion accessions.

Accession	Cytoplasms			Accession	Cytoplasms		
	N	S	T		N	S	T
ACeP-1	0.33	0.17	0	ACeP-16	0.79	0	0.21
ACeP-2	0.40	0.20	0	ACeP-17	0	0	0
ACeP-3	0.54	0	0.08	ACeP-18	0.83	0	0
ACeP-4	0.64	0	0	ACeP-19	0.31	0	0
ACeP-5	0.46	0.27	0	ACeP-20	0.92	0	0
ACeP-6	0.25	0	0.25	ACeP-21	0.89	0	0
ACeP-7	0.46	0	0.31	ACeP-22	0.14	0.01	0
ACeP-8	0.83	0	0.17	ACeP-23	0	0.50	0.10
ACeP-9	0	0	0	ACeP-24	0	0.10	0
ACeP-10	0.39	0.08	0	ACeP-25	0.86	0	0
ACeP-11	0.15	0.15	0	ACeP-26	0	0	0
ACeP-12	0.39	0.15	0	ACeP-27	0.70	0	0
ACeP-13	0.08	0.17	0	ACeP-28	1.00	0	0
ACeP-14	0	0.07	0	ACeP-29	0.50	0	0
ACeP-15	0	0.67	0				

3.2. Evaluation of the male fertility restoration (Ms) locus.

Figure 2 shows an example of the molecular profiles found with the AcPMS1 marker used for the analysis of the genotype present in the *Ms* locus of the nucleus. As can be seen, the amplicons obtained are those expected according to what has been reported in the literature (Kim *et al*., 2015; Ferreira *et al*., 2017).

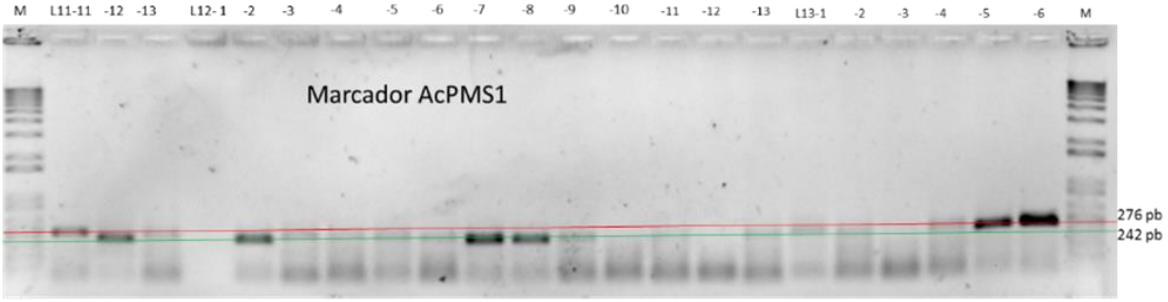

Figure2: Molecular profile for the AcPMS1 marker, showing the 242 and 276pb amplicons, in a 2.5% agarose gel electrophoresis in 1X TBE, M= 1Kb Plus marker.

These molecular profiles were used to identify the genotype present in the *Ms* locus of the cell nucleus, according to what was proposed in the works of Kim *et al*. (2015) and Ferreira *et al*. (2017). The different genotypes were quantified based on their frequency in the different accessions analyzed. This analysis indicated that the *Ms* and *ms* alleles were detected in the 29 genotypes evaluated, both in the homozygous and heterozygous state, the most frequently found genotype being the homozygous recessive (*msms*) that was found in 25 of the 29 families (86%), followed by the dominant homozygous (*MsMs*) found in 22 of the 29 families (76%), with the least frequent heterozygous genotype (*Msms*) occurring in 8 accessions (28%) (Table 3).

The higher frequency of the *ms* allele is explained by Little *et al*. (1944), who suggested that the occurrence of the most recessive allele in most onion and shallot populations, indicates that the mutation from *Ms* to *ms* must have occurred early in onion evolution or has occurred many times. In plants carrying the 'T' cytoplasm (Tables 3), the presence of the marker linked to the fertility-restoring *MsMs* genotype was found in the CMS-S system.

*Corresponding Author: gonzalez.enrique@inifap.gob.mx

These results coincide with what was found by Kim (2014) and Ferreira *et al*. (2017) who report similar results. Kim (2014), concluded that the restoration of fertility in this cytoplasm may not be controlled by three independent genes, as proposed by Schweisguth (1973), but by the segregation distortion of a single gene.

Table 3. Frequencies of the genotypes of the fertility restorer locus in 29 onion accessions.

Accession	Genotype			Accession	Genotype s		
	MsMs	***Msms***	***msms***		***MsMs***	***Msms***	***msms***
ACeP-1	0	0	0.17	ACeP-16	0	0	0.14
ACeP-2	0	0	0.80	ACeP-17	0	0	0
ACeP-3	0.15	0	0.23	ACeP-18	0.50	0	0
ACeP-4	0.27	0	0.09	ACeP-19	0.13	0.19	0.31
ACeP-5	0.36	0	0.46	ACeP-20	0.08	0.42	0.50
ACeP-6	0.33	0	0.67	ACeP-21	0.45	0.33	0.22
ACeP-7	0.31	0	0.54	ACeP-22	0.50	0.21	0.29
ACeP-8	0.50	0	0.33	ACeP-23	0	0.20	0.80
ACeP-9	0.15	0	0.54	ACeP-24	0	0.30	0.70
ACeP-10	0.08	0	0.08	ACeP-25	0.29	0.14	0.57
ACeP-11	0.23	0	0.08	ACeP-26	0.07	0.14	0.79
ACeP-12	0.69	0	0	ACeP-27	0.20	0	0.80
ACeP-13	0.25	0	0.33	ACeP-28	0.10	0	0.60
ACeP-14	0.21	0	0	ACeP-29	0.60	0	0.40
ACeP-15	0	0	0.42				

In this way, he hypothesizes that the restoration is conferred by the same gene or by two strongly linked genes. Considering that the CMS-S system is preferred by breeders, since the inheritance of a single locus is easier and more stable under various environmental conditions (Havey, 2000). Based on the results obtained in this study, it can be noted that the onion germplasm of the CE-Bajío Vegetable Program has male-sterile (S) lines, since 29 individuals were found with this genetic condition, representing 8.3% of the population of the 348 individuals analyzed, and distributed in 12 of the 29 populations (41.4%) (Table 4).

Table 4. Number of individuals with phenotype "S", "B" or "C", found in 29 onion populations of the CE-Bajío Vegetable Program.

Accession	Phenotype			Accession	Phenotype		
	S (S*msms*)	**B (N*msms*)**	**C (*MsMs*)**		**S (S*msms*)**	**B (N*msms*)**	**C (*MsMs*)**
ACeP-1	2			ACeP-16		2	
ACeP-2	1	2		ACeP-17			
ACeP-3			4	ACeP-18			9
ACeP-4		1	4	ACeP-19		2	
ACeP-5	3	2	2	ACeP-20		6	2
ACeP-6		3		ACeP-21		2	6
ACeP-7		2	6	ACeP-22	1		4
ACeP-8		4	8	ACeP-23	5	1	
ACeP-9				ACeP-24	1		
ACeP-10	1	1	2	ACeP-25		4	2
ACeP-11	2		4	ACeP-26			
ACeP-12	2		4	ACeP-27		6	2
ACeP-13	2	1		ACeP-28		6	2
ACeP-14	1			ACeP-29		3	4
ACeP-15	8			**Totales**	**29**	**48**	**65**

For the purposes of genetic improvement focused on the production of hybrids, sterility-maintaining lines (B) are required, with this genetic condition 48 individuals were found, representing 13.8% of the individuals analyzed, distributed in 17 of the 29 populations. (58.6%).

An important aspect to highlight is that, in 5 of the accessions, there are both "A" plants and "B" plants, which will help to maintain male sterility in these populations, in the rest of the populations that present male sterility it is necessary start the backcross process to obtain the isogenic lines "B". On the other hand, 18.7% of the individuals analyzed presented a "fertility restorer" (C/R) genotype, distributed in 16 of the 29 populations,

*Corresponding Author: gonzalez.enrique@inifap.gob.mx

indicating that in 55% of the populations it was feasible to identify this genotype (Table 4). These populations will allow the CE-Bajío Vegetable Improvement Program to start the process of formation of onion hybrids.

IV. CONCLUSSIONS

It is possible to characterize the types of cytoplasm present in 29 onion accessions using the markers *5'Cob, orfA501*. The AcPMS1 marker allowed us to characterize the genotype of the *Ms* nuclear locus in the 29 onion genotypes. In the germplasm analyzed, the three types of cytoplasm S, N and T are present, with N being the most frequent and T the least frequent.

In the analyzed germplasm, the most frequently found genotypic condition at the *Ms* nuclear locus was homozygous recessive (*msms*) and the least frequent was heterozygous (*Msms*). With the germplasm that the CE-Bajío Vegetable Improvement Program possesses, it is possible to start a scheme for the formation of onion hybrids.

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*Corresponding Author: gonzalez.enrique@inifap.gob.mx

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*Corresponding Author: gonzalez.enrique@inifap.gob.mx