

Genetic improvement of faba bean (*Vicia faba* L.) genotypes selected for resistance to chocolate spot disease

Mejoramiento genético de genotipos de haba (*Vicia faba* L.) seleccionados con resistencia a la enfermedad de la mancha chocolate

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ABSTRACT

Inter-varietal hybridization is a powerful tool for genetic improvement and production of new genotypes for a trait of interest. Four parents of faba beans (*Vicia faba* L.) were hybridized using agromorphological and molecular characterization to obtain genotypes resistant to the chocolate spot disease. The study was done at the Nubaria Research Station, Giza, Egypt. Eight traits including resistance to chocolate spot, days to flowering, plant height (cm), number of branches/plant, number of pods/plant, number of seeds/plant, 100-seed weight (g), and seed yield/plant were estimated during the three growth seasons of 2016/2017, 2017/2018, and 2018/2019. Genetic parameters revealed by RAPD and ISSR markers assessed the genetic variation of genotypes with their generations. Crosses 1 (P₁ "Nubaria-1" x P₂ "Sakha-1"), 2 (P₁ "Nubaria-1" x P₃ "TW"), and 3 (P₁ "Nubaria-1" x P₄ "Camolina") revealed high resistance to disease with high yield. Markers patterned specific loci of resistant parents at a length of 360, 470, 450, 660, and 140 bp in RAPD and 1100, 810, 650, 700, 480 bp in ISSR. Inter-varietal hybridization between the resistant and susceptible genotypes is considered one of the most promising methods to obtain germplasm with resistance and high yield.

Key words: hybridization, ISSR, genetic resistance, plant breeding, RAPD, yield components.

RESUMEN

La hibridación intervarietal es una herramienta poderosa para el mejoramiento genético y la producción de nuevos genotipos prometedores para un rasgo de interés. Cuatro progenitores de haba (*Vicia faba* L.) fueron cruzados para obtener genotipos resistentes a la enfermedad de la mancha chocolate mediante caracterización agromorfológica y molecular. El estudio de campo se llevó a cabo en la Granja Experimental de la Estación de Investigación de Nubaria, Giza, Egipto. Se estimaron ocho características, incluidas el grado de resistencia a la mancha chocolate, los días a floración, la altura de planta (cm), el número de ramas/planta, el número de vainas/planta, el número de semillas/planta, el peso de 100 semillas (g) y el rendimiento de semillas/planta, durante las tres temporadas de crecimiento de 2016/2017, 2017/2018 y 2018/2019. Los parámetros genéticos se estimaron mediante marcadores RAPD e ISSR para evaluar la variación genética de los genotipos con sus generaciones. Los cruces 1 (P₁ "Nubaria-1" x P₂ "Sakha-1"), 2 (P₁ "Nubaria-1" x P₃ "TW") y 3 (P₁ "Nubaria-1" x P₄ "Camolina") revelaron alta resistencia a la enfermedad de la mancha chocolate con alto rendimiento. Los marcadores modelaron loci específicos de padres resistentes a una longitud de 360, 470, 450, 660 y 140 pb en RAPD y 1100, 810, 650, 700, 480 pb en ISSR. La hibridación intervarietal entre los genotipos resistentes y susceptibles es considerada uno de los métodos más promisorios para obtener germoplasma con resistencia y alto rendimiento.

Palabras clave: hibridación, ISSR, resistencia genética, fitomejoramiento, RAPD, componentes de rendimiento.

Introduction

Faba beans (*Vicia faba* L.; 2n=12) are one of the most common field pulses in Egypt (Bakry *et al.*, 2011; Mohamed *et al.*, 2012) and one of the oldest crops cultivated worldwide (Link *et al.*, 1995; Zong *et al.*, 2009). They belong to the Fabaceae family, the subfamily of Papilionoideae, and the

tribe of Viceae (Duc, 1997). Faba beans are consumed for their green pods and dried seeds (Duc *et al.*, 2010). They are considered a main source of cheap protein and energy in Africa and some parts of Asia and Latin America, where many people cannot afford a meat source of protein (Duc, 1997; Alghamdi, 2009).

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A fungal disease called chocolate spot, caused mainly by *Botrytis fabae* (Sardina) and by *B. cinerea* (Pers.), is one of the most damaging diseases to this plant (Harrison, 1988; El-Komy *et al.*, 2015; Haile *et al.*, 2016; Aguilar-Luna *et al.*, 2021). Symptoms oscillate from some spots on leaves to complete covering of the plant. Under severe conditions, the disease spreads from affected leaves into stems, flowers, and pods, causing damages (Bernier *et al.*, 1993; Rahman *et al.*, 2002; Villegas-Fernández *et al.*, 2012; Haile *et al.*, 2016). Various approaches are employed to control the disease, including genetic improvement (Wilson, 1937; Sahile *et al.*, 2008; Abou-Zeid & Hassanein, 2000; Aguilar-Luna *et al.*, 2021).

Quantitative trait loci (QTL) utilizes our knowledge of the effect of genetic control tools for selection in crop breeding programs. Various approaches are used for selecting promising traits, especially those for adapting to the local environment (Mahdy & El-Sharabasy, 2021). Phenotypic-revealed markers assess genetic diversity and performance of germplasm versus the attribute (Mahdy, 2012). These markers are based on visual observations (El-Sharabasy *et al.*, 2021; Mahdy & El-Sharabasy, 2021) and screen the quantitative traits to increase field crop production.

PCR (polymerase chain reaction) - based markers are applied for genetic improvement and breeding, genetic diversity, and genetic relationships (Chen *et al.*, 2008; Tomás *et al.*, 2016). PCR is fast, reproducible, simple, and low-cost procedure. PCR-based markers are very practical in multi-disciplines, including genetic diversity and genetic improvement programs (González *et al.*, 2005). Various PCR-based markers were applied on crops, *i.e.*, Random Amplified Polymorphic DNA (RAPD) (Link *et al.*, 1995), Inter-simple Sequence Repeats (ISSR) (Terzopoulos & Bebeli, 2008; Aguilera *et al.*, 2011; Abdel-Razzak *et al.*, 2012; Mahdy, 2012; Asfaw *et al.*, 2018) on jew's mallow, Restriction Fragment Length Polymorphism (RFLP) (Torres *et al.*, 1993), Start Codon Target (SCoT) (Mahdy *et al.*, 2021) on cowpea, and Conserved DNA-Derived Polymorphism (CDDP) (Ghazzawy *et al.*, 2021) on date palm.

We chose four faba bean parents for (1) improving genetic resistance against the chocolate spot, (2) determining the

genetic variations, (3) evaluating the performance under the infection of chocolate spot, (4) measuring genetic distance, and (5) generating a molecular profile using agromorphological traits and RAPD markers.

Materials and methods

Faba bean materials, planting, and field experiment

Four parents, as shown in Table 1, were sown on the experimental farm at the Nubaria Research Station, Agricultural Research Center (ARC), Egypt, during the seasons of 2016/2017, 2017/2018, 2018/2019, to evaluate their performance via the measurement of agromorphological traits. There is a high incidence of chocolate spot disease in Nubaria; moreover the disease is spread widely in the northern region of the Nile Delta of Egypt, with low temperatures and high relative humidity (Khalil *et al.*, 1993). Four parents were hybridized in 2016/2017 to secure F₁ hybrid seeds in the 2016/2017 season. In the 2017/2018 season, parents were re-hybridized; their F₁ hybrids were grown in a randomized complete block design with three replicates under insect-free cages. In the 2018/2019 season, parents with F₁ and F₂ generations were artificially inoculated with *Botrytis fabae* fungus, under insect-free cages, then purified and identified according to Morgan (1971). Each plot comprised six rows 3 m long, with 0.60 m distance between rows, and 0.2 m between mounds with two seeds in each.

Measurement of agromorphological traits

Eight traits were measured: plant height (cm), number of branches/plant, days to flowering, seed number per plant, pod number per plant, seed yield per plant (g), 100-seed weight (g), and reaction to chocolate spot disease. An assessment scale of response to chocolate spot disease was estimated using a quantitative scale of 0-5, where 0 (very highly resistance) indicates no visible chocolate spot, 1 (high resistance) indicates a few chocolate spots, 2 (resistance) indicates increased and scattered spots, 3 (moderately resistance) indicates larger spots, 4 (susceptible) indicates necrotic spots reaching half of the leaf, 5 (highly susceptible) indicates majority of necrotic spots and leaf abscission (ICARDA, 2005). Homogeneity of the

TABLE 1. Pedigree and origin of four parents.

ID	Parents	Origin	Pedigree	Botanical group	Foliar disease reaction
P1	Nubaria1	Egypt	Single plant selection form Giza Blanka	Large	Resistant
P2	Sakha1	Egypt	620/283/85x716/724/88	Medium	Resistant
P3	TW	Sudan	Sudan	Medium	Susceptible
P4	Camolina	Spain	Imported from Spain	Small	Susceptible

variance across environments was tested according to the Bartlett test (Steel & Torrie, 1980). Parents and their F₁ and F₂ hybrids were evaluated for the experiment traits and measurements without reciprocal.

Molecular analysis

DNA extraction

DNA was extracted from twenty samples of the faba beans (4 parents + 6 hybrids F₁ + 10 hybrids F₂) using the DNeasy Plant Kit (Qiagen, Germany). Nanodrop was used to determine the DNA concentration and quality.

PCR analysis

Six RAPD primers and five ISSR primers were used to detect the polymorphism among the twenty samples, which were synthesized by Metabion Corp., Germany (Tab. 2).

The amplification reaction was done in 25 µl reaction volume containing 12.5 µl Master Mix (sigma), 2.5 µl primer (10 pmol), 3 µl template DNA (10 ng), and 7 µl dH₂O, according to Ibrahim *et al.* (2019). The PCR was processed with a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems, USA) adjusted to fulfill 40 cycles. The initial denaturation cycle was for 5 min/94°C. Each was at 94°C in 45 s for the denaturation step and 72°C in 60 s for the elongation step. The annealing temperature was adjusted as in Table 2 for 50 s. The extension was adjusted at 72°C in 7 min in the final cycle. The amplified products were run in a 1.5% agarose gel containing ethidium bromide (0.5 µg ml⁻¹) in 1X TBE buffer at 95 V. A 100 bp DNA ladder (Promega, USA) was standardized to determine the PCR product sizes. Gel images were visualized using a UV transilluminator and photographed using a Gel

Documentation System (BIO-RAD 2000, USA). A binary matrix as present (1) or absent (0) was scored for the PCR products. The final data sets included both polymorphic and monomorphic bands.

Statistical analysis

A randomized complete block design (RCBD) with three replicates was used according to Gómez and Gómez (1984) and analyzed using MSTATC computer software. Average values and analysis of variance were conducted for all studied traits of 20 faba bean genotypes (four parents: (1) P₁, (2) P₂, (3) P₃, (4) P₄, six F₁ generation: (5) P₁xP₂, (6) P₁xP₃, (7) P₁xP₄, (8) P₂xP₃, (9) P₂xP₄, (10) P₃xP₄, and ten F₂ generation: (11) P₁xP₂, (12) P₁xP₃(R), (13) P₁xP₃(S) (14) P₁xP₄(R), (15) P₁xP₄(S) (16) P₂xP₃(R), (17) P₂xP₃(S), (18) P₂xP₄(R), (19) P₂xP₄(S) and (20) P₃xP₄(SxS)) in each generation according to Steel *et al.* (1997). The least significance difference (LSD) test ($P \leq 0.05$) was calculated (Steel & Torrie, 1980).

We calculated the number of total bands, unique bands, polymorphic bands, and the percent of polymorphism. We also estimated some genetic parameters. Shannon information index was calculated $\{I = -1 \times (p \times \ln(p) + q \times \ln(q))\}$ according to Shannon (1948). The observed number of alleles (N_a), effective number of alleles $\{N_e = 1 / (p^2 + q^2)\}$, Nei genetic diversity $\{h = 1 - \sum (p^2 + q^2)\}$, and Unbiased Diversity $\{u h = (N / (N - 1)) \times h\}$; where p = band frequency and $q = 1 - p$ estimated according to Hartl and Clark (1997) and Liu and Muse (2005). Nei's genetic identity and distance used Nei (1972) and Nei (1978). Nei's genetic identity was calculated with the following formula: $NeiI = \frac{J_{xy}}{\sqrt{J_x J_y}}$, where $J_{xy} = \sum_{i=1}^k P_{ix} P_{iy}$, $J_x = \sum_{i=1}^k P_{ix}^2$, $J_y = \sum_{i=1}^k P_{iy}^2$. Nei genetic distance was estimated with the formula $NeiD = -\ln I$, where I is the genetic identity. F-test was determined trait-marker associations. Power marker software V3.0 was fed. The association analysis selected markers with a high P -value ($P > 0.01$).

Results and discussion

Phenotype-based traits

The average values of the agromorphological traits were calculated (Tab. 3). The results showed broad significance, as evidenced by the characteristic ranges for chocolate spot disease (1.30 to 5.63, resistant to susceptible, respectively), days to flowering (40.80 to 63.66), plant height (93.30 to 131.66 cm), number of branches per plant (3.10 to 10.80), number of pods per plant (25.46 to 111.36), number of seeds per plant (83.13 to 295.83), 100 seed weight (47.30 to 137.20 g), and seed yield per plant (56.37 to 199.96).

TABLE 2. List of primers and their nucleotide sequence.

No	Name	Sequence	Temperature, °C
1	OPA-07	5'-GAAACGGGTG-3'	36
2	OPA-10	5'-GTGATCGCAG-3'	36
3	OPA-17	5'-GACCGCTTGT-3'	36
4	OPB-05	5'-TGCGCCCTTC-3'	36
5	OPG-19	5'-GTCAGGGCAA-3'	36
6	OPG-20	5'-TCTCCCTCAG-3'	45
7	ISSR-1	5'-AGAGAGAGAGAGAGAGTC-3'	45
8	ISSR-2	5'-AGAGAGAGAGAGAGAGTG-3'	45
9	ISSR-3	5'-ACACACACACACACAT-3'	45
10	ISSR-4	5'-ACACACACACACACTG-3'	45
11	ISSR-5	5'-GTGTGTGTGTGTGTAG-3'	45

TABLE 3. Performance of growth and yield characteristics.

Genotypes	Chocolate spot	Days to flowering	Plant height (cm)	No. of branches/plant	No. of pods/plant	No. of seeds/plant	100-seed weight (g)	Seed yield/plant
Parents								
P ₁	1.30	63.66	130.80	10.80	44.50	152.33	129.30	187.16
P ₂	1.87	42.93	112.20	4.50	42.33	131.73	100.36	132.70
P ₃	5.63	42.56	102.63	3.10	38.73	104.03	55.20	56.73
P ₄	5.40	40.80	102.17	5.10	46.56	137.80	47.30	64.93
F₁ generation								
P ₁ ×P ₂	1.86	41.90	125.80	6.53	40.73	119.00	137.20	155.66
P ₁ ×P ₃	2.73	51.83	127.47	5.63	44.33	132.13	93.26	129.00
P ₁ ×P ₄	2.20	44.96	126.10	7.56	82.46	250.86	78.10	199.96
P ₂ ×P ₃	2.30	46.63	119.13	5.90	67.46	205.20	71.83	147.96
P ₂ ×P ₄	2.86	44.63	111.90	6.96	98.50	295.83	62.53	181.20
P ₃ ×P ₄	3.86	42.06	103.83	5.53	111.36	265.96	54.53	134.06
LSD _{0.05}	1.18	3.33	5.35	1.03	9.27	35.34	7.61	30.35
F₂ generation								
P ₁ ×P ₂ (R×R)	1.73	50.20	131.66	6.90	47.40	147.30	132.13	172.36
P ₁ ×P ₃ (R)	2.44	49.63	125.38	5.42	44.33	132.13	93.26	129.00
P ₁ ×P ₃ (S)	3.53	47.00	122.20	4.20	25.46	83.13	76.40	62.60
P ₁ ×P ₄ (R)	2.22	49.43	136.18	7.56	73.58	242.08	78.16	169.88
P ₁ ×P ₄ (S)	3.53	48.63	132.43	6.56	53.86	175.73	76.40	123.06
P ₂ ×P ₃ (R)	2.34	45.83	117.73	5.92	65.77	203.82	71.93	149.76
P ₂ ×P ₃ (S)	3.40	42.93	116.63	4.46	55.86	173.13	70.46	119.80
P ₂ ×P ₄ (R)	2.48	45.13	113.82	5.87	91.73	276.91	61.84	171.68
P ₂ ×P ₄ (S)	4.50	42.86	111.06	4.60	73.06	158.90	60.03	95.76
P ₃ ×P ₄ (S×S)	5.20	43.33	93.30	5.36	61.40	181.70	51.90	93.50
LSD _{0.05}	1.01	4.10	6.13	1.44	8.07	32.92	6.52	29.82

As shown in Table 3, Nubaria 1 recorded the highest mean values of 100-seed weight, seed yield/plant, and plant height (cm). Meanwhile, Camolina recorded a higher number of pods/plant, seed/plant, and branches/plant than the other parents. On the other hand, TW recorded the lowest mean values of the number of pods and seeds per plant. The two parents, Nubaria 1 and Sakha 1, gave the highest mean values of plant height. The parents, Nubaria 1 and Sakha 1, were considered highly resistant; their estimated mean values were (1.30 and 1.80) for chocolate spot infection. The parents TW and Camolina had the highest susceptible values (5.63 and 5.40) for the chocolate spot.

F₁ generation

The mean values of the sex-tested hybrids were calculated (Tab. 3). Results indicated that the cross (P₁×P₂) had the highest mean value of 100-seed weight (137.20 g). The two crosses, P₁×P₃ and P₁×P₄, produced the last cross. For plant height, the crosses, P₁×P₃ and P₂×P₄, had the highest mean values (127.47 cm and 126.10 cm, respectively) of plant height and the lowest value (103.83 cm) obtained from the

cross (P₃×P₄). The genotypes Camolina, Sakha1, and TW were the parents which flowered quickest. Also, cross P₁×P₂ (41.90 d) was the cross which flowered quickest, followed by the cross P₃×P₄ with a mean value of 42.06 d. Concerning the number of branches/plant, the results showed that three crosses (P₁×P₄), (P₂×P₄), and (P₁×P₂) had the highest values.

Concerning the number of pods per plant, the parental variety Camolina (P₄) showed the highest mean value (46.56), whereas cross (P₁×P₂) gave the lowest number of pods/plant. For the number of seeds per plant, the cross P₂×P₄ gave the highest mean value (295.83), followed by the two crosses P₃×P₄ (265.96) and P₁×P₄ (250.86).

The two crosses (P₁×P₂) with 137.20 g and (P₁×P₃) with 93.26 g had the highest mean of 100-seed weight and highest seed yield per plant.

The highest seed yield per plant could be attributed to the high number of seeds and seed weight/plant. The two mentioned crosses were the most promising for yielding ability and tended to combine high seed yield and its components.

F₂ generation

The cross (P₁xP₂) gave the highest mean values of the number of seeds per plant, seed yield per plant, and 100-seed weight and gave the lowest infection value for chocolate spot (1.73) (Tab. 3). The cross (P₁xP₃) had the lowest mean values for number of pods per plant and number of seeds per plant and the highest infection value of the cross P₁xP₂ (3.53). The cross (P₃xP₄) had a low mean value for 100-seed weight, and the reaction for chocolate spot gave the highest susceptible value (5.20).

Three crosses, P₂xP₃, P₂xP₄, and P₃xP₄, were the earliest crosses for the flowering date. The cross (P₂xP₄) gave the highest number of pods per plant, number of seeds per plant, and the highest susceptible value for reaction chocolate spot (4.50). The lowest values were scored by the two crosses, P₁xP₄ and P₂xP₃, which also gave the lowest value for chocolate spot infection (3.53 and 3.40, respectively). Those genotypes considered promising for chocolate spot resistance and the best performing F₁ crosses had mean values slightly better than those of F₂ crosses in most of the studied traits.

Significant differences among faba bean genotypes in all studied traits were considerable evidence for the existence of a suitable amount of genetic diversity valid for further assessments (Ahmed *et al.*, 2016; Abdalla *et al.*, 2017; Hamza & Khalifa, 2017; Abou-Zaid & El-Gendy, 2019; El-Abssi *et al.*, 2019).

The results suggest that these genotypes carry genes for resistance to chocolate spot disease, coming from their parents according to their pedigree (Tab. 1). Similar results have been reported for faba bean yield traits and components, as well as for disease resistance traits (Abid *et al.*, 2015; Zakaria *et al.*, 2015; Beyene *et al.*, 2016; Eldemery *et al.*, 2016; Belal *et al.*, 2018; El-Rodeny *et al.*, 2020).

Polymorphism revealed via RAPD markers

Performance among twenty faba bean genotypes was analyzed by PCR-based markers, RAPD and ISSR (Figs. 1-2). Table 4 summarizes the data resulting from all analyzed loci that resulted from 148 amplified fragments ranging from 60 for ISSR and 88 for RAPD. The RAPD markers ranged from 130-2000 bp while the ISSR markers oscillated from 160-1500 bp. The polymorphism averaged a score of 74%, oscillating from 57% for ISSR to 74% for RAPD. RAPD markers revealed a high percentage of polymorphism compared with ISSR. The RAPD markers scored four positive unique bands by the primer OPA-07. Unique bands were at 1350 and 140 bp for P₁xP₂ (F₁), 920 and 130 bp for P₁xP₄ S and P₁xP₃ S (F₂), respectively. On the contrary, the ISSR revealed negative unique bands across the F₂ generation. The primer ISSR-1 scored a negative unique band at a distance of 160 bp (P₁xP₄ R); ISSR-3 scored at 650 bp (P₂xP₄ R), 360 (P₁xP₃ S), and 250 bp (P₂xP₃ R); and ISSR-5 scored 529 bp (P₂xP₄ R).

TABLE 4. Polymorphism revealed by ISSR and RAPD markers.

Primers	MW (bp)	Total (AF)	M _b	U _{b+}	U _{b-}	P _b	P (%)
RAPD							
OPA-07	130 -1350	19	5	4	0	14	74
OPA-10	160 - 1500	14	3	0	0	11	79
OPA-17	140 - 860	14	5	0	0	9	64
OPB-05	140 - 820	10	3	0	0	7	7
OPG-19	160 - 2000	15	2	0	0	13	87
OPG-20	180-1800	16	5	0	0	11	69
Total_{RAPD}	130-2000	88	23	4	0	65	74
ISSR							
ISSR-1	1530 -1100	14	4	0	1	10	71
ISSR-2	170 - 800	12	6	0	0	6	5
ISSR-3	200 - 1500	14	4	0	3	10	71
ISSR-4	160 - 530	11	8	0	0	3	27
ISSR-5	239 - 950	9	4	0	1	5	56
Total_{ISSR}	160 - 1500	60	26	0	5	34	57
Total		148	49	4	5	99	67

MW=molecular weight, AF=amplified fragment, M_b=monomorphic bands, U_{b+}=positive unique bands, U_{b-}=negative unique bands, P_b=polymorphic bands, P=polymorphism percentage.

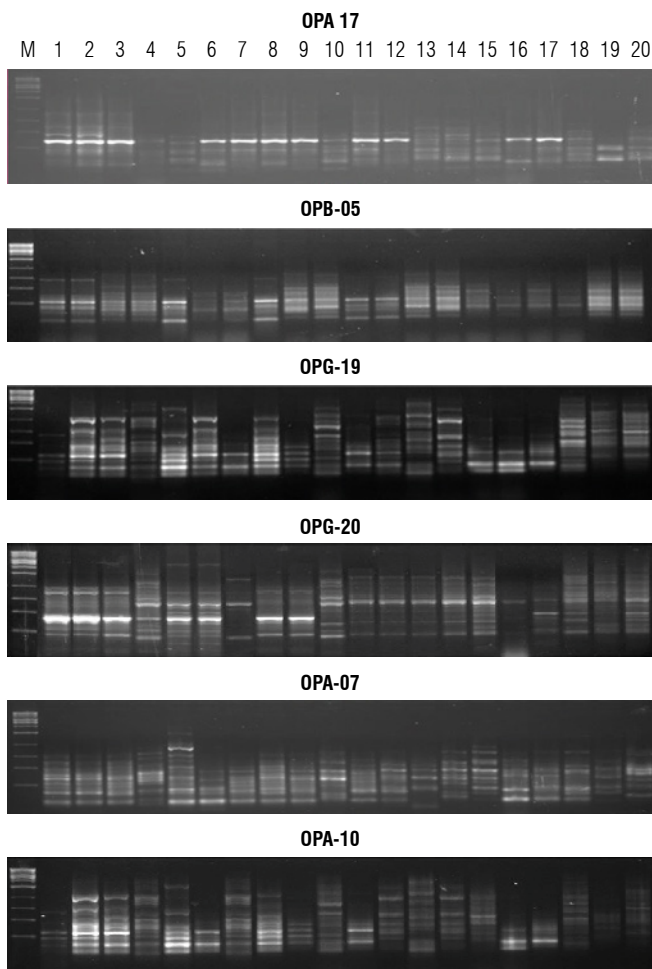


FIGURE 1. Profiles of twenty faba bean genotypes revealed via RAPD.

Table 5 summarizes specific loci in parents segregated during subsequent generations. These loci can be used directly for the breeding program for resistance to chocolate spot disease. RAPD markers patterned specific loci of resistant parents at a length of 360 bp for OPA-07, 470 bp (P_2), and 450 bp (P_1 and P_2) for OPA-17, 660 and 140 bp (P_1 and P_2) for OPB-05. Primers, OPA-10, OPG-19, and OPG-20, patterned specific loci of susceptible P_4 except the primer OPA-17 owing to a specific locus for susceptible parent (P_3) at a distance of 400 bp. ISSR patterned resistant specific loci at a length of 1100 & 810 bp by ISSR-1, 650 bp by ISSR-2, and 700 & 480 bp by ISSR-3 for parents 1 and 2. The primers ISSR-2 and ISSR-5 patterned susceptible specific loci at 800 & 170 bp for P_3 and P_4 and 390, 281 and 239 bp, respectively. The primer ISSR-1 characterized P_3 only at a length of 200 bp.

We found the RAPD markers showed higher polymorphism than the ISSR, in contrast to other results detected in wheat (Nagaoka & Ogihara, 1997) and vigna beans (Ajibade *et al.*, 2000). Terzopoulos and Bebeli (2008) found 68%

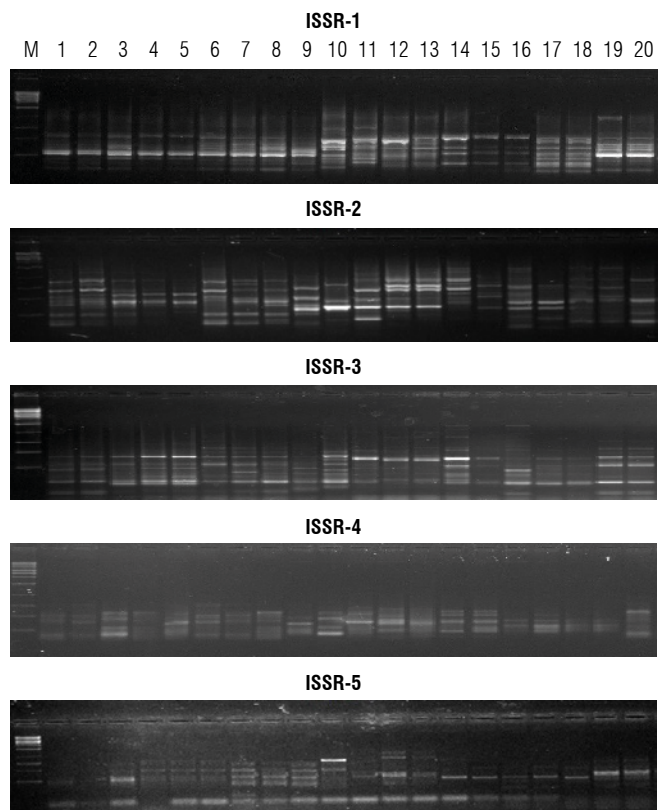


FIGURE 2. Profiles of twenty faba bean genotypes revealed via ISSR.

polymorphism between the Greek faba bean populations using eleven ISSR primers. Khalaf *et al.* (2015) found 73% polymorphism between seven faba bean genotypes using fifteen ISSR primers.

Genetic parameters, identity, and distance

Genetic parameters are vital for the efficiency of a marker technique used in discriminatory objects based on polymorphism. The number of genetic parameters was estimated to evaluate the informative and discriminatory power during subsequent faba beans generation, as shown in Table 6. Heterozygosity (h) averaged 0.18, oscillating from 0.16 to 0.20 for RAPD, from 0.15 to 0.23 for ISSR, and from 0.16 to 0.21 for both markers. h reveals important polymorphism information content (PIC), with high informative marker value.

The Shannon index (I) revealed an average of 0.271 for both markers used. The RAPD markers ranged from 0.229 for Parents to 0.304 for F_1 , with an average of 0.275. The ISSR markers oscillated from 0.23 (F_1) to 0.33 (F_2), with an average of 0.266. The effective number of alleles (N_e) of RAPD markers ranged from 1.275 (Parents) to 1.311 (F_1), while ISSR revealed the range from 1.26 (F_1) to 1.1 (F_2), with a grand average of 1.306.

TABLE 5. Specific loci related to performance of resistance and susceptible genotypes.

Primer	MW	P _R	P _S	F ₁	F ₂
OPA-07	360	P ₁ , P ₂		P ₁ xP ₃	P ₁ xP ₂ , P ₂ xP ₃ (R), P ₂ xP ₃ S, P ₃ xP ₄
OPA-10	1200	---	P ₄	P ₃ xP ₄	P ₁ xP ₃ S
	810	---	P ₄	P ₁ xP ₄ & P ₃ xP ₄	P ₁ xP ₃ S & P ₁ xP ₄ S & P ₂ xP ₄ R & P ₃ xP ₄
OPA-17	470	P ₂	---	----	P ₂ xP ₄ S & P ₃ xP ₄
	450	P ₁ & P ₂	---	P ₁ xP ₄ & P ₂ xP ₃ & P ₃ xP ₄	P ₁ xP ₂ & P ₁ xP ₃ R & P ₁ xP ₃ S & P ₁ xP ₄ R & P ₂ xP ₄ R
	400		P ₃	P ₁ xP ₂	P ₁ xP ₃ S & P ₁ xP ₄ R & P ₁ xP ₄ S
OPB-05	660	P ₁ & P ₂	---	P ₁ xP ₂ & P ₂ xP ₃	P ₂ xP ₄ S & P ₃ xP ₄
	140	P ₁ & P ₂	---	P ₁ xP ₂ & P ₂ xP ₃	P ₁ xP ₂ & P ₁ xP ₃ R & P ₂ xP ₄ S & P ₃ xP ₄
OPG-19	2000	---	P ₄	----	P ₂ xP ₄ R
	840	---	P ₄	P ₂ xP ₄ & P ₃ xP ₄	P ₁ xP ₃ R & P ₁ xP ₃ S & P ₂ xP ₄ R & P ₂ xP ₄ S
	600	---	P ₄	P ₂ xP ₄	P ₁ xP ₃ S
OPG-20	1500	---	P ₄	P ₃ xP ₄	P ₁ xP ₂ & P ₂ xP ₄ R & P ₂ xP ₄ S
	1050	---	P ₄	P ₃ xP ₄	P ₂ xP ₄ R & P ₂ xP ₄ S & P ₃ xP ₄
	210	---	P ₄	P ₁ xP ₄ & P ₃ xP ₄	----
ISSR-1	1100	P ₁ & P ₂	---	P ₁ xP ₂	P ₁ xP ₄ R
	810	P ₁ & P ₂	---	P ₁ xP ₂ & P ₂ xP ₄	P ₁ xP ₂ & P ₂ xP ₄ R & P ₂ xP ₄ S
	200	---	P ₃	P ₁ xP ₃ & P ₁ xP ₄ & P ₂ xP ₃ & P ₂ xP ₄ & P ₃ xP ₄	P ₁ xP ₃ R & P ₁ xP ₄ S & P ₂ xP ₃ R & P ₂ xP ₄ R & P ₃ xP ₄
ISSR-2	800	---	P ₃ & P ₄	P ₃ xP ₄	P ₁ xP ₃ R & P ₁ xP ₃ S & P ₁ xP ₄ R & P ₁ xP ₄ S & P ₂ xP ₃ R & P ₂ xP ₃ S & P ₂ xP ₄ R & P ₂ xP ₄ S
	650	P ₁ & P ₂	---	P ₁ xP ₂ & P ₂ xP ₄	P ₁ xP ₂ & P ₂ xP ₄ R & P ₂ xP ₄ S
	170	---	P ₃ & P ₄	P ₃ xP ₄	P ₃ xP ₄
ISSR-3	700	P ₁ & P ₂	---	P ₁ xP ₃ & P ₁ xP ₄ & P ₂ xP ₃ & P ₂ xP ₄ & P ₃ xP ₄	P ₁ xP ₂ & P ₁ xP ₃ R & P ₁ xP ₃ S & P ₁ xP ₄ R & P ₁ xP ₄ S & P ₂ xP ₃ R & P ₂ xP ₃ S & P ₂ xP ₄ R & P ₂ xP ₄ S
	480	P ₂	---	P ₂ xP ₃ & P ₂ xP ₄	P ₁ xP ₂ & P ₁ xP ₃ R & P ₁ xP ₄ R & P ₂ xP ₃ R & P ₂ xP ₃ S & P ₂ xP ₄ S
ISSR-5	390	---	P ₃ & P ₄	P ₂ xP ₃ & P ₃ xP ₄	P ₁ xP ₃ R & P ₁ xP ₃ S & P ₁ xP ₄ R & P ₁ xP ₄ S & P ₂ xP ₃ R & P ₂ xP ₃ S & P ₂ xP ₄ R & P ₂ xP ₄ S & P ₃ xP ₄
	281	---	P ₃ & P ₄	P ₂ xP ₄ & P ₃ xP ₄	P ₁ xP ₃ S & P ₁ xP ₄ S & P ₂ xP ₃ S & P ₂ xP ₄ R & P ₂ xP ₄ S & P ₃ xP ₄
	239	---	P ₃ & P ₄	P ₃ xP ₄	P ₁ xP ₃ S & P ₁ xP ₄ S & P ₂ xP ₃ S & P ₂ xP ₄ S & P ₃ xP ₄

TABLE 6. Genetic parameters calculated among parents and their generations.

Generation	Na	Ne	I	He	uHe	%P
RAPD						
P	1.148	1.275	0.229	0.16	0.178	38
F ₁	1.568	1.311	0.304	0.20	0.213	64
F ₂	1.568	1.297	0.292	0.19	0.197	64
Total	1.428	1.294	0.275	0.18	0.196	56
ISSR						
P	1.33	1.30	0.24	0.17	0.19	0.38
F ₁	1.38	1.26	0.23	0.15	0.17	0.42
F ₂	1.57	1.41	0.33	0.23	0.24	0.57
Total	1.428	1.324	0.266	0.18	0.199	45.56
Grand markers						
P	1.223	1.286	0.232	0.16	0.183	39.19
F ₁	1.493	1.291	0.272	0.18	0.194	54.73
F ₂	1.568	1.342	0.309	0.21	0.216	60.81
Total	1.428	1.306	0.271	0.18	0.197	51.58

Na=number of alleles, Ne=effective number of alleles, I=Shannon index, He=expected heterozygosity, uHe=unbiased expected heterozygosity, and %P=percentage of polymorphism.

Duc *et al.* (2010) reported an enormous genetic variability for faba beans, useful for breeding purposes. The results of h agree with those of Suresh *et al.* (2013), who recorded PIC values of 0.45 in faba beans genotypes revealed by developing 55 novel polymorphic cDNA-SSR markers. Also, Oliveira *et al.* (2016) recorded PIC values from 0.07 to 0.66, with an average of 0.33. Hemeida (2008) used PIC to evaluate the primer efficiency of ISSR and established the relationships, and successfully discriminated among the genotypes tested.

The difference between the mean diversity (h) of both markers and between markers was undoubtedly due to mirror inbreeding or selection method against heterozygotes. The nature of used markers might be due to the level of observed heterozygosity resulting in the non-detection of homozygotes from heterozygotes because of the presence of null alleles. The heterozygosity represents the direct count of heterozygosity in the population and is estimated based on the allele frequency of individuals in that population according to the Hardy-Weinberg equilibrium. The PIC evaluates the informative potential of markers in different germplasm (Grativol *et al.*, 2011; Mahdy, 2018). The h value of a marker with many amplicons desirable for variation splits into three main classes based on Botstein *et al.* (1980). The PIC values are more than 0.5 for highly informative markers, between 0.25-0.5 for reasonably informative markers, and less than 0.25 for slightly informative markers. The Shannon information index (I) is one of the most important genetic diversity measurements (Sherwin *et al.*, 2006). The effective number of alleles (N_e) is a reciprocal of gene homozygosity (Hartl & Clark, 1997). The N_e is used as a corollary to h ; when h is high, N_e will be the high.

Table 7 summarizes the estimates of genetic distance and genetic identity between generations and their parents according to the Nei coefficient (Nei, 1972; Nei, 1978). It reflects the genetic relationships and the direction of the genetic improvement process. Results show that the RAPD markers revealed high genetic identity and distance values. The highest genetic distance value scored by RAPD was 0.089 on parents versus F_1 , and the lowest value scored 0.028 on F_1 versus F_2 . Genetic identity oscillated from 0.914 (parent versus F_1) to 0.972 (F_1 versus F_2), as revealed by RAPD.

Nei genetic identity ranges from 0 to 1. Consequently, Nei genetic distance ranges from 0 to infinity (Nei 1972; Nei, 1978). ISSRs are more efficient markers for polymorphism and potent for intra- and inter-genomic diversity than other arbitrary markers like RAPDs (Zietkiewicz *et al.*, 1994). Both markers target different portions of the genome.

TABLE 7. Genetic and identity distance among generations and parents.

Parameter	P versus F_1	P versus F_2	F_1 versus F_2
RAPD			
Nei genetic identity	0.924	0.914	0.972
Nei unbiased genetic identity	0.947	0.932	0.989
Nei genetic distance	0.079	0.089	0.028
Nei unbiased genetic distance	0.055	0.070	0.011
ISSR			
Nei genetic identity	0.951	0.942	0.954
Nei unbiased genetic identity	0.973	0.964	0.969
Nei genetic distance	0.050	0.059	0.047
Nei unbiased genetic distance	0.028	0.037	0.031
All			
Nei genetic identity	0.935	0.925	0.964
Nei unbiased genetic identity	0.957	0.945	0.981
Nei genetic distance	0.067	0.077	0.036
Nei unbiased genetic distance	0.044	0.057	0.020

Genetic variations in genotypes of interest may be more directly due to polymorphism detected by technique rather than which techniques are employed.

Cluster analysis and similarity between twenty genotypes

Cluster analysis derived from both markers based on UPGMA in accordance with Jaccard (1908; Fig. 3). The twenty genotypes divided into two main groups at a distance of 0.65. Cluster I (right cluster) divided into two subgroups at a distance of 0.668. The first subgroup (left subgroup) consists of P_1 and P_2 in the sub-sub group and $P_1 \times P_2$ (F_1) only in a sub-sub group. The second subgroup (right subgroup) split further into sub-sub groups, which include the genotypes of $P_1 \times P_2$ (F_2) and $P_2 \times P_4$ (F_1) grouped in a cluster, $P_1 \times P_3$ (F_1) only in a group, $P_2 \times P_3$ (F_1) only in a group, and $P_2 \times P_3$ S(F_2) and $P_2 \times P_3$ R(F_2) together in a group.

Cluster II (left cluster) separated into two subgroups at a distance of 0.672. Each split further into sub-sub groups. The first sub-sub group (right one) includes $P_1 \times P_2$ R(F_2) and $P_2 \times P_4$ R(F_2) together, P_4 and $P \times P_4$ (F_1) in a cluster, and $P_1 \times P_4$ R(F_2) and $P_1 \times P_4$ S(F_2) in a group. The second one consists of $P_2 \times P_4$ S(F_2), $P_1 \times P_3$ S(F_2), and $P_3 \times P_4$ (F_2) in a separated cluster for each and P_3 and $P_3 \times P_4$ (F_1) in a cluster.

El-Ghadban *et al.* (2017) and Mahdy *et al.* (2021) reported similar results. The differences in the clustering pattern of genotypes may be due to marker sampling error, the level of polymorphism, or the number of loci and their coverage across the genome (Loarce *et al.*, 1996).

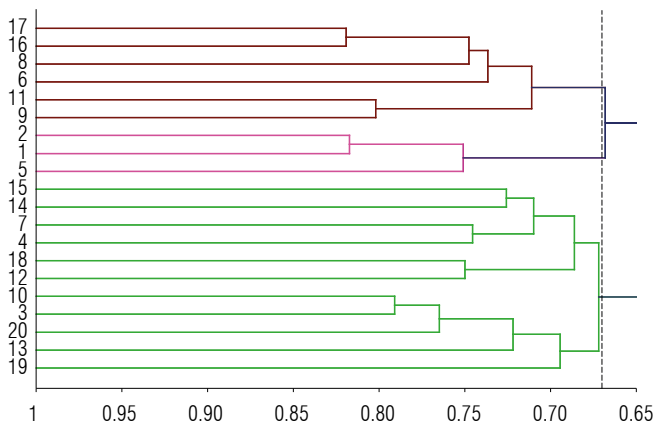


FIGURE 3. Dendrogram tree constructed using UPGMA according to Jaccard similarity. Cluster analysis based on RAPD and ISSR markers; (1)-P₁, (2)-P₂, (3)-P₃, (4)-P₄, “six F₁” (5)-P₁xP₂, (6)-P₁xP₃, (7)-P₁xP₄, (8)-P₂xP₃, (9)-P₂xP₄, (10)-P₃xP₄, “ten F₂” (11)-P₁xP₂, (12)-P₁xP₃ (R), (13)-P₁xP₃ (S) (14)-P₁xP₄ (R), (15)-P₁xP₄ (S) (16)-P₂xP₃ (R), (17)-P₂xP₃ (S), (18)-P₂xP₄ (R), (19)-P₂xP₄ (S) and (20)-P₃xP₄ (S×S)”.

Association analysis

Trait-marker association was analyzed using a single locus F-test module in Power maker software (Fig. 4). The results show the property of one trait for one locus characterized. The characterized locus generated by OPG-20 was associated uniquely with seed yield per plant. Moreover, several traits were associated with one or more than one locus. Amplified loci of OPA-07 were associated with the different characterized chocolate spots, days to flowering, the number of branches per plant, and seed yield per plant.

Previous results have suggested the high potential use of molecular markers in search for genes affecting crop productivity and resistance to chocolate spots. Also, defense mechanisms against biotic and abiotic stress factors identified statistical associations between the genetic markers and the traits of interest.

Marker-trait associations were generally diverse in the amount of genetic variation valid for assessment. The results exhibited that a greater number of primers was possibly involved in controlling traits at resistance to chocolate spot disease. This variation described by identified associations for each trait may be attributed to the role of many minor genes controlling the trait, performance, and reaction of faba bean genotypes to chocolate spot disease, markers exhibiting minor quantitative effect, rare alleles, and complex allelic interactions (Yang *et al.*, 2010; Debibakas *et al.*, 2014). These results correspond with the findings of Lou *et al.* (2015) and Sun *et al.* (2015). Association data can

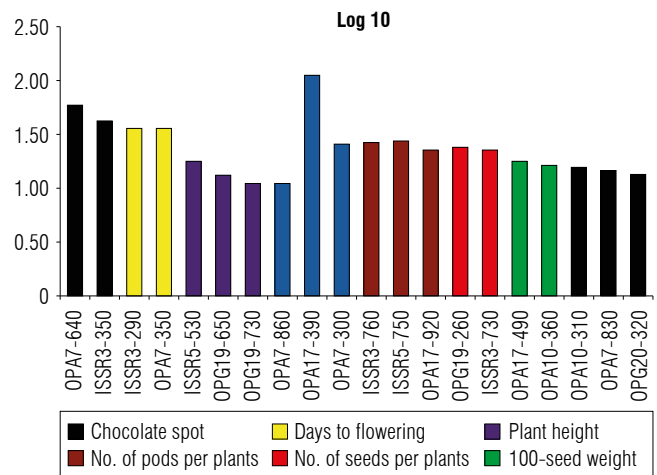


FIGURE 4. Association analysis. Trait-marker association was achieved through F-test analysis. Power marker software V3.0 was used and markers with high *P*-value (*P*>0.01) were selected in the association analysis. X and Y axes refer to -Log P values and the name of primers associated with certain traits.

be used for faba beans breeding, especially in terms of its resistance to biotic and abiotic stresses.

Conclusion

The parents Nubaria1, Sakha1, TW, and Camolina could be considered good for crossing for resistance to foliar chocolate spot disease. Crosses 1, 2, and 3 (P₁xP₂ Nubaria-1 x Sakha-1, P₁xP₃ Nubaria-1 x T.W, P₁xP₄ Nubaria-1 x Camolina, respectively) showed resistance to disease during both generations, F₁ and F₂ with high values for yield and its components, especially date of flowering and 100-seed weight per plant. Both markers exhibited interest specific loci relating to performance of chocolate spot that are 360 bp (OPA-07), 450 and 470 bp (OPA-17), 140 and 660 bp (OPB-05), 810 and 1100 bp (ISSR-1), 650 bp (ISSR-2), and 480 and 700 bp (ISSR-3). The genetic distance valued by RAPD was 0.089 on parents versus F₁, and the lowest value scored 0.028 on F₁ versus F₂. Genetic identity oscillated from 0.914 (parent versus F₁) to 0.972 (F₁ versus F₂), as revealed by RAPD. Estimating genetic relationships and differences generated by both markers could be moderately clarified by the product number of PCR, the number of bands, and coverage along the genome with the association of agromorphological traits. The current research could provide information on the morphological and molecular characteristics of faba beans chocolate spot disease. Inter-varietal hybridization (resistant and susceptible) is still considered one of the most promising methods for obtaining germplasm with resistance and high yield.

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Conflict of interest statement

The authors declare that there is no conflict of interest regarding the publication of this article.

Author's contributions

EM, MI, AM, and HEH designed the experiments. HEH, EM, and MI carried out the formal analysis. MI and HEH provided all resources. EM, MI, and AM oversaw and led the research activity and execution. All authors wrote the original draft. EM, HEH, and EMBM authorized, edited, and reviewed the manuscript.

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