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Partial success of marker-assisted selection of 'A' and 'B' onion lines in Brazilian germplasm

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ABSTRACT

This work aims to identify 'A' and 'B' onion lines in Brazilian germplasm, associating marker-assisted selection (MAS) of cytoplasm type and the *Ms* locus with pollen viability analysis. MAS and pollen viability tests presented a complete agreement for the lines Alfa SF, BRS 367, BRS 367, Cascuda T7, Cascuda T5, EHCEB 20102017 and EHCEB 20142027. MAS was not validated by the pollen viability tests in some plants of the lines Alfa SF, EHCEB 20142028, EHCEB 20141028, EHCEB 20141027 e EHCEB 20141017. Plants with fertile pollen within a specific 'A' line were eliminated before anthesis. The methods used to evaluate pollen viability did not present agreement in the sterile and fertile classification in five onion accessions. Six pairs of 'A' and 'B' lines identified in the present study [Alfa SF (T*msms*) x Alfa SF (N*msms*), BRS 367 (T*msms*) x BRS 367 (N*msms*), Cascuda T7 (N*msms*) x Cascuda T5 (S*msms*), EHCEB 20142027 (N*msms*) x 20141027 (T*msms*), EHCEB 20102017 (N*msms*) x EHCEB 20141017 (T*msms*), and EHCEB 20142028 (N*msms*) x EHCEB 20141028 (S*msms*)] have potential for onion hybrid development.

1. Introduction

Onion (*Allium cepa* L.) stands out as one of the main vegetable produced in Brazil, with an estimated production of 1.6 million tons for the year of 2015 (IBGE, 2015). The states of Bahia and Pernambuco, in the northeast region of the country, account for almost 20% of the national production, where the use of open-pollinated cultivars predominates (Santos et al., 2012). Despite the increase in the use of hybrids in the Southeast, Midwest, and part of the Northeast (Faria et al., 2012), open-pollinated cultivars account for almost 70% of the Brazilian onion production (Santos and Oliveira, 2011).

The CMS-S and CMS-T systems of nuclear-cytoplasmic sterility are employed in the production of onion hybrids seeds. The first one is the most widely used, due to the great stability in the different environments (Havey, 2000). In the CMS-S system, only one gene (*Ms*) restores fertility, while alleles of three genes are involved in fertility restoration in the CMS-T system (Kim et al., 2015). These systems require the use of male-sterile lines ('A' lines), maintainer lines ('B' lines), and lines with good combining ability ('C' lines) (Santos et al., 2008).

The obtainment of onion hybrids by the conventional method is costly and takes almost 20 years in temperate regions, including the identification of 'A' lines (male-sterile lines) and 'B' lines (maintainer lines) in the open-pollinated populations (Pike, 1986). Previous attempts to identify 'A' and 'B' lines in the Brazilian germplasm by conventional methods were not successful in the development of onion hybrids (Santos et al., 2010).

The full assisted selection of 'A' and 'B' lines by molecular markers became possible with cytoplasm identification by the markers 5'*cob* and *orf*A501 (Sato, 1998; Engelke et al., 2003) or *orf*725 (Kim et al., 2009), and with the possible identification of the male-sterility restorer nuclear locus (*Ms*) by the markers AcSKP1 (Huo et al., 2015) or AcPMS1 (Kim et al., 2015).Thus, it is expected that marker-assisted identification of 'A' and 'B' lines takes three years in tropical environments, where the seed-to-seed cycle is one year, considering at least one test cross. This result will lead to significant reduction of the time required by classic methods.

The stability of fertile and sterile pollen grains in 'B' and 'A' lines, respectively, which is the basis of the production of onion hybrids, can be proven with several reagents, such as acetic carmine and Alexander's staining solutions, or *in vitro* germination of the pollen grain (Abdelgadir et al., 2012). Khar and Saini (2016) reported marker-assisted selection of 'A' and 'B' lines in an Indian onion germplasm, associating pollen viability with acetic carmine staining. For the Brazilian germplasm, no similar studies associating marker selection for 'A' and

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Table 1
Cytoplasm type identified with the marker *orf725* and genotyping of the male-fertility restorer locus by the marker AcSKP1 in plants (n) of onion accessions with potential for 'A' and 'B' lines.

Accession	Cytoplasm				Genotyping			
	N	N	S	T	n	*MsMs*	*Msms*	*msms*
Alfa SF linha 'A'	12	–	–	12	12	–	–	12
Alfa SF linha 'B'	5	5	–	–	5	–	–	5
BRS 367	9	6	1	2	9	3	2	4
Cascuda T7 ('B')	13	13	–	–	13	7	4	2
Cascuda T5 ('A')	23	–	23	–	23	11	10	2
EHCEB 20142028	13	13	–	–	13	–	–	13
EHCEB 20141028	42	–	42	–	42	–	–	42
EHCEB 20142027	3	3	–	–	3	–	–	3
EHCEB 20141027	14	–	–	14	14	–	–	14
EHCEB 20101017	10	–	–	10	10	–	–	10
EHCEB 20102017	12	12	–	–	12	–	–	12

'B' lines with pollen viability analysis have been reported yet.

The objective of this work was to identify 'A' and 'B' lines in Brazilian onion germplasm, associating marker-assisted selection of the cytoplasm type and the *Ms* locus with pollen viability analysis as an initial stage for the development of onion hybrids.

2. Material and methods

2.1. Plant material

Eleven accessions (Table 1) from the onion germplasm bank of EMBRAPA (Brazilian Agricultural Research Corporation) were evaluated in this study. The accessions had been previously identified (Ferreira et al., 2017) with potential for selection of 'A' and 'B' lines, as they presented either mixed cytoplasm and recessive *ms* alleles or 100% of plants at N*msms* and S*msms* or T*msms* condition. Onion seedlings were transplanted in May/2016, in the experimental field of Embrapa Semiárido, Petrolina, PE, and harvested in September/2016.

Bulbs were stored and vernalized in a cold chamber for 120 days, at 8 °C, and approximately 60% air humidity, for further planting and emission of floral scape, aiming at the evaluation of pollen viability and analyses with cytoplasmic markers and male-fertility restorer locus.

2.2. Evaluation of cytoplasm type and male-fertility restorer locus (Ms)

Genomic DNA was extracted using the 2x CTAB protocol (Doyle and Doyle, 1990), as described by Ferreira et al. (2017).

The cytoplasm type was identified with the marker *orf*725 (Kim et al., 2009), for a final volume of 10 µl: 0.2 µM of each primer, 0.2 mM of each dNTP, 1x of PCR buffer, 2.0 mM $MgCl_2$, 1.0 unit of Taq DNA polymerase, and 50 ng total DNA. The thermocycler was programmed for the amplification of this marker, as follows: initial denaturation cycle of 5 min at 94 °C, 40 cycles of 30 s at 94 °C, 1 min at 60 °C, and 90 s at 72 °C, and a final cycle of 5 min at 72 °C. PCR products were visualized and classified on 1.5% agarose gels: 833 bp fragment = 'N' cytoplasm; 833 bp and 628 bp fragments = 'T' cytoplasm, and 628 bp fragment = 'S' cytoplasm.

The alleles of the male-sterility restorer locus were identified by the marker AcSKP1 (Huo et al., 2015), for a total volume of 25 µl: 50 ng of total DNA, 0.4 µM of FU898, 0.4 µM of FD898, 0.2 µM of SU628, 0.2 µM of SD628, 0.15 mM of each dNTP, 1x of PCR buffer, 2.0 mM $MgCl_2$, and 2.0 units of Taq DNA polymerase. The amplification protocol adopted for AcSKP1 followed the description of Huo et al. (2015). Amplified products were separated and classified on 1.5% agarose gels. The evaluation for the fertility restorer locus of the marker AcSKP1 considered: 898 bp fragment = dominant homozygote (*MsMs*), 898 and 628 bp fragment = heterozygote (*Msms*), and 628 bp fragment = recessive homozygote (*msms*).

2.3. Analysis of pollen viability

Plants identified with cytoplasm *Smsms* or *Tmsms* and *Nmsms* were evaluated for pollen grain fertility or sterility. Four freshly opened flowers were randomly collected from the umbel of each plant for the analysis of pollen viability using 2% acetic carmine and modified Alexander's solution (Peterson et al., 2010) and *in vitro* germination.

2.3.1. Staining with 2% acetic Carmine and modified alexander's solution

Freshly opened flower buds were individually collected from plants of each accession. For the preparation of the slides, pollen grain was removed by crushing the anthers immersed in a drop of dye deposited on a histological slide covered with coverslips. Four slides per inflorescence were prepared for each dye, and in each slide, 100 pollen grains were counted, using a light microscope at 100× magnification, totaling 400 pollen grains for the four replications per dye.

For 2% acetic carmine, pollen grains were classified as fertile when they presented 100% reddish coloration, and as sterile when they showed 100% brownish coloration, no coloration, or partial coloration. For the modified Alexander's solution, pollen grains were considered as viable when they presented purple coloration and as non-viable when they presented green or diffuse coloration.

2.3.2. In vitro germination

Pollen grains were collected during the anthesis of the inflorescence flowers and inoculated on excavated slides containing 200 g L^{-1} of sucrose, 50 mg L^{-1} of boric acid, and 1 g of agar, after being heated to total agar dilution (Gomes et al., 2000). The medium was distributed in the wells of the excavated slides after being heated to total agar dilution.

Pollen grain was spread on the culture medium for a homogeneous distribution of the material. Slides containing pollen grain and culture medium were kept in Petri dishes with moistened paper, simulating a moist chamber, to avoid the drying of the medium, and stored at room at 25 °C temperature with exposure to bright light for a minimum period to start the evaluation of 4 h. Germination was evaluated by measuring the pollen tube length, with the aid of an optical microscope with 100x magnification (Karak and Hazra, 2012). To facilitate the counting of pollen grains, each well of the slide was divided into two vision fields, corresponding to two replications. For each plant, two wells were prepared, totaling four replications. A hundred pollen grains was counted in each replication. Pollen grains were considered as fertile when they presented a tube length equal to or greater than the diameter of the pollen grain itself.

3. Results

Gels were easily identified, producing fragments of the expected sizes, both for the cytoplasmic marker (*orf*725) (Kim et al., 2009) (Fig. 1) and for the marker of the restorer locus (AcSKP1) (Huo et al., 2015) (Fig. 2).

'A' and 'B' pure lines were observed among the evaluated accessions, which presented 100% *Smsms* or T*msms* and 100% N*msms*, respectively, for the accessions selected for this allele-cytoplasmic combination: Alfa SF line 'A'/Alfa SF line 'B', EHCEB 20141028/EHCEB 20142028, EHCEB 20141027/EHCEB 20142027, and EHCEB 20101017/EHCEB 20102017 (Table 1). The accession 'BRS 367' presented a mixture for cytoplasmic type, as well as genotypic mixture for the fertility restorer locus, which may allow the identification of 'A' and 'B' lines (Table 1). The accessions Cascuda T7 ('B') and Cascuda T5 ('A') presented a single type of cytoplasm and genotypic mixture for fertility restoration (Table 1), indicating the possibility of using Cascuda T7 plants as maintainers of Cascuda T5, considering that both accessions belong to the Valencian onion group.