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SCoT molecular markers are efficient in genetic fingerprinting of pomegranate (*Punica granatum* L.) cultivars

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Abstract. The pomegranate is an economically important fruit plant species which has been utilized since ancient time as a source of food and medicine by mankind. This plant although is cultivated in certain geographical regions, but its fruits are imported and sold throughout the world. Iran is the center of origin for pomegranate and contains huge number of known cultivars. However, genetic studied on these cultivars are very limited and much detailed information has to be produced for better hybridization and breeding tasks in the country. A fingerprinting study was performed on 178 *Punica* trees in 47 known cultivars by using SCoT molecular markers. We obtained 61 SCoT bands/ loci which were used for genetic diversity analyses and grouping of the cultivars. A low genetic variability was obtained within and among *Punica* cultivars, but as revealed by AMOVA, this was quiet enough to produce significant genetic difference among them. DAPC analysis revealed a trace of genetic admixture among the cultivars either due to gene flow or as a result of common ancestral shared alleles. Discriminating SCoT loci may be used in germplasm evaluation of *Punica*. The genetic difference of these cultivars can be utilized for hybridization and breeding programs.

Keywords: AMOVA, DAPC, genetic diversity, pomegranate, SCoT.

INTRODUCTION

Pomegranate (*Punica granatum* L., family = Lythraceae) is an ancient fruit species originated from Iran (Graham et al., 1998). This plant species is native of Iran and Mediterranean region, and is also cultivated in tropical and subtropical regions (Gundogdu & Yilmaz, 2012, Fischer et al., 2010, Patil et al., 2020). One of the amazing features of pomegranate is its adaptation to wide climatic conditions and it can grow in light and heavy soils, but

it is the only limiting factor of cold winters (Vazifeshenas et al., 2012).

Recent investigations have shown medicinal value of pomegranate plant and particularly, its antioxidant activity and polyphenol content (Li et al., 2006). Interestingly enough, different parts of pomegranate tree, such as fruit peel, seeds, fruits, leaves, and flower have different and special healing (Li et al., 2006). The pomegranate juices, seeds and extracts are used for treating cardiovascular disease, diabetes and prostate cancer (Patel et al., 2008).

Based on morphological features as well as agronomical properties, several pomegranate cultivars are known in Iran (Beghè et al., 2019, Khadivi et al., 2020, Shahsavari et al., 2021a), moreover, there are two main pomegranate germplasm collection centers located in Yazd, and Saveh cities, which contain about 700-1000 labelled pomegranate cultivars. In spite of this huge number of pomegranate cultivars and accessions, we have very little information on their genetic structure and fingerprinting.

Germplasm evaluation requires a suitable system to identify parental lines, genotypes, wild relatives and released varieties. Although the variability in morphological and biochemical characters are useful for the task due to easy scoring and their economical nature, they have some weakness like low variability, environmental influence, epistasis, and complex inheritance pattern. With the advent of molecular markers, DNA fingerprinting became a more objective sensible, and less error-prone method of identifying plant varieties than traditional methods (Poets et al., 2020).

Several factors together may determine the type and number of molecular markers for plant accessions' genotyping. The number of loci required to discriminate verity depends on the diversity of the crop and its genome size. Moreover, the polyploidy level as well as breeding system or pollination mechanism of the plant can affect the genetic variability of the target plant species. The estimated genome size of *Punica granatum* is about 320. Mb (Luo et al., 2020), which is almost a small genome size and shows open pollination as we can obtain hybrids among different pomegranate plant trees.

Recent studies which are concerned with molecular assessment of different plant cultivars and accessions produce data not only on genetic finger printing of target plants but also some information may be obtained on the cultivars' relationship, degree of gene flow among the studied samples, identify discriminating molecular loci or bands, and even identify the loci with potential adaptive value (see for example (Saboori et al., 2020, 2021, Sepahian et al. 2021).

Till present time, different DNA markers, have been utilized to investigate genetic diversity within pomegranate cultivars and illustrate their relationship as well as genetic fingerprinting. These markers are Random Amplified Polymorphic DNA (RAPD, (Sheidai et al., 2007, Hasnaoui et al., 2010, Noormohammadi et al., 2012)), Amplified Fragments Length Polymorphism (AFLP, (Jbir et al., 2008, Moslemi et al., 2010)), Inter-Simple sequence Repeats (ISSR, (Narzary et al., 2010, Ahmed, 2018)), simple sequence repeats (SSR, (Noormohammadi et al., 2012, Zarei et al., 2018, Patil et al., 2020, 2021, Shahsavari et al., 2021a, 2021b)) and Start Codon Targeted polymorphism marker (SCoT, (Ahmed, 2018)).

SCoT technique has been successful in identifying cultivars and analyzing genetic diversity within and between plant species, in many different plant species including crop plants such as wheat (Abdel-Lateif & Hewedy, 2008, Collard & Mackill, 2009), barley (Dora et al., 2017) and potato (Gorji et al., 2011) and also fruit trees such as mango (Luo et al., 2010), grapes (Guo et al., 2012) and date palm (Saboori et al., 2020).

Recently breeding program on pomegranate cultivars in Iran has been focused on producing hybrids with the aim to obtain elite genotypes in Iran. In this regard, attempts are made to evaluate the standing genetic variability of the parental genotypes and their hybrids.

The present study is a part of such investigations with the following tasks: 1- Produce data on genetic structure of the parental genotypes and their hybrids. 2- Estimating the standing genetic diversity within our germplasm collection. 3- Illustrate genetic affinity of the studied samples. 4- Compare discriminating power of SCoT and SSR molecular markers.

MATERIALS AND METHODS

Plant materials

This study was performed on 187 *Punica* tress collected randomly from 47 genotypes, which were grown in Agricultural and Natural Resources Research and Training Center, Yazd, Iran. Among the studied samples, we had also 7 hybrid genotypes, each having four replicates. These were collected from the trees cultivated in Mazandaran province, Iran (Sahebi Pomegranate Cooperative Company -Sari). Details of some of these cultivars are provided in Table 1.

DNA extraction and SCoT PCR amplification

Total genomic DNA was extracted from fresh leaves by CTAB with some modification based on Krizman et

Table 1. Genetic diversity parameters determined in *Punica* genotypes studied.

No	Cultivar name	Geographical location	Accession code	Na	Ne	I	He	%P
1	Rabab Poostghermez	Fars	68-119-1	0.525	1.063	0.061	0.039	13.11%
2	Vahshi Poost ghermez	Roodbar	67-210-2	0.393	1.038	0.034	0.023	6.56%
3	Goojagh Shahpar Vramin	Vramin	69-181-1	0.508	1.082	0.067	0.046	11.48%
4	Makhmal shar Reza	Esfahan	69-143-1	0.492	1.049	0.034	0.025	4.92%
5	Marmar Ramhormoz	Ramhormoz	69-161-2	0.295	1	0	0	0.00%
6	Ardestani torsh Semnan	Semnan	69-179-4	0.475	1.075	0.058	0.04	9.84%
7	Golnar Farsi Shahdad	Kerman	68-541-3	0.525	1.069	0.06	0.04	11.48%
8	Poostsiyah Abrand Abad	Yazd	67-233-1	0.262	1	0	0	0.00%
9	Zaghe Yazdi	Yazd	68-602-1	0.475	1.092	0.07	0.049	11.48%
10	Shirin Shahvar Yazdi	Yazd	70-680-1	0.475	1.043	0.036	0.024	6.56%
11	Goroch Shahvar Yazdi	Yazd	68-546-1	0.656	1.106	0.097	0.064	18.03%
12	Vashik malas	Sistan	67-614-1	0.426	1	0	0	0.00%
13	Bihaste khafri	Jahrom	67-215-1	0.623	1.143	0.111	0.077	18.03%
14	Savehie torsh	Esfahan	67-209-1	0.295	1	0	0	0.00%
15	Togh Gardan Yazdi	Yazd	67-203-1	0.508	1.071	0.057	0.039	9.84%
16	Malas Dane Ghermez Yazdi	Yazd	67-191-1	0.525	1.061	0.054	0.036	9.84%
17	Faroogh Ij Estahban	Fars	67-204-1	0.541	1.066	0.05	0.035	8.20%
18	Malas Pishva Vramin	Vramin	69-173-1	0.41	1	0	0	0.00%
19	Sefid Pooste Dezfooli	Dezfool	69-131-1	0.426	1.016	0.011	0.008	1.64%
20	Shirin Poost ghermez Ramsar	Gilan	69-168-1	0.443	1	0	0	0.00%
21	Tabolarze Aban Mahi	Yazd	69-144-1	0.541	1.103	0.075	0.053	11.48%
22	Vahshi Jangali Sisangan	Sisangan	69-138-1	0.344	1	0	0	0.00%
23	Siyah Dane Shahvar Kan	Tehran	69-132-1	0.361	1	0	0	0.00%
24	Dane Siyah Ramhormoz	Khoozestan	69-120-1	0.541	1.058	0.05	0.034	8.20%
25	Barge Moordi	Charmahl Bakhtiyari	69-108-1	0.426	1.058	0.05	0.034	8.20%
26	Zaghe Droshte Hrabarjan	Yazd	69-151-1	0.475	1.021	0.018	0.012	3.28%
27	Bagh Malek Ize	Khorasan	69-113-1	0.344	1	0	0	0.00%
28	Shirin Poost nazok Darjezin	Semnan	69-174-1	0.656	1.114	0.098	0.065	18.03%
29	Vahshi Shirin Behbahan	Behbahan	69-171-1	0.295	1	0	0	0.00%
30	Golabi haste nazok Sangan	Sangan	67-226-1	0.361	1	0	0	0.00%
31	Fereshte ghermez	Sari	95-3-1	0.393	1	0	0	0.00%
32	Ghandehar	Afghanistan	95-1-1	0.344	1	0	0	0.00%
33	Totsh Miankale	Sari	95-6-47	0.508	1.092	0.07	0.049	11.48%
34	Narm Haste Andarab	Afghanistan	95-7-1	0.344	1	0	0	0.00%
35	Molar	Spain	95-4-1	0.59	1.105	0.077	0.055	11.48%
36	Wonderful zoodras	USA	95-5-1	0.557	1.087	0.063	0.045	9.84%
37	Wonderful dirras	USA	95-9-1	0.377	1.005	0.006	0.004	1.64%
38	Malas Saveh	Saveh	92-29-1	0.361	1	0	0	0.00%
39	Malas Yazdi	Yazd	67-299-1	0.377	1	0	0	0.00%
40	Sefid Pooste Rabi Ardel	Boroojen	69-137-1	0.393	1	0	0	0.00%
41	Code 6	Sari	95-11-1	0.328	1	0	0	0.00%
42	Code 16	Sari	95-23-1	0.311	1	0	0	0.00%
43	Code 17	Sari	95-22-1	0.328	1	0	0	0.00%
44	Code 18	Sari	95-24-1	0.279	1	0	0	0.00%
45	Code 33	Sari	95-16-1	0.295	1	0	0	0.00%
46	Code 40	Sari	95-18-1	0.328	1	0	0	0.00%
47	Code 48	Sari	95-19-1	0.311	1	0	0	0.00%
Total				0.427	1.034	0.028	0.019	4.78%

Na = No. of Different Alleles, Ne = No. of Effective Alleles = 1 / (p^2 + q^2, I = Shannon's Information Index = -1* (p * Ln (p) + q * Ln(q), He = Expected Heterozygosity = 2 * p * q, %P = Percentage of Polymorphic Loci.

al. (2006) (32). We used activating charcoal and polyvinyl pyrrolidone (PVP) for binding of polyphenolics during extraction. The genomic DNA was examined for quality and quantity by using 0.8% agarose electrophoresis and Nanodrop spetrophotometer respectively

Five primers (SCOT5, SCOT6, SCOT7, SCOT8, SCOT8) were selected based on high polymorphic genetic indices (Collard & Mackill, 2009).

For SCoT amplification, 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Parstous, Iran); 2 X PCR buffer, 1.5 mM MgCl2; 0.2 mM of each dNTP (Parstous, Iran) with 0.2 µM of each primer, was implemented for 20µL polymerase chain reaction (PCR). The reactions were amplified in Technethermocycler (Bio-Rad, USA) using the following procedure 5 min at 95°C, 40 cycles of 1 min and 15 sec at 94°C, 1 min and 30 sec at 46.9-52.9°C (SCoT-5 46.9°C, SCoT-6 49.7 °C, SCoT-7 50 °C, SCoT-8 52.6 °C, SCoT-9 52.9°C) and 1 min at 72°C and a final cycle of 5 min at 72°C. All PCR products were visualized on 2 % agarose gel followed by the SYBR Green staining. For fragment size, we used 100-base pair (bp) molecular size ladder (Fermentas, Germany).

Data analysis

In total 61 SCoT bands/ loci were obtained in this study. These bands were coded as binary data (presence = 1, absence = 0), for further analyses. The genetic diversity parameters like, number of alleles (Na), effective number of alleles (Ne), Shannon index (I), Nei's genetic diversity (He), unbiased He (UHe), and percentage of polymorphism (P%)) were determined for the studied cultivars by using GeneAlex ver. 6.4 (Peakall & Smouse, 2006).

Genetic differentiation of the studied genotypes was examined by Analysis of Molecular Variance (AMOVA) as implemented in GeneAlex ver. 6.5 (Peakall & Smouse, 2006). The genetic distinctness of the genotypes and their replicates was determined by TCS- networking as implemented in POPART ver. 3 (Hammer et al., 2001).

We used principal coordinate analysis (PCoA), of PAST ver.3 to differentiate the genetic groups, and discriminant analysis of principal components (Hammer et al., 2001). (DAPC), to identify discriminating SCoT loci among *Punica* genotypes (Jombart et al., 2010). The assignment test of the same program was used to reveal genetic admixture in *Punica* genotypes. These analyses were performed by adegenet package of R (Jombart, 2008).

RESULTS

In total we obtained 61 SCoT bands/ loci in this study. The genetic diversity parameters determined in *Punica*. genotypes based on SCoT markers are provided in Table 1. The mean number of effective alleles (Ne) was almost alike in all *Punica* genotypes studied. However, the mean Shanon index and gene diversity differed in these genotypes. The same holds true for genetic polymorphism as it. varied from 0.0. (complete genetic uniformity within a cultivar), to about. 18%, which is still a low value for within cultivar genetic variability.

AMOVA produced significant genetic difference among the studied *Punica* cultivars. The analysis showed that about 9% of total genetic variability is due to within population diversity, while 91% of genetic difference. occurs due to among cultivar genetic difference.

TCS network constructed based on SCoT loci obtained revealed a high degree of genetic uniformity within replicates of each genotype studied (Fig. 1). This particularly holds true for the genotypes 12, 18, 30, 8, 27, 14, 20, 41, and 42. The replicates of these genotypes were 100% alike in SCoT loci and were positioned on each other in TCS network nodes.

The other genotypes which showed some level of within population genetic variability were also separated from the other genotypes, and their replicates were placed closer to each other than the other genotypes. This result indicates that SCoT markers can differentiate the studied tunica genotypes from each other.

PCoA plot of tunica genotypes (Fig. 2), placed them in four different genetic groups. For example, the genotypes 2, 3, 6, 7, 9, 13, 14, 20, and 29, comprised the first genetic group due to their genetic similarity. The other genotypes formed the rest of genetic groups.

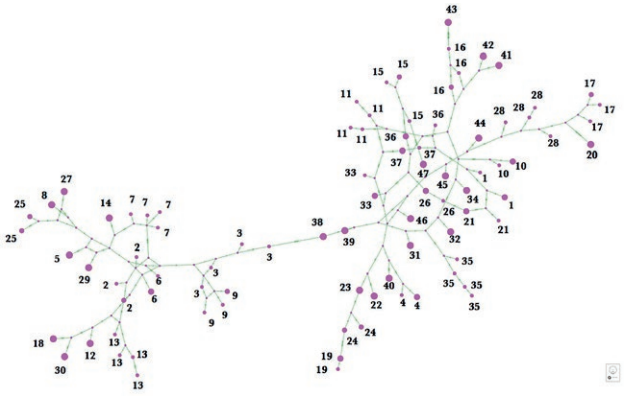

Figure 1. TCS network of *Punica* genotypes based on SCoT data showing that these markers can differentiate the replicates of tunica genotypes from each other.

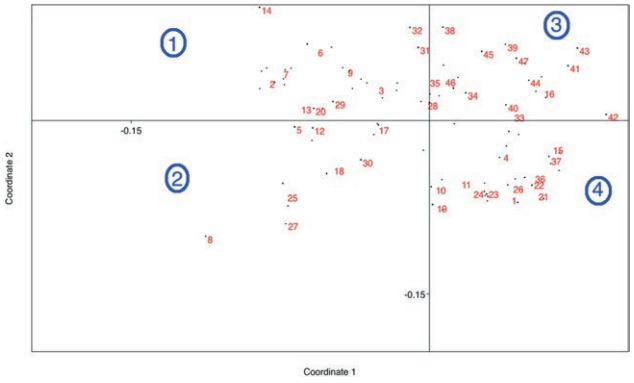

Figure 2. PCoA plot separating *Punica* genotypes in four main genetic groups.

To illustrate the genetic distance between these four genetic groups, we determined Dice' genetic similarity (S), between representative genotypes of each group and from that, we estimated the genetic distance by reducing 1-S. For example, genetic distance between genotypes number 14 and. 8, from the genetic groups. 1 and 2, produced genetic distance D = 0.55. Similarly, D value between genotypes 14 and 42, was 0.62, and between genotypes 8 and 15 was 0.70. Finally, D value between 10 and 42, was 0.40. Therefore, genetic distance obtained between the four genetic groups ranged from 40-70%, which i a high magnitude of genetic dissimilarity, and we can use these genetic differences for further breeding tasks in *Punica*.

DAPC analysis of SCoT data, revealed that the first five Linear discriminant axes (LDA), comprise the highest percentage of discrimination factors (Fig. 3).

LDA plot constructed based on the first two LA axes, grouped the studied *Punica* genotypes in 3-4 genetic groups (Fig. 4), which is in agreement with PCoA analysis presented before.

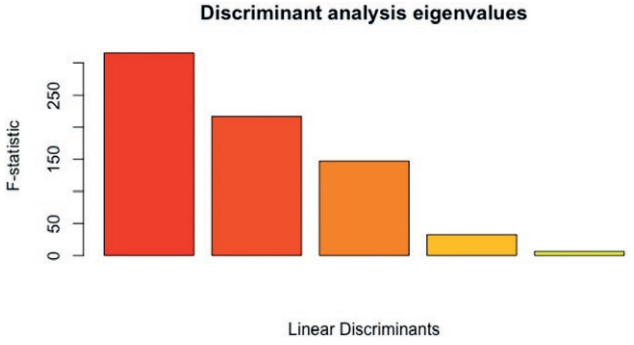

Figure 3. LDA. analysis of. SCoT data in *Punica* genotypes, showing. the first five LDA axes as discriminating factors among them.

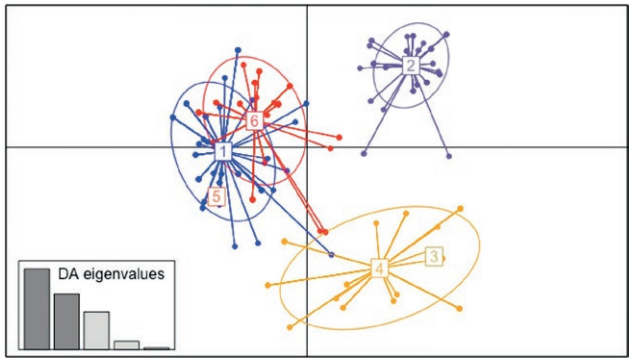

Figure 4. DLA plot of *Punica* genotypes based on SCoT data.

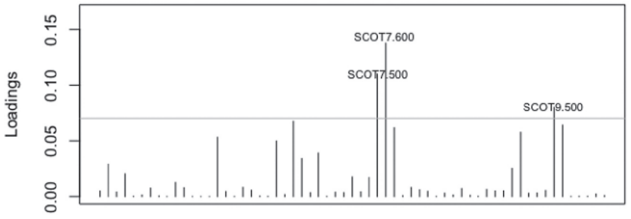

Figure 5. LDA loading of SCoT loci showing important loci of the first LDA axis.

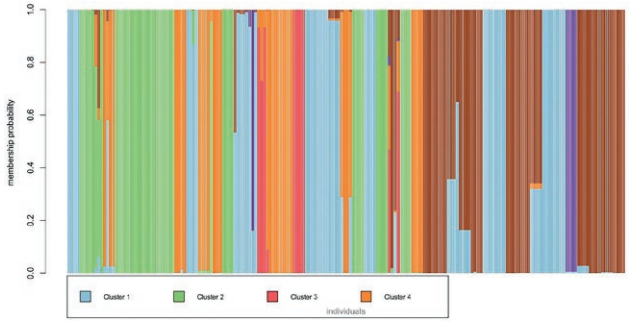

Figure 6. Assignment plot of *Punica* genotypes based on SCoT markers. Similarly colored individuals have similar genetic content, while admixed colors. indicated gene flow or ancestral shared alleles.

LDA analysis identified the SCoT loci with the highest discriminating power (see for exapmle, Fig. 5). SCoT loci 7-500, and 600, as well as SCoT loci9-500, are important. loci of the first LDA axis. Similarly, SCoT loci5-950, and 1000, SCoT7-400, and. SCoT8-800, are important loci of the second LDA axis.

In the third LDA axis, SCoT loci 5-200, 300, and 950, as well as SCoT7-400.

Therefore, of 61 SCoT loci obtained, a combination of SCoT. 5 and 7, may be used for genetic fingerprinting of *Punica* genotypes.

Assignment test of DAPC. analysis (Fig. 6), revealed genetic affinity of the studied *Punica* genotypes (similarly colored individuals). Almost four genetic groups can be identified based on genetic content (similar colors). This plot also revealed some degree of genetic admixture (mixed colors) among *Punica* genotypes studied. The genetic admixture may be due to cross pollination of the genotypes or due to ancestral common shared alleles

DISCUSSION

Genetic fingerprinting as a mean for genetic equation of plants germplasm are very important and of immediate use for planning selection and hybridization programs. Data obtained from these investigations illustrate molecular basis of cultivar differences and if such differences are also accompanied to important agronomic characteristics, then plant breeders have a very good source of genetic material for improvement of that target plant (Nandakumar et al., 2004, Gorji et al., 2011, Nybom et al., 2014, Saboori at al., 2021).

Finding the proper molecular markers for genetic fingerprinting is an essential step in genetic evaluation. For this reason, different molecular markers are used and compared in genetic fingerprinting of economically important plant species. This also holds true for *Punica* plant.

The molecular markers can be assayed for their utility in the cultivar differentiation, and also revealing genetic affinity. The present study revealed that SCoT markers are very efficient markers for both showing within cultivar/ population genetic variability, and also for differentiation *Punica* cultivars. A previous study on twelve pomegranate cultivars grown in Egypt (Ahmed , 2018) showed a high level of genetic variability among the cultivars by using SCoT markers. They reported that none of SCoT primers were able to identify all cultivars independently while our findings on Iranian pomegranate cultivars identified different alleles of SCoT loci that successfully isolated some cultivars. For instance, alleles in SCoT 5, SCoT 6, SCoT 7 and SCoT 9 loci distinguished Poostsiyah Abrand Abad cultivar from other cultivars.

The magnitude of genetic diversity obtained may differ in different molecular markers. However, an interesting similar results are obtained when we compare SCoT and SSR markers results obtained in the same genotypes. Shahsavari et al. (2021), studied the same *Punica* cultivars by SSR molecular markers and reported that these cultivars have a high genetic similarity with genetic distance ranging from 0.005 to 0.52, which is in close agreement with our study based on SCoT markers (10). They also reported significant genetic difference among *Punica* cultivars and that 8% of total genetic variability was due to among genotype difference, while 92% was due to. between cultivar genetic differences, which is almost similar to the present study results of SCoT markers.

These authors (Shahsavari et al. 2021), based on SSR data, reported some degree of genetic admixture among *Punica*. cultivars and also identified discriminating SSR loci to differentiate *Punica*. cultivars. We could also identify a few SCoT loci which can discriminate *Punica* cultivars (10). Also barcoding and mini-barcode analyses based on *trnH-psbA* and *matK* sequences on the same cultivars were provided better resolution of pomegranate cultivars' assignment by Shahsavari et al. 2021b (25).

Therefore, in conclusion we suggest that a combination of SCoT and SSR molecular markers may be used in *Punica* germplasm evaluation and the results obtained on the genetic grouping and genetic difference of these cultivars can be utilized for hybridization and breeding tasks of this important fruit crop.

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AUTHOR CONTRIBUTION STATEMENT

Z.N.: conceptualization of the project**,** data analyses; **M.Sh:** analyses of data; **Sh.Sh** and **F.F.:** data collection and lab work; **MR.V.:** providing samples; **Z.N, M.Sh** project design.

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