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Pollen characteristics of blood oranges and determination of hybrids with SRAP markers

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Abstract

*Anther numbers, pollen numbers, the ratios of pollen viability and germination tests were performed in 11 parent blood orange parents in 2011. Hybridizations between 'Clementine' mandarin (*Citrus reticulata *Blanco) and different blood oranges (*C. sinensis *Osbeck), namely 'Moro', 'Sanguinello', 'Tarocco' cultivars, and A1, A2, A3, H1, H2, H3, K1, K2 local genotypes, were conducted in 2010 and 2011. The highest pollen viability was found in H3 blood orange genotype with 43.38% in TTC (2, 3, 5-triphenyl tetrazolium chloride) test. The highest pollen germination was obtained at H3 blood orange genotype on 1% agar+ 25% sucrose. While the highest anther count was found in 'Tarocco' with 22.17, the highest total pollen count/flower was found in A1 with 139421 on the hemacytometer. At hybridization experiments, 1397 and 580 flowers were crossed, and 27 and 43 fruit, and 86 and 348 seeds were obtained in 2010 and, 2011, respectively. From these seeds, 42 and 161 hybrid plants were grown. The 13 alive hybrids and two parents ('Clementine', K1) from the crosses in 2010 were used to obtain diversity with SRAP (sequence-characterized amplified polymorphism) molecular markers. While a total of 66 bands were obtained, 24 of which were polymorphic. Polymorphism ratio was 36.4%.*

Keywords: *Citrus reticulata*, *C. sinensis*, hybridization, pollen number, viability, germination, SRAP markers

1. Introduction

Citrus (Rutaceae, 2*n*=2*x*=18), including oranges, mandarins (=tangerines), lemons, limes, grapefruits, pummelos (=shaddock), citrons, and others, are specialty crops and one of the main vitamin and nutrition sources in human health. Although they are originated from South-East Asia, citrus trees have been grown in suitable climatic agricultural regions between 40° north and 40° south latitudes [4]. With 131 283 333 t total and 68 223 759 t orange production in 8 785 549 ha and 3 816 692 ha area, respectively, citrus occupy the largest fruit group produced worldwide. Turkey has 3 556 407 t citrus and 1 662 000 t orange production in 104 239 ha and 45 733 ha area, respectively [8]. Besides introduction, chance seedling selections, mutations, genetic engineering breeding techniques, citrus cultivar improvement has been extensively relied on natural and man-made cross hybridizations. Since most genotypes have problems with female and/or male sterility/incompatibility, choosing appropriate parents for hybridization is an important task for saving time and money. Blood, known as pigmented in some places, oranges are adapted to Mediterranean climate with hot days and cool nights for fruit to development deep red flesh color due to anthocyanin [4]. 'Doble Fina', 'Maltaise Sanguine', 'Moro', 'Sanguinelli', 'Sanguinello', 'Tarocco', 'Tomango', and 'Washington Sanguine' (or 'Doublefine Ameliorée') are important blood

orange cultivars [20]. Recently some mandarin blood orange hybrids, namely 'Alkantara' ('Oroval clementine × 'Tarocco'), 'Mandared' ('Nules' Clementine × 'Taracco'), 'Tacle' and 'Clara' ('Monreal clementine × 'Tarocco'), 'Camel' ('Nules' clementine × 'Avana' mandarin), 'Reale' ('Monreal Clementine × *Fortunella hindsii*), 'Mandalate' ('Fortune' mandarin × 'Avana' mandarin) have been released from Italy to world fresh fruit market [16, 19]. Between 1978 and 1980, Starrantino (1992) [21] made a series of hybridization and obtained triploid hybrids from hand-pollination between diploid Oroval clementine and tetraploid Moro NL 58-8D-1 (OMO) and Tarocco 57-1E-1 (OTA) blood oranges [18]. The number of pollen grains could be counted using a hemocytometer. The pollen viability is the first and most important issue for hybridization. Different methods can be used to assess pollen viability namely safranin [13], acetocarmine [1, 10], Alexander's stain [3], TTC (2, 3, 5-triphenyl tetrazolium chloride) [3, 10, 13], FDA (fluorescein diacetate) [3], and IKI (iodine potassium iodide) [7, 13]. The pollen germination tests can be performed with solidified agar in Petri plate and hanging drop methods incorporating different sucrose concentrations [7, 10, 15]. SRAP (sequence-characterized amplified polymorphism) markers have been certified first time in mapping and gene tagging in *Brassica* [17] and afterwards used to identify and discriminate germplasms, molecular genetic diversity, and population structure analyses in variety of organisms including *Citrus* spp. [12, 23], *Morus* sp. [24], *Ficus* sp. [14], *Abelmoschus* sp. [11], *Cucurbita* sp. [9], and *Ganoderma* sp. [22]. The aims of this study were (1) to investigate pollen numbers, viability and germination of blood oranges and (2) to determine the hybrid nature of the plants obtained from 'Clementine' × blood orange cross with SRAP markers. These pollen characteristics of blood oranges were determined at the first time with this research.

2. Materials and Methods

Plant Material: 'Clementine' mandarin (*Citrus reticulata* Blanco) located at the Faculty of Agriculture, Adnan Menderes University, South Campus, Aydın, Turkey was used as a female parent. 'Moro', 'Sanguinello', and 'Tarocco' located at West Mediterranean Agricultural Research Institute (BATEM), Antalya, Turkey, and A1, A2, A3, K1 and K2 located at Aydın province, H1, H2, and H3 located at Söke district of Aydın were used as male parents. The flowers were collected at the white balloon stage, just before opening.

Anther Numbers, Pollen Number, Viability, and Germination Rate of Blood Oranges: In 2010, the flowers of 'Moro', 'Sanguinello', and 'Tarocco' were collected from the fruit collection orchard in BATEM on 07 April 2010. The flowers of K1 and K2 were collected from an orchard in Aydın on 05 April 2010. In 2011, the flowers of 'Moro', 'Sanguinello', and 'Tarocco' were collected from BATEM on 11 April 2011. The flowers of H2 and H3 were collected on 16.04.2011. The flowers of K1 and K2 were collected on 17 April 2011. The flowers of A1, A2, A3 were collected on 20 April 2011 in different orchards in Aydın. In both years, anthers were burst overnight spreading onto a white paper under a lamp in a room temperature. Pollen grains were stored in a small glass vials in the refrigerator until they are used for pollination, and pollen viability and germination tests [7]. Anther numbers were counted from fifteen flowers from the blood oranges of 'Moro', 'Sanguinello', and 'Tarocco' were used in 2010 and that of 'Moro', 'Sanguinello', and 'Tarocco' (Group 1), and A1, A2, A3, H1, H2, H3, K1, and K2 (Group 2) were used in 2011. Number of pollen grains was calculated using 2×10 flowers and hemocytometer modified from Eti [6]. The viability test of pollen grains was performed by TTC (2, 3, 5 triphenyl tetrazolium chloride) according to Eti [7]. The red-colored pollen grains were recorded as viable after 2 h incubation observed at 10×10 magnification. Pollen grain germination test was performed with agar+sucrose method using 0, 5, 10, 15, 20, 25% sucrose with 1% agar. The germination was recorded 24 h after pollen plating. The germination ratios were determined by dividing

the number of pollens germinated by the total number pollens in the field of light microscope view [7, 10, 13]. Data were analyzed as completely randomized design with three readings in three replicates. Differences between means were determined by LSD test at P=0.05 level.

Hybridization between 'Clementine' and Blood Oranges: Hand pollination was performed at the white balloon stage with 'Moro', 'Sanginello', 'Tarocco', K1, and K2 blood oranges on 07-18 April 2010, and that with 'Moro', 'Sanguinello', 'Tarocco', A1, A2, A3, H1, H2, H3, K1, and K2 on 02-08 May 2011. Self-incompatible 'Clementine' mandarin was used as a female parent in all crosses. Fruit set data were recorded after June drop period on 22 June 2010 and 18 June 2011. Fruit were harvested on 25 October 2010 and 19 November 2011. The seeds obtained were sown in peat : perlite (3:1) medium in styrofoam vials on 29 October 2010 and 19-25 December 2011.

SRAP Analysis: DNA from the young leaves of 15 plants was extracted from parents ('Clementine' and K1) and putative hybrids (13) following the manufacturer's directions (AxyPrep^{TM} Multisourse Genomic DNA Miniprep Kit, Axygen). The PCR was performed by modified from Li and Quiros [17] and Gulsen et al. [12]. The 15 µl PCR mix contained 1.5 µl 10× buffer, 1.1 µl $MgCl_2$, 0.33 µl dNTP, 1.2 µl BSA, 0.2 µl *Taq* DNA polymerase (5 µg/µl), 0.6 µl forward an reverse primes (Table 1), and 1.0 µl DNA template. A drop of mineral oil was added to the samples. The PCR program consisted of a degeneration step of 3 min at 95°C; five cycles of 45 s at 94°C, 30 s at 35°C, 1 min at 72°C; 35 cycles of 45 s 94°C, 30 s 50°C, 90 s 72°CC; and a final extension of 7 min at 72°C. Seven Me-Em SRAP primer combinations were tested. The PCR products were separated by 2.0% agarose gel using 3.2 µl ABM Safewave stain with 100 V, 200 mA for 30-60 min photographed under UV light. The present (1) and absent (0) bands were recorded.

3. Results and Discussions

Anther Numbers, Pollen Number, Viability, and Germination: The highest number of anthers per flower, that of pollens per anther, and that of pollen per flower were observed in 'Tarocco' (22.17), A1 (6375), and A1 (139421), respectively (Table 2) while the lowest of that were recorded in H1 and H2 (20.37), 'Tarocco' (1750), and 'Tarocco' (38798). Eti (1990) [6] recorded 20.9 anthers, 13420 pollens per anther, and 276706 pollens per flower in a local orange genotype. Although anther number is higher in blood oranges than a local orange, both number of pollen per anther and flower are lower in blood oranges than a local orange. This difference is most probably caused by the different genotypes used. Within the blood orange cultivars 'Sanguinello' (22.15%) and genotypes H3 (43.38%) gave the highest pollen viability in TTC test. The lowest pollen viability was obtained from 'Moro' (7.43%) and K2 (1.32%) (Table 2). The highest pollen viability was obtained from Alexander's stain (90.8%) comparing to FDA (60.0%) and TTC (37.5%) [3]. The pollen viability using acetic carmine was found 16.3% in 'Blood Oval' (syn. 'Doble Fina'), 62.4% in 'Sangüínea de Piracicaba', 68.7% in 'Ruby Blood', and 77.7% in 'Sangüínea de Mombuca' [5]. The low pollen viability results were obtained in the current study since genotype and climate of the flowering season are different from previous studies. There was not any pollen germination observed in 0% sucrose. 'Moro' (6.13%), 'Tarocco' (6.51%), H2 (12.55%), H3 (12.76%), and A3 (8.28%) gave the highest pollen germination ratio at 25% sucrose (Table 2). There was no pollen germination obtained from K1 and K2 in all sucrose concentrations. The highest pollen germination ratio was obtained from grapefruits 20% sucrose with 0.5% agar (52.3%) and 0.8% agar (51.7), and that from pummelo 20% sucrose with 0.8% agar (44.9%) [2]. In another study, the highest pollen germination in *Murraya koenigii* L. was observed in Brewbaker and Kwack's medium (50.0%) comparing to 15% sucrose (33.3%) [3].

Table 1. SRAP primers and bands used with 'Clementine' and K1 parents, and 15 putative hybrids

İleri	(5'→3')	Geri	(5'→3')	Total	Polymorphic	Polymorphism (%)	Band size (bp)
Me1	TGAGTCCAAACCGGATA	Em14	GACTGCGTACGAATTCTT	10	3	0.30	300-950
Me3	TGAGTCCAAACCGGAAT	Em1	GACTGCGTACGAATTAAT	8	4	0.50	350-1700
Me3	TGAGTCCAAACCGGAAT	Em2	GACTGCGTACGAATTTGC	8	4	0.50	800-1400
Me3	TGAGTCCAAACCGGAAT	Em3	GACTGCGTACGAATTGAC	7	1	0.14	900
Me3	TGAGTCCAAACCGGAAT	Em9	GACTGCGTACGAATTCAG	13	6	0.46	700-1300
Me11	TGAGTCCAAACCGGAAC	Em9	GACTGCGTACGAATTCAG	9	3	0.33	650-2000
Me11	TGAGTCCAAACCGGAAC	Em10	GACTGCGTACGAATTCAT	11	3	0.27	1200-2700
			Total	**66**	**24**	**0.36**	

Table 2. Number of anthers per flower and number of pollen grains per anther and flower in blood oranges

Genotype	Number of anthers per flower	Number of pollen grains per anther	Number of pollen grains per flower	TTC (%)	Pollen germination in 1% agar (%)					
					0	5	10	15	20	25
Group 1										
Moro	21.93*a	3625	79496	7.43 b	0.00	0.00	0.00	1.88	0.00	6.13
Sanguinello	22.10 a	4000	88400	22.15 a	0.00	0.00	4.79	2.77	3.51	0.52
Tarocco	22.17 a	1750	38798	17.46 ab	0.00	0.00	0.00	2.39	0.00	6.51
Group 2										
A1	21.87 ab	6375	139421	21.92 bc	0.00	0.00	0.00	0.00	2.64	2.01
A2	21.63 abc	5685	122967	18.33 bc	0.00	0.00	0.00	0.00	3.79	3.82
A3	20.57 bc	4875	100279	36.96 a	0.00	0.00	2.23	1.12	6.44	8.28
H1	20.37 c	4375	89119	43.38 a	0.00	0.00	0.00	1.93	0.00	2.28
H2	20.37 c	3250	66203	16.16 c	0.00	1.12	2.89	8.08	5.45	12.55
H3	20.50 c	5000	102500	29.13 ab	0.00	1.66	5.27	6.64	4.18	12.76
K1	21.40 abc	4185	89559	4.38 d	0.00	0.00	0.00	0.00	0.00	0.00
K2	22.00 a	2750	60500	1.32 d	0.00	0.00	0.00	0.00	0.00	0.00

*: $P<0.05$

Hybridization between 'Clementine' and Blood Oranges: The total numbers of 'Clementine' flowers pollinated with different blood oranges were 1397 and 580, respectively, in 2010 and 2011 (Table 2). The highest fruit set was observed in 'Clementine' × K1 (4.30%) in 2010 and in 'Clementine' × A3 (56.52%) in 2011. There was a correlation between TTC pollen viability test (36.96%) and pollen germination (6.44%) in 20% sucrose which resulted high fruit set (56.52%) in 'Clementine' × A3 combination. The highest number of fruit was harvested from 'Clementine' × K1 (8) in 2010 and from 'Clementine' × H3 (14) in 2011 (Tables 3, 4). While 'Frost Dancy' mandarin × 'Mosambi' orange (15.8%) gave the highest fruit set, 'Frost Dancy' × 'Kinnow' mandarin (9.7%) and 'Frost Dancy' × 'Duncan' grapefruit (9.0%) combination were yielded lower fruit set [1]. Thus, different pollen parents can give different fruit set ratios. The highest number of seeds were obtained from 'Clementine' × 'Sanguinello' (29) in 2010 and from 'Clementine' × A3 (142) in 2011. The seed numbers were changed 0-29 in 2010 and 0-24 in 0- 2011.

Table 3. Flower, fruit, and seed numbers in hybridization in 2010

Male parent	Flowers pollinated	Fruit set	Fruit set ratio (%)	Fruit number harvested	Fruit harvested/flower polllinated ratio (%)	Seeds obtained	Seeds min-max	Seed/Fruit
Moro	342	9	2.63	6	1.75	5	0-2	0.83
Sanguinello	346	7	2.02	7	2.02	39	0-29	5.57
Tarocco	324	5	1.54	4	1.23	5	0-2	1.25
K1	186	8	4.30	8	4.30	35	0-8	4.38
K2	199	4	2.01	2	1.00	2	0-2	1.00
Total	1397	33		27		86		
Average			2.36		1.93			3.18

Table 4. Flower, fruit, and seed numbers in hybridization in 2011

Male parent	Flowers pollinated	Fruit set	Fruit set ratio (%)	Fruit number harvested	Fruit harvested/flower polllinated ratio (%)	Seeds obtained	Seeds min-max	Seed/Fruit
Moro	99	20	20.20	1	1.01	10	10	10.00
Sanguinello	131	4	3.05	4	3.05	26	1-11	6.50
Tarocco	122	15	0.00	0	0.00	0	0	0.00
A1	31	14	45.16	7	22.58	23	1-5	3.29
A2	31	15	48.39	1	3.22	4	4	4.00
A3	23	13	56.52	12	52.17	142	7-20	11.83
H1	30	13	43.33	1	3.33	4	4	4.00
H2	30	15	50.00	1	3.33	2	2	2.00
H3	40	21	52.50	14	35.00	135	3-24	9.64
K1	22	7	31.82	2	9.09	2	0-2	1.00
K2	21	8	38.10	0	0.00	0	0	0.00
Total	580	141		43		348		
Average			24.31		7.41			8.09

SRAP Analysis: Forty two monomorphic, 24 polymorphic, and the total of 66 bands were obtained from seven SRAP primer pairs in 'Clementine' × K1 cross (Table 1). While 9.4 markers per amplification were obtained, the polymorphic markers per amplification recorded were 3.4. Polymorphism ratio was 36.4% in the study. All hybrids were found to be true hybrids between 'Clementine' mandarin and K1 blood orange. They have at least one band difference (Fig 1). Uzun et al. [23] reported that the total of 376 polymorphic fragments was obtained from 21 SRAP primers with 17.9 markers per primer using 83 *Citrus* and relatives. Gulsen et al. [12] used 134 SRAP primers and scored 385 markers with 2.9 polymorphic markers per amplification in 164 F_1 hybrids between 'Clementine' × 'Orlando' cross. Polymorphic band ratio in the current study is lower than Uzun et al. [23] and higher than Gulsen et al. [12]. The reason for that might the differences between laboratory and analysis conditions.

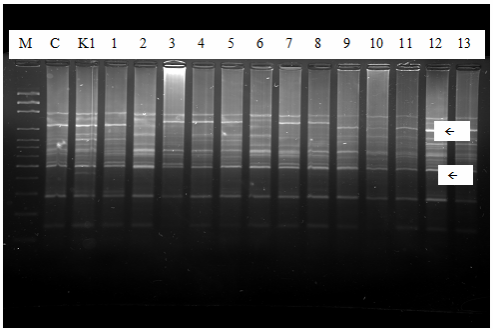

Figure 1. Banding pattern of Me3-Em9 SRAP primer combination with parents and hybrids
M: Marker (100 bp)
C: 'Clementine'
K1: blood orange (♂ parent)
Lanes 1-13: hybrid plants
Arrow (←) indicates polymorphic bands.

4. Conclusions

Although different pollen viability and germination ratios were obtained, this study presented some useful results for blood orange genotypes used for their maternal contribution in cross breeding experiments. It was observed that 'Clementine' mandarin has been a good maternal parent included in different blood orange crosses. SRAP markers effectively differentiated the genetic variation among hybrid plants cross between 'Clementine' mandarin and blood orange. The hybrids resulted from hand-made crosses need to be evaluated for their plant morphological and fruit pomological characteristics.

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