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Perspectives on the Extension and Diversification of Basil (*Ocimum basilicum* L.) Assortment by Exploiting Genetic Resources Conserved at The Plant Genetic Resources Bank of Buzău

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RESEARCH ARTICLE

Abstract

The present study aims to evaluate the diversification of the current indigenous assortment of the genus *Ocimum* by obtaining new distinct genotypes with superior quality, following classical intraspecific hybridization and determination of their chemical composition. The germplasm collection contains 63 genotypes of which 27 are genetic stable. Among the stable genotypes, 4 genitors were studied as breeding material. The breeding procedures used were classical hybridization, negative mass selection and segregation. Laboratory analyses were also carried out in terms of chemical composition description. In the present research work, as a result of intraspecific hybridization, 4 new genotypes were obtained to enrich the local assortment of aromatic and medicinal plants of the genus *Ocimum* in Romania. The new morphotypes obtained have distinct characteristics and superior qualities.

Keywords: preservation; hybridization; genotype; aromatic; medicinal.

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INTRODUCTION

Sweet basil (*Ocimum basilicum* L.) is a widely grown aromatic crop cultivated either for production of essential oil, dry leaves for the fresh market, or as an ornamental. Within this species, there is a significant variation in phenotype and chemotype in terms of oil content and composition. Historically, due to its pleasant aroma that suppresses other scents, basil has been widely used in religious rituals in various cultures and times. Fresh basil is used as an ingredient in various dishes and food preparations, especially in the Mediterranean cuisine (Zheljazkov et al., 2008). Basil exhibits a great variety of cultivars grown for various purposes (Varga et al., 2017). Basil is an herbaceous aromatic plant that can be grown as an annual or perennial according to the region where the plant is cultivated (Costa et al., 2014). The taxonomy of *Ocimum* is complex due to interspecific hybridization and polyploidy of the species in the genus (Telci et al., 2006). The still existing uncertainty in the classification within the genus depends on the fact that species identification relied on morphological characters whose expression is known to be affected by developmental and environmental factors (Labra et al., 2004). The genus *Ocimum* has several species that are used to treat

different types of ailments from ancient time, especially the species *O. basilicum* (Purushothaman et al., 2018). Species of the genus *Ocimum* are widely used and appreciated due to their essential oil that includes several components of interest (Costa et al., 2014). The recurring polymorphism determines a large number of subspecies, different varieties and forms producing essential oils with varying chemical composition, some present a high camphor content others are characterized by citral, geraniol, methylchavicol, eugenol, thymol, etc. (Khalid et al., 2006). *Ocimum* species are a rich source of diverse specialized metabolites (including monoterpenes, sesquiterpenes, and phenylpropanoids) and have numerous pharmacological activities as well as insecticidal properties (Mahajan et al., 2015). Polyphenols are secondary plant metabolites that have a variety of structures and functions in the plants and are well known as antioxidants in the human diet (Filip, 2017). For the incredible aroma, basil is also called" king of herbs" (Filip, 2017). Basil is classified according to its aroma into categories such as sweet, lemon, cinnamate or cinnamon, camphor, anise, and clove (Costa et al., 2014). Basil is a condimental plant cultivated and used frequently in soups, desserts, pickles, pizza, spaghetti sauce, egg, cheese dishes, tomato juice, dressings, confectionery, salads, meat products etc. as a flavouring agent (Özcan and Chalchat, 2002). Breeding programs for basil in the United States have explored the natural diversity and the presence of chemotypes in sweet basil to develop basil cultivars with specific aromas and fragrances for niche markets (Zheljazkov et al., 2008). The production of hybrids by crossing cultivars contributes to the creation of new essential oils for the world market. The most important decision for the hybridization programs the choice of parents; this decision depends on the characteristics to be improved, the type of inheritance of the characteristics, and the available source of germplasm (Costa et al., 2014). The availability of various chemotypes offers the opportunity for production of basil to meet the market requirements of specific basil oils or individual compounds such as (-)-linalool, eugenol, methyl chavicol, methyl cinnamate, or methyl eugenol (Zheljazkov et al., 2008). The present study aims to evaluate the diversification of the current indigenous assortment of the genus Ocimum by obtaining new distinct genotypes.

MATERIALS AND METHODS

The germplasm collection studied in this paper includes 63 genotypes that have been systematized into 3 groups according to genetic stability: 27 genetically stable genotypes, 14 advanced genotypes and 22 segregants. The morphotypes in the germplasm core collection belong to subspecies: *Ocimum basilicum* spp., *Ocimum kilimascharicum*, *Ocimum rubrum*, *Ocimum basilicum* var. *purpurascens*, *Ocimum tenuiflorum*, *Ocimum basilicum* var. *bulatum*, *Ocimum basilicum* var. *crispum*, *Ocimum basilicum* var. *citriodorum*, *Ocimum gratissimum*, *Ocimum basilicum* var. *thyrsiflora*.

The crop was established in the open field of PGRB in Buzau by planting them on 5th of May, respecting the isolation distances of 2000 m between families, given the high degree of allogamy. The genetically stable genotypes studied were: G1, G3, G4, G7, G8, G9, G10, G11, G12, G13R, G13P, G14, G15 si G16. Genetically stable morphotypes have been studied according to the international UPOV descriptors, an international system also followed by Carović et al., (2011) and also IPGRI standards. Morphological analysis were made to examine phenotypic characteristics including leaf shape, leaf margin, leaf color, leaf surface, stem color, and flower color, according to the study made by Javanmardi et al. (2002). The main biometric measurements were done on fully developed plants including the flowering stems. Observations were made on the main flowering stem.

Four parents with phenotypic distinctiveness and qualitative traits essential for the breeding process were identified and selected: G12, G 11, G 13 R and G7. G13 R genitor is the oldest local population cultivated in Romania, Calarasi county, known as "de Radovanu". G12 genitor comes from var. *thyrsiflora*, being chosen as parent for the characteristic anthocyanin colouring of the inflorescence. G11 is distinguished by the lemon aroma of the entire vegetative part and G7 genitor is part of var. *rubrum* subspecies, being the richest in antioxidants and anthocyanin and flavonoid pigments.

There were carried out 2 crosses as follows: G13R ♀ X G 12 ♂ and G11 ♀ X G 7 ♂. To realize hybridization, inflorescences of each plant were selected as female and marked; different color was used for the male genitor as pollen donor. Every morning the crosses were performed using collected inflorescences. The picked flowers with mature pollen were touched against the stigmas of emasculated flowers. After hand pollination, the inflorescences functioning as female were protected with paper bags to prevent the flowers from self-pollinating.

Biochemical analyses were performed to identify the volatile oil composition for the two parents, G 13R and G11 to estimate the potential of the two genitors in terms of this qualitative character. The chemical composition of the volatile oil was carried out using the Agilent Technologies 6890N type gas chromatograph coupled with the mass detector MSD 5975 type XL Mass Selective Detector. For the determination of the volatile oil composition, Neo Clavenger hydro distillation was performed for 100 g of naturally dried plant material, resulting in a volume of 0.72 ml volatile oil, followed by gas chromatographic analysis coupled with mass spectrometry similar to the study performed by Phippen and Simon (1998). The chromatograms of each of the oil samples were compared to the chromatograms from standard sample. The target peaks were confirmed by both retention time and mass spectra. There were compared and analysed two samples: G13R and G11 with controls, Trial test 1 (0,72 ml/100 g) and

Trial test 2 (0,82 ml/100 g).

Statistical analysis was performed using IBM SPSS STATISCS BASE software. Analyses of variance ANOVA was made followed by Duncan's post-hoc test with 95% confidence interval and p-values < 0.05.

RESULTS AND DISCUSSIONS

Evaluation of the germplasm core collection was carried out by performing biometric measurements for the main characters based on the international UPOV and IPGRI descriptors. From the germplasm collection, 4 morphotypes with specific characters were selected to be imprinted on the F1 generation: G13R, G12, G11 and G7. As a result of hybridization, the following morphotypes were genetically stabilized: G3, G15, G13P, G10, G9, G16.

Ocimum basilicum L. core collection biometric evaluation

The germplasm core collection includes 63 genotypes in advanced stages of breeding. From these, lines with a high degree of genetic stability and distinct phenotypic characteristics were selected for the present study. For the establishment and evaluation of genotypes, biometric measurements of plants grown in the open field during 5 years of culture were carried out (Table 1).

Table 1. Main biometric characteristics of genotypes -averaged open field values

Genotypes	Plant height (cm)	Canopy width (cm)	Main shoots no. (piece)	Leaf lenght (cm)	Leaf width (cm)	Petiole lenght (cm)	Flowering stem (piece)	Flowering stem lenght (cm)	Florets no./flowering stem (piece)
G1	50.5±1.4^{b}	31.8±1.7^{a}	5.8±0.7^{a}	4.4±0.4^{a}	2.4±0.5^{a}	2.3±0.1^{bc}	8.8±2.3^{a}	13.4±2.7^{ab}	15.4±1.02^{de}
G3	52.3±6.9^{b}	76.4±4.8^{f}	7.8±0.7^{a}	4.5±0.8^{a}	2.8±0.5^{ab}	1.2±0.3^{a}	68.2±4.4^{c}	21.6±0.9^{de}	18±2.10^{ef}
G4	70.4±1.7^{d}	97.6±3.7^{g}	13.8±2.4^{c}	13.8±2.1^{e}	11.9±0.9^{d}	6.9±0.5^{f}	93±3.7^{d}	28±2.1^{f}	20.8±4.02^{f}
G7	52.7±2.7^{b}	46.8±4.4^{c}	7.6±1.0^{a}	7.4±0.5^{cd}	4.2±0.1^{c}	2.3±0.8^{bc}	44.4±2.5^{b}	17.3±4.4^{c}	18.6±2.58^{ef}
G8	54.8±2.1^{bc}	51.8±4.9^{c}	10.2±0.9^{b}	7.4±0.7^{cd}	4.4±0.4^{c}	3.9±1.0^{d}	93.2±8.7^{d}	18.5±4.5^{cd}	17.8±1.17^{ef}
G9	52.7±4.5^{b}	51.0±8.3^{c}	5.8±0.7^{a}	5.4±0.1^{ab}	3.0±0.2^{ab}	2.5±0.3^{bc}	89.4±20.2^{d}	22.8±1.8^{e}	16.8±1.47^{de}
G10	53.3±5.7^{bc}	40.6±3.3^{b}	6.4±1.0^{a}	4.5±1.0^{a}	2.8±0.9^{ab}	1.7±0.7^{abc}	39.6±4.7^{b}	11.5±1.5^{a}	11.6±2.06^{bc}
G11	72.5±2.3^{de}	68.9±2.4^{e}	13.2±1.1^{c}	5.4±1.0^{ab}	2.2±0.4^{a}	1.6±0.3^{ab}	182.8±9.6^{f}	22.2±1.4^{e}	15.80±1.72^{de}
G12	76.3±2.0^{e}	78.0±2.9^{f}	18.4±2.0^{d}	7.1±0.5^{cd}	2.2±0.1^{a}	2.7±0.1^{c}	80.6±2.0^{cd}	27.4±1.6^{f}	5.8±0.84^{a}
G13R	58.6±3.9^{c}	68.2±2.1^{e}	29±1.5^{e}	7.7±0.4^{d}	2.9±0.5^{ab}	2.6±0.1^{c}	47.2±1.3^{b}	11.4±1.0^{a}	8.8±1.17^{ab}
G13P	50.1±1.3^{b}	37.9±4.7^{b}	12.2±1.0^{c}	5.4±0.7^{ab}	2.9±0.5^{ab}	2.0±0.8^{abc}	292.4±35.7^{h}	10.7±2.0^{a}	9±1.6^{ab}
G14	73.4±5.3^{e}	50.2±1.7^{c}	7.6±1.2^{a}	12.6±1.0^{e}	11.6±1.0^{d}	4.8±0.1^{e}	26.4±1.0^{ab}	10.6±1.0^{a}	8.6±1.02^{ab}
G15	43.8±1.4^{a}	58.0±4.6^{c}	8.0±2.3^{a}	6.5±0.7^{bcd}	2.9±0.3^{ab}	2.4±0.5^{bc}	272±23.7^{g}	13.7±1.6^{ab}	14.4±3.3^{cd}
G16	76±6.9^{e}	51.8±2.9^{d}	12.2±0.7^{c}	6.0±0.7^{bc}	3.6±0.4^{bc}	2.6±1.0^{c}	134.2±26.2^{e}	15.6±3.0^{bc}	16.2±3.4^{de}

Note: Different letters between cultivars denote significant differences (Duncan test, $p < 0.05$, 95% confidence level)

The mean values recorded for the main measurable characters were subjected to the Duncan test for assessing biodiversity and differences between the 14 morphotypes. Thus, a very high variability was identified in the number of florets per inflorescence, with genotypes G4 and G12 being significantly distinct. As for the degree of similarity, it was identified by statistical calculation in the case of the number of main branches per plant, which shows that the plants have similar main branches in number but differ in the degree of higher order branches, II, III, IV.

The main biometric measurements were done on fully developed plants including the flowering stems. Observations were made on the main flowering stem.

G1 is distinguished by the clove-like aroma of the vegetative part, the leaf blade is broad ovate, the appearance of the plant is of a particular specificity with narrow filiform inflorescences and small and sparse leaves.

G3 is distinguished by a globular, uniform habit with light green leaves, long flower stalks compared to the height of the plant and a specific, strong camphor aroma.

G4 is distinguished by its curly, deeply serrate leaves on the edge of the limb, of a raw green colour.

G7 is the genotype with the strongest anthocyanin coloration on the entire vegetative part, leaves and stems are purple-black. The purple-lilac flowers show the purple colour style.

G8 also shows anthocyanin coloration, but less uniform than genotype G7. The leaves at the base of the plant are mostly green, the upper leaves dark purple.

G9 has a strong aniseed and camphor aroma, with green leaves and purple-lilac flowers.

G10 has the same aroma as G9 but the flowers are green.

G11 is a special genotype, distinguished by its narrow-elliptic leaf blade and distinct specific grey-green colour. Also, the glandular peristomes are visible and prominent on the entire vegetative part. Both on the leaves and on the flower. The oil secreted by this plant has a lemon aroma.

G12 is the genotype with a strong anthocyanin coloured bouquet with purple-lilac flowers, compact and globular inflorescence with an aniseed aroma.

G13R is the classic basil, old local population, medium green colour and fine aroma.

G13P is a morphotype with light green foliage but globular inflorescences, small spikelets with an average of 9 florets per spike.

G14 is distinguished by its strongly embossed, raw green leaves and flowers with both lips completely lilac.

G15 is distinguished by strongly coloured dark purple inflorescences. Also, the stems show a strong anthocyanin colouring unlike the leaves which are completely green.

G16 is distinguished by purple inflorescences, the rest of the vegetative part is green.

Figure 1 shows details of the leaves of both the upper and lower leaf face, the lower leaf face magnified under a microscope to reveal the glandular cells containing the essential oil characteristic of the species. In the case of the G9 morphotype, a multitude of such secretory cells is noted, being the richest genotype in essential oil. Also shown are the microscopically enlarged flowers, the smallest flower being G1 and the largest flowers of genotypes G7 and G16.

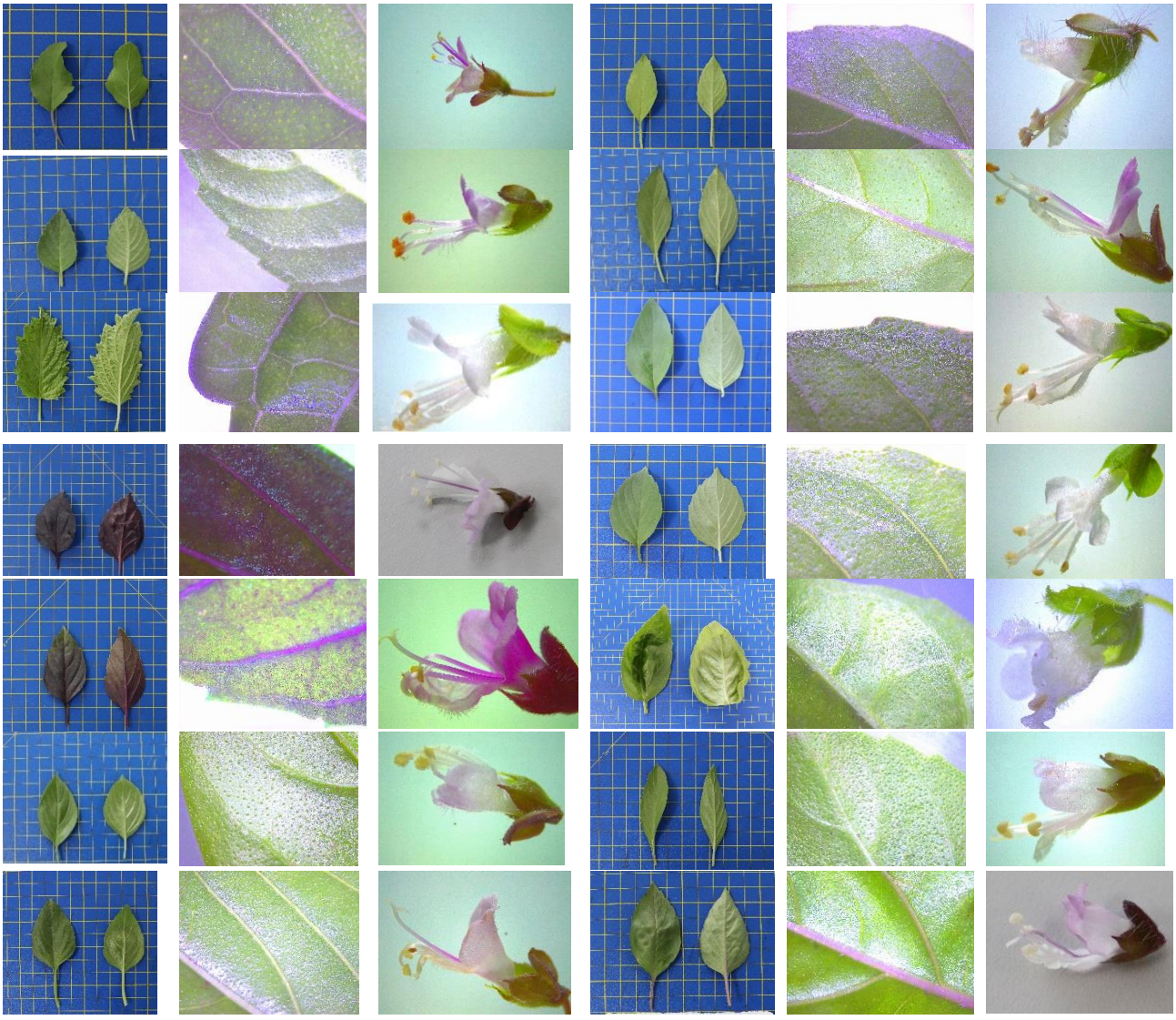

Figure 1. The studied morphotypes (top to bottom, left to right): G1, G3, G4, G7, G8, G9, G10, G11, G12, G13R, G13P, G14, G15 and G16. (a) adaxial and abaxial leaf surface; (b) microscope leaf detail; (c) flower

New morphotypes obtained by crosses

As a result of G13R ♀ X G 12 ♂ cross 3 descendants of major interest were identified: G3, G15 and G13P. In F2, the percentages with which the selected forms were found in the culture were G3 with 12%, G15 with 11% and G13P with 14%. In F3 the percentages increased respectively 22% G3, G15-21% and G13P with 24%. The process of stabilization and elimination of atypical forms (Figure 3) continued until the three progenies were genetically stabilized and produced 100% uniform plants. The stabilization of G3 in F6 was achieved, being the first genetically stabilized morphotype, followed by G15 in F7 and G13P in F9.

The results of the G11 ♀ X G 7 ♂ cross are also 3 progenies, namely, G10, G9 and G16, similar to the parental forms, with distinct qualitative characteristics. Thus, following selection, the percentages of typical forms in F2 were determined: G10 with 16%, G9 with 9% and G16 with 12%. Atypical forms accounted for 63%. In F3, the percentages of typical forms increased slightly to 26% for G10, 19% for G9 and 22% for G16. Atypical forms accounted for 33% in F3. The descendants were stabilized in F7, genotypes G10 and G16 and genotype G9 in F8, when no more atypical forms appeared.

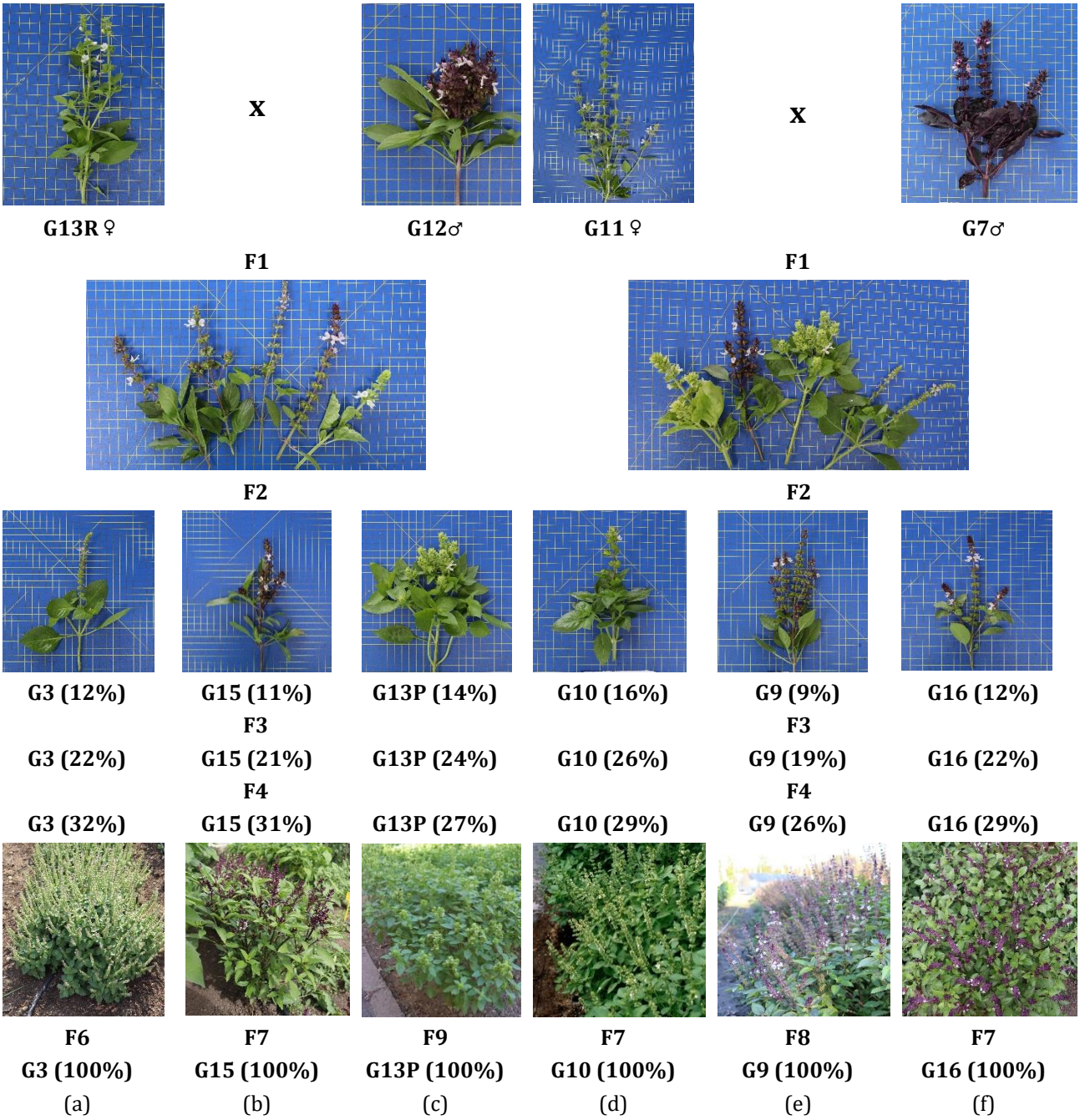

Figure 2. The two crosses that produced the following F1 descendants: G3, G15, G13P, G10, G9, G16

Figure 3. Atypical forms that were eliminated throughout the research

Chemical analyses of G13R and G11 genitors

The volatile oil composition for the two genitors, G13R and G11, was analysed by gas chromatographic method coupled with GC/MS mass spectrophotometry. Thus, the analyzed samples have common compounds, which occur in much higher percentages in the case of the old local population G13R, namely linalool-38.47%, estragole-45.89%. The two chemical compounds in which G11 is superior to the other parent are caryophyllene-7.08% and germacrene D with a percentage of 2.65% (Table 2). Also, Srivastava et co. found that some accessions are highly rich in linalool and can be used for selection of linalool rich chemotype/lines.

Table 2. Concentration of major compounds (%) in G13R and G11 genitors

Compounds	Trial test 1	G13R	RT (min)	Difference from trial test (%)	Trial test 2	G11	RT (min)	Difference from trial test (%)
Linalool	40.4	38.47	11.26	5.0	0.39	6.18	11.22	1484
Estragole	41.09	45.89	15.25	12.0	0.4	8.11	15.22	1927
Caryophyllene	0.45	0.6	24.5	44.0	3.53	7.08	24.5	100
Germacren D	1.28	2.29	27.05	78.0	0.59	2.65	2.05	338

Note: TR-retention time

Also, G13R is mainly characterized by a high content of estragole and linalool unlike G11 which is characterized by a high content of geranial-22.7% and carveol-17.7%. Carović-Stanko et co. also had a study where identified high levels of both morphological and chemical variability exist within the species due to the intraspecific hybridization and long-term uses.

CONCLUSIONS

The germplasm collection was evaluated phenotypically and biometrically. All 14 morphotypes analysed show characteristic distinctiveness composing a diverse range in terms of main morphological characteristics.

Four parents were selected from the germplasm collection: G13R, G12, G11 and G7. Two crosses were carried out and the descendants of major interest were selected in F1: G3, G15 and G13P respectively G10, G9 and G16, being stabilized in F6, F7, F8 and F9, according to each morphotype.

Biochemical analyses were also carried out to determine the volatile oil composition of the parents of major interest: G13R and G11. G13R is mainly characterized by a high content of estragole and linalool, unlike G11 which is characterized by a high content of geranial -22.7% and carveol -17.7%. Research will continue by obtaining other distinct morphotypes, using the genetic resources conserved at the Buzau Plant Genetic Resources Bank.

Author Contributions: C.V. coordinated the entire study, being the holder of the *Ocimum* spp. germplasm collection, B.M., C.B. and G.N. contributed to the phenotypic observations, biometric measurements and M.P. and A.P. performed the laboratory analyses.

Conflicts of Interest

The authors declare that they do not have any conflict of interest.

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