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Genetic Variability, Structure Analysis, and Association Mapping of Resistance to Broomrape (*Orobanche aegyptiaca* Pers.) in Tobacco

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ABSTRACT

Broomrape is a debilitating holoparasiting weed in tobacco (*Nicotiana tabacum* L.) fields with devastating effects on its production. In this study, the reaction of 89 tobacco genotypes was evaluated against broomrape (*Orobanche aegyptiaca*) in randomized complete block design with three replications during two years. In each year, genotypes were planted in both non-inoculated and inoculated conditions where the soil of pots was mixed with 0.06 g of broomrape seed. Considering the average data of two years, studied genotypes did not show infection to broomrape at non-inoculated condition, whereas in inoculated condition, the majority of genotypes showed infection to broomrape. Two genotypes including 'TB 22' and 'Kramograd NHH 659' did not show any infection to broomrape in inoculated condition. In a molecular experiment, the fingerprint of tobacco genotypes was prepared with 26 SSR loci. Using model-based Bayesian approach, the studied association panel was divided into three subgroups. The D′ was used to test the LD between pairs of SSR loci using the software package TASSEL. 7.08% of possible SSR locus pairs showed significant level of linkage disequilibrium (P<0.01). By using mixed linear model, 5 SSR loci from linkage groups 2, 10, 11 and 18 of tobacco reference map were identified as DNA markers to be linked to gene(s) controlling broomrape resistance in tobacco.

Keywords: Association analysis, Broomrape, Linkage disequilibrium, Tobacco.

INTRODUCTION

Orobanche genus (from Orobanchaceae family), commonly known as broomrape, are achlorophyllous annual or perennial plants, which parasitize the roots of various dicotyledonous plants (Roman *et al.,* 2003; Schneeweiss *et al.,* 2004). The *Orobanche* genus has more than 150 species (Musselman, 1980), with *O. aegyptiaca* having the widest host range and parasitizing many Solanaceous crops such as potato (*Solanum tuberosum* L.), tobacco (*Nicotiana tabacum* L.), tomato (*Lycopersicon esculentum* Mill.), members of Brassicaceae and Fabaceae, and several other families. Also, there are other invasive species of *Orobanche* such as *O. crenata* which parasitize forage legumes including *Lathyrus sativus* L., *L. cicera* L., *Vicia sativa* L., *V. villosa* L. (Linke *et al.,* 1993) and *O. cumana* Wallr. which is the most important parasite in sunflower (Pacureanu-Joiţa *et al.,* 2009).

Being the most important nonfood crop, tobacco (*Nicotiana tabacum* L.) is cultivated in more than 100 countries on approximately 4.2 million hectares of crop lands (Davalieva *et al.,* 2010). Beside leaves as the economic part of the plant, tobacco seeds contain 38% of nonedible oil, which could be an appropriate substitute for diesel fuel (Giannelos *et al.,* 2002).

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Several strategies, such as cultural practices and chemical control, have been developed to control broomrape, but none were successful (Mariam and Suwanketnikom, 2004; Rubiales *et al.,* 2009). For instance, applying herbicides is extremely difficult because the holoparasite is directly connected to the host plants and predominantly subterranean and produces numerous, durable, and small seeds (Buschmann *et al.,* 2005). Given such a scenario, the development of Orobanche-resistant varieties is a more efficient way of limiting the effects of this parasitic genus on host plants (Perez-de-Luque *et al.,* 2008). Breeding for resistance is the most economic, feasible, and environmental friendly method of control (Slavov *et al.,* 2005). Host genetic resistance is generally considered critical to successful integrated pest management programs (Goldwasser *et al.,* 1999). Parker and Riches (1993) reported significant genetic variability for resistance to broomrape (*O. aegyptiaca*) among tobacco varieties.

Plant breeding based on molecular tools, such as DNA markers, has revolutionized conventional breeding activities in recent years. Identification of DNA markers linked to interested trait could improve the efficiency of selection via marker-assisted selection. Among DNA markers, SSRs or microsatellites as multiallelic, chromosome-specific, and evenly distributed along chromosomes, have been developed and widely used in tobacco genetic diversity and mapping studies (Davalieva *et al.,* 2010; Bindler *et al.,* 2011; Darvishzadeh *et al.,* 2013; Hatami Maleki *et al.,* 2013).

Two approaches are mostly used for Quantitative Trait Loci (QTL) mapping in crop species: Family-Based Linkage (FBL) mapping and linkage disequilibrium-based Association Mapping (AM) (Mackay and Powell, 2007). FBL mapping is a classical approach in which LD is created by developing a population and crossing few founders. It is very costly, has low resolution, and evaluates few alleles simultaneously in a relatively longer time scale (Flint-Garcia *et al.,* 2003; Gupta *et al.,* 2005; Ross-Ibarra *et al.,* 2007). The method of AM, also known as LD-based mapping, has been proven to be useful and powerful for genetic dissection of complex traits (Adhikari *et al.,* 2011). This method attains a higher resolution because of the use of all meiosis accumulated in the breeding history and Linkage Disequilibrium (LD). AM analysis has been successfully applied to identify marker–trait associations in different crops such as maize (Thornsberry *et al.,* 2001), barley (Ivandic *et al.,* 2003; Kraakman *et al.,* 2006), hexaploid wheat (Breseghello and Sorrels, 2006), chickpea (Saeed *et al.,* 2013), and long life span forest plants (Wilcox *et al.,* 2007). In genomic studies of tobacco (Julio *et al.,* 2006; Tong *et al.,* 2012; Vontimitta and Lewis, 2012; Hatami Maleki *et al.,* 2013), FBL mapping was mainly used for the detection of QTL controlling several agromorphological and chemical characteristics and disease resistance; there is no report yet on genetic analysis of resistance to broomrape especially through the AM approach.

The objectives of the present study were as follows: i) evaluation of genetic variation of tobacco germplasm for resistance to broomrape, ii) fingerprinting of tobacco genotypes using SSR markers and inferring population structure, and iii) detection of genomic regions associated with resistance to broomrape (*O. aegyptiaca*) through association mapping analysis.

MATERIALS AND METHODS

Plant Material and Phenotypic Analysis

A total of 89 tobacco genotypes (Table 1) were used in the present experiment. Among the studied genotypes, the "SPT" lines, known as 'Chopogh', were selected from our local landraces using the Single Seed Descent (SSD) method. Genotypes: 'Isfahan', 'Jahrom', 'Borazjan', 'Lengeh', 'Shahroudi', 'Saderati', and 'Balouch' are known as water pipe's tobacco and selected

Table 1. Name and origin of studied tobacco genotypes, membership percentage of each genotype to constructed subgroups (Q-matrix), mean and standard error values for broomrape growth characteristics considering average data of two consecutive years in inoculated condition.

Genotype	Origin	*Q*-matrix				Character [a]		
		S.P.1	S.P.2	S.P.3	Sub population	*NEB* [a]	*FWB* [b] (g)	*DWB* [c] (g)
GD 165	Bulgaria	0.07	0.80	0.13	Green	0.17 ± 0.17	0.17 ± 0.17	0.05 ± 0.05
Kharmanli 163	Turkey	0.56	0.27	0.17	Mix	2.33 ± 0.76	63.38 ± 38.12	23.18 ± 15.12
Nevrokop	Bulgaria	0.03	0.10	0.87	Blue	2.33 ± 0.61	13.97 ± 6.50	4.82 ± 2.24
Trabozan	Turkey	0.02	0.03	0.96	Blue	2.33 ± 0.56	19.25 ± 4.62	4.80 ± 1.12
Krumovgraid	Bulgaria	0.09	0.54	0.36	Mix	0.50 ± 0.22	0.97 ± 0.46	0.20 ± 0.09
Basma S. 31	Greece	0.75	0.19	0.05	Red	3.00 ± 0.93	25.89 ± 12.02	6.30 ± 2.80
Triumph	Yugoslavia	0.23	0.02	0.75	Blue	2.50 ± 0.62	51.52 ± 16.39	18.13 ± 5.75
Xanthi	-	0.07	0.90	0.03	Green	4.83 ± 1.25	62.13 ± 25.25	23.92 ± 9.81
Matianus	-	0.05	0.71	0.24	Green	2.50 ± 0.62	23.40 ± 8.00	13.03 ± 4.83
Immni 3000	-	0.30	0.58	0.12	Mix	2.00 ± 0.58	10.88 ± 5.45	2.83 ± 1.62
Melkin 261	Bulgaria	0.06	0.89	0.05	Green	2.50 ± 0.50	71.62 ± 5.61	16.18 ± 1.60
Tyk-Kula	-	0.06	0.92	0.02	Green	2.33 ± 0.42	43.48 ± 17.27	6.12 ± 2.47
Ss-289-2	-	0.01	0.01	0.98	Blue	2.67 ± 0.95	54.67 ± 21.86	15.32 ± 7.39
Ohdaruma	Yugoslavia	0.03	0.03	0.94	Blue	0.50 ± 0.34	23.08 ± 16.40	6.72 ± 6.62
Ploudive 58	Bulgaria	0.11	0.79	0.10	Green	1.00 ± 0.00	1.33 ± 0.11	0.47 ± 0.02
Line 20	-	0.06	0.78	0.16	Green	2.83 ± 0.79	21.20 ± 6.54	4.72 ± 1.44
TB 22	-	0.10	0.43	0.47	Mix	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Ts 8	-	0.19	0.20	0.61	Mix	0.33 ± 0.21	14.72 ± 14.04	5.37 ± 5.33
Alborz 23	Iran	0.03	0.95	0.02	Green	1.67 ± 0.33	5.12 ± 1.66	1.43 ± 0.42
FK 40-1	Iran	0.02	0.76	0.22	Green	2.33 ± 0.61	20.40 ± 5.40	6.78 ± 3.18
PI7	Bulgaria	0.15	0.64	0.21	Mix	0.83 ± 0.31	28.27 ± 11.05	6.12 ± 2.22
KPHa		0.03	0.55	0.43	Mix	2.33 ± 0.76	35.43 ± 13.40	9.90 ± 3.43
KB		0.04	0.29	0.67	Mix	1.00 ± 0.37	2.30 ± 1.15	1.10 ± 0.63
HTI	-	0.09	0.89	0.02	Green	0.50 ± 0.34	10.50 ± 7.05	2.53 ± 1.64
Kramograd NHH 659	Bulgaria	0.50	0.41	0.10	Mix	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
TK 23	-	0.02	0.95	0.03	Green	2.50 ± 0.62	9.13 ± 2.18	3.00 ± 0.88
L16	Iran	0.05	0.94	0.01	Green	0.67 ± 0.33	1.97 ± 1.27	0.58 ± 0.41
Izmir 7	Turkey	0.11	0.82	0.07	Green	1.00 ± 0.00	13.70 ± 4.53	3.50 ± 1.16
Mutant 3	Iran	0.10	0.84	0.07	Green	1.17 ± 0.31	4.23 ± 0.91	0.87 ± 0.20
Mutant 4	Iran	0.72	0.19	0.09	Red	1.00 ± 0.37	7.77 ± 5.54	1.42 ± 0.82
Pobeda 1	Russian	0.41	0.47	0.12	Mix	0.67 ± 0.42	4.27 ± 2.70	1.97 ± 1.24
Pobeda 2	Russian	0.42	0.28	0.29	Mix	2.00 ± 0.37	42.70 ± 20.56	4.04 ± 1.35
Rustica	-	0.06	0.37	0.58	Mix	1.50 ± 0.34	13.40 ± 2.94	4.60 ± 1.13
Samsun 959	Turkey	0.38	0.31	0.31	Mix	1.00 ± 0.45	8.15 ± 3.64	3.25 ± 1.49
Samsun dere	Turkey	0.29	0.69	0.02	Mix	1.33 ± 0.33	6.38 ± 1.45	2.92 ± 0.94
OR-205	Iran	0.83	0.13	0.04	Red	1.83 ± 0.48	48.00 ± 10.77	12.22 ± 2.73
OR-345	Iran	0.94	0.04	0.02	Red	2.17 ± 0.31	16.00 ± 3.18	4.58 ± 1.41
OR-379	Iran	0.63	0.07	0.30	Mix	1.33 ± 0.49	1.65 ± 0.59	0.40 ± 0.15
CHT 209.12e	Iran	0.02	0.02	0.96	Blue	0.50 ± 0.22	1.60 ± 0.76	0.13 ± 0.06
CHT 209.12e×F.K.40-1	Iran	0.01	0.02	0.97	Blue	0.83 ± 0.40	4.27 ± 2.77	4.23 ± 1.95
CHT 266-6	Iran	0.04	0.14	0.82	Blue	0.50 ± 0.22	16.85 ± 8.04	4.58 ± 2.58
CHT 283-8	Iran	0.24	0.03	0.73	Blue	2.33 ± 0.56	6.35 ± 0.60	1.78 ± 0.81
CHT 273-38	Iran	0.11	0.13	0.76	Blue	0.83 ± 0.31	34.08 ± 10.93	11.55 ± 3.74
Basma 12-2	Iran	0.20	0.04	0.76	Blue	1.67 ± 0.21	5.95 ± 1.91	2.67 ± 0.94
Basma 16-10	Iran	0.61	0.08	0.31	Mix	1.00 ± 0.37	7.15 ± 2.19	2.15 ± 0.74
Basma 104-1	Iran	0.91	0.02	0.07	Red	1.17 ± 0.31	7.80 ± 2.98	4.12 ± 1.58
Basma 181-8	Iran	0.49	0.05	0.46	Mix	1.67 ± 0.21	7.88 ± 1.90	1.73 ± 0.36
Zichna	-	0.68	0.30	0.02	Mix	1.83 ± 0.48	10.75 ± 3.51	5.02 ± 1.37
Izmir	Turkey	0.90	0.08	0.03	Red	2.17 ± 0.65	9.90 ± 2.06	2.17 ± 0.45
PD 324	Iran	0.21	0.11	0.69	Mix	1.50 ± 0.43	41.35 ± 8.63	16.12 ± 3.90
PD 325	Iran	0.93	0.05	0.02	Red	1.17 ± 0.40	3.47 ± 1.19	2.22 ± 0.81

[a] Number of Emerged Broomrapes; [b] Fresh Weight of Broomrapes, [c] Dry Weight of Broomrapes.

Table 1. Continued…

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Genotype	Origin	*Q*-matrix				Character [a]					
		S.P.1	S.P.2	S.P.3	Sub population	*NEB* [a]		*FWB* [b] (g)		*DWB* [c] (g)	
PD 406	Iran	0.02	0.71	0.27	Green	1.33	±0.33	0.87	± 0.39	0.37	±0.14
PD 328	Iran	0.95	0.03	0.02	Red	2.17	±0.48	14.45	± 4.88	4.35	±1.01
PD 329	Iran	0.92	0.05	0.03	Red	1.50	±0.43	16.43	± 3.91	4.50	±2.30
PD 336	Iran	0.91	0.08	0.02	Red	1.33	±0.33	1.48	± 0.53	1.68	±1.31
PD 345	Iran	0.97	0.02	0.01	Red	1.17	±0.17	10.20	± 5.31	0.47	±0.12
PD 364	Iran	0.95	0.04	0.01	Red	1.67	±0.49	3.12	± 1.30	1.15	±0.50
PD 365	Iran	0.91	0.03	0.06	Red	2.33	±0.21	25.03	± 7.17	8.30	±1.61
PD 371	Iran	0.98	0.01	0.01	Red	2.00	±0.00	22.10	± 7.70	10.27	±3.01
PD 381	Iran	0.93	0.03	0.04	Red	2.00	±0.26	12.63	± 6.47	4.97	±1.01
SPT 403	Iran	0.68	0.07	0.25	Mix	1.50	±0.22	31.92	± 8.63	4.28	±1.55
SPT 405	Iran	0.30	0.50	0.20	Mix	0.83	±0.40	3.28	± 1.57	1.75	±0.85
SPT 406	Iran	0.05	0.14	0.81	Blue	1.00	±0.37	37.87	± 16.40	9.29	±4.47
SPT 408	Iran	0.19	0.79	0.02	Green	2.00	±0.63	26.33	± 14.08	8.12	±3.36
SPT 409	Iran	0.22	0.70	0.08	Green	3.00	±0.00	28.08	± 8.18	3.78	±1.31
SPT 410	Iran	0.19	0.22	0.58	Mix	4.00	±0.58	40.85	± 13.12	13.37	±4.87
SPT 412	Iran	0.27	0.20	0.53	Mix	1.83	±0.17	20.98	± 4.11	5.28	±1.37
Isfahan 5	Iran	0.08	0.81	0.11	Green	2.50	±0.50	31.92	± 7.68	7.13	±1.75
SPT 420	Iran	0.02	0.70	0.28	Green	2.67	±0.49	10.77	± 2.50	1.18	±0.23
SPT 430	Iran	0.06	0.52	0.42	Mix	3.00	±0.68	4.72	± 0.64	2.63	±0.68
SPT 432	Iran	0.18	0.70	0.12	Green	1.00	±0.37	12.63	± 7.18	2.58	±2.18
SPT 433	Iran	0.13	0.82	0.05	Green	1.83	±0.40	7.55	± 1.97	3.63	±1.44
SPT 434	Iran	0.14	0.83	0.02	Green	2.67	±0.61	27.95	± 13.75	7.13	±3.61
SPT 436	Iran	0.84	0.08	0.08	Red	2.67	±0.21	20.13	± 7.38	5.05	±1.94
SPT 439	Iran	0.03	0.95	0.02	Green	2.33	±0.42	25.90	± 6.25	3.57	±1.09
SPT 441	Iran	0.03	0.95	0.02	Green	1.00	±0.37	6.67	± 3.15	0.23	±0.08
Isfahan 2	Iran	0.73	0.26	0.02	Red	1.67	±0.21	2.93	± 1.32	4.47	±0.82
SPT 413	Iran	0.03	0.94	0.03	Green	1.67	±0.67	17.70	± 4.26	3.27	±1.39
Isfahani	Iran	0.14	0.85	0.02	Green	1.33	±0.21	7.47	± 2.60	0.85	±0.27
Jahrom 14	Iran	0.44	0.50	0.06	Mix	1.33	±0.21	4.07	± 1.47	1.67	±0.52
Borazjan	Iran	0.07	0.85	0.08	Green	5.00	±0.97	15.50	± 4.92	2.83	±0.83
L17	Bulgaria	0.39	0.60	0.01	Mix	0.33	±0.11	1.27	± 0.54	0.14	±0.08
Balouch	Iran	0.56	0.35	0.09	Mix	0.67	±0.21	0.38	± 0.14	0.43	±0.14
Lengeh	Iran	0.46	0.52	0.02	Mix	3.00	±0.97	14.20	± 5.22	1.27	±0.71
Saderati	Iran	0.18	0.80	0.01	Green	2.33	±1.17	11.83	± 7.32	2.47	±1.31
Iraqi	Iran	0.20	0.78	0.02	Green	1.00	±0.37	11.73	± 3.79	2.37	±0.75
Shahroudi	Iran	0.45	0.51	0.04	Mix	0.67	±0.42	6.83	± 4.32	1.80	±1.14
TKL	-	0.10	0.46	0.44	Mix	2.33	±0.42	23.50	± 2.93	6.10	±0.57
CHT 269-12e	Iran	0.02	0.04	0.94	Blue	0.67	±0.21	1.73	± 0.83	0.83	±0.44

[a] Number of Emerged Broomrapes; [b] Fresh Weight of Broomrapes, [c] Dry Weight of Broomrapes.

from our local landraces using the SSD method. The 'PD' and 'OR' lines are Recombinant Inbred Lines (RILs) coming from the cross between 'Basma seres 31' and 'Dubec 566'. Genotypes, including the 'CHT' suffix, are semi-oriental tobacco and known as 'Tikolak'. Other genotypes used in this study are inbred lines from different countries introduced from the Cooperation Center for Scientific Research Relative to Tobacco (http://www.coresta.org) collection or pure lines provided by the Iranian Tirtash Tobacco Research Center.

The screening experiment was arranged in randomized complete block design with three replications under both non-inoculated (control) and inoculated states during two consecutive years. Each replication consisted of one ceramic pot. Seeds of genotypes were sown at a rate of approximately 5 g m^{-2} in bed. After sowing the seeds, a fine layer of well-fermented and sieved sheep manure was spread on top of

the beds. Tobacco seedlings were transplanted to pots filled with 10 kg of sterilized soil when plant averaged about 12 cm in height. We used soil that was sampled from Alfalfa farm and was representative of northwest Iran where the tobacco is planted. In inoculated conditions, the soil of each pot was mixed with 0.06 g of Egyptian broomrape (*O. aegyptiaca*) seeds. Standard agrotechnical practices for this tobacco type were applied during the growing season. Traits such as the Number of Emerged Broomrapes (NEB), Dry Weight of Broomrapes (DWB), and Fresh Weight of Broomrapes (FWB) were recorded in each pot.

Genotyping with SSR Markers

Genomic DNA was extracted from the leaves of 89 genotypes, following the method described by Doyle and Doyle (1987). Twenty-six of 278 SSR primer pairs from the tobacco SSR database (Bindler *et al.,* 2007 and 2011) were used for fingerprinting. The choice of SSR primer pairs was based on their known genetic locations to obtain near-uniform coverage of the tobacco genome and clarity of produced bands (Bindler *et al.,* 2011). PCR amplifications were performed in a 20-µl volume using a 96-well Eppendorf Mastercycler Gradient (Type 5331; Eppendorf AG, Hamburg, Germany). The reaction mixture contained 2.5 mM of each reverse and forward primers, 0.4 Unit of Taq DNA polymerase (Cinna Gen Inc., Tehran, Iran), 100 µM of each dNTP (BioFluxbiotech, http://biofluxbiotech.com), 2 µl of 10X PCR buffer, 2 mM $MgCl_2$ (CinnaGen, Tehran, Iran), ddH_2O, and 25 ng template DNA. Amplification was carried for 35 cycles consisting of a denaturation step at 94ºC for 1 minute, annealing at 55ºC for 1 minute, and an extension step at 72ºC for 1.5 minutes. An initial denaturation step at 94ºC for 4 minutes and a final extension step at 72ºC for 10 minutes were also included. The amplification products were visualized on 3% (w/v) ultrapure agarose (Invitrogen) with ethidium bromide (1 µg ml^{-1}) and photographed using a gel documentation (Gel Logic 212 PRO, USA) system.

Statistical Analysis

Descriptive statistics was calculated for recorded phenotypic data across two years using Excel 2007. The SSR genotyping data were scored as codominant markers to distinguish homozygotes and heterozygotes for each locus. Major Allele Frequency (MAF), Polymorphic Information Content ($\hat{PIC}_l$), and the levels of gene Diversity ($\hat{D}_l$) were calculated using Power Marker 3.25 software package (Liu and Muse, 2005). Gene diversity, referred to as expected heterozygosity, is defined as the probability of difference between two randomly chosen alleles from the population.

$\hat{D}_l = (1-\sum_{u=1}^{k} \tilde{P}_{lu}^2)/(1-\frac{1+f}{n})$, where f is the inbreeding coefficient and estimated from the data using the method of moments.

Polymorphism Information Content ($\hat{PIC}_l$) is estimated following the formula proposed by Botstein *et al.* (1980): $\hat{PIC}_l = 1-\sum_{u=1}^{k} \tilde{P}_{lu}^2 - \sum_{u=1}^{k-1}\sum_{v=u+1}^{k} 2\tilde{P}_{lu}^2 \tilde{P}_{lv}^2$.

A model-based Bayesian approach in the software package Structure ver. 2.3.4 (Pritchard *et al.,* 2000) was used for population structure analysis. Five independent runs were performed in the structure with the number of subpopulations (K) from 1 to 10, the admixture model, a burn-in period of 100,000 and 100,000 Markov Chain Monte Carlo (MCMC) repetitions. Inferred ancestry estimates of individuals (Q-matrix) were derived for the selected subpopulation (Pritchard *et al.,* 2000). The estimation of kinship coefficients based on SSR markers was calculated using

the TASSEL version 2.1 software (Bradbury *et al.,* 2011). Average phenotypic data of each year and average data of two years were used for marker–trait association analysis. Association studies considering *K* and *Q*-matrices as covariates in the Mixed Linear Model (MLM) function were performed with TASSEL software.

RESULTS

Genetic Variation and Population Structure in Tobacco Germplasm

Mean values related to growth characteristics of broomrape across two years in inoculated condition are shown in Table 1. Considering the average data of two years, studied genotypes did not show any infection by broomrape in non-inoculated condition, whereas in inoculated condition, most genotypes showed infection to broomrape. In inoculated condition, the Number of Emerged Broomrapes (NEB) ranged from 0.0 to 5.0 (Table 1). Fresh Weight of Broomrapes (FWB) ranged from 0.0 to 71.6 g and Dried Weight of Broomrapes (DWB) ranged from 0 to 23.1 g (Table 1).

In Table 1, some of the studied genotypes, including 'GD165', 'Krumovgraid', 'Ohdaruma', 'Ts8', 'HTI', 'CHT 209.12e', 'L17', and 'CHT 266-6', possessed minimum value of *NEB* (below 0.5) and genotypes 'TB 22' and 'Kramograd N.H.H. 659' had no infection via broomrape (Table 1). In this study, the 'Basma seres 31' as the maternal line of RIL subpopulation ('PD' and 'OR' lines in Table 1), showed the *NEB* value of 3.00±0.93. However, in all of its progenies (Table 1), the broomrape growth characteristics were less than it (Basma seres 31). In 'SPT' subpopulation known as 'Chopogh', *NEB* fluctuated between 0.83±0.4 in 'SPT405' and 4.00±0.58 in 'SPT410' (Table 1). In the 'Tikolak' group, the maximum and minimum infection was observed in 'CHT 283-8', 'CHT 266-6' and 'CHT 209.12e', respectively (Table 1). It is inferable from the results (Table 1) that among the studied water pipe's tobaccos, 'Borazjan' genotype possessed maximum infection to broomrape (*O. aegyptiaca*).

The molecular genetic diversity among 89 tobacco genotypes was assessed using 26 SSR loci, and a total number of 66 alleles with an average of 2.53 alleles per locus were detected. Regarding the SSR data, the Major Allele Frequency (MAF) ranged from 0.38 to 0.80. Polymorphic Information Content (PIC) and gene diversity fluctuated from 0.27 (PT30094) to 0.60 (PT30014) and 0.33 to 0.66, respectively. The model-based Bayesian clustering approach was used to analyze the genetic structure of the association panel. The group of 89 tobacco genotypes was partitioned in three subgroups (K) (Supplementary data 1). The membership percentage of each genotype to identified subgroups is presented in Table 1. These subgroups matched the three germplasm groups: 'Chopogh' (SPTs), RIL ("PD" and "OR" lines), and 'Tikolak' (Supplementary data 2). In this study, 81% of RIL, 70.58% of SPT, and 70% of 'Tikolak' genotypes were assigned into the corresponding subgroups, and the remaining ones were categorized into the "mixed" subgroups based on their *Q* values (Supplementary data 2 and Table 1).

Linkage Disequilibrium and Marker-trait Association

The triangle plot for pairwise LD between markers in the genome is shown in Supplementary data 3. In Supplementary data 3, above the diagonal display are the D' (as a predictor of linkage disequilibrium) values and below the diagonal display are the correspondence *P*-values from 1000 permutation test. In the collection under investigation, D' ranged from 0.015 to 1 with the average value of 0.243; 7.08% of possible SSR locus pairs ($\frac{n(n-1)}{2} = \frac{26(26-1)}{2} = 325$ pairs) showed a significant level of Linkage Disequilibrium (LD) ($P<0.01$) (Supplementary data 3).

To understand a reliable and possible association between SSR markers and gene(s) responsible for resistance to broomrape, association mapping was performed using Mixed Linear Model (MLM) approach in TASSEL 2.1 accounting for population structure, and kinship relatedness (Q+K model). Three SSR loci including PT30250, PT30285 and PT30067 and two including PT30285 and PT30008 were identified to be significantly associated with *NEB* in the years 2009 and 2010, respectively (Table 2). In the year 2010, locus PT30094 was detected as a linked marker with *FWD* and *DWB* traits (Table 2). AM analysis based on combined data of two consecutive years (Table 2) revealed four SSR loci including PT30250, PT30285, PT30067, and PT30008 to be significantly associated with *NEB* trait. Locus PT30094 also showed strong association with *FWB* and *DWB* traits based on combined two-year data (Table 2).

DISCUSSION

Breeding for resistance is straightforward when a good source of resistance is available, and an efficient, easily controlled, and practical screening procedure exists to provide good selection pressure (Rubiales and Fernandez-Aparicio, 2012). In this study, high genetic variability was observed among studied tobacco genotypes for resistance to broomrape (*O. aegyptiaca*) regarding growth characteristics of parasite in terms of host and SSR marker data. Qasem and Kasrawi (1995) ranked 25 tomato cultivars accompanied with one wild tomato from relatively high to moderate considering their resistance reaction to branched broomrape (*O. ramosa*). Similarly, Fernandez-Aparicio *et al.* (2008) reported a wide range of responses to broomrape (*O. crenata*) in a Spanish germplasm of lentil (*Lens culinaris*). In contrast with the findings of Fernandez-Aparicio *et al.* (2008), here complete resistance was observed in the interaction of some tobacco genotypes with broomrape (*O. aegyptiaca*). In this study, results pertaining to phenotypic data of "SPT" lines, revealed the genetic variability for resistance to broomrape in local landraces of tobacco. In our previous investigations (Darvishzadeh *et al.,* 2010 and 2011), the "SPT" lines also presented high variability for different characters, such as resistant to powdery mildew and chloride accumulation rates in leaves. Deferential reaction of RILs ("PD" and "OR" lines) population coming from the cross 'Basma Seres 31×Dubec 566' is expectable because of a phenomenon known as transgressive segregation. In transgressive segregation, the alleles with positive or negative additive effects are accumulating in the offspring (Zhang *et al.,* 2012). In the present work, there was no resistance source in water pipe's tobacco lines which are specific to

Table 2. SSR loci associated with growth characteristics related to broomrape by MLM method in inoculated condition.

Markers	Linkage group	Genetic distance (CM)	Trait	P-Marker (2009)	P-Marker (2010)	P-Marker (2009+2010)
PT30250	10	90.7	NEB [a]	0.0289*	ns	0.0406*
PT30285	18	55.3	NEB	0.0074**	0.0145*	0.0071**
PT30067	2	29.2	NEB	0.0119*	ns	0.0214*
PT30008	11	39.9	NEB	ns	0.0051**	0.0306*
PT30094	18	0	FWB [b]	ns	0.0041**	0.006**
PT30094	18	0	DWB [c]	ns	0.0057**	0.0067**

[a] Number of Emerged Broomrapes; [b] Fresh Weight of Broomrapes, [c] Dry Weight of Broomrapes.
^{ns}, * and **: Are non-significant, significant at %5 and %1 probability level respectively.

Iran.

In the present study, analysis of population structure showed that this association panel possessed a diverse genetic variation and therefore, could be used for association analysis. An appropriate germplasm collection with phenotypic and underlying genetic variation for the traits of interest is mandatory for successful association mapping (Zhu *et al.,* 2008). However, the efficiency of association mapping is significantly influenced by the population structure (Sharbel *et al.,* 2000). Many models were used to minimize the false-positive of association analysis produced from the admixture of populations. It has been shown that *K* and *Q* matrices incorporated into the MLM were sufficient to minimize false-positive associations (Zhang *et al.,* 2012).

Albeit there is no report about genetic control of resistance to broomrape in tobacco, both simple (Fernández-Martínez *et al.,* 2008) and digenic (Velasco *et al.,* 2007) inheritance were reported for resistance to broomrape in studied crop species. Complication of inheritance often results from the segregation of multiple genetic factors. Molecular tools facilitate the dissection of complex inheritance through studying marker-trait association. The success of association mapping depends on the possibility of detecting LD between marker alleles and alleles affecting phenotypic traits (Stich *et al.,* 2005). Several statistics have been developed for quantification of LD; choosing the appropriate LD measures depends on the objective of the study, and one performs better than the other in particular situations and cases (Abdurakhmonov and Abdukarimov, 2008). *D′* is one of the most commonly used measures of LD. In this study, a low range of LD was seen between markers. Low LD indicates that marker density in the study is not sufficient for detection of QTLs in the genome. However, similar to findings of Shehzad *et al.* (2009), some markers still captured the signal of QTL even in such density.

There are narrow studies about molecular markers related to broomrape resistance in plants, such as sunflower (Perez-Vich *et al.,* 2004), pea (Fondevilla *et al.,* 2010; Valderrama *et al.,* 2004), and faba bean (Diaz-Ruiz *et al.,* 2010), only through the FBL mapping approach. However, there is no report about DNA markers linked to broomrape resistance genes in tobacco. Our results (Table 2) reveal the efficiency of association mapping in detecting tobacco genomic regions conferring resistance to broomrape. Here, among newly identified loci linked to resistance to broomrape (*O. aegyptiaca*), locus PT30094 is common for *DWB* and *FWB*. Similarly, through family-based linkage mapping analysis, co-localized QTLs were reported for several chemical characteristics related to smoking characteristics in tobacco (Julio *et al.,* 2006). In addition, locus PT30285 is linked with *NEB* and is stable over the years, which imply its important value in tobacco breeding programs.

In conclusion, there are promising sources of resistance to broomrape (*O. aegyptiaca*) in the studied germplasm. The existent genetic variation could facilitate identification of DNA markers linked to broomrape resistance genes through association mapping. In the present study, 5 SSR loci were detected for broomrape growth characteristics. Although, for the association study, a large number of molecular markers are suitable, but our study can serve as initial effort of association mapping for broomrape resistance in tobacco.

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تنوع ژنتیکی، تجزیه ساختار و مکان یابی ارتباطی مقاومت به گل جالیز (*Orobanche aegyptiaca* Pers.) در توتون

ر. درویش زاده

چکیده

گل جالیز علف هرز انگل در مزارع توتون (*Nicotiana tabacum* L.) است که دارای اثرات مخرب شدید بر عملکرد آن می باشد. در این مطالعه، عکس العمل ۸۹ ژنوتیپ توتون در مقابل علف هرز گل جالیز (*Orobanche aegyptiaca*) در قالب طرح بلوک کامل تصادفی با سه تکرار و در طی دو سال زراعی ارزیابی شد. در هر سال، ژنوتیپ ها در دو شرایط غیرتلقیح شده و تلقیح شده بواسطه

مخلوط نمودن ۰/۰۶ گرم بذر گل جالیز در گلدان ها کشت شدند. با توجه به میانگین داده های دو سال، ژنوتیپ های مورد مطالعه هیچ گونه آلودگی در شرایط غیر تلقیح شده نداشتند در حالی که در شرایط تلقیح شده، اکثر ژنوتیپ ها آلودگی نشان دادند. در شرایط تلقیح شده، دو ژنوتیپ 'T.B.22' و 'N.H.H. 659' هیچ گونه آلودگی به گل جالیز نشان ندادند. در آزمایشات مولکولی، انگشت نگاری ژنوتیپ های توتون با ۲۶ نشانگر SSR انجام گرفت. با استفاده از مدل بیزین، ژنوتیپ های مورد مطالعه در سه زیرگروه قرار گرفتند. ۷/۰۸٪ از ۳۲۵ ترکیب دو تایی ممکن از نشانگرهای SSR، نامتعادلی پیوستگی ژنتیکی معنی دار نشان دادند ($P<0.01$). با استفاده از مدل خطی مخلوط، ۵ مکان ریزماهواره ای از گروه های پیوستگی ۲، ۱۰، ۱۱ و ۱۸ در نقشه پیوستگی مرجع توتون به عنوان نشانگرهای مرتبط با مقاومت به گل جالیز در توتون شناسایی شدند.