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Morpho-agronomical and Biochemical Traits Screening and Genetic Variability in Selected Black Cumin (*Nigella sativa*) Mutant Lines

(Ciri Morfo-agronomi dan Biokimia Penyaringan serta Kebolehubahan Genetik pada Garis Mutan Jintan Hitam *(Nigella sativa)* Terpilih)

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ABSTRACT

The production of new Nigella sativa *cultivars by plant breeding programs is difficult due to its narrow genetic base. A number of induced morphological traits, yield components and percent content of fatty acid methyl esters in the parent line and nine selected mutants (Mt1-Mt9) have been reported in two generations (M_3 and M_4) of* N. sativa *to determine the best genotype to release as a new cultivar. The results showed that Mt2 plants were the tallest (118.3 and 149.7 cm in M_3 and M_4, respectively). The highest seed yield per plant was measured for Mt5; Mt4 showed the highest per cent of palmitic and stearic acids, 11.93% and 13.70%, respectively; whereas Mt8 had the highest percent content (45.67%) of linoleic acid. Five Inter Simple Sequence Repeat (ISSR) markers were used to investigate genetic variability within mutant lines and their parent. These primers generated 71 reproducible and scorable amplification products across the genotypes tested. Fifty-eight of these fragments were highly polymorphic (81.7%). The proportion of common bands (13) was low (18.3%). All primers produced unique fragments and generated 33 specific alleles. The average number of amplification products per primer was 14.2. The size of ISSR amplified fragments varied from 1109 to 148 base pairs (bp). The similarity between each mutant and the parent line varied from 0.56% to 100%. Finally, the present investigation indicated that mutants Mt5 and Mt6 are promising high yielding genotypes which can be recommended as new cultivars, whereas Mt3 and Mt7 possess an attractive phenotype appropriate for ornamental use.*

Keywords: Fatty acid; ISSR; mutant; Nigella sativa*; yield*

ABSTRAK

Penghasilan kultivar Nigella sativa *baharu melalui program pembiakan tumbuhan adalah sukar kerana asas genetik yang sempit. Beberapa ciri morfologi yang diinduksi, komponen hasil dan kandungan peratus metil ester asid lemak dalam titisan induk dan sembilan mutan terpilih (Mt1-Mt9) telah dilaporkan dalam dua generasi (M_3 dan M_4)* N. sativa *untuk menentukan genotip terbaik yang boleh dikeluarkan sebagai kultivar baharu. Keputusan menunjukkan bahawa tumbuhan Mt2 adalah tertinggi (118.3 dan 149.7 cm masing-masing dalam M_3 dan M_4). Hasil bijian tertinggi telah diukur bagi tumbuhan Mt5, Mt4 menunjukkan kadar tertinggi asid palmitik dan stearik, masing-masing 11.93% dan 13.70%, manakala Mt8 mempunyai kandungan peratus tertinggi (45.67%) bagi asid linoleik. Sebanyak lima penanda* Inter Simple Sequence Repeat (ISSR) *digunakan untuk mengkaji kebolehubahan genetik dalam titisan mutan dan induk mereka. Pencetus ini menjanakan 71 produk amplifikasi yang boleh diulang dan penskoran merentas genotip yang diuji. Sejumlah 58 fragmen ini sangat polimorfik (81.7%). Perkadaran jalur biasa (13) adalah rendah (18.3%). Semua pencetus menghasilkan serpihan unik dan menghasilkan 33 alel khusus. Purata bilangan produk amplifikasi bagi setiap pencetus adalah 14.2. Saiz serpihan ISSR yang diperkuat bervariasi daripada 1109 hingga 148 pasangan asas (bp). Kesamaan antara setiap mutan dan garis induk adalah antara 0.56% hingga 100%. Akhirnya, kajian ini menunjukkan bahawa mutan MT5 dan Mt6 merupakan genotip yang memberikan hasil yang tinggi dan boleh disyorkan sebagai kultivar baru, manakala Mt3 dan Mt7 mempunyai fenotip yang sesuai untuk kegunaan tumbuhan hiasan.*

Kata kunci: Asid lemak; hasil; ISSR; mutan; Nigella sativa

INTRODUCTION

Nigella sativa L. (Ranunculaceae), commonly known as black cumin, is an annual herbal crop with a wide range of medicinal uses (Pruthi 1998). An interesting quote by Prophet Mohammed, peace be upon him, says 'Use the black seed, for indeed, it is a cure for every disease, except death'. The grain yield of black cumin is ranged from 449.62 to 612.98 kg/ha according to variety (Assefa et al. 2015). It contains 32% to 40% fixed oil consisting of saturated and unsaturated fatty acids and 0.40% to 0.45% volatile oil. Effective utilisation of *N. sativa* for medicinal purposes as well as for trade largely depends upon its yield (raw plant product, seeds, bioactive compounds, essential oil) and quality. Black cumin may also be grown as an

ornamental plant in gardens for its feathery, finely cut leaves and delicate white flowers (Subrahmanyam 2009).

Mutation breeding is more effective than hybridisation when natural variation does not provide the genes for desired trait or genes are present but tightly linked to undesirable genes (Chahal & Gosal 2002). Induced mutation is the best method of creating genetic variability in many species, especially in those of narrow genetic base. These variations can be passed on as heritable changes to the progeny of an individual (Baake & Gabriel 1999). Iqbal (2012) reported that genetic diversity in *N. sativa* L. germplasm was narrow based on SDS-PAGE marker. Mutation work has already been conducted on *N. sativa* and mutants with desired economic plant characteristics have been produced using colchicines (Biswas & Chatterjee 1971; Biswas & Datta 1982), gamma radiation (Datta et al. 1986; Kumar & Gupta 2007; Mitra & Bhowmik 1997; Saha & Datta 2002) and x-rays (Datta & Biswas 1983). There are a number of desirable economic traits in black cumin to be improved such as seed and oil yield, oil quality and ornamental quality for ornamental gardens. Mutation breeding is considered an important technique in improving the specific characteristics and a possible short cut for inducing desired genetic alterations in economically important crops. Voluminous work has been done worldwide for improvement of both seed and vegetatively propagated crops through induced mutation. The prospects of utilisation of induced mutations, and list of released mutant varieties in different ornamentals/crop plants have been published extensively (Ahloowalia et al. 2004; Broertjes & Van Harten 1988; Datta 2014; Datta et al. 2012; Maluszynski et al. 1995; Micke 1991; Micke et al. 1990; Schum & Preil 1998; Shu 2009).

Assessment of the genetic diversity in black cumin is therefore, of crucial importance for developing a breeding and conservation strategy for this economically important species. Molecular markers have been gradually replacing traditional morphological and agronomic characterisation, since they virtually cover the whole genome, the environment does not influence them and the process of characterization is less time-consuming (Aga et al. 2005). Molecular markers have been applied to increase our understanding of the distribution and extent of genetic variation within and between species. Markers, such as inter simple sequence repeats (ISSR) (Zietkiewicz et al. 1994), are widely used in genetic diversity studies, because they need no prior DNA sequence information, development costs are low and laboratory procedures can easily be adapted to any plant species. The ISSR technique may be highly informative if we are interested in detecting a new variety (Rout & Aparajita 2009). A large number of markers are easily generated using reliable and highly reproducible technique (Araújo et al. 2016).

The purpose of this study was the creation and evaluation of valuable black cumin mutants through appropriate methods for crop genetic improvement. Therefore, the aim of this study was to evaluate different morphological, yield, biochemical and genetic traits in nine black cumin mutant lines to determine which might be the best for seed production and for ornamental use.

Materials and Methods

Plant material

Local variety of *N. sativa* L. was obtained from the experimental farm of Kafrelshiekh University with a code No. NS-101 and used in our mutation breeding program. Dry and soaked seeds (five years' self-pollination) were treated with different mutagenes (laser, 2,4 Dinitroanaline and gamma rays) and sown in the field to develop second (M_2) generation (Maamoun et al. 2014). Nine promising variants/mutants (Mt1- Mt9) were selected from M_2 generation (El-Mahrouk et al. 2015). These nine variants/mutants, selected from different treatments (Table 1) and parent lines were used as the experimental materials.

Experimental design and field management practices

M_3 and M_4 experiments were set up in a complete randomized block design with three replicates and each replicate was represented by 60 seeds rendering a group of 180 seeds per genotype. Self-pollinated seeds of experimental materials were sown on a clay soil during

TABLE 1. Code and source of the nine mutants

Mutant code	Source of mutant
Mt1	60 min Laser of soaked seeds
Mt2	45 min Laser of dry seeds
Mt3	10 ppm 2,4Dinitroanaline
Mt4	5 k rad gamma radiation of dry seeds
Mt5	5 k rad gamma radiation of dry seeds
Mt6	5 k rad gamma radiation of dry seeds
Mt7	20 k rad gamma radiation of dry seeds
Mt8	10 k rad gamma radiation of soaked seeds
Mt9	10 k rad gamma radiation of soaked seeds

2013 (M_3) and 2014 (M_4). Each plot consisted of three rows (5 m × 0.7 m). Distance between plants was 25 cm. All experiments were maintained for 7 months. Standard cultural practices were followed for proper growth of plants (Rajeswara et al. 1989). Sixty plants were selected from three replicates to determine the morphological and yield traits, in terms of plant height, number of branches per plant, days to flowering, number of flowers per plant, shoot fresh and dry weights, seed yield per plant and 100-seed weight. Oil percentage was determined according to Folch et al. (1957).

BIOCHEMICAL ANALYSIS

M_4 seeds were subjected to biochemical analysis. Preparation of fatty acid methyl ester (FAME) from *N. sativa* oil was carried out according to Siew et al. (1995). The fatty acid composition of black cumin was determined using the corresponding fatty acid methyl esters for identification and quantisation by injection into a gas chromatograph (Hewlett Packard 6890) equipped with a flame ionisation detector (FID) and an HP-5 Column, (30 m × 0.32 mm, ID= 0.25 µm film thickness coated with 5% diphenyl and 95% dimethylpolysiloxane). Nitrogen was used for carrier gas at a flow rate of 1 $mLmin^{-1}$ with a split ratio 50:1. The volume of samples injected was 3 µL. Injector and detector temperatures were 220 and 250°C, respectively. Fatty acid methyl ester (FAME) was reported as percentage of seed oil.

GENOMIC DNA EXTRACTION AND INTER SIMPLE SEQUENCE REPEATS (ISSR) ANALYSIS

DNA was extracted from young leaves of parent and nine M_4 mutant lines based on the procedure described by Doyle and Doyle (1990). ISSR-PCR reactions were carried out using the five primers shown in Table 2. Amplification was conducted in a 25 µL reaction volume containing 2.5 µL 2.5 mM dNTPs, 2.5 µL 2.5 mM $MgCl_2$, 2.5 µL 10X buffer, 3.0 µL primer (10 pmol), 3.0 µL template DNA (25 $ngµL^{-1}$), 1 µL Taq polymerase ($1UµL^{-1}$) and 12.5 µL sterile, deionized distilled H_2O. Conditions for PCR were as follows: an initial denaturing step at 94°C for 4 min followed by 45 cycles at 94°C for 1 min, then a 1-minannealingcycle at 57°C, followed by 2-minextension at 72°C and final extension at 72°C for 10 min. All reactions were performed on an Eppendorf Mastercycler ep384 (Eppendorf, Germany). PCR products were separated on 1.5% agarose gels and fragment size was estimated with the 100bp ladder marker.

STATISTICAL ANALYSIS

Data were analysed with one-way ANOVA using the statistical program (SPSS version 20). Means separation was carried by the least significant difference (LSD) and Duncan's multiple range tests (Duncan 1955) with significance determined at $P \leq 0.05$. The DNA bands generated by each primer were counted and their molecular sizes were compared with that of the DNA markers. The bands scored from DNA profiles generated by each primer were pooled together. ISSR fragments were scored visually from the gel photograph. The fragments were scored as presence (1) or absence (0) of a particular molecular size to compile a binary matrix, which was then subjected to cluster analysis. The similarity matrix was estimated using the Jaccard genetic similarity coefficient (Jaccard 1908). PAST statistics software, version 2.17 package was used to calculate genetic diversity for each genotype (Hammer 2003).

RESULTS AND DISCUSSIONS

MORPHOLOGICAL TRAITS

On average, Mt2 plants were the tallest among all materials tested, with a mean height of 118.3 and 149.7 cm in generations M_3 and M_4, respectively (Table 3). On the other hand, Mt3 and Mt7 plants were the shortest among all mutants, in both generations. The number of branches and flowers displayed a similar trend. The highest number of branches and flowers were observed in Mt4 and Mt5 mutants, while the lowest were recorded for Mt3 and Mt7, in both generations (Table 3). Days to flowering varied significantly among the various mutants. The parent line and Mt3 plants were the earliest to flower when compared with other mutants in both generations. In contrast, Mt2 and Mt9 mutants showed maximum late in days to flowering (Table 3). Although there were variations in morphological traits of the same mutant in generations M_3 and M_4, the trend was the same. The occurrence of a number of changed characters occurring in one mutant suggested that the changes were perhaps induced at several independent loci. However, pleiotropic or epistatic effect of a mutated gene affecting yield and branch could not be

TABLE 2. Codes and nucleotides sequence of primers used in ISSR markers

Primer code	Primer sequence
44B	5′ CTC TCT CTC TCT CTC TGC 3′
HB-10	5′ GAG AGA GAG AGA CC3′
HB-12	5′CAC CACCAC GC 3`
HB-14	5` CTC CTCCTC GC 3`
HB-15	5`GTG GTGGTG GC 3`

ruled out. Mutation technique has been successfully employed in *N. Sativa* to isolate good mutants with desired economic plant characters (Datta & Biswas 1985; Datta et al. 2012). Previous studies indicated that re-irradiation of mutants can increase the variation in flower traits such as color, number, size and days to flowering (Yamaguchi 2018). In addition, the use of ion beams improved the traits that affect the production of cut flowers, such as fewer malformed flowers (Hisamura et al. 2016) and the ability for early flowering (Sakamoto et al. 2016). Mutagenesis increased mitotic activity of cambial cells, which improved morphological traits and increased the number of productive branches (Chandorkar & Dangler 1987). Mahmoud and Ibrahem (2000) found that laser treatments increased gibberellic acid and nitrogen contents, which led to increased protein and plant organs. Mt3 is a tetraploid mutant derived by 2,4-Dinitroaniline treatment and it had big flowers and a beautiful architecture (El-Mahrouk et al. 2015). Polyploidy is often associated with several changes in plant physiological parameters, such as increase in mesophyll cell volume and thickness of leaves, in contrast to diploids (Shafieizargar et al. 2013). Environmental condition during the growth season had clear effect on the morphological and yield traits of black cumin (Safaei et al. 2017).

AGRONOMICAL TRAITS

The mean values of yield traits are shown in Figure 1. The highest seed yield per plant was found in Mt5, with 51.83 and 41.73 g, while the lowest was observed in Mt3, with 4.33 and 5.00 g in generations M_3 and M_4, respectively (Figure 1(A)). The largest 100-seed weight was obtained from Mt1, Mt3 and Mt8 in both generations, compared to the parent line and the rest of the mutants (Figure 1(B)). On the other hand, the lowest 100-seed weight was recorded for the parent line and Mt9 in both generations. Mt4 had the highest oil percent content with 38.31% and 35.97%, followed by Mt6 with 32.38% and 30.04% in generations M_3 and M_4, respectively (Figure 1C). Conversely, the lowest oil percent content was observed in Mt3, with 17.11% and 18.02% in both generations, respectively. The present study showed that different traits of black cumin varied in generations M_3 and M_4 of the same mutant according to environmental condition. Kara et al. (2015) indicated that seed yield of black cumin depended on the location and environmental condition of

TABLE 3. Morphological traits of nine selected mutants of *Nigella sativa* in M_3 and M_4 generations

Mutants	Plant height (cm)	Number of branches/plant	Number of flowers/plant	Days to flowering
		M3 Generation		
Parent line	79.0 e	62.33 f	72.0 d	76.0 f
Mt1	90.0c	71.7 e	81.0 d	86.0 f
Mt2	118.3a	110.6 c	115.0 b	125.7 a
Mt3	81.4 de	26.0 h	26.8 f	76.0 f
Mt4	94.7bc	162.0 b	178.0 a	113.0 c
Mt5	96.6 b	171.0 a	186.5 a	114.0 c
Mt6	84.3 d	109.0 c	119.0b	97.0 e
Mt7	48.0 f	39.0 g	43.4 e	85.0 f
Mt8	84.8 d	94.0 d	102.7 c	102.0 d
Mt9 Sig	92.2bc**	107.3 c**	112.3 b**	120.0 b**
LSD (0.05)	4.52	9.15	10.14	1.62
		M4 Generation		
Parent line	100.3 c	43.8 e	49.0 f	92.7 f
Mt1	73.4d	58.4 d	69.4 e	103.6 de
Mt2	149.7 a	92.7 b	100.7 c	148.5 ab
Mt3	56.8 e	24.0 f	26.8 g	99.0 ef
Mt4	100.2 c	99.3ab	130.0 a	142.3 bc
Mt5	124.6 b	110.8 a	114.0 b	143.0 bc
Mt6	73.5 d	75.0 c	82.4 d	138.7 c
Mt7	67.0 d	35.5ef	40.0 f	108.0 d
Mt8	72.8 d	96.3 b	102.5 c	142.7 bc
Mt9 Sig.	117.2 b**	78.0 c **	86.7 d**	150.6 a**
LSD (0.05)	11.37	11.96	9.97	6.75

Mean separation within columns by LSD and Duncan's multiple range tests at 5% level

agriculture year regardless the variety. In addition, the combined analysis of variance of different black cumin traits indicated that the genotype × environment interaction was highly significant (Fufa 2018). Hence, different traits of black cumin varied in generations M_3 and M_4 of the same mutant according to environmental condition. Dixit et al. (2013) observed that NSM 8 mutant line of *N. sativa* was significantly increased in total oil content compared to its respective control. Seed weight reflects total yield per plant. It was interesting to note that 100 seed weight was maximum in Mt1, Mt3 and Mt8, but the total seed yield per plant was much less in these mutant lines. This was due to less number of branches per plant. Morad et al. (2011) studied the effect of mutation on yield and yield components in four wheat cultivars and concluded that gamma radiation induced greater variability and improved different traits, such as 1000-grain weight. Furthermore, 100-seed weight is dependent on seed size and correlates negatively to seed yield per plant. Aytac and Kinaci (2009) observed a negative relationship between seed yield and 100-seed weight, which was negatively correlated with oil percent content. Some yield components significantly affect total seed yield, either directly or indirectly, through other components. Seed yield per plant is an important component of total seed yield, which depends on genotype and environmental conditions. Although genetic variation in seed yield per plant exists, it may have negative effects on other yield components, such as oil percent content (Diepenbrock 2000). Thus, Mt4, Mt5 and Mt6 mutants could be promising lines for seed yield, compared to the parent line; while Mt3 and Mt7 are promising ornamental lines, because they have beautiful flowers and architecture.

OIL ANALYSIS BY GAS CHROMATOGRAMS

Gas chromatograms of mixed methyl esters showed presence of different saturated fatty acids in the seed oil of *N. sativa* (Table 4). It is interesting to note that palmitic acid (11.93%) and stearic acid (13.68%) were maximum in the Mt4. In addition, all nine mutants showed higher values of palmitic acid percentage over the parent line. All mutant oils, except for Mt4, had higher myristic acid content than the parent line. The highest percentage of myristic acid was 0.59% and was found in Mt8. Similarly, the highest content in caprylic acid (0.11%) was observed in Mt1, while it was absent from Mt3 and Mt5 mutants. Caproic acid percentage was found highest in Mt1 and Mt6, with 1.17% and 1.11%, respectively, but it was under detection level in Mt5 and Mt8. Moreover, caproic, lauric, pentadecylic, and margaric acids were present only in Mt8 and at low percentage. Total volatile fatty acids (TVFA) were recorded highest in Mt1 and Mt6, with 1.28% and 1.18%, respectively, followed by Mt7, with 0.79%. On the other hand, TVFA were absent from Mt5. The value for TVFA content is considered an indicator of volatile oil percentage in the plant. On this basis, Mt2 and Mt6 likely had the highest volatile oil percent content in compares onto the parent line and the other mutants. In general, total saturated fatty acid (TS) percentage ranged from 19.64% for Mt2 to 25.90% for Mt4. Although linoleic (LA) and oleic (OA) acids represent the majority of unsaturated fatty acid components, LA was much more abundant than OA. Additionally, Mt8 plants had the highest LA percentage (45.67%), followed by the parent line and Mt9, with 43.29% and 43.26%, respectively; while the lowest percentage (37.00%) was observed in Mt4. On the other hand, OA percent content ranged from 25.63% for Mt9 to 33.93% for Mt2. The highest percent contents of eicosadienoic acid, 5.60% and 5.60%, were observed in Mt6 and Mt3, respectively; while the lowest contents, 4.11% and 4.12%, were recorded in Mt9 and Mt8, respectively. In contrast, palmitoleic acid was present in small quantities in all mutants and in the parent line. On the other hand, heptadecenoic acid appeared only in Mt9 mutant in minute amounts (0.02%). In general, the highest total unsaturated fatty acid (TU) percent content (78.00%) and TU:TS ratio (3.97) were observed in Mt2, followed by Mt3, with 77.42% and 3.74%, respectively; while the lowest values, 71.89% and 2.77%, respectively, were observed in Mt4. The results showed that Mt2 had the highest OA:LA ratio (0.86), followed by Mt4 with 0.80%; while the lowest value (0.59%) was observed in Mt9. It was possible in the recent experiment to induce genetic variability for various fatty acids by using different mutagens. Mondal and Badigannavar (2010) induced large seeds and higher oleic acid content in groundnut mutants. Gamma rays are known to influence plants by inducing genetic and biochemical changes in cells and tissues (Gunckel & Sparrow 1961). Low radiation doses produced long-term effects on the stimulation of lipid degradation through the action of free radicals generated after irradiation (Voisine et al. 1991). The resulting free radicals engaged in various reactions leading to the formation of stable radiolysis products, which have been classified as primary, recombination and secondary products, according to the mode of their formation; while, in the case of unsaturated fatty acids, the dimerization reaction appeared to be of major importance (Nawar 1978). Presence of short-chain fatty acids and increased long-chain saturated fatty acids to reach maximum levels may be due to inter conversion between fatty acids, which occurs under the irradiation treatment (Afify et al. 2013). Doo et al. (2008) reported that lipid content and unsaturated oleic acid increased from 0.2% to 5.7% and from 1.3% to 14.0%, respectively, in mutants, over the parent genotype. Additionally, they found that some mutant lines had the highest palmitic acid content and the ratio of oleic to linoleic acid ranged from 0.9 to 2.2. Chu et al. (2011) reported that a high OA:LA ratio is desired by the peanut industry, because it expresses high oxidative stability to the oil. Aitzetmuller et al. (1997) discovered eicosadienoic acid (C20:2) in *Nigella* species. Matthaus and Ozcan (2011) reported that eicosadienoic fatty acid is a chemotaxonomic characteristic of the *Nigella* genus, a trait which could be used as a criterion for identification of genuine black cumin seed oil. On the other hand, resent results do not support

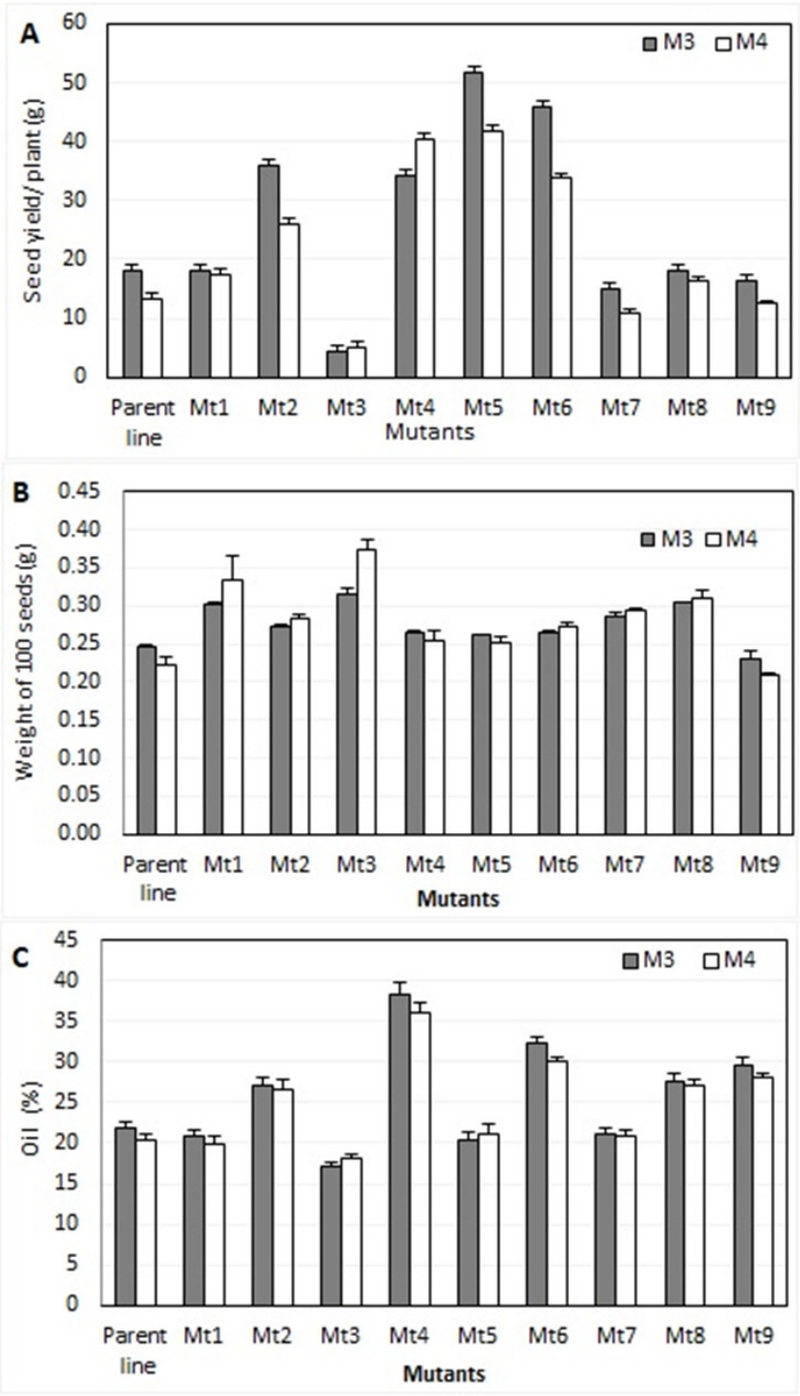

FIGURE 1. Yield component in tested mutants and parent line in M_3 and M_4 generations, A) seed yield/ plant (g); B) weight of 100 seeds (g); C) oil percentage

the findings by Üstün et al. (1990) who observed that C20:2 acids were absent from three samples of *N. sativa* seed, which contained high levels of free fatty acids.

CHANGES IN ISSR MARKERS PATTERN

Densitometric analysis of ISSR markers showed qualitative and quantitative variations among the parent and mutant genotypes (Figure 2 and Table 5). The 44B primer showed seven positive specific markers, two of them 507 and 204 bp for the parent genotype, a 481 bp fragment for Mt5 genotype, another 551 bp fragment for Mt7 genotype, two fragments, 879 and 598 bp, for Mt8 and one more specific marker, 858 bp, for Mt9. Additionally, two more bandss, 375 and 292 bp, were present in all genotypes. The HB-10 primer resulted in 10 positive specific markers at 365 bp for the parent genotype, two markers, 415 and 226 bp, for

TABLE 4. The percentage of fatty acid methyl ester (FAME) in seed oil of nine selected mutants of *Nigella sativa* in M_4 generation

FAME	Parent line	Mt1	Mt2	Mt3	Mt4	Mt5	Mt6	Mt7	Mt8	Mt9
					Saturated fatty acids%					
Caproic acid (C6 : 0)	0.3572	1.1735	0.1023	0.0408	0.0366	-	1.1154	0.70984	-	0.3824
Caprylic acid (C8 : 0)	0.0402	0.1100	0.0279	-	0.0872	-	0.0701	0.0793	0.0232	0.0463
Capric acid (C10 : 0)	-	-	-	-	-	-	-	-	0.1038	-
TVFA	0.3975	1.2835	0.1301	0.0408	0.1238	-	1.1856	0.7891	0.1269	0.4287
Lauric acid (C12 : 0)	-	-	-	-	-	-	-	-	0.0971	-
Myristic acid (C14 : 0)	0.1826	0.2752	0.1959	0.1996	0.1677	0.3803	0.2973	0.4399	0.5892	0.2426
Pentadecylic acid (C15 : 0)	-	-	-	-	-	-	-	-	0.0645	-
Palmitic acid (C16 : 0)	10.6975	11.3154	10.8135	11.5205	11.9291	11.7602	10.3967	11.5307	11.5447	11.0863
Margaric acid (C17 : 0)	-	-	-	-	-	-	-	-	0.0601	-
Stearic acid (C18 : 0)	12.0365	9.2550	8.5028	8.9267	13.6801	11.7828	10.2473	10.8905	9.2399	11.4504
Total saturated fatty acids	23.3140	22.0434	19.6422	20.6876	25.8997	23.9233	22.1268	23.6503	21.7223	23.2080
					Unsaturated fatty acids%					
Palmitoleic acid (C16 :1)	0.1049	0.1628	0.1212	0.1393	0.1225	0.1088	0.0932	0.1081	0.1166	0.1453
Heptadecenoic acid (C17:1)	-	-	-	-	-	-	-	-	-	0.0223
Oleic acid (C18:1)	28.3078	27.9484	33.9299	31.3373	29.5688	28.2869	29.1003	27.7631	25.8392	25.6324
Linoleic acid (C18 :2)	43.2875	40.6799	39.5373	40.3426	37.0044	39.7817	39.7280	38.9477	45.6742	43.2593
Eicosadienoic acid (C20 :2)	4.1568	4.4430	4.4140	5.5995	5.1924	5.0915	5.6005	5.3986	4.1195	4.1128
Total unsaturated fatty acids	75.8570	73.3147	78.0023	77.4187	71.8881	73.2689	74.5220	72.2174	75.7494	73.1720
(OA)/(LA)	0.6540	0.6870	0.8582	0.7768	0.7991	0.7111	0.7325	0.7128	0.5657	0.5925
(TU)/(TS)	3.2537	3.3259	3.9712	3.7423	2.7755	3.0627	3.3680	3.0536	3.4872	3.1529
Un known	0.8290	4.6368	2.3555	1.8937	2.2111	2.8078	3.3512	4.1323	2.5283	3.6200

FAME= Fatty acid methyl ester, Mt1 to Mt9 means number of mutants from 1 to 9, TVFA= total volatile fatty acids.
OA= Oleic acid, LA= linoleic acid, TU= Total unsaturated fatty acids, TS= Total saturated fatty acids

Mt1, a 405 bp fragment for Mt2, a 259 bp fragment for Mt6, two markers, 725 and 427 bp for Mt7, two more fragments, 232 and a 193 bp fragmen for Mt8, and a 239 bp band for Mt9. The three common fragments, 635,500 and 320 bp, were present in all tested genotypes. HB-12 primer produced five positive specific markers at 192 bp for the parent genotype, one marker 299 bp for the Mt5 genotype, a 781 bp marker for the Mt7 genotype and two specific markers, 617 and 224 bp, for Mt7, while the two fragments were present in all genotypes. On the other hand, the HB-14 primer amplified seven positive specific markers, two, 479 and 393 bp for the parent genotype, a 429 bp fragment for the Mt1, a 493 bp marker for Mt2, two positive specific markers, 1001 and 538 bp, for the Mt3 genotype and one specific marker 508 bp long for the Mt6 genotype. The HB-15 primer showed four positive specific markers, two, 516 and 456 bp, for Mt4, a 245 bp fragment for Mt8 and a 250 bp marker for Mt9, while there were three fragments, each 570, 413 and 324 bp, present in all genotypes. In the present study, there was a significant variation between the mutants and the parent genotype. Some fragments were missing in the mutants and some new fragments appeared. For instance, for primer 44B, fragments 507.6 and 204.4 bp were missing in the mutants,

while a new band, 717.12 bp, appeared in all mutants. A Similar variation was also shown for primers HB10, HB12, and HB14 (Table 5).

In this study, some mutants showed unique agronomic and molecular characteristics. Mt3 mutant showed the lowest values for all agronomic characters, which could be fingerprinted with two positive specific markers, 1001 and 538 bp for primer HB4.On the other hand, Mt4 and Mt5 mutants showed the highest agronomic values which could be linked to a specific unique band, 456 and 516 bp

TABLE 5. Distribution of ISSR markers among the parent line and its induced mutants of *Nigella sativa*

Primer code	Amplicons No.	Polymorphic amplicons	Polymorphism (%)	Unique bands
44B	12	10	83.33	7
HB-10	14	11	78.57	10
HB-12	14	12	85.71	5
HB-14	17	14	82.35	7
HB-15	14	11	78.57	4
Total	71	58		33

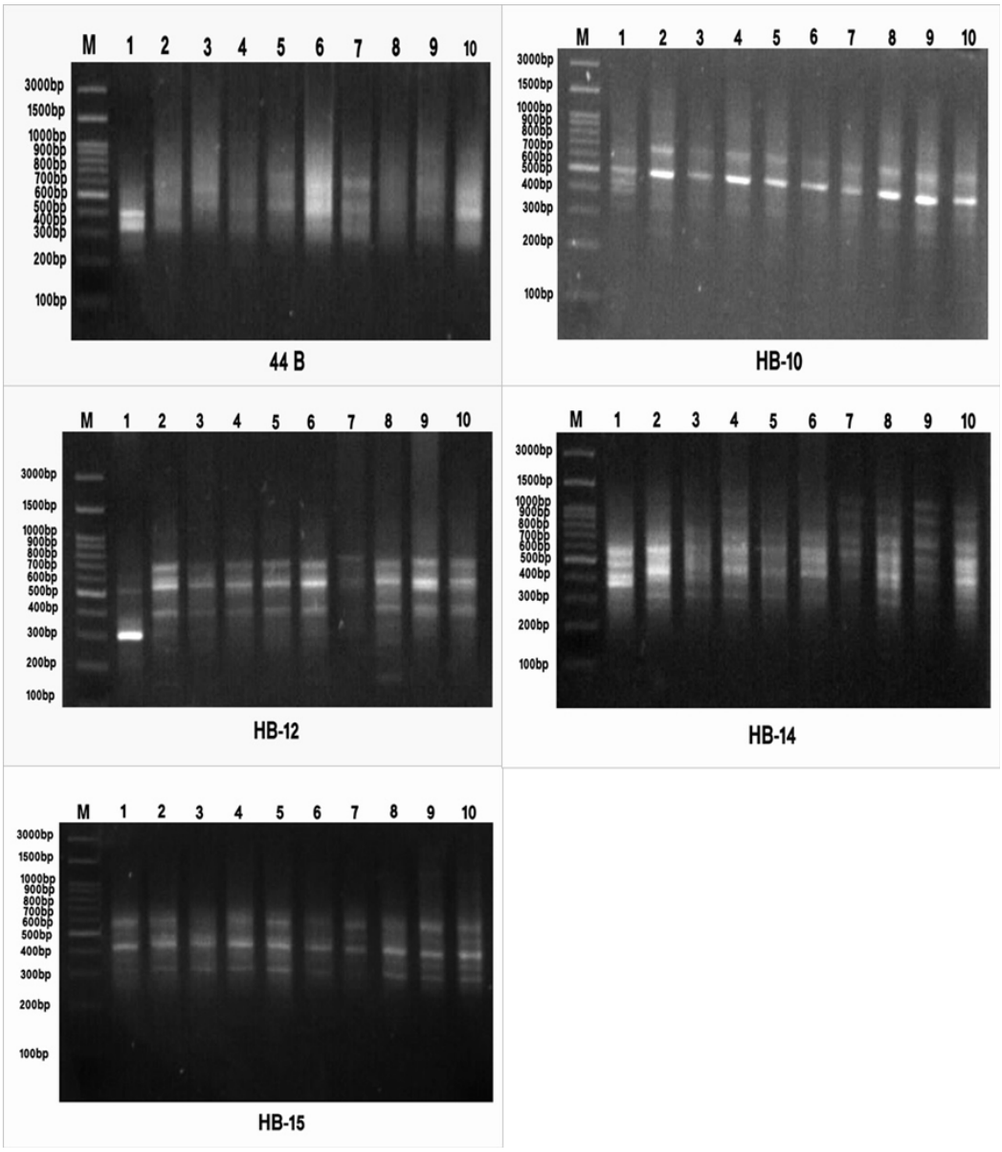

FIGURE 2. ISSR-PCR analysis of the parent line of *N. sativa* and its induced mutants (1=Parent line, 2= Mt1, 3= Mt2, 4= Mt3, 5= Mt4, 6- Mt5, 7=Mt6, 8=Mt7, 9=Mt8, 10=Mt9)

produced by primer HB15 for the mutant Mt3 and markers 481 and 299.5 bp produced by primers 44B and HB12 for mutant Mt5, respectively. Similarly, Mt8 mutant distinguished itself for high branching and flowering ability associated with six unique bands which could be used as markers for these characters and, also, as a genetic fingerprint for this mutant. ISSR fingerprinting for the other mutants showed a sufficient number of unique bands corresponding to each mutant, something which would be very useful for mutant characterisation and identification. The genetic variation through molecular markers has been highlighted in a number of plant species, including ornamental plants (Kumar et al. 2016). A modification of the SSR-based marker system, namely ISSR analysis (Wolff & Peters-Van 1993) has demonstrated wide applicability in a variety of plants, as it provides highly reproducible results and generates abundant polymorphisms in many systems (Liu & Wendel 2001). ISSR markers are useful in studies on genetic diversity, phylogeny, gene tagging, genome mapping and evolutionary biology, reasons for which they are widely applied in plant genetic analyses (Kapital et al. 2015). Analyses of genetic diversity among different accessions and mutants using ISSR were reported in crops, such as sugarcane (Srivastava & Gupta 2008), mulberry (Vijayan et al. 2006) and cotton (Liu & Wendel 2001). Results obtained from ISSR analysis does not always conform to the conventional classification, merely relying on the forms of yield contributing traits such as seed yield (Mitra et al. 2006).

POLYMORPHISM AND GENETIC SIMILARITY BASED ON ISSR MARKERS

Polymorphism levels differed from one primer to another, for example, primers HB-12 and 44B exhibited high levels of polymorphism (85.71% and 83.33%, respectively), whereas HB-10 and HB-15 exhibited a low level of polymorphism (78.57%). The lowest number of polymorphic fragments was detected for primer 44B (10 bands), while the highest number of polymorphic fragments was detected for primer HB-14 (14 bands). The tested ISSR primers generated specific markers for the parent and the mutant genotypes (Table 6). Thirty-three, out of 71 ISSR fragments were found to be useful as specific markers. Primer 44B produced the highest number of unique markers (10), whereas primer HB15 produced the lowest number (4). According to the ISSR results, the most closely related mutant genotype to the parent genotype, was Mt8 (Table 7), which had the highest similarity index (0.99). On the other hand, the most distant genotypes from the parent genotype were Mt2 and Mt3, with a low similarity index (0.02), whereas the two genotypes located very far were Mt2 and Mt5 (similarity index of 0.01) (Table 7). In the present study, we observed that mutants and their parent line possessed an intensive polymorphism, as shown by this analysis (Table 6). Results of morphological and ISSR analyses showed that mutants with similar phenotypes were not necessarily as close (Tables 6 and 7) at the molecular level. Ortiz (1997) reported poor correlation between diversity based on morphological traits and molecular markers, when a large number of morphological traits were used. Markers from ISSR may accurately assay the degree of genetic change differentiating two genomes, but they may not necessarily reflect divergence in terms of changes in traits of agronomic importance. The similarity index (Table 7) showed low percentages of similarity among some mutant lines and between the mutant lines and their parent line. This finding resulted from the mutagenic effects of the different mutagenic treatment used to generate these mutant lines. These divergent mutant lines could be used in plant breeding program for gene pyramiding of yield and oil content traits. Although it was expected that variation in the mutant lines will exist, but the environment markedly influences the expression of most phenotypic traits, while detection of molecular markers is not confounded by environmental effects. Consequently, ISSR primers are used to confirm the genetic variations at the molecular level and to find some specific marker linked to some mutants could be used for genetic fingerprinting of those mutants.

TABLE 6. Survey of the five ISSR tested primer fragments in the *N. sativa* parent line and the induced mutants) (1 =present bands; 0 = absent bands)

ISSR Primers	Molecular size(bp)	Origin	Mt1	Mt2	Mt3	Mt4	Mt5	Mt6	Mt7	Mt8	Mt9
44B	879.881	0	0	0	0	0	0	0	0	1	0
	858.228	0	0	0	0	0	0	0	0	0	1
	598.980	0	0	0	0	0	0	0	0	1	0
	553.735	0	0	1	0	1	1	1	0	0	1
	551.450	0	0	0	0	0	0	0	0	0	0
	507.692	1	1	1	1	1	1	1	1	1	1
	481.129	0	0	0	0	0	1	0	0	0	0
	442.951	0	0	0	0	0	0	1	0	1	0
	375.443	1	1	1	1	1	1	1	1	1	1
	292.972	1	1	1	1	1	1	1	1	1	1
	252.462	1	1	1	1	1	1	1	1	1	1
	204.473	1	1	1	1	1	1	1	1	1	1

HB-10	725.810	0	0	0	0	0	0	0	1	0	0
	635.691	1	1	1	1	1	1	1	1	1	1
	500.734	1	1	1	1	1	1	1	1	1	1
	438.561	1	1	1	1	1	1	1	1	1	1
	427.085	0	0	0	0	0	0	0	1	0	0
	415.910	0	0	0	0	0	0	0	0	0	0
	405.027	0	0	1	0	0	0	0	0	0	0
	365.651	1	1	1	1	1	1	1	1	1	1
	320.251	1	1	1	1	1	1	1	1	1	1
	259.040	0	0	0	0	0	0	0	0	0	0
	239.233	0	0	0	0	0	0	0	0	0	1
	232.973	0	0	0	0	0	0	0	0	1	0
	226.876	0	1	0	0	0	0	0	0	0	0
	193.507	0	0	0	0	0	0	0	0	1	0
HB-12	781.659	0	0	0	0	0	0	1	0	0	0
	722.734	1	1	1	1	1	1	1	1	1	1
	617.877	0	0	0	0	0	0	0	1	0	0
	601.943	0	1	0	0	0	1	0	0	0	0
	556.566	1	1	1	1	1	1	1	1	1	1
	378.936	0	1	1	1	1	1	0	1	1	1
	315.604	1	1	1	1	1	1	1	1	1	1
	301.781	0	0	1	1	1	0	0	0	0	0
	299.537	0	0	0	0	0	1	0	0	0	0
	274.897	0	0	0	0	0	0	0	1	1	1
	269.815	1	1	1	1	1	1	1	1	1	1
	224.720	0	0	0	0	0	0	0	1	0	0
	192.835	1	1	1	1	1	1	1	1	1	1
	148.499	0	1	0	0	0	0	0	1	0	0
HB-14	1109.905	0	0	0	1	0	0	1	1	1	1
	1001.521	0	0	0	1	0	0	0	0	0	0
	907.442	0	0	0	0	0	0	1	1	1	1
	717.912	0	1	1	1	1	1	1	1	1	1
	584.547	1	1	1	1	1	1	1	1	1	1
	538.416	0	0	0	1	0	0	0	0	0	0
	523.146	0	1	0	0	0	1	0	0	0	0
	508.309	0	0	0	0	0	0	1	0	0	0
	493.892	0	0	1	0	0	0	0	0	0	0
	489.849	0	0	0	0	0	0	0	1	1	1
	479.885	1	1	1	1	1	1	1	1	1	1
	429.478	0	1	0	0	0	0	0	0	0	0
	424.215	0	0	1	1	1	1	1	1	1	1
	393.963	1	1	1	1	1	1	1	1	1	1
	331.500	1	1	1	1	1	1	1	1	1	1
	272.145	1	1	1	1	1	1	1	1	1	1
	223.418	1	1	1	1	1	1	1	1	1	1
HB-15	635.992	1	1	1	1	1	1	1	1	1	1
	626.909	0	0	1	0	0	1	1	0	1	0
	570.953	1	1	1	1	1	1	1	1	1	1
	516.266	0	0	0	0	1	0	0	0	0	0
	508.893	0	1	1	1	0	1	0	0	0	0
	483.908	1	1	1	1	1	1	1	1	1	1
	456.852	0	0	0	0	1	0	0	0	0	0
	450.328	0	1	1	1	0	1	0	0	0	0
	413.093	1	1	1	1	1	1	1	1	1	1
	380.302	1	1	1	1	1	1	0	1	1	1
	324.649	1	1	1	1	1	1	1	1	1	1
	282.168	0	0	0	0	1	0	0	1	1	1
	250.595	0	0	0	0	0	0	0	0	0	1
	245.246	0	0	0	0	0	0	0	0	1	0

TABLE 7. Similarity indices among the parent line and its induced mutants of *Nigella sativa* based on ISSR markers

	Parent	Mt1	Mt2	Mt3	Mt4	Mt5	Mt6	Mt7	Mt8
Parent									
Mt1	0.569								
Mt2	0.924	0.442							
Mt3	0.700	0.232	0.024						
Mt4	0.752	0.528	0.051	0.107					
Mt5	0.777	0.202	0.01	0.183	0.348				
Mt6	0.924	0.959	0.388	0.574	0.480	0.528			
Mt7	0.798	0.604	0.691	0.368	0.403	0.682	0.569		
Mt8	0.99	0.915	0.528	0.569	0.485	0.646	0.279	0.234	
Mt9	0.566	0.718	0.569	0.368	0.279	0.450	0.446	0.057	0.234

CONCLUSION

Mutagens, in the present experiments, have successfully created wide range of heritable diversity in different characters which bred true in M_3 and M_4 suggesting their recessive nature. Mutations were randomly induced and each mutant line harbored distinct mutations. Modern techniques for plant characterisation, particularly those of molecular biology, were integrated with conventional methods to generate and characterise useful induced mutations. The present study elucidated morphological, biochemical and genetic characteristics of parent genotype and nine selected mutants to determine the best genotype. Mt4 and Mt5 are promising mutants, which had the highest number of flowers and branches. In this regard, the highest seed yield per plant was measured in Mt5, while Mt4 had the highest oil, palmitic and stearic acid percent content. On the other hand, Mt8 had the highest linoleic acid percent content. In addition, Mt3 and Mt7 are promising ornamental lines due to their attractive phenotype. The markers generated by primers 44B, HB-10, HB-12, HB-14 and HB-15 allowed detection of genetic polymorphism and genetic fingerprinting among the mutants of *N. sativa* tested. In doing so, they have shown to be useful for the construction of a germplasm collection and for providing additional information that could form the basis for the rational design of breeding programs. The present characterization strategies will help to exploit different mutant lines for developing F_1 hybrids with desired traits.

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