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Research Article

ASSESSMENT OF THE DIVERSITY OF SOME CENTER AND SOUTH IRANIAN ONION (*ALLIUM CEPA* L.) POPULATIONS IN RESPECT OF BOLTING RESISTANCE AND ITS RELATIONSHIP WITH SSR MARKERS

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ABSTRACT
Formation of flowering branches at the time of bulb production reduces the quality and quantity of product due to food stored in the bulb. So, studying on how this phenomenon occurs is important to identify the genotypes resistant to bolting. To achieve this aim, the seeds of 16 onion populations of Iran and two foreign cultivars were planted in the pots. After reaching to eight-leaf stage, the plants were kept at a mean temperature of 8°C in cold room for 45 and 70 days. 12 SSR primers were used to investigate the genetic diversity of the studied populations. 10 primers showed acceptable reproduction. The average polymorphism information (PIC) for these primers was 0.38 and the average observed and expected heterozygosity were estimated 0.49 and 0.59, respectively. Cluster analysis based on molecular data using Jaccard's similarity coefficient and UPGMA method, classified the studied onion populations into four major groups. The results of logistic regression showed that there is likely correlation of some alleles and morphological and physiological characteristics that may be related to bolting resistance.

Keywords: *PCR, Cluster Analysis, Flowering, Logistic Regression*

INTRODUCTION
The onion plant (*Allium cepa* L.) is a biennial crop. Onion has great nutritional value because of the minerals, sugar, vitamin C and volatile sulfur compounds (Fritsch and Friesen, 2002). After the potato and tomato, onion is placed in the third place of production in the world. It is consumed as vegetable. It is perishable due to high humidity and it is costly to store it and also, its quality cannot be maintained during storage. Accordingly, continuous production of fresh product is one of the challenges faced with the problem of bolting. Although, flower production is one of the main stages of seed production, but during formation of flowering food stored in the bulb is consumed by the flowering branches. This reduces the quality and quantity of products. So, studying on how this phenomenon occurs and determining the factors causing such loss are important. The objective was to identify the bolting-resistant genotypes and to combine them with native cultivars to produce hybrid seeds leading to more stability of product yield. Critical size of plants for stimulating the production of flowering branches depends on genotype (Brewster, 1985). The times required to vernalize the different genotypes are different. The cultivars which grow in low latitude require shorter time to be vernalize but the ones grown in high latitude relatively require longer time (Alemzadeh-Ansari, 2010). Temperature required to stimulate the flower production varies between 5 to 12 degree centigrade (Khokhar *et al*., 2007; Wiebe, 1990; Brewster, 1982).
Many studies have been performed on the use of molecular marker to examine the diversity. Fisher and Bachmann (2000) identified total 30 microsatellite motifs, most of them have GT motifs and were used them to investigate the germplasm diversity of onion. Their results showed that only 15 0f 30 SSR primer pairs can be used in separating the diploid cultivars of this genus. Dannequin *et al.,* (1997), McCallum (2006) and Mahajan *et al.,* (2009) also used SSR markers for investigation of genetic diversity in onion. Moosavizadeh *et al.,* (2006) examined the diversity within and between of 20 Iranian onion landraces using RAPD markers. Ahmadi-Meshgnany *et al.,* (2015) used 11 RAPD primers to investigate the genetic diversity in thirteen onion populations of Iran and compared with two commercial cultivars. Karimi-Nafchi *et al.,* (2011) investigated the genetic relationships between onion landraces of Iran and compared

them with some commercial cultivars using 15 SSR markers. Their results showed that there is high heterozygosity among evaluated populations. Yun Hyun *et al.*, (2009) performed genetic and molecular studies on onion with crossing between two different varieties in respect of resistance to flower production (MOS8 sterile and resistant to flower production and Guikum sensitive to flower production). Segregation ratios in F2 population indicated that the characteristic of delay in flower production controlled by a dominant gene.

Since no studies have been done on genetic diversity of onion populations of Iran with respect to bolting resistance by SSR markers and with respect to the applications of this marker, useful genetic information can be obtained by investigating the populations genetically and this information can be used in onion breeding program. So, this study aimed to investigate the diversity of some onion (*Allium cepa* L.) populations of center and south Iran with respect to bolting resistance and its relationship with SSR markers.

MATERIALS AND METHODS

The seeds of six onion populations of Iran including Lorestan, Qom, Tehran, Kerman, Hormozgan and Sistan (received from Iranian Gene Bank), 10 indigenous populations including Ramhormoz (Khuzestan), Kazeron (Fars), Sarbiare (Kohgiluyeh and BoyerAhmad), Tashon (Khuzestan), Dil (Kohgiluyeh and BoyerAhmad), Ghalereysi (Kohgiluyeh and BoyerAhmad), Badrod (Isfahan), Bahraghan (Fars) dorcheh (Isfahan) and Natanz (Isfahan) were collected from different regions of center and south of Iran, as well as two foreign commercial cultivars including Golden and Gardesko were planted in Randomized Complete Block Design in pots in greenhouse of Agriculture school of Yasouj University (10-12 seeds of each population planted in a pot consisting of soil, sand, manure in the ratio of 1:2:2). After reaching to eight leaf stage, plants were transferred to the cold room. The average temperature of cold room was 8 °C and photoperiod was 11 hours brightness to simulate winter condition. To evaluate the need of cold for bolting, two different treatment times (45 and 70 days) were considered. After that treatment, pots were transferred to the greenhouse. Morphological traits such as plant height, weight, diameter, length and volume of bulbs and total soluble solids of leaves (by refractometer) and total soluble protein of leaves (by method provided by Kara and Mishra, (1980)) were measured. For molecular analysis, the sample leaves obtained from 4-leaf stage were used and DNA was extracted by Murray and Thompson (1980) method. The quality and quantity of extracted DNA were evaluated through spectrophotometry and electrophoresis on agarose gel. 12 SSR primer pairs (Table 1) were used and their characteristics were listed in Table 1. All of them were selected based on the study by Fischer and Bachman (2000) and synthesized by Takapozist Company (Tehran, Iran).

To perform PCR, a tube with 4 uL template DNA (10 ng/L), 0.75 uL $MgCl_2$ (50 mM), 0.5 uL dNTP (10 mM), 0.125 uL Taq DNA polymerase (5 unit/uL), 2 uL primer (10 ppm), 2.5 uL buffer PCR (1x). PCR program included denaturation of genomic DNA for 2 minute at 94°C, and denaturation of DNA for 30 second at 94°C. The annealing temperature of each primer pairs was set according to table 1 and continued with extension for 60 second at 72°C with 35 replications and final extension was done for 10 minute at 72°C and finally; electrophoresis of PRC was performed on 2 percent agarose gel. Then, to analyze the data, the patterns of bands were identified based on the presence and absence of band (Quoted by Asgarinia *et al*., 2011). Molecular analysis was done using NTSYS-pc (version 2.02e) and GENALEX (version 6.5) and Popgene1.32 software. To draw dendrogram of genetic relationships between local onions populations and commercial cultivars, Jaccard's coefficient was chosen as the most proper coefficient to draw dendogram after comparing three coefficients of Dice, Jaccard and Simple Matching through Mantel test. According to Jaccard's similarity matrix, different methods of clustering were compared and given to cophenetic correlation coefficient between similarity matrices and dendogram (r=0.769), UPGMA clustering method created the most appropriate classification. To identify positive markers associated with morphological characters and to examine the relationship between the markers and the most important features of resistance to bolting, non-linear logistic equation (for binary observations) was used. In this part, SPSS V.20 software was used.

Table 1: Characteristics of Used Primers

Primer	Sequence of Primer (5'→3')	Annealing Temperature
AMS 04	F: TAT GTT TTC AGC TGC GAT GTG AG R: AAA TCT AAG CAC GGA TAC CAA GTG	56
AMS 06	F: TGC GAA TGT GAG GTT TTC TGC R: CGA CCC GGA AAT TTC GAT C	55
AMS 08	F: GCC ACG ATG TTG AGA TTT CG R:CCC GAA TAT CCC ACC AGT TC	56
AMS 10	F: TTC ATG TTG TAT TGA GAT TTG G R: GAA GGA ATG GAA GCA GTT C	52
AMS 12	F: AAT GTT GCT TTC TTT AGA TGT TG R: TGC AAA ATT ACA AGC AAA CTG	56
AMS 13	F: CCC CTG AGT AAA TTC AAA ATC C R: TCC TTA GTA TAA TTT CGG GGT AAC	58
AMS 14	F: CCC CTG AGT AAA TTC AAA ATC C R: TCC TTA GTA TAA TTT CGG GGT AAC	60
AMS 16	F:CTG CAT TAA AAC AAC CAA ACT TG R: GAG CTC CAC TTC TTC CAA ACT AG	58
AMS 23	F: GCT GTT CAC TGG TCT ATC TGG R: ATT CGG TGC TGA TTT TCG	58
AMS 26	F: ATC TAA TCA AAG CAT ATG TG R: TTG TCC AAG TAG TTG TGA	52
AMS 29	F: CAT CAG AAA ATC GAC TCA C R: TTG AAA CTT GGA AGG TTG TC	54
AMS 30	F: CAC TAA TGG GGT AAA TAA TGT TCT AC R: TTG CCT TGA AAT CCA GAC	57

RESULTS AND DISCUSSION

10 of 12 SSR primer pairs had ability to reproduce the right microsatellite regions and produce the right bands in genotypes of Iran and foreign commercial cultivars (Figure 1).

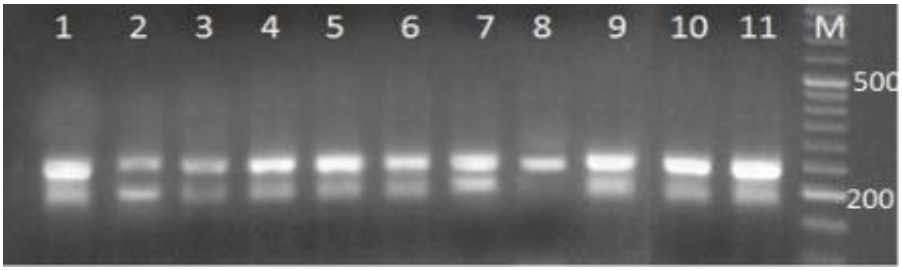

Figure 1: Banding Patterns of AMS16 Primer for Ramhormoz (1), Kazeron (2), Sarbiare (3), Tashon (4), Dil (5), Ghalereysi (6), Badrod (7), Natanz (8), Bahraghan (9), Lorestan (10), Qom (11) Populations, M: 50 bp Weight Marker (Cinnagen Company, Iran)

AMS04 and AMS08 primers were not suitable. In this study, except AMS06 primer, other primers were polymorphic. These 10 primers totally produced 19 alleles with average of 1.9 alleles per locus (Table 2). Karimi-Nafchi *et al.*, (2011) reported 43 alleles of 13 SSR loci in 20 different onion genotypes. Differences in the number of alleles in different studies may be due to different number of genotypes and differences in their genetic basis. The number of efficient alleles per locus varied from 1 in AMS06 to 2 in AMS26 and their average was 1.73. The mean expected and observed heterozygosity was estimated 0.41 and 0.49, respectively. Heterozygosity per locus is the frequency of heterozygous individuals for the

identified position to total number of individuals in the general population (Falconer, 1989). The highest and lowest observed heterozygosity were obtained in AMS26 and AMS06 loci, respectively. This heterozygosity in more markers caused by the reproduction form of plant. Since onion is cross-pollinated crop, so the expected heterozygosity is high. Karimi-Nafchi *et al.*, (2011) also mentioned that a high percentage of heterozygosity in a cross pollinate onion is justified with its cross pollinate nature, culture conditions, the presence of pollinator insects and even naturally occurrence of cytoplasmic male sterility (although low frequency). Gene diversity or expected heterozygosity show that the probability of difference between random alleles of two genotypes in the highest and lowest expected heterozygosity was between 0.52 and zero and obtained for AMS26 and AMS06 primers, respectively (Table 2). Polymorphism information content (PIC) varied from 0 to 1.00 with an average of 0.38, indicating a relatively high capacity of some markers in separation of genotypes. In the study by Karimi-Nafchi *et al.*, (2011), polymorphism information was reported 0.52. Polymorphism information content is one of the important indices to compare different markers in terms of power and its content can vary from 0 to 1, which uses allele frequencies to determine the separation power of markers (Anderson, 1993). High levels of this index indicate the high polymorphism at one locus and also, separation power of marker (Robeiro-Carvalho *et al*., 2004). As can be seen, the markers with high polymorphism have higher diversity and this shows the correlation between PIC and genetic diversity. Shannon index shows the diversity of each primer. In this study, the mean of Shannon index was 0.56. Except AMS06 and AMS30 loci that their indices were estimated 0.47 and 0, respectively, and showed low diversity, all loci showed the high diversity and the highest Shannon index (0.69) belonged to AMS26 primer (table 2). Analysis of molecular variance showed that in terms of genetic diversity, the difference between and within populations and foreign cultivars was not significant (Table 3). Also, 12% of the total variation related to diversity between populations and foreign cultivars and 88% of it was related to diversity within them. Karimi-Nafchi *et al.*, (2011) also performed similar study on 18 landraces of onions and two foreign cultivars using SSR markers, analysis of molecular variance showed that genetic diversity between and within genotypes was significant.

Table 2: Genetic Diversity Indices Obtained from Assessed Onoio Populations by Using SSR Marker

Primer	Observed Heterozygosity	Expected Heterozygosity	Polymorphism Information Content	Observed Allels	Effective Allels	Shannon`s Index
AMS06	0.00	0.00	0.00	1	1	0.00
AMS10	0.50	0.51	0.08	2	1.97	0.68
AMS12	0.88	0.51	0.79	2	1.98	0.69
AMS13	0.62	0.44	0.35	2	1.75	0.62
AMS14	0.31	0.49	0.16	2	1.93	0.67
AMS16	0.88	0.51	0.79	2	1.97	0.69
AMS23	0.27	0.46	0.39	2	1.8	0.64
AMS26	1.00	0.52	1	2	2	0.69
AMS29	0.18	0.34	0.13	2	1.48	0.51
AMS30	0.35	0.29	0.11	2	1.41	0.47
Mean	0.49	0.41	0.38	1.9	1.73	0.56

Table 3: Analysis of Molecular Variance for Evaluated Onion Populations

Source of Variance	Degree of Freedom	Mean of Square	Variance	Variance Percent	Significant Level
Between Populations and Cultivars	1	3.22	0.29	12	0.19 ^{ns}
Within Populations	16	2.18	2.18	88	0.12

Also, 42% of the total variation was related to the diversity between populations and foreign cultivars and 58% of it is related to the diversity within populations. It shows the high differentiation of onion landraces of Iran.

Cluster analysis divided onion genotypes into 6 groups (Figure 2). Ramhormoz, Sarbiare, Tashon populations were placed in a sub-group of first group and also Kazeron population was placed the same group. Ramhormoz and behbahan population are cultivated in a similar climate condition and it was expected that they were placed in the same group, but Sarbiareh population belongs to Kohgiloyeh and Boyer Ahmad and cultivated in different climatic condition. The second group was divided into two subgroups.

The first subgroup includes Ghalereysi, Hormozgan, Sistan and Dorcheh populations and second sub-group includes Gardesko and Golden foreign commercial cultivars. Iranian populations were placed in the second group and also, foreign commercial cultivars were placed in the same subgroup, this showed the similarity of these populations with foreign commercial cultivars and confirms the results of the study by Dannequin *et al.,* (1997) that mentioned to the migration of onion from Asia to Africa and Europe and also confirms the results of the study by Ahmadi-Meshgnany *et al.,* (2015). In the third group, Badrod and Dil populations were placed in same subgroup and Bushehr and Qom populations were placed in another subgroup.

The fourth group included Lorestan and Kerman populations. Natanz and Bahraghan populations were placed in two groups separately. These results indicate that these populations have less genetic similarity with others. Clustering analysis can help breeder in two cases: finding the real groups of individuals based on genetic similarity between them and reducing data and selecting limited individuals of each group. Clustering of the Iranian populations in different groups showed that there is high genetic diversity among Iranian populations.

As expected, the two foreign commercial cultivars were placed in the same group. Although, these results cannot show the relationship between molecular variation and traits associated with bolting resistance because the foreign commercial cultivars and the genotypes that have resistance to bolting and do not produce flower (Golden, Gardesko and Ghalereysi) and the populations that have high percentage of flower production were placed in the second group (Lorestan, Dorcheh and Sistan), but it can show the similarity of one Iranian population that resistance to bolting (Kerman population) with foreign commercial cultivar.

Chen *et al.,* (2012) reported that there are two reasons for lack of consistency between the classification pattern and geographic origin: the number of used markers is not sufficient to cover the entire genome and duplication of germplasm after long-term storage and reuse of them causes some mistakes and separates the genotypes from each other.

Shourvazdi *et al.,* (2014) and Abdollahi-Sisi (2012) also said that the reason for non-placement of the native populations with geographic origin in same group is related to their vast geographic distribution and also, the impact of different evolutionary forces on the genetic structure of them.

Analysis of Main Component showed that the first and second components explained 75.13 and 5.79 percent of variance, respectively. These two components totally explained 80.92 percent of total variance (Table 4).

In the study of genetic diversity through molecular data, it is better that markers have a homogenous distribution in genome. So, if the marker is selected from different parts of the genome, the correlation between them will be low and therefore, a greater number of them are necessary to explain diversity (Pirseyedi *et al*., 2006).

Cumulative arrangement of onion genotypes using marker-based genetic similarity and these two components are shown in figures 3.

Distribution of genotypes in this figure is largely based on population's distribution in dendogram branches. In general, distribution of the studied genotypes showed high diversity between Iranian genotypes and as expected, two foreign cultivars were placed beside each other and far away from other genotypes.

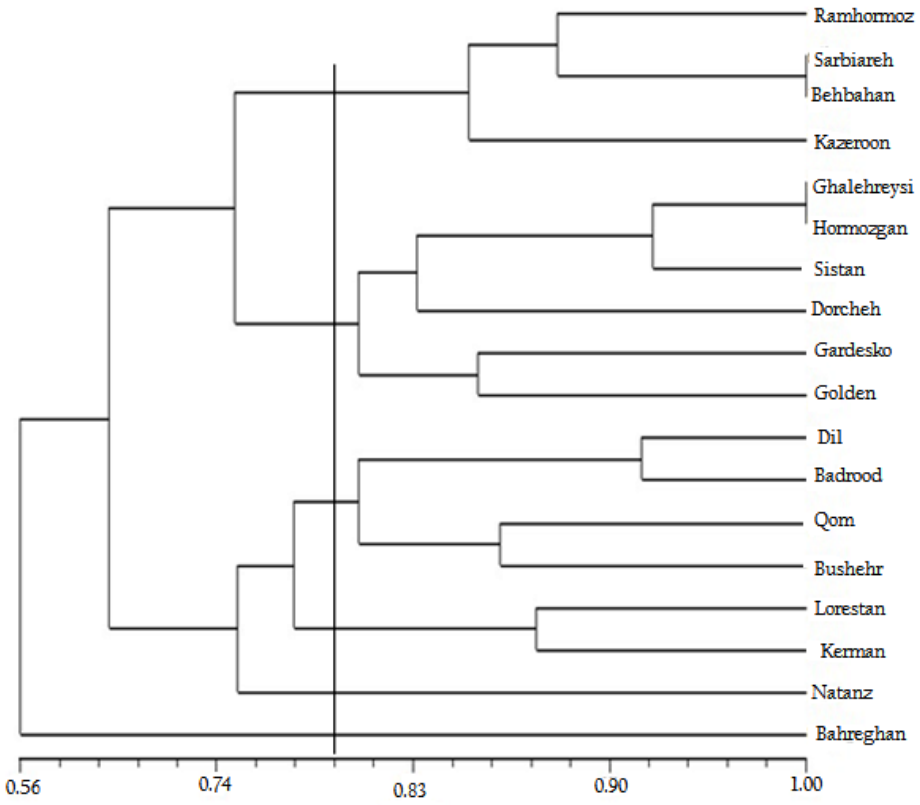

Figure 2: Clustering of Onion Populations Based on SSR Markers

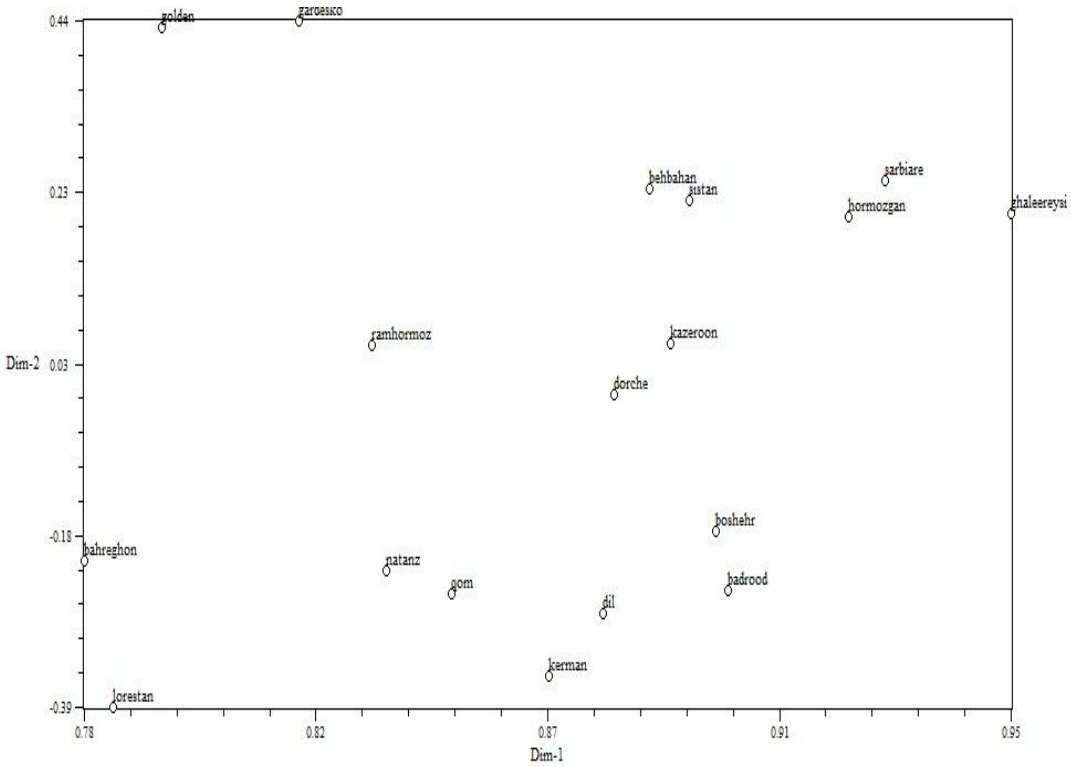

Figure 3: Two Dimensional Graphs of 18 Onion Populations Based on SSR Markers Data

Table 4: Characteristics of Six First Components Based on Molecular Data

Components	Eigen Values	Component Variance	Accumulative Variance
PC1	13.52	75.13	75.13
PC2	1.04	5.79	80.92
PC3	0.71	3.94	84.87
PC4	0.42	2.31	87.18
PC5	0.36	1.98	89.15
PC6	0.32	1.81	90.96

Logistic analyses based on molecular data showed that evaluated traits have not significant effects on alleles because they have not high standard error. In this study, coefficient B was used to examine the relationship between markers and traits. When coefficient B of an independent variable on the dependent variable is positive and large, it is associated with the presence of band and if it is negative, it is associated with the absence of bands. Table 5 shows coefficient B obtained from logistic regression for the measured traits in terms of vernalization during 45 days. According to Table 5, there are relationships between ALL2-P2 Micro-satellite locations and the traits of bulb volume, total soluble protein, soluble sugar and flowering. ALL1-P9 andALL2-P9 alleles are related to the bulb volume, soluble sugar of leaf. The relationships between ALL1-P10 and bulb volume, total soluble solids and total soluble protein were significant. These results showed that in 45-day vernalization, there are relationships between flowering and ALL1-P2, ALL1-P5 and ALL2-P5 and the maximum impact coefficient (432.28) belongs to ALL1-P5. Also, there are relationships between the trait of soluble solids and ALL2-P2, ALL2-P5, ALL1-P9, ALL2-P9 and ALL1-P10.

Table 5: Effect Coefficients B in Binary Logistic Analysis between Some Onion Traits and SSR Alleles in 45 Day Vernalization Conditions

Primer / Traits	ALL 1-P2	ALL 2-P2	ALL 2-P3	ALL 1-P4	ALL 1-P5	ALL 2-P5	ALL 2-P6	ALL 1-P7	ALL 2-P7	ALL1 -P9	ALL 2-P9	ALL 1-P10
Plant height	0.097 -	-9.7	-1.61	0.163	0.176	32.05 -	-0.64	0.057	0.023 -	3.8	-3.5	-3.08
Bulb diameter	0.102	12.01	-1.46	-0.45	1.77	-6.48	-1.55	0.078 -	0.07	0.97	-1.4	26.7
Bulb weight	1.62	-46.2	48.86	4.36	13.8	-21.3	75.2	-1.9	4.6	-54.4	21.9	105.5 -
Bulb length	0.062	-26.5	0.54	0.37	-8.11	19.2	-2.24	00.34	0.497 -	14.6	-3.5	2.2
Bulb volume	-3.62	246.3	45.67 -	0.47	160.1 2	10.3	13.3	-4.4	5.6	142.0 -2	22.5	276.8 -
Total soluble Protein	-0.3	165.5 -	6.98	-3.32	172.9 -	178.9	9.9	-1.14	0.284	45.2	-4.7	131.1
Total Soluble Solids	-0.39	209.1 -	-3.7	-2.18	82.97	172.9	14.6	-2.2	1.5	-100.4	130.4	605.2 -
Flower production	1.84	313.7 -	43.3	-8.13	432.2 8	224.9	64.03	-1.2	4.5	69.3	83.4	-42.9

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