Article

Validating Single Nucleotide Polymorphism Markers for Fusarium Basal Rot Resistance in Short-Day Onion Cultivars through Kompetitive Allele-Specific PCR

Suman Sharma, Christopher S. Cramer *

Department of Plant and Environmental Sciences, New Mexico State University, N127 Skeen Hall, MSC 3Q, Las Cruces, NM 88003-8003, USA

* Correspondence: Christopher S. Cramer, Email: cscramer@nmsu.edu.

ABSTRACT

Fusarium Basal Rot (FBR) caused by *Fusarium oxysporum* f. sp. *cepae* is a detrimental disease affecting onions and leading to significant bulb yield losses. Understanding the genetic mechanisms underlying FBR resistance is crucial for developing FBR-resistant cultivars. This study investigated whether the favorable alleles associated with FBR resistance have been fixed in the onion cultivar populations selected for FBR resistance, utilizing eight single nucleotide polymorphism (SNP) markers. DNA was extracted from cultivar populations including susceptible and partially-resistant check cultivars and subjected to Kompetitive allele-specific PCR (KASP) genotyping. Allelic frequencies were calculated for each marker across all cultivar populations. Among the eight SNP markers, *Isotig_33746_1093* exhibited a clear allelic discrimination for check cultivars and presented a negative correlation with FBR incidence (%), suggesting its potential association with FBR resistance. Another marker *Isotig_44683_192* presented near fixation of the favorable allele in the most advanced selections, including the partially-resistant check, suggesting a strong link to the loci on chromosome 4A for FBR resistance. Additionally, *Isotig_33439_640* predominantly featured the desired allele in the most advanced selections, indicating a potential connection with FBR resistance. Moreover, *Isotig_30594_1021* exhibited an abundance of the desirable allele in the most advanced selections, proposing its possible link to the genomic region on chromosome 8 for FBR resistance. However, the small sample size warrants caution, and future studies with larger sample sizes are required to validate the association of other potential SNP markers with FBR resistance.

KEYWORDS: *Allium cepa* L.; *Fusarium oxysporum* f. sp. *Cepae*; FBR resistance; single nucleotide polymorphisms; marker-assisted selection

Received: 23 May 2024
Accepted: 05 September 2024
Published: 09 September 2024

INTRODUCTION

Fusarium basal rot (FBR), which is caused by a soilborne fungus, *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *cepae* (H.N. Hans.) W.C. Snyder & H. N. Hans. (FOC), is a major threat to onion (*Allium cepa* L.) cultivation worldwide [1]. The precise estimation of yield losses due to FBR is not yet fully understood. However, empirical evidence suggests a 45% reduction in bulb yield postharvest, with an additional loss of 12% to 30% of bulbs during storage in shallot (*Allium cepa* L. var. aggregatum G. Don), that closely resembles the common bulb onion [2]. Various strategies are employed to control FBR including soil solarization, crop rotation, application of soil fumigants and fungicides, biological control agents, and exploiting host plant resistance. Among these, host plant resistance has been suggested as the most efficient and cost-effective method due to constraints associated with other control measures [3].

Traditional breeding methods involve selecting resistant germplasm through seedling and mature bulb screening assays that can be laborious and time-consuming [4,5]. Genetic markers associated with FBR resistance can accelerate the process of developing FBR-resistant cultivars. Identifying single nucleotide polymorphism (SNP) markers associated with FBR resistance can select individuals with the resistant alleles for further breeding efforts. Taylor et al. [6] extracted DNA from one of ten half-sib (HS) mother plants per accession from a total of 91 founder onion accessions that were utilized for subsequent genotyping to assess genetic diversity. Additionally, genotyping using 892 published SNP markers was conducted on the remaining nine HS mother plants of four selected onion accessions, along with ten individual plants from each of the four selected accessions. They identified five SNP markers that exhibited a strong association with FBR resistance, and three of them were mapped to chromosomes 1, 6, and 8. These markers were: *Isotig30594_1021*, *Isotig34519_442*, *Contig00676_1004*, *Isotig33746_1093* and *Isotig33439_640*. Two different SNP markers, *Isotig38484_281* and *Isotig44683_192* on chromosomes 2B and 4A, respectively, were linked to FBR resistance as revealed by Straley et al. [7]. In this study, leaf tissue was harvested from 33 F_2 plants derived from a cross between a FBR-resistant inbred, W440, and a FBR-susceptible inbred, B5351. Random selections were made from each of the resulting F_3 families for DNA extraction and SNP genotyping. A SNP marker, *Isotig38484_281*, on chromosome 2B exhibited a strong association with FBR resistance. For each additional allele inherited from the resistant parent, there was a 21% increase in seedling survival against the disease. Similarly, SNP marker *Isotig44683_192* on chromosome 4A was linked to FBR resistance, with the presence of a dominant allele from the resistant parent resulting in a 17% increase in seedling survival. However, an allele inherited from the resistant parent at *Isotig31106_505* on chromosome 4C had a negative effect, leading to a 15% decrease in seedling survival. A SNP marker, *NQ0257570*, reported in a plant patent [8] was located 6.2 cM away from the most significant SNP (*Isotig44683_192*)

on chromosome 4. Based on the results of the study by Straley et al. [7] and the previous research, it was suggested to assign the loci names of *Foc1* and *Foc2*, proposed by Bacher [9], to the putative FBR-resistant loci found on chromosomes 2 and 4, respectively.

The incorporation of markers associated with favorable alleles can enhance breeding strategies aimed at selecting FBR-resistant individuals using marker-assisted selection. Vu et al. [10] explored FBR resistance mechanisms using *Allium fistulosum* L., shallot, and monosomic alien addition lines (MAALs). MAALs can be produced via the addition of a single chromosome of an alien donor species to the entire chromosome complement of the recipient species [11]. MAALs are powerful tools for elucidating genome structure and transferring genes. In the study, Vu et al. [10] found that the monosomic line FF+2A, with chromosome 2 from shallot, revealed the highest FBR resistance, suggesting the role of chromosome 2 in conferring resistance. This line also accumulated alliospiroside A, a saponin linked to FBR resistance [12]. These observations suggested that chromosome 2 and 4 loci were involved in FBR resistance, as evidenced by different studies.

While these studies provided valuable insights into the genetic factors contributing to FBR resistance in onion, there is still a need for a deeper understanding of the genetic basis of FBR resistance. The eight SNPs identified in two studies linked to FBR resistance require further validation in additional populations to confirm their association with FBR resistance. This association can facilitate the implementation of marker-assisted selection strategies for identifying and breeding resistant germplasm. For validation of SNP markers, a homogeneous, fluorescence-based SNP genotyping platform known as kompetitive allele-specific PCR (KASP) genotyping assay (KASP™, LGC Genomics, London, UK) is popular for bi-allelic discrimination of known SNPs [13]. In our effort to improve FBR resistance, the NMSU onion breeding program employed an artificial inoculation mature bulb screening. After mature bulbs were inoculated with a virulent pathogen isolate, resistant onion bulbs were selected, genetically recombined, and repeated over multiple selection cycles. Recently, our breeding program has realized significant progress in improving FBR resistance levels in the most advanced selected populations for each cultivar. Comparing the resistance levels in the initial population to the advanced selections revealed substantial improvement with the current resistance levels comparable to those of the partially-resistant cultivar [3]. For the current study, it was hypothesized that the susceptible and the partially-resistant check cultivars would exhibit distinct allelic discrimination for the SNP markers associated with FBR resistance. Also, it was hypothesized that our advanced selected populations would exhibit higher frequencies of the alleles associated with FBR resistance compared to the initial cultivar population, indicating the progress in fixing favorable alleles for improved FBR resistance. Therefore, the objective of this study was to identify specific SNP markers

associated with FBR resistance that can distinguish between susceptible and partially-resistant germplasm in New Mexican short-day onion cultivars through KASP genotyping using eight SNP markers.

MATERIALS AND METHODS

Plant Material and DNA Extraction

The selected populations of three autumn-sown, open-pollinated short-day onion cultivars (NuMex Camino, NuMex Mesa, and NuMex Luna) [14–16] were field planted at the Fabian Garcia Research Center in Las Cruces, NM, USA (32.2799° N, 106.7725° W) for the growing season of 2022–23 in a randomized complete block design with four replications along with two check cultivars, Serrana (partially-resistant) ['Baia Periforme', the originator cultivar of 'Serrana' (Monsanto Vegetable Seeds, Woodland, CA, USA)] [8] and NuMex Crimson (susceptible) [17]. 'NuMex Camino' was developed involving a cross between 'Excel 986B' and a pink root and bolting-resistant variety, 'Texas Early Grano 502' [14]. 'NuMex Mesa' was developed through reciprocal crosses between 'NuMex BR1' and 'Buffalo' in 1984, followed by intercrossing F_1 progenies and recurrent selections until 1992, resulting in a cultivar likely inheriting 100% S cytoplasm from its parent lines and exhibiting desirable traits such as bolting and pink root resistance, bulb firmness, shape, scale color, single-centered bulbs, and maturity [15]. 'NuMex Luna' emerged from a cross between 'NuMex BR1' and bolting-resistant selections from 'Ben Shemen' in 1984 and after several years of intercrossing, and bulb selections, it was released in 1991 [16]. 'NuMex Crimson' was developed through intercrossing parent lines including 'Kurenai', 'Rojo', and selections from 'Texas Early Grano 502 PRR', 'Peckham Yellow Sweet Spanish', and Henry's Special in 1989 [17]. In the current study, three populations of each cultivar, including FBR1 (first/initial), FBR1-5, and FBR6 (the most advanced selections), were generated by advancing the resistant bulbs to the next generation in every selection cycle using an artificial inoculation mature bulb screening (AIMBS) [3]. In the procedure of AIMBS, the evaluation of selected populations for FBR resistance involved inoculating mature onion bulbs with a highly-virulent locally obtained *Fusarium oxysporum* f. sp. *cepae* (FOC) isolate 'CSC 515', selected for its ability to distinguish two check cultivars. A spore suspension, with a concentration of 3.0×10^4 spores/mL^{-1}, was combined with potato dextrose agar and applied to transversely-cut basal plates of mature onion bulbs by preparing 1-cm diameter plugs [3]. After a 20-day incubation period, the basal plates of twenty bulbs per plot were rated for FBR severity on a scale from 1 (no disease) to 9 (≥70% of basal plate infected). The resistant bulbs (rating 1) were advanced to form the next generation [3].

For three KASP trials, leaf samples were collected from the most-recently, fully-expanded plant leaf for all cultivar populations on the day of DNA extraction. In the first and second trials, leaf samples were

collected from one randomly-selected plant per population of three cultivar populations and check cultivars. In the third sampling, leaf samples were collected from three randomly-selected plants from planted bulbs for seed production in cages. These bulbs were selected for their resistance after artificial inoculation for the most advanced selected populations (FBR1-5 & FBR6) of each cultivar. DNA was extracted using NucleoSpin® Plant II Midi kit (MACHEREY-NAGEL GmbH & Co. KG, Germany). The eluted DNA was stored at −20 °C until further analysis. DNA concentration was measured with a NanoDrop Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

SNP Markers and Reaction Setup

Eight markers were selected due to their association with FBR resistance, as demonstrated in the previous research [6,7] and marker sequences were converted to KASP primer sequences (Table 1).

Table 1. Primers producing amplicons and revealing single nucleotide polymorphisms (alleles) in onion using the KASPar platform.

cDNA	Primer for allele X	Primer for allele Y	Common primer	Allele	
				X	Y
Isotig44683_192	CCATAGTACAGCCGCCGCCA	CATAGTACAGCCGCCGCCG	CCTATAGCCGGAAAGGGAAGAACTA	A	G
Isotig38484_281	GTTTTCGCAATTGGGGCCGCT	GTTTTCGCAATTGGGGCCGCG	CCATCAAGCACAATAGATCCAACAAACAA	A	C
Isotig34519_442	ACTGCGCTTCATCATCATGGGC	ACTGCGCTTCATCATCATGGGT	CTCTTACTTGGGTCTGGTAGGCTTT	C	T
Isotig33746_1093	CACCAAATAAGCTGTTCCAAAATTCATG	CACCAAATAAGCTGTTCCAAAATTCATA	TGGAGTTACTGGCACAGTCAAATATAGTT	C	T
Isotig33439_640	ATCACTACGATCCTTTTCCATGTCC	GATCACTACGATCCTTTTCCATGTCT	GATGGTGCTTCCACTGTTGCCATTT	C	T
Isotig31106_505	CACTTGGGTAACACTTTTAGTCCTCA	ACTTGGGTAACACTTTTAGTCCTCG	CGAGGTTTCGAACATGGATGGAAGAA	A	G
Isotig30594_1021	CCTCTTCTAACCATCTTCATTTGAGAT	CTCTTCTAACCATCTTCATTTGAGAC	TAATAGATAGCTCTAGACCTACCGCTAT	A	G
Contig00676_1004	CAACTTCTGAAATAACAGCCTGACTTTTA	CAACTTCTGAAATAACAGCCTGACTTTTT	CCCAACTGATGCTGAATTGGTATTATACTA	A	T

Each SNP marker had its separate KASP Assay Mix with two competitive allele-specific primers and one common reverse primer (LGC, Biosearch Technologies™, London, UK) [18]. The allele-specific primers are customized with unique tail sequences, each tailored to correspond with a specific universal Fluorescence Resonant Energy Transfer (FRET) cassette [19]. These cassettes consist of one primer tagged with FAM™ dye and another with HEX™ dye. In the KASP-TF Master Mix, these universal FRET cassettes are combined with ROX™ passive reference dye, Taq polymerase, free nucleotides, and $MgCl_2$, all carefully formulated in an

optimized buffer solution [19]. KASP-TF Master Mix with standard ROX (2.5 mL) was used to prepare the reaction mix (LGC, Biosearch Technologies™, London, UK). The quantitative polymerase chain reaction (qPCR) was implemented for KASP genotyping using the BIO-RAD CFX-96 real-time PCR (Bio-Rad Laboratories, Inc., CA, USA). All samples were standardized to a uniform concentration, each containing the equivalent of 30 ng of DNA used for genotyping. No template control (NTC) without any DNA sample was included to detect any contamination or non-specific amplification that might occur during the PCR reaction. The constituent reagent volumes were prepared for all the samples and the reaction volume was set to 10 μL with 5 μL DNA and 5 μL genotyping mix per well. In each well of a 96-well plate, 5 μL of DNA was combined with 5 μL of 2X KASP-TF Master Mix, and 0.14 μL of KASP Assay Mix, resulting in a total reaction volume of 10 μL. Eight genotyping mixes were prepared for eight SNP primers by mixing each KASP Assay Mix with Master Mix (LGC, Biosearch Technologies™, London, UK). PCR plates were covered with a lid and centrifuged before running in the BIO-RAD CFX-96 real-time PCR. Before running, FAM and HEX fluorophores for channel 1 and channel 2 were selected, respectively and protocol for the KASP assay was added in the CFX manager software (LGC, Biosearch Technologies™, London, UK) (Table 2).

Table 2. Thermal cycling conditions for the KASP assay [20].

Step	Description	Temperature (℃)	Time	Number of cycles/step
1	Activation	94	15 min	1
2	Denaturation	94	20 sec	10 cycles
	Annealing/elongation	58	60 sec (drop 0.6 ℃ per cycle)	
3	Denaturation	94	20 sec	26 cycles
	Annealing/elongation	55	60 sec	

Additional cycling conditions for compact clustering included denaturation at 94 ℃ for 20 seconds, repeated for 3 cycles, followed by annealing/elongation at 58 ℃ for 60 seconds [20]. Plates were read after the cycles were finished and allelic discrimination was observed.

Data Analysis

Clustering based on different markers was performed using built-in CFX manager software for the BIO-RAD CFX-96 real-time PCR. Allele frequencies for each SNP marker were calculated based on the observed genotype in individual plant samples representing each population. For the X allele, a frequency of 1 indicates its presence, while a frequency of 0 means its absence. Similarly, for the Y allele, a frequency of 1 indicates its presence, while a frequency of 0 means its absence. In the case of

heterozygous genotype (H), both X and Y alleles have a frequency of 0.5. FBR incidence was determined by calculating the proportion of infected bulbs out of 20 random bulbs per plot. Moreover, mean FBR incidence (%) was calculated using Proc MEANS in SAS Studio in the web-based environment, SAS® OnDemand for Academics (SAS Institute Inc., Cary, NC, USA). Correlation analysis between the allele frequencies of each SNP marker and FBR incidence across all cultivar populations was conducted using Proc CORR.

RESULTS

To identify the most informative primer sets for revealing chromosomal regions associated with FBR resistance in short-day onion cultivars, eight SNP markers previously implicated in FBR resistance were selected for validation within our cultivar populations. It was observed that the selected cultivar populations differed in their FBR incidence (%). All populations exhibited a reduced FBR incidence compared to the susceptible check cultivar ($p < 0.05$) (Figure 1). Additionally, the most advanced selected populations of cultivars demonstrated a lower FBR incidence (%) compared to the initial population, except L5 (an advanced selected population of 'NuMex Luna'), which exhibited comparable levels to the initial population (L1).

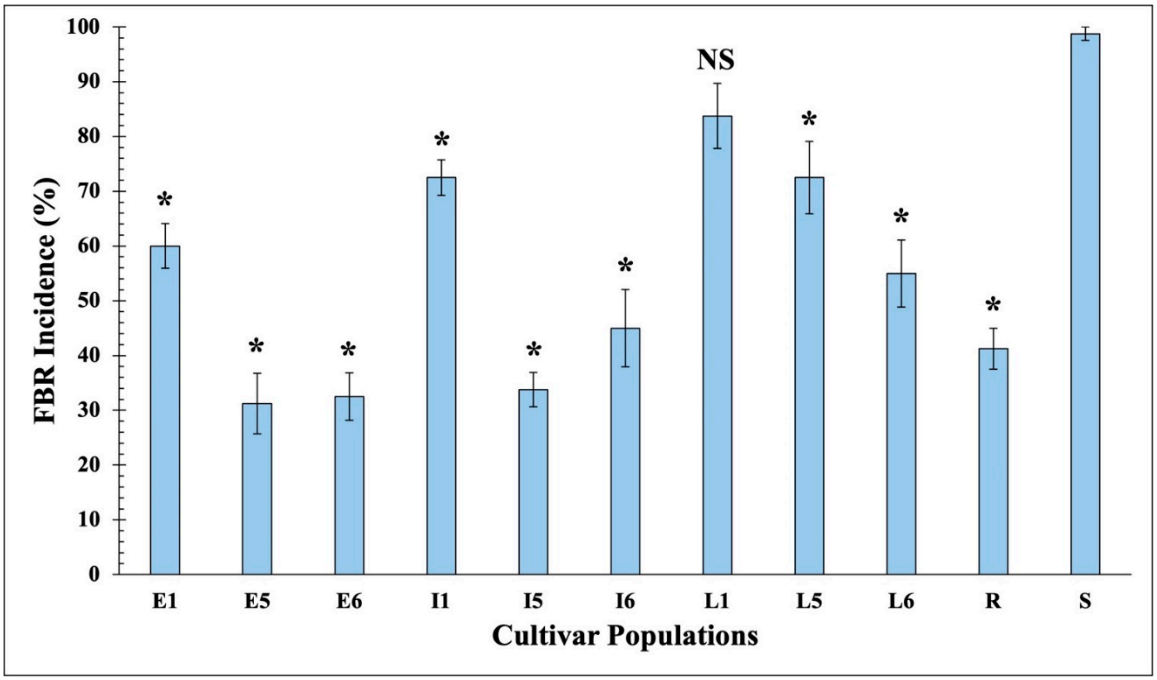

Figure 1. Average FBR incidence (%) for all cultivar populations. E1, I1, and L1 are the initial populations of 'NuMex Camino', 'NuMex Mesa', and 'NuMex Luna', respectively. E5 & E6, I5 & I6, and L5 & L6 are the most advanced selected populations of 'NuMex Camino', 'NuMex Mesa', and 'NuMex Luna', respectively. 'R' is the partially-resistant check, 'Serrana', and 'S' is the susceptible check, 'NuMex Crimson'. FBR incidence (%) was determined by calculating the proportion of infected bulbs out of 20 bulbs per plot. Error bars are the standard errors of the mean for FBR incidence (%). *—Significantly different from the susceptible check (S) and NS—non-significant difference ($p < 0.05$).

In exploring the genetic basis of FBR resistance among various cultivar populations, including susceptible and partially-resistant check cultivars, genetic variation was observed. Our analysis aimed to identify genotypic differences and SNP markers associated with FBR resistance, by distinguishing fixed alleles from those exhibiting heterozygosity. For the initial two KASP runs involving a single plant sample from each population, consistent biallelic discrimination was observed across cultivar populations, except for minor inconsistencies for two SNP markers (*Contig_00676_1004* and *Isotig_33746_1093*), possibly due to inherent plant-to-plant variation within open-pollinated populations (Table 3). Moreover, For the first trial, mature plants that were close to being harvested were sampled for DNA extraction which did not yield high-quality DNA. Improved DNA quality was obtained in the second run by sampling young seedlings for leaf samples. The second run contained two technical replicates for performing SNP genotyping. Among the eight SNP markers tested, most of the markers showed inter-population genetic diversity. For instance, *Isotig_44683_192* exhibited the prevalence of allele X in populations E1, I5, I6, I1, L6, and the partially-resistant check, while heterozygosity was observed in populations E5, E6, L5, L1, and the susceptible check (Table 3).

Table 3. Bi-allelic scoring of eight SNP markers across different short-day onion cultivar populations including check cultivars.

	NuMex Camino[x]			**NuMex Mesa[y]**			**NuMex Luna[z]**			**NuMex Crimson[1]**	**Serrana[2]**
SNPs*	**E5**	**E6**	**E1**	**I5**	**I6**	**I1**	**L5**	**L6**	**L1**	**S**	**R**
Isotig_44683_192	H	H	X	X	X	X	H	X	H	H	X
Isotig_38484_281	X	X	Y	H	H	H	Y	H	X	X	H
Isotig_34519_442	X	X	X	X	X	X	H	Y	Y	X	X
Isotig_33746_1093	X	X	X	X	Y	H	X	H	X/H[#]	Y	X
Isotig_33439_640	X	X	X	X	H	X	H	X	X	Y	H
Isotig_31106_505	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Isotig_30594_1021	X	X	X	X	Y	X	Y	X	Y	Y	H
Contig_00676_1004	Y	Y	H	H	H	Y	H	X	X	Y	H/Y[#]

[x]NuMex Camino populations where E5 & E6 are the most advanced selected populations and E1 is the initial population. [y]NuMex Mesa populations where I5 & I6 are the most advanced selected populations and I1 is the initial population. [z]NuMex Luna populations where L5 & L6 are the most advanced selected populations and L1 is the initial population. [1]NuMex Crimson (S) is the susceptible check cultivar. [2]Serrana (R) is the partially-resistant check cultivar. *For allelic discrimination at each SNP, X is allele 1, Y is allele 2 and H is heterozygous (XY). [#]Differences in the first and second KASP runs for bi-allelic discrimination.

Isotig_38484_281 also exhibited varied allelic composition, with allele X predominating in populations E5, E6, L1, and the susceptible check, whereas heterozygosity was noticed in populations I5, I6, I1, L6, and the partially-resistant check (Table 3). *Isotig_34519_442* showed the predominance of the X allele in 'NuMex Camino' (E6, E5, and E1) and 'NuMex Mesa' (I6, I5, and I1) populations including both check cultivars. Further, *Isotig_33746_1093* exhibited allele X prevalence in the majority of cultivar populations including E6, E5, E1, I5, L5, and the partially-resistant check, while populations I6 and the susceptible check demonstrated allele Y (Table 3). Moreover, allele X frequencies for this SNP marker exhibited a negative correlation (−0.55) with FBR incidence (%) across all cultivar populations (p < 0.1). Additionally, a greater number of populations clustered closely with the partially-resistant check in the cluster plot (Figure 2).

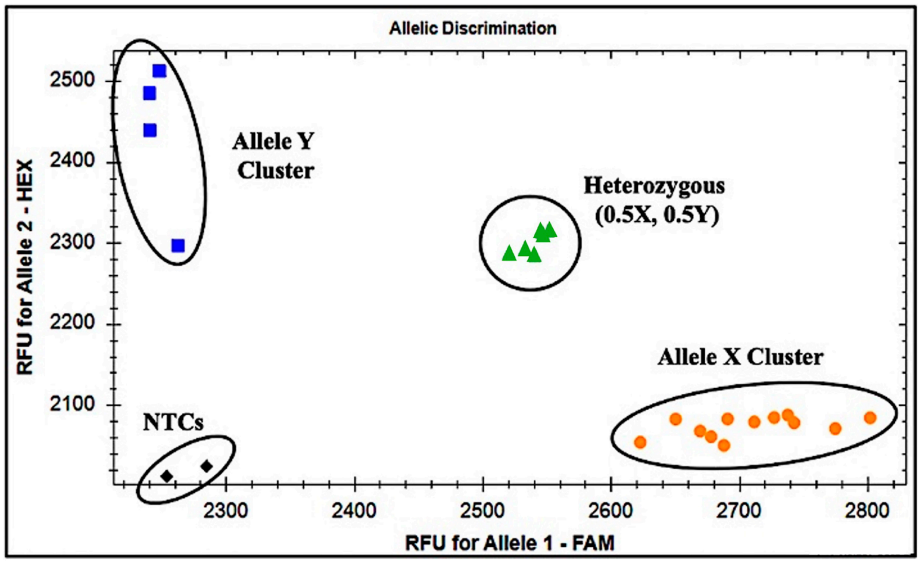

Figure 2. Cluster analysis for *Isotig_33746_1093* from the second KASP run including two replicates of a plant sample from each cultivar population including check cultivars. Relative Fluorescence Units (RFU) values for allele 1 (allele X) (labeled with FAM) and allele 2 (allele Y) (labeled with HEX) measure the fluorescence emitted by a fluorophore sensed by a detector. Cluster plot showing KASP genotyping results: Blue = HEX homozygotes, Green = Heterozygotes, Orange = FAM homozygotes. Black dots indicate no template controls (NTCs).

Isotig_33439_640 had an abundance of allele X in most of the cultivar populations, such as E6, E5, E1, I5, I1, L6, and L1, while Y allele in the susceptible check. *Isotig_31106_505* consistently presented allele Y across all populations, indicating monomorphism, and was excluded from allele frequency calculations. *Isotig_30594_1021* exhibited allele X prevalence in E6, E5, E1, I5, I1, and L6 populations, whereas allele Y in I6, L5, L1, and the susceptible check. Allelic frequencies were calculated to observe the prevalence and distribution of specific alleles within populations, thereby providing important insights into the fixation of alleles responsible for FBR resistance (Figure 3a,b).

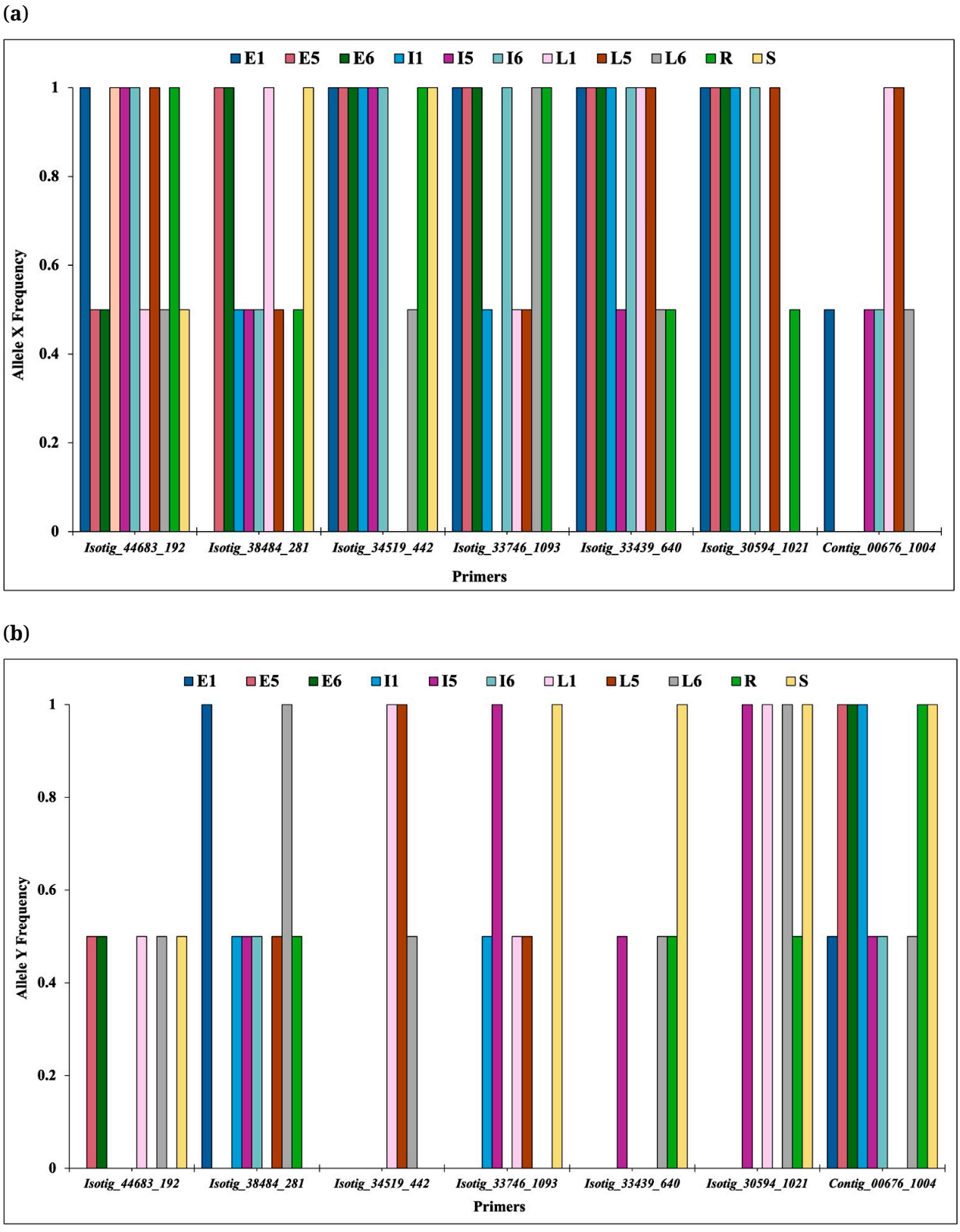

Figure 3. Comparison of allelic frequencies at eight SNPs, (**a**) allele X frequency, (**b**) allele Y frequency, across all different cultivar populations including check cultivars. E1, I1, and L1 are the initial populations of 'NuMex Camino', 'NuMex Mesa', and 'NuMex Luna', respectively. E5 & E6, I5 & I6, and L5 & L6 are the most advanced selected populations of 'NuMex Camino', 'NuMex Mesa', and 'NuMex Luna', respectively. 'R' is the partially-resistant check, 'Serrana', and 'S' is the susceptible check, 'NuMex Crimson'.

The third run included the most advanced selected populations from three cultivars, revealing intra-population diversity (Table 4). Interestingly, the SNP marker *Isotig_44683_192* exhibited variation among plants in populations E5, E6, and L5. Similarly, *Isotig_38484_281* presented differences among plants for I5, I6, and L6 populations. Conversely, the SNP marker *Isotig_34519_442* demonstrated differences only in the L5 population. Furthermore, within-population differences were evident in populations I6 and L6 for the SNP marker *Isotig_33746_1093*. Moreover, no differences were observed for the SNP marker *Isotig_31106_505*, which remained consistent across the three KASP runs. However, *Isotig_33439_640* exhibited variations in populations I6 and L5, with three plants showing variation. Additionally, differences were detected for the SNP marker *Isotig_30594_1021* in populations I6, L5, and L6, while the SNP marker *Contig_00676_1004* presented plant-to-plant variations in populations E5 and I5.

Table 4. Bi-allelic scoring of eight SNP markers for three plants from the most advanced selected populations of three short-day onion cultivar populations.

	NuMex Camino[x]						**NuMex Mesa[y]**						**NuMex Luna[z]**					
SNPs*	**E5**			**E6**			**I5**			**I6**			**L5**			**L6**		
Isotig_44683_192	X	H	H	H	H	X	X	X	X	X	X	X	X	H	Y	X	X	X
Isotig_38484_281	X	X	X	X	X	X	X	Y	Y	Y	Y	H	Y	Y	Y	H	H	Y
Isotig_34519_442	X	X	X	X	X	X	X	X	X	X	X	X	H	H	Y	H	H	H
Isotig_33746_1093	X	X	X	X	X	X	X	X	X	X	H	H	X	X	X	H	H	Y
Isotig_33439_640	X	X	X	H	H	H	X	X	X	X	Y	Y	X	H	H	X	X	X
Isotig_31106_505	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Isotig_30594_1021	X	X	X	X	X	X	X	X	X	X	H	H	Y	Y	H	H	Y	X
Contig_00676_1004	Y	H	Y	Y	Y	Y	H	H	Y	Y	Y	Y	H	H	H	X	X	X

[x]NuMex Camino populations where E5 & E6 are the most advanced selected populations. [y]NuMex Mesa populations where I5 & I6 are the most advanced selected populations. [z]NuMex Luna populations where L5 & L6 are the most advanced selected populations. *For allelic discrimination at each SNP, X is allele 1, Y is allele 2 and H is heterozygous (XY).

DISCUSSION

In this study, we sought to identify the presence of the eight SNP markers associated with FBR resistance corroborated by findings from two separate studies using our New Mexican short-day onion cultivar populations selected for FBR resistance. These two independent investigations identified distinct chromosomal regions associated with FBR resistance. Among SNP markers associated with FBR resistance and seedling vigor in the previous study by Taylor et al. [6], *Isotig_33746_1093*,

Isotig_33439_640, and *Isotig_30594_1021* were the most informative ones in our cultivar populations. *Isotig_33746_1093* stands out as the only SNP marker exhibiting a clear allelic discrimination between the susceptible and the partially-resistant check cultivars. It also showed near fixation of allele X within the most advanced selected populations (E5, E6, I5, and L5*)*. Moreover, a negative correlation of allele X frequencies with FBR incidence (%) along with a greater number of populations clustering with the partially-resistant cultivar highlighted the potential utility of the SNP marker *Isotig_33746_1093* in effectively distinguishing between the resistant and the susceptible genotypes. However, *Isotig_33746_1093* has yet to be mapped on one of the onion chromosomes, despite demonstrating significant associations with root and shoot growth, including FBR resistance [6].

Within the partially-resistant check, a few bulbs showed complete rotting suggesting the existence of individuals in the population (heterozygosity) with FBR-susceptibility at the genotypic level which might have made it difficult for two SNP markers *Isotig_33439_640*, and *Isotig_30594_1021* to distinguish the partially-resistant check from the susceptible one clearly. Based on this information, the SNP marker *Isotig_30594_1021* which showed fixation of allele X in most of the advanced selected populations, suggested its potential link to the genomic region on chromosome 8 for FBR resistance. Similarly, the SNP marker *Isotig_33439_640* showing a higher abundance of allele X in most of the advanced selected populations revealed its role in FBR resistance. However, this marker has yet to be mapped to an onion chromosome similar to *Isotig_33746_1093*. In their study, Taylor et al. [6] employed a seedling screening assay at the young and mature bulb stages. There was a significant correlation between these two stages for FBR resistance. In contrast, our study conducted a mature bulb screening by artificially inoculating transversely-cut basal plates with a virulent FOC isolate and identifying resistant bulbs with intact basal plates. These different phenotyping methods were correlated with genotypic results to elucidate associations between FBR resistance and SNP markers, that potentially contributed to variation in the observed results.

An additional promising marker, *Isotig_44683_192* identified in a study by Straley et al. [7], was linked to the genomic regions on chromosome 4A for FBR resistance. The SNP marker *Isotig_44683_192*, that showed near fixation of allele X in most of the advanced selected populations including the partially-resistant check, proposed its strong association with the loci on chromosome 4A involved in FBR resistance. This SNP showed an additive effect on FBR resistance by increasing seedling survival [7]. On the other hand, the SNP marker *Isotig_31106_505* with predominance of allele Y across all cultivar populations including check cultivars, suggested no genetic variation at the SNP locus on chromosome 4C associated with FBR susceptibility, as previously demonstrated by Straley et al. [7]. Such fixation may yield positive effects by excluding the detrimental allele in

our cultivar populations. In their study, Straley et al. [7] conducted a seedling screening by inoculating silica sand with a conidial suspension of a virulent FOC isolate before sowing seeds, and seedling survival was calculated for phenotypic evaluation. The difference in screening for phenotypic performance was integrated with genotypic results to understand the association between FBR resistance and the SNP markers, that potentially accounted for variability in the results. Additionally, the diversity among FOC isolates might pose challenges in selecting FBR-resistant germplasm and conducting genetic analyses. In our study, FBR-susceptibility was assessed using a local virulent FOC isolate with its ability to distinguish between FBR-resistant and FBR-susceptible genotypes. Taylor et al. [6] used their most aggressive FOC isolate, FUS2 collected from a grower's field in Lincolnshire, UK, and Straley et al. [7] isolated the highly virulent FOC isolate, ID4ss2 from an FBR-infected onion bulb in Payette, ID USA.

Besides onion, KASP genotyping has also been successfully utilized in other crops for enhancing disease resistance. For instance, QTL analysis identified two major genomic regions on chromosome arms 2BS and 3BS associated with stripe rust resistance in wheat [21]. KASP markers linked to these regions in the study, such as *IWA5377*, *IWA2674*, *IWA5830*, *IWB6491*, and *IWB57990*, are reliable for marker-assisted selection in wheat breeding programs. Moreover, association studies highlighted that KASP markers, RGA-G3Ap103, and PsC8780P118 were strongly linked to Ascochyta blight resistance in peas [22]. Further, *Phytophthora* blight in *Capsicum annuum* L. was mapped on chromosome 5, resulting in the development of 30 KASP markers. Seven of these markers showed 82.7% accuracy in predicting resistance, offering a valuable tool for marker-assisted selection in pepper breeding programs [23]. Besides the successful implementation of the KASP genotyping assay to identify beneficial SNP markers in onions and other crops, it is being improved for more complex genotyping scenarios. Currently, the KASP assay is commonly used for biallelic discrimination where the standard protocol works well when both alleles are almost equally prevalent in the DNA template. However, to detect rare alleles in pooled samples or distinguish more than three genotypes, such as tri-allelic loci, modifications are required. Brusa et al. [24] have presented some modifications to the protocol for these non-traditional applications, including reaction conditions for improving the fluorophore signals from rare alleles which can help increase the KASP assay sensitivity. Moreover, different ways of analyzing this data have been explored to enhance the statistical power of genotyping which will help for complicated scenarios in plant breeding programs.

All the SNPs used in this study for FBR resistance serve as a valuable tool for selection, despite not achieving complete accuracy in distinguishing between susceptible and resistant genotypes. Their contribution can be crucial in marker-assisted selection, where even partial accuracy can still significantly improve the efficiency of breeding

programs by helping identify more robust and reliable markers for FBR resistance. In the future, studies can investigate marker-trait associations for FBR resistance using different virulent isolates of FOC to validate the existing and novel SNP markers associated with FBR resistance. FBR resistance is quantitative in nature and controlled by several quantitative trait loci (QTLs). Given this complexity, the resistant alleles at some SNPs are not completely fixed and plant-to-plant variation still exists, so it is important to further improve FBR resistance in the studied populations. Future efforts should focus on increasing the selection intensity and conducting additional cycles of selection to fix resistant alleles at all identified SNPs. Moreover, employing comprehensive genotyping and genomic selection techniques will help identify and select for additional FBR resistance-associated alleles.

CONCLUSIONS

Certain SNP markers demonstrated near fixation of a specific allele associated with FBR resistance within the most advanced selected populations that suggested a selection pressure favoring FBR-resistant alleles in the populations. Despite allelic fixation for some SNPs, genetic variability was still observed within some populations that demands further selection within those populations. In addition, the sample size for each population is crucial in studying the allelic frequencies. In the future, a greater sample size for each population will improve the potential of statistical analysis for allelic distribution within a population to achieve more accurate results. Two previous studies [6,7] identified SNP markers linked to FBR resistance across distinct chromosomal regions that suggested the presence of multiple QTLs governing FBR resistance in onion. The SNP markers especially, *Isotig_33746_1093*, *Isotig_44683_192*, *Isotig_33439_640*, and *Isotig_30594_1021* offer valuable tools for marker-assisted selection at the early stage of selection after several validations, allowing breeders to combine independently inherited resistance sources and potentially reduce yield losses caused by FBR in onion cultivation.

DATA AVAILABILITY

All data generated from the study are available in the manuscript.

AUTHOR CONTRIBUTIONS

SS and CSC planned this study. SS conducted the experiment and wrote the first draft of the manuscript. CSC reviewed and edited the manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

ACKNOWLEDGMENTS

The authors would like to thank Jennifer Randall for her assistance in the BIO-RAD CFX-96 real-time PCR.

REFERENCES

1. Brayford D. *Fusarium oxysporum* f. sp. *cepae*. Mycopathologia. 1996;133(1):39-40.
2. Sintayehu A, Sakhuja PK, Fininsa C, Ahmed S. Management of fusarium basal rot (*Fusarium oxysporum* f. sp. *cepae*) on shallot through fungicidal bulb treatment. Crop Prot. 2011;30(5):560-5.
3. Sharma S, Cramer CS. Selection Progress for Resistance to Fusarium Basal Rot in Short-Day Onions Using Artificial Inoculation Mature Bulb Screening. Horticulturae. 2023;9(1):99.
4. Mandal S, Saxena A, Cramer CS, Steiner RL. Comparing efficiencies of two selection approaches for improving Fusarium Basal Rot resistance in short-day onion after a single cycle of selection. Horticulturae. 2020;6(2):26.
5. Mandal S, Cramer CS. Improving Fusarium basal rot resistance of onion cultivars through artificial inoculation and selection of mature bulbs. Horticulturae. 2021;7(7):168.
6. Taylor A, Teakle GR, Walley PG, Finch-Savage WE, Jackson AC, Jones JE, et al. Assembly and characterization of a unique onion diversity set identifies resistance to Fusarium basal rot and improved seedling vigour. Theor Appl Genet. 2019;132(12):3245-64.
7. Straley E, Marzu JC, Havey MJ. Genetic analyses of resistance to Fusarium basal rot in onion. Horticulturae. 2021;7(12):538.
8. Black L, Chan EK, Colcol JF, Jones R, Kramer C, Xiang W. QTLs conferring resistance to fusarium basal rot, pink root and complementary pinks in onions. Available from: https://patents.google.com/patent/US11457582B2/en. Accessed on 5 Sep 2024.
9. Bacher JW. Inheritance of resistance to *Fusarium oxysporum* f. sp. *cepae* in cultivated onions [dissertation]. East Lansing (US): Michigan State University; 1989.
10. Vu HQ, El-Sayed MA, Ito SI, Yamauchi N, Shigyo M. Discovery of a new source of resistance to *Fusarium oxysporum*, cause of Fusarium wilt in *Allium fistulosum*, located on chromosome 2 of *Allium cepa* Aggregatum group. Genome. 2012;55(11):797-807.
11. Tan GX. Monosomic alien addition lines: a new tool for studying and using plant genomics. Yi Chuan Hereditas. 2008;30(1):35-45.
12. Abdelrahman M, El-Sayed M, Sato S, Hirakawa H, Ito S, Tanaka K, et al. RNA-sequencing-based transcriptome and biochemical analyses of steroidal saponin pathway in a complete set of *Allium fistulosum—A. cepa* monosomic addition lines. PLoS One. 2017;12(8):e0181784.
13. Semagn K, Babu R, Hearne S, Olsen M. Single nucleotide polymorphism genotyping using Kompetitive Allele Specific PCR (KASP): Overview of the

technology and its application in crop improvement. Mol Breeding. 2014;33:1-14.
14. Cramer CS, Corgan JN. 'NuMex Camino'Onion. HortScience. 2003;38(6):1251-2.
15. Corgan JN. 'NuMex Mesa' Onion. NM Agr Expt Sta Var Rel Not. 1996;1-4.
16. Corgan JN. 'NuMex Vado'and 'NuMex Luna' onion varieties. NM Agr Expt Sta Rel Not. 1995;1-6.
17. Cramer CS, Corgan JN. 'NuMex Crimson'Onion. HortScience. 2003;38(2):306-7.
18. Duangjit J, Bohanec B, Chan AP, Town CD, Havey MJ. Transcriptome sequencing to produce SNP-based genetic maps of onion. Theor Appl Genet. 2013;126:2093-101.
19. Rahman MZ, Hasan MT, Rahman J. Kompetitive allele-specific PCR (KASP): An efficient high-throughput genotyping platform and its applications in crop variety development. In: Kumar N, editor. Molecular Marker Techniques: A Potential Approach of Crop Improvement. Singapore (Singapore): Springer Nature; 2023. p. 25-54.
20. LCG. User guide: KASP end-point genotyping trial kit. Available from: https://biosearch-cdn.azureedge.net/assetsv6/KASP-genotyping-trial-manual.pdf. Accessed on 9 Sep 2024.
21. Wu J, Wang Q, Kang Z, Liu S, Li H, Mu J, et al. Development and validation of KASP-SNP markers for QTL underlying resistance to stripe rust in common wheat cultivar P10057. Plant Dis. 2017;101(12):2079-87.
22. Jha AB, Gali KK, Banniza S, Warkentin TD. Validation of SNP markers associated with Ascochyta blight resistance in pea. Can J Plant Sci. 2019;99(2):243-9.
23. Zhang Z, Cao Y, Wang Y, Yu H, Wu H, Liu J, et al. Development and validation of KASP markers for resistance to *Phytophthora capsici* in *Capsicum annuum* L. Mol Breeding. 2023;43(3):20.
24. Brusa A, Patterson E, Fleming M. Modifications of Kompetitive Allele-Specific PCR (KASP) Genotyping for Detection of Rare Alleles. In: Shavrukov Y, editor. Plant Genotyping: Methods in Molecular Biology. New York (US): Springer; 2023. p. 173-89.

How to cite this article:
Sharma S, Christopher CS. Validating Single Nucleotide Polymorphism Markers for Fusarium Basal Rot Resistance in Short-Day Onion Cultivars through Kompetitive Allele-Specific PCR. Crop Breed Genet Genom. 2024;6(3):e240006. https://doi.org/10.20900/cbgg20240006