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Mutation induction in sweet basil (*Ocimum basilicum* L.) by fast neutron irradiation

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Summary: Basil species are highly sensitive to exterior environmental conditions and its consequences lead to great economic and agronomic losses. In this research, a mutation method was optimized out for creating a new variety of *Ocimum basilicum* L., which could tolerate the extreme/extraordinary climatic circumstances or biotic stresses, such as fungal diseases. Fast neutron irradiation was performed on the Hungarian commercial variety seeds with doses of 5 to 60 Gray and grown into fully developed plants. Numerous phenotypical changes like deformed congestion, leaf mutation, and low growth occurred, especially at higher dosages. Then to confirm whether the plantlets had mutation or not, and to detect the molecular variation and relationship, fingerprinting profiles of the developed mutant regenerants and donor plant have been assessed using ISSR markers. 115 loci were yielded, ranging from 0.2 to 1.5 kb, out of which 110 loci were polymorphic in nature, representing 95.6% polymorphism. The most suitable primer to determine the genetic diversity within the *Ocimum* species was the UBC-856 with 0.42 PIC and 4.1 MI values.

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Key words: mutation induction, fast neutron, radiation, sweet basil, *Ocimum basilicum* L.

Introduction

Ocimum basilicum L. is one of the species which is used in food products, beverages, medicines, cosmetics as well as in perfumery. This species is widely grown through the Mediterranean region to the United States. From the genus, more than 60 species are cultivated, which are annual, perennial, or shrubs, native to Asia, Africa and over the tropical regions of the world. The main cultivated varieties belong to *O. basilicum* L., but there are many other cultivars with economic significance, for example, *O. americanum* (hoary basil) and *O. gratissimum* (clove basil). The determination and differentiation of all varieties from each other is particularly challenging because the basil cross-pollinates easily, and drawing boundaries between species are fairly difficult. Recent studies carried on the determination of the phylogeny of *Ocimum* L. genus (Pyne et al., 2018, Kumar et al., 2016).

The breeding of this species has been limited to the classical method (selection and crossing). The wide diversity of the genus, the huge numbers of unstable traits and segregates within the progenies all make the breeding procedures extremely difficult. But at the same time, the wideness of the gene pool offers lots of opportunities which could be beneficial for the breeding programme of new and better cultivars. The main goal of the basil breeding is to gain resistance against plant pathogens and fungus diseases, like *Cercospora* leaf spot (*Cercospora ocimicola)*, Downy mildew (*Peronospora belbahrii)*, Fusarium wilt (*Fusarium oxysporum*), which destroy plant development and cause great yield loss.

Although there are many publications that deal with the basil *in vitro* propagation (Sahoo et al., 1997; Phippen & Simon, 2000; Siddique & Anis, 2007; Siddique & Anis, 2008; Saha et al., 2010; Shahzad et al., 2012; Ekmekci & Aasim, 2014), the efficient modern method to generate new valuable varieties like disease and abiotic stress resistant, or has higher essential oil content etc, were not fully developed until now.

Only a few resistant varieties were introduced/developed, mainly through selection of the resistant genotype within a variety (Dudai et al., 2002; Pyne et al., 2015), and an efficient method for the Agrobacterium-mediated transformation of *Ocimum tenuiflorum* was performed (Vyas & Mukhopadhyay, 2014; Khan et al., 2015). There is no modern transformation technique developed regarding the *O. basilicum* L. breeding programme.

The mutation induction has been a perspective tool in breeder's hands since the 1970s. According to the FAO/IAEA Mutant Variety Database, there are 3281 mutant varieties or breeding sources within the cultivated plants. However, basil species were not involved deeply in a breeding programme based on mutation induction (IAEA, 2015, FAO/IAEA, 2015).

We found a couple of sources that mention mutation induction of *Ocimum* L. Unfortunately, the genetic diversity or the phenotypic observations were not included in the papers (Asante et al., 2016).

Since mutations occur spontaneously in nature, at a very low frequency, in order to create comprehensively diverse novel mutations, mutagens are being utilized. Different kinds

of irradiation (X-ray, Gamma Ray, Fast neutron ray, Heavy ion beam) are mainly used for mutagenesis. These mutations can affect single nucleotide pairs, causing point mutations, or result in large scale duplications and deletions throughout the genome, since they typically occur randomly.

Depending on the type, the dosage, and the time of exposure to the mutagen, the effectiveness and degree of mutation, and the sensitivity of the species against the radiation is very variable. In most cases, the mutation caused by physical mutagenesis appear in the next generations, and the inheritance of these traits are unknown.

The ion beam is a comparatively new mutagen, and mostly due to its biological effects, it has gradually gained more popularity. The ion implantation mutation merges energy, charge and mass factors, and the occurred damage to the biological substances (including genetic materials), predominantly displaces, blends and recombines the biological molecules and atoms. Due to the mentioned properties, the biotechnological employment of ion beams is satisfactory for cell processing, gene transfer and overall for mutation breeding (Abe et al., 2015; Ishii et al., 2016).

When performing mutation induction for practical breeding purposes, since the radiosensitivity of the plant species and materials under study may vary, before any large scale mutagenic treatment, most necessarily, preliminary experiments must be run to carefully determine the optimal doses for every system. According to Kazama et al. (2008) in general, the objective is to use a dosage and rate in which 50% of the M1 seeds will germinate and produce seed for the next generation".

The primary necessity in plant breeding is the maintenance of genetic stability during the process of adoption of new varieties, germplasm conversation, and transgenic plant production. The molecular basis of the valuable traits, including the genes and pathways have to be elucidated.

Inter simple sequence repeat (ISSR) technique is a quick and simple PCR based method that combines most of the advantages of microsatellites (SSRs) and amplified fragment length polymorphism (AFLP) with the universality of random amplified polymorphic DNA (RAPD). It associates DNA segment amplification that is present at an amplifiable distance between two identical microsatellite repeat regions oriented in opposite direction (Reddy et al., 2002).

The possibility of spontaneous mutation within microsatellites is considerably higher in nature as compared to most of the other site of the genome, which increases the probability of polymorphism in these sequences. Since microsatellite markers are present in both nuclear and organellar genomes, they are accountable for the genesis of highly polymorphic loci. The genomic fingerprint created by ISSR markers are suitable for the characterization of germplasm collections and the exploration of genetic diversity within species (Roy et al., 2015), cultivar determination (Chowdhury et al., 2002), genomic stability determination within a species or a cultivar, however, they are not entirely suitable for phylogeny connections determination at the species level because of their dominant behavior. The ISSR technique is cost effective compared to RFLPs and AFLPs at the same time it gives higher reproducibility results than RAPDs. Individuals within the same varieties normally show only barely differences between their ISSR profiles, as they are closely related taxa, but subspecies and different populations may give a specific banding pattern that can be used to clarify the phylogenetic relationship.

In the microsatellites technique, customarily, 16–25 bp long primers have been used in a single primer PCR reaction targeting multiple genomic loci to amplify mainly the inter-SSR sequences of different sizes. The primers applied in microsatellite assays can be di-nucleotide, tri-nucleotide, tetra-nucleotide or penta-nucleotide. The primers are usually anchored at 3' or 5' end with 1 to 4 degenerate bases extended into the flanking sequences (Zietkiewicz et al., 1994), but they also can be unanchored (Gupta & Varshney, 2000, Meyer et al., 1993). ISSRs assays are highly reproductive, probably due to the use of longer primers (16–25 mers) as compared to RAPD primers (10- mers), which allows the use of high annealing temperature (45–60 °C) leading to higher fidelity. Based on the results dealing with the reproducibility of ISSR, only the faintest bands are not reproducible. In polyacrylamide gel about 92–95% of the gained bands could be repeated within the samples of the same cultivar and across different PCR runs (Fang & Roose, 1997).

Numbers of authors and breeders consider that the ISSRs alleles segregate mostly as dominant inherit, based on the simple Mendelian laws (Wang, 2011; Wang et al., 1998). However, some studies reported the co-dominant segregations in case of some ISSR markers. Hence sometimes it's possible to make the distinction between homozygotes and heterozygotes (Wu et al., 1994; Akagi et al., 1996; Wang, 2011; Sankar & Moore, 2001).

Polymorphic information content (PIC) analysis is considered a very useful tool for appropriate marker selection of phylogenetic analysis and genetic mapping. This assay provides an estimation of the discriminating ability of the marker based on the number of noticeable bands and their distribution (Mishra et al., 2015; Powell et al., 1996). The other expedient indicator for the discrimination potential of any marker system is called Resolving power (RP) (Kumar et al., 2016; Prevost & Wilkinson, 1999).

In case of this experiment, we performed fast neutron irradiation with different doses on *Ocimum basilicum* L. species, to monitor the spontaneous mutations and determine the plant's radial sensitivity. Unlike the electrons and protons, neutrons don't ionize the atoms (excite the electron) in the same manner due to their absence of charges. Nevertheless, neutron interactions are broadly ionizing, as it can be observed when a recoiled nucleus causes a number of traditional ionization in other atoms, or when an electron is removed from an atom by photon (gamma ray), due to gamma emission caused by neutron absorption. Neutron radiation is much more penetrating than alpha or beta radiations, even in some cases than gamma radiation, which is impeded in materials of high atomic number. A low energy gamma ray could be more penetrating than a high energy neutron, in the case of materials like hydrogen, because of the low atomic number (Yue et al., 2013).

In this regard, we have evaluated fingerprinting profiles of the developed mutant regenerants and the original *Ocimum basilicum* variety using ISSR markers to confirm the genetical stability of the plantlets.

Figure 1. The fast neutron irradiation experiment of *Ocimum basilicum* L. at ATOMKI-DE MÉK-2018.05-06.

Materials and methods

Plant material

Three repetitions of irradiation were performed on Hungarian commercial variety *Ocimum basilicum* L. seeds (200 seeds per treatment) at the following doses 5 Gray, 7.5 Gray, 12.5 Gray, 15 Gray, 17.5 Gray, 20 Gray, 30 Gray and later 40-50-60 Gray doses were added to complete the experiment.

The irradiation treatment was performed at MTA Atomki (Bem tér 18/c, H-4026 Debrecen, Hungary) on 29.03.2018. The high-intensity neutron irradiation facility (Fenyvesi, 2004) at the MGC-20E cyclotron was used. Fast neutrons with broad energy spectrum in the E_n = 0 – 18 MeV neutron energy range were produced by bombarding a beryllium target with Ep = 16.65 MeV protons. For nuclear physics reasons, the irradiation field of the neutrons was accompanied by a field of gamma photons. A twin ionization chamber technique (Broerse et al., 1981) was used for monitoring the separate neutron and gamma dose rates. The dosimetry calculations for the seeds were based on the recommendations of the International Atomic Energy Agency (IAEA, 1967). The material of the seeds was modeled by a medium that contains equal number of hydrogen and carbon atoms. The absorbed neutron (Dn) and gamma (Dg) doses were calculated for this medium. The contribution of the gamma dose to the total dose (Dtot) was Dg/Dtot = Dg/(Dn + Dg) = (14 ± 3) % for each irradiated seed sample. It is important to emphasize that the biological effectiveness of 1 Gy neutron dose is 2-11 times higher than that of 1 Gy gamma dose depending on the neutron energy.

Sowing of irradiated seeds

300 seeds per each dose (3 x 100) were sown into equal sized containers to germinate. Repeat doses were reported in irradiated seeds at different positions (A, K or F / number), which was treated separately during the evaluation. Thus, for each dose, the positions illustrated repetitions, so for example, when 100 seeds from the 30GY-A7 treatment were sown, for the same dose of K7 and F7 treatments have been also 100-100 seeds.

After sowing the seeds went through a 0.2% Dithane (Indofil Industries Limited -Via Filippo Turati, 6-20121, Milan, Italy) fungicide treatment (04.2 / 2762-1 / 2012 NÉBIH). The germination percentage was evaluated 14 days after sowing (***Figure 1/A***).

Nursery operations

40 days post-sowing, the height above the ground surface, the number of adult leaves and the number of lateral shoots of each treatment were recorded from 20 randomly selected plants at each point of the tray (***Figure 1/B-C***). Growth Reduction Value was calculated in the percentage of the plants' height reduction compared to the controls.

12 plants from each treatment were placed in 10cm diameter pots in which Kekkilä brown peat was used, supplemented with Siforga (granulated fertilizer-3kg/200). The remaining plant material was subjected to field conditions (Nurse in trade) (***Figure 1/D***).

Plant material of molecular assisted selection

Young shoots (15-20 cm) were collected from phenotypically selected 6-month-old fast neutron irradiated (15GY, 17.5GY, 40GY, and 50GY) mutants of *Ocimum basilicum* L. maintained in the demonstration plant garden of the Department of Botany, Physiology and Biotechnology, University of Debrecen, Hungary. The nodal segments with axillary and apical buds were separated and ground with liquid nitrogen, in order to prepare a homogenized sample.

DNA isolation

Total genomic DNA was isolated from the prepared plant sample using the NucleoSpin Plants II kit and its protocol (***Figure 2***) described by Macherey-Nagel (2014). The quantity of isolated genomic DNA samples were quantified spectrophotometrically (Thermo Scientific NanoDrop 2000 full-spectrum, UV-Vis spectrophotometer) by measuring absorbance at 260 nm and 280 nm for the OD_{260}/OD_{280} ratio. Also, the quality was checked by visualizing it under UV light after electrophoresis on 0.8% agarose gel. The stock DNA was diluted to make a required working solution of 10 ng/µl.

ISSR-PCR amplification

Seven ISSR primers were used for PCR (polymerase chain reaction) for DNA amplification. DNA finger printing profiles were compared to evaluate clonal fidelity and genetic stability. Amplification was performed in 50 µL using PCR mixture of consisting of 47µL DreamTaqGreen Mastermix (which contains DreamTaqgreen DNA polymerase, dNTP (dATP: dTTP: dCTP: dGTP in 1:1:1:1 parts) and DNA polymerase buffer with $MgCl_2$), 2µL genomic DNA and 1µL ISSR primer (***Table 1***).

*Table 1.*The PCR circumstances and used ISSR primers

	Sequence 5'-3'	Melting temp. (°C)	Num. of cycles	Denat. temp. (°C)	Program	End
UBC 807	AGAGAGAGAGAGAGAGT	47	40	94 °C-1 min,	94°C-20 sec, a.h-20 sec, 72°C-2 min	72°C – 6 min
UBC 810	GAGAGAGAGAGAGAGAT	51	40	94 °C-1 min,	94°C-20 sec, a.h-20 sec, 72°C-2 min	72°C – 6 min
UBC 818	CACACACACACACACAG	52	40	94 °C-1 min,	94°C-20 sec, a.h-20 sec, 72°C-2 min	72°C – 6 min
UBC-835	AGA GAG AGA GAG AGA GYC	50,2	40	94 °C-1 min,	94°C-20 sec, a.h-20 sec, 72°C-2 min	72°C – 6 min
UBC-840	GAG AGA GAG AGA GAG AYT	47,4	40	94 °C-1 min,	94°C-20 sec, a.h-20 sec, 72°C-2 min	72°C – 6 min
UBC-841	GAG AGA GAG AGA GAG AYC	48,5	40	94 °C-1 min,	94°C-20 sec, a.h-20 sec, 72°C-2 min	72°C – 6 min
UBC-856	ACA CAC ACA CAC ACA CYA	52,8	40	94 °C-1 min,	94°C-20 sec, a.h-20 sec, 72°C-2 min	72°C – 6 min

The PCR conditions were carried out using a thermal cycler (MJ Research PTC-150 Thermal Cycler) using the parameters above in Table 1. Amplicons were electrophoresed on 1.5% agarose gel and stained with ethidium bromide (5 µL/gel). 100bp plus DNA Ladder (ThermoFisher Scientific Co.) was used as the DNA marker, and bands were visualized under UV light and photographed using the Gel Documentation equipment (Bio Rad Laboratories Inc.).

Amplified ISSR products were analyzed with a software called PhylElph. The binary matrixes were scored for band presence or absence for each accession (presence=1, absence=0), and a binary data matrix was formed. Primer banding characteristics such as the number of total bands (TB), the number of polymorphic bands (PB), and the percentage of polymorphic bands (PPB) were acquired.

To check the genetic relation among the *Ocimum basilicum* and its mutants, data matrix of ISSR markers was converted into genetic similarity matrix using Jaccard coefficient (Jaccard, 1908) (JACCARD, 1908) in SPSS 17.0 (SPSS Inc.) and NTSYS-PC 2.02j (Rohlf, 1998).

The performance of markers were measured in order to analyze the suitability of ISSR markers to determine the genetic profiles of *Ocimum basilicum* mutants, using three parameters:

- polymorphic information content (PIC)
- effective multiplex ratio (EMR)
- marker index (MI)

The PIC value for each locus was calculated as proposed by Roldan-Ruiz et al. (2000);

$$PIC_i = 2fi * (1 - fi);$$

where PIC_i is the PIC of the locus *i*, f*i* is the frequency of the amplified fragments (band present), and (1 - f*i*) is the frequency of non-amplified fragments (band absent).The frequency was calculated as the ratio between the number of amplified bands at each locus and the total number of accessions (excluding missing data). The PIC of each primer was calculated using the average PIC value from all loci of each primer.

The marker index (MI) was calculated as described by Varshney et al. (2007);

$$MI = EMR * PIC;$$

where EMR is the product of the fraction of polymorphic loci and the number of polymorphic loci for an individual assay.

The resolving power (RP) of each primer was calculated according to Prevost and Wilkinson (1999);

$$RP = \sum Ib;$$

where I*b* represents the informative fragments. The I*b* can be represented on a scale of 0–1 by the following formula:

$$Ib = 1 - [2 * (0{,}5 - p)];$$

where p is the proportion of cultivars containing the band.

Results and discussion

There were significant differences in the germination percentage in most of treatment, except in 20 and 30 Gy in comparison to the control seeds (***Figure 2***). Even the highest dose of irradiation did not inhibit significantly the germination process. However, at the higher dose, progression stopped in cotyledons, no complete plants developed from 60 GY doses seeds. The lowest germination percentages were detected within the 20 and 30 GY treatments.

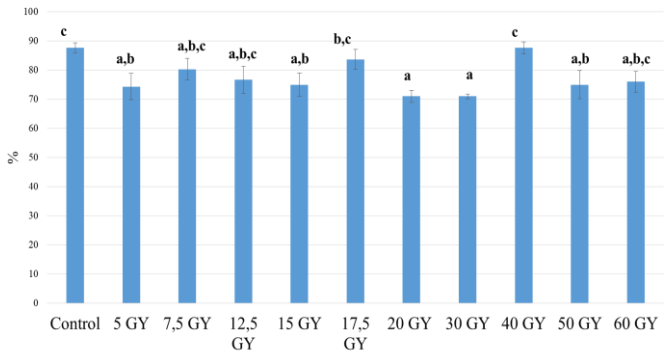

Figure 2. Germination percentage of the plants at different doses of radiation. Legend: Different letters mean that there are significant differences between the measured characteristics at the level of α = 0.05 based on the TUKEY test.

Different dosage of irradiation treatments showed high morphology heterogeneity (***Figure 7***). A very unique and totally uniform treatment, the 17.5 Gray K2 was observed, whose morphology and growth habit significantly differed from the others. The same dose of irradiated seeds at other positions do not exhibit such uniformity.

The higher dose of fast neutron strongly inhibits the plants' growth (***Figure 3-4***). The Growth reduction percentage was more than 50% in the cases of 17.5 Gray and higher irradiation dosage.

The number of adult leaves were reduced by 40 and 50 Gy treatment (***Figure 5***). The control plants had more adult leaves than the treated plants. The control plants have 5.5 leaves per plant in average, in contrast the genotype in higher dose has only 2 or 3 leaves per plants. In addition, we observed several morphological deformations at higher dose of irradiation (30-40-50 GY).

In case of lateral shoot number, only the higher dosage reduced the shoots growth significantly (***Figure 6***), where the average lateral shoot number decreased from 3.5 to 1-0.5 per plants.

Asante et al. (2016) found that the mutation induction is effective applying 15-20 Gray gamma irradiation and can cause detectable mutation in the M2 generation. Based on our studies, in case of *Ocimum basilicum*, the same or higher dosage of fast neutron irradiation is also suitable for the new genetic variety establishment. We observed many phenotypic polymorphism in the M0 generation.

With higher doses (between 15 and 50 GY), the following aberrations were observed (in the order of frequency) (***Figure 7***):

- deformed congestion,
- leaf mutations, spearmint/spiky and split leaves (30 GY, 40 GY, 50 GY),
- low growth* (17,5GYK2-12,5GY, 40GY)
- tissue chimeras (yellow-green leaf) (20GY-40GY),
- spearmint/spiky leaf shape (15 GY),
- flower anthocyanins (so far 15-30GY)

Molecular markering of mutant

Phenotype of the selected plants

Table 2 contains some morphology parameters of the selected plants for the molecular assays. The mutant marked with 'T' are markedly different in one or more phenotypic characters.

Performance of ISSR markers

In order to boost the polymorphism and specificity, all the used dinucleotide primers were anchored at their 3′-end with one or two degenerate nucleotide(s). The seven ISSR primers used in this study yielded 115 loci ranging from 0.4 to 1.7 kb, out of which 110 loci were polymorphic in nature representing 95.6% polymorphism. The number of loci reproduced by these arbitrary primers was found to range from 10 to 22 of different sizes with an average frequency of 16.43 loci per ISSR primer (***Table 4***).

The primer UBC 835 that gave the maximum number of bands (22) were the most informative, while the primer UBC 810 that gave the minimum number of products were the least informative.

Based on the similarity matrix (***Table 4***), there are no stronger genetic relationships within the same dosage, mainly in the lower dosage. Interestingly, the mutant in higher dosage treatment (40-50 Gray) showed higher genetic (83.3-100) homogeneity compared to the lower dosage treated mutant (46-72). Since these mutations will be detectable in the M1 to M4 generations, in the next generation we are going to determine the genetic stability of the mutation.

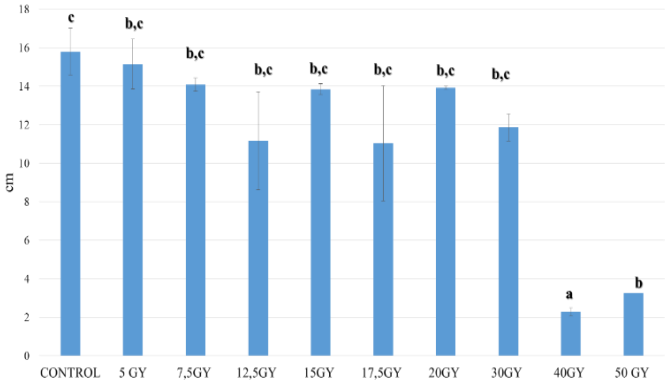

Figure 3. Height of the plants at different doses of radiation

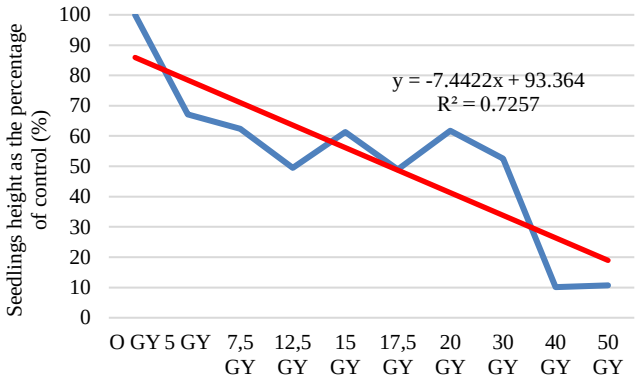

Figure 4. Growth reduction effect of the fast neutron on *Ocimum basilicum*

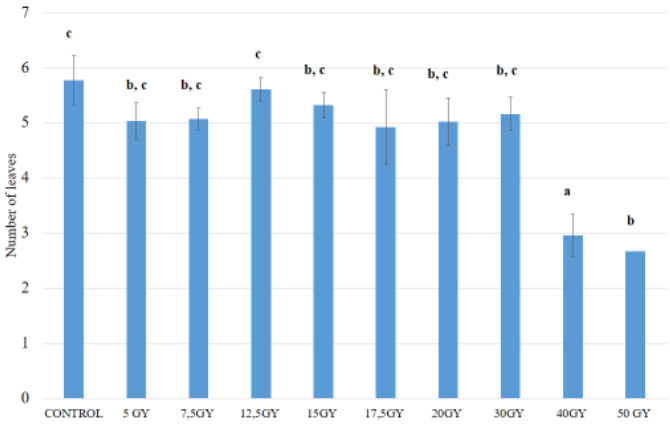

Figure 5. Number of adult leaves of the plants at different doses of radiation

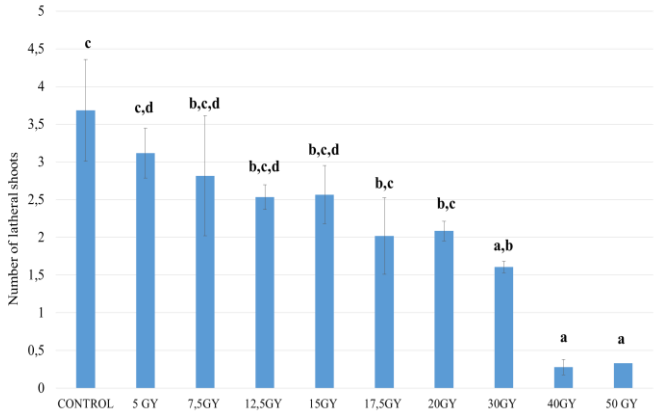

Figure 6. Number of lateral shoots of the plants at different doses of radiation

Table 2. Some morphology parameters of the selected *Ocimum basilicum* mutant

	Height	Shape of leaves	Colour of petals	Colour of sepals
K	52	Ovate	white	green
15 Gy T	23	Lancolate	pink	purple
15 Gy 1	43	Ovate	white	green
15 Gy 2	45	Ovate	white	green
17,5 Gy T	30	Ovate	white	purple
17,5 Gy 1	40	Ovate	white	green
17,5 GY 2	39	Ovate	white	green
40 Gy T	20	Lancolate	white	green
40 Gy 1	35	Ovate	white	green
40 Gy 2	39	Ovate	white	green
50 Gy T	25	Ovate, deform	white	green
50Gy 1	38	Ovate	white	green
50 Gy 2	38	Ovate	white	green

Table 3. The genetic similarity matrix of *Ocimum basilicum*, gained by the UBC 856 primer

	K	15 Gy T	15 Gy 1	15 Gy 2	17,5 Gy T	17,5 Gy 1	17,5 Gy 2	40 Gy T	40 Gy 1	40 Gy 2	50 Gy T	50 Gy 1	50 Gy 2
K	100.00	57.14	61.54	50.00	46.15	46.15	57.14	57.14	61.54	66.67	50.00	46.15	46.15
15 Gy T	57.14	100.00	61.54	50.00	46.15	61.54	57.14	57.14	46.15	50.00	66.67	76.92	76.92
15 Gy 1	61.54	61.54	100.00	72.73	66.67	66.67	76.92	76.92	83.33	90.91	72.73	66.67	66.67
15 Gy 2	50.00	50.00	72.73	100.00	54.55	90.91	83.33	83.33	90.91	80.00	80.00	72.73	72.73
17,5 Gy T	46.15	46.15	66.67	54.55	100.00	66.67	76.92	76.92	66.67	72.73	72.73	66.67	66.67
17,5 Gy 1	46.15	61.54	66.67	90.91	66.67	100.00	92.31	92.31	83.33	72.73	90.91	83.33	83.33
17,5 Gy 2	57.14	57.14	76.92	83.33	76.92	92.31	100.00	100.00	92.31	83.33	83.33	76.92	76.92
40 Gy T	57.14	57.14	76.92	83.33	76.92	92.31	100.00	100.00	92.31	83.33	83.33	76.92	76.92
40 Gy 1	61.54	46.15	83.33	90.91	66.67	83.33	92.31	92.31	100.00	90.91	72.73	66.67	66.67
40 Gy 2	66.67	50.00	90.91	80.00	72.73	72.73	83.33	83.33	90.91	100.00	80.00	72.73	72.73
50 Gy T	50.00	66.67	72.73	80.00	72.73	90.91	83.33	83.33	72.73	80.00	100.00	90.91	90.91
50Gy 1	46.15	76.92	66.67	72.73	66.67	83.33	76.92	76.92	66.67	72.73	90.91	100.00	100.00
50 Gy 2	46.15	76.92	66.67	72.73	66.67	83.33	76.92	76.92	66.67	72.73	90.91	100.00	100.00

Table 4. Marker parameters calculated for each ISSR primer used with *Ocimum basilicum* mutant

	TB	NPB	PIC	% OF POLYMORPHIC BAND	EMR	MI
UBC-807	20	20	0.37	100.0	5.10	1.88
UBC-810	10	9	0.33	90.0	5.18	1.73
UBC-818	15	13	0.32	86.7	4.50	1.44
UBC-835	22	22	0.33	100.0	4.00	1.32
UBC-840	14	12	0.25	85.7	6.58	1.64
UBC-841	19	19	0.36	100.0	6.46	2.32
UBC-856	15	14	0.41	93.3	8.13	3.32

TB: Total number of bands, NPB: Number of polymorphic band, PIC: Polymorphic information content EMR: Effective multiplex ratio MI: Marker index

The ISSR loci were almost all polymorphic at the species level for each primer except UBC 810, UBC 840 and UBC 856 (***Table 4***). In this study, the highest PIC value of 0.41 was observed for primer UBC 856 (***Figure 8***), and the lowest PIC value of 0.25 was observed for primer UBC 840, with an overall average PIC value of 0.344 per primer.

The fraction of polymorphic loci impact the ISSR effective multiplex ratio. In this study, the highest effective multiplex ratio (EMR = 8.13) was observed with the primer UBC-856 with a mean EMR of 5.00 per primer (***Table 4***). To determine the general usefulness of the system of markers used, we calculated the MI (marker index) for each ISSR primer (***Table 4***).

The highest MI was observed with the primer UBC-856 (3.32) and lowest in the primer UBC-835 (1.35). We gained similar results as Kumar et al. (2016), who studied the phylogenetic relationship between the *Ocimum* taxa using similar primers.

Conclusions

We found that fast neutron irradiation is highly effective for mutation induction in case of *O. basilicum* seeds. The higher dosage caused phenotypically detectable deformation/mutation in the M0 generation, however in the next generations, the

Figure 7. Post-treatment mutations A: deformed congestion, B: tissue chimeras, C: spearmint/spiky leaf shape, D: anthocyanin flowering shaft and flower scrubs (D-1 normal, D-2 mutant 30GY A7), E- spearmint/spiky leaf shape with split/toothy edges, F: spearmint leaf shape

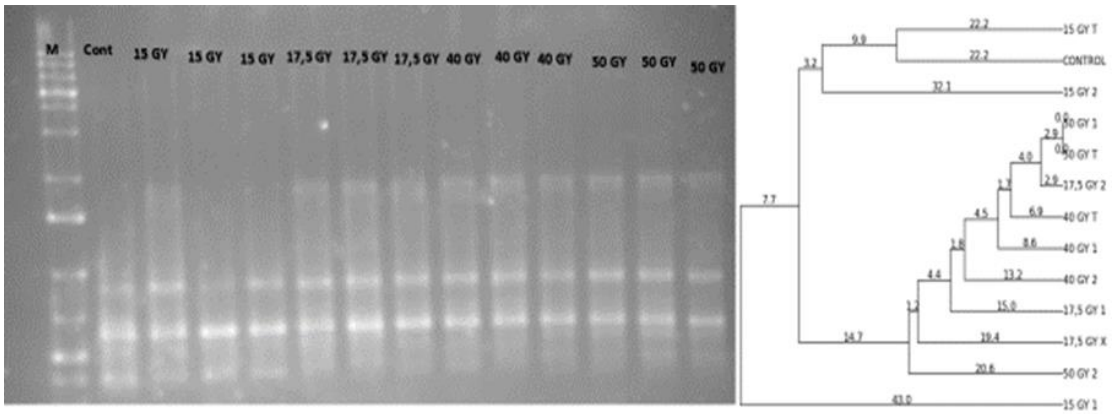

Figure 8. The agarose gel picture of ISSR (UBC-856) markers PCR with 12 *Ocimum basilicum* samples

mutation will be stabilized. The 60 Gray fast neutron dosage inhibits the plant development after the germination. The 50 Gray has also damaged the plant development, but we were able to grow some mutant plant from it. We detected other phenotypic polymorphism within the mutant population like deformed congestion, leaf mutations, spearmint/spiky and split leaves, low growth, tissue chimeras, and flower anthocyanin.

According to the study of Asante et al. (2016), the mutation induction is effective in applying 15-20 Gray gamma irradiation and can cause detectable mutation in the M2 generation. Based on our studies, in the case of *Ocimum basilicum*, the same or higher dosage of fast neutron irradiation is also suitable for the new genetic variety establishment. We observed many phenotypic polymorphisms in the M0 generation. As it is well-known, the induced mutation becomes detectable in the next mutant generations (M1-M4-5), so the unique mutant genotypes were isolated, and the self-pollinated following mutant generations will be further examined in the next years.

The analysis clearly shows that ISSR markers are a robust means for creating fingerprinting keys and have the potential to identify mutation specific markers for *Ocimum basilicum*. The most vital resources of the breeding and management of *O. basilicum* germplasm include a lot of elements. The modern technologies provide opportunity of the generation of fingerprinting keys, the identification of species and cultivar-specific ISSR markers and the development of function or phenotype specific marker in order to the elucidation of the inheritance of the valuable traits.

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