Israel Journal of Plant Sciences *Vol. 59* *2010* *pp. 183–189*
DOI: 10.1560/IJPS.59.3-4.183

Estimation of nuclear DNA content of cultivated *Ocimum* species by using flow cytometry

Adolfina R. Koroch,[a] Wenqin Wang,[b] Todd P. Michael,[b] Nativ Dudai,[c] James E. Simon,[b,d] Faith C. Belanger[b,d,*]
[a]*CUNY, Borough of Manhattan Community College Deptment of Science, 199 Chambers Street, New York, New York 10007, USA*
[b]*Department of Plant Biology and Pathology, Rutgers, The State University of New Jersey, 59 Dudley Road, New Brunswick, New Jersey 08901, USA*
[c]*Newe Ya'ar Research Center, Agricultural Research Organization, P.O. Box 1021, Ramat Yishay 30095 Israel*
[d]*New Use Agriculture and Natural Plant Product Program (NUANPP), School of Environmental and Biological Sciences, Rutgers, The State University of New Jersey, New Brunswick, New Jersey 08901, USA*

(*Received July 13, 2009*; *accepted in revised form October 12, 2009*)

ABSTRACT

Ocimum spp. are cultivated worldwide for culinary and medicinal uses. Some species are the subjects of improvement in breeding programs while others are still collected from the wild. Polyploidy and dysploidy are common features of the cultivated species. Taxonomical confusion has arisen in the classification of *Ocimum* spp., in part due to the ease with which many of the species intercross and the morphological similarities between several of the species. Flow cytometry is a fast and easy method to measure nuclear DNA content and could be a useful tool to assess germplasm accessions in a breeding program. Here we report the first estimates of nuclear DNA content of eight *Ocimum* spp., as determined by flow cytometry. The 2C nuclear DNA content ranged from 928 (*O. campechianum)* to 5515 Mbp (*O. americanum)*. The nuclear DNA content levels may be useful for better understanding of the taxonomical relationships among species.

Keywords: basil, flow cytometry, nuclear DNA content, *Ocimum*

INTRODUCTION

The genus *Ocimum* L. (Lamiaceae) includes many aromatic species that are cultivated for culinary and medicinal uses (Naithani and Kakkar, 2002; Makri and Kintzios, 2007). The most well known species is *O. basilicum*, or sweet basil. Some of the other species in the genus are also commonly referred to as basils. Many *Ocimum* spp. are rich in essential oil, which is comprised largely of phenylpropanoids and terpenes (Hiltunen and Holm, 1999). The essential oil is synthesized in peltate glandular hairs located on the leaf surface (Werker et al., 1993). Some species are also a source of rosmarinic acid (Juliani et al., 2008). Significant variation in essential oil content and composition between and within *Ocimum* spp. has been observed (Simon et al., 1990; Grayer et al., 1996; Naithani and Kakkar, 2002; Vieira and Simon, 2006; Dudai and Belanger, 2008). Although basil has been used for thousands of years, formal breeding of basil is a relatively new practice. There are active basil breeding programs in Israel, Italy, and the United States.

The genus has a wide range of geographic distribution. It probably originated in tropical Africa and from there was introduced to tropical Asia (Paton et al.,

*Author to whom correspondence should be addressed.
E-mail: belanger@aesop.rutgers.edu

2004). *Ocimum* spp. were introduced to tropical South America by European colonists and through the African slave trade (Vieira and Simon, 2000), and yet there also appear to be species in the Americas not reported in Africa. Polyploidy and interspecific hybridization are common among the *Ocimum* spp., which has led to taxonomic confusion and misidentification of important chemotypes of commerce (Simon et al., 1990).

Sobti and Pushpangadan (1982) classified *Ocimum* spp. into two broad groups based on morphology and base chromosome number. The Basilicum group contained species with a base chromosome number of 12 and the Sanctum group contained species with a base chromosome number of 8. Paton et al. (1999) suggested that the Basilicum/Sanctum classification system should not be used since it does not comply with the International Code of Botanical Nomenclature. Paton et al. (1999) developed a revised taxonomy of the genus *Ocimum* in which they described 64 species. They divided the genus *Ocimum* into three subgenera, *Ocimum* (with 3 sections), *Nautochilus*, and *Gymnocimum* (with 2 sections).

Chromosome numbers for several *Ocimum* spp. have been reported (Table 1). Variation between species is likely due to variations in ploidy levels. However, even within a species there is variation in reported chromosome numbers, which may be due in part to the above-mentioned taxonomic confusion and the ease with which several of the species intercross. For example, Paton et al. (1999) suggested that the *O. americanum* (citral type) examined by Pushpangadan and Sobti (1982) is actually *O. x citriodorum* according to their revised taxonomy. The variation within species may also be due to instances of dysploidy that have become fixed within different populations (Pushpangadan and Sobti, 1982; Guerra, 2008).

Determination of nuclear DNA content can provide additional data useful in understanding species relationships. The method of flow cytometry is widely used because large numbers of samples can be screened in a short period of time. Samples can be prepared from any plant tissue and only small amounts are needed (Dolezel et al., 2007). This technique has been successfully used in determining nuclear DNA content in many species (Bennett and Leitch, 2005). Flow cytometry can provide plant breeders with a quick and simple method of assessing DNA content of their germplasm accessions. The Angiosperm DNA C-values database (http://data.kew.org/cvalues/) currently lists 4427 species, but no *Ocimum* spp. are included. In the present study, flow cytometry analysis was used to estimate nuclear DNA content in some commercially important *Ocimum* spp.

MATERIALS AND METHODS

Plant material

Plant samples were obtained from the North Central Regional Plant Introduction Station, Ames, Iowa (accessions with PI numbers), and from commercial sources (Table 2). Plants were grown from seeds under standard and uniform greenhouse conditions. Herbarium specimens of the plants used have been deposited in the Chrysler Herbarium, Rutgers University, New Brunswick, New Jersey.

Flow cytometry measurement of nuclear DNA content

The preparation of nuclei and flow cytometry were modified from procedures described previously (Galbraith et al., 1983; Peterson et al., 1997; Dolezel et al., 2007). All stages of the extraction and staining were performed on ice. Approximately 10 mg of fresh young healthy leaves were excised from each of the plants and used for the determinations. Plant nuclei were mechanically released by chopping the leaves with sharp razor blades in a plastic Petri dish containing 1 ml of ice-cold MEB buffer (1 M 2-methyl-2,4-pentanediol, 10 mM PIPES-KOH, 10 mM $MgCl_2$, 2% PVP-10, 10 mM sodium bisulfite, 5 mM 2-mecaptoethanol, 0.5% sodium diethyldithiocarbamate, 6 mM EGTA, 200 mM L-lysine-HCl, adjusted to pH 5.0 with HCl).

The chopped leaves were filtered through a CellTrics® 30 µm nylon mesh (Partec, Münster, Germany) into a microfuge tube and then centrifuged for 2 min at 9000 rpm. Nuclei were resuspended in 500 µl of homogenization buffer [45 mM $MgCl_2$ 6 H_2O, 20 mM MOPS, 30 mM sodium citrate, 0.1 % (v/v) Triton X-100, adjusted to pH 7.0 with HCl] by gentle shaking. The suspension of nuclei was stained with propidium iodide. To prepare the staining solution, 50 µl of 1 mg ml^{-1} propidium iodide was mixed with 5 µl of 5 mg ml^{-1} RNase and then added to the suspension of nuclei.

Samples were analyzed with a Coulter Cytomics FC500 Flow Cytometer (Beckman Coulter, Inc., Miami, FL). At least three independent measurements were performed for each of the samples. Only measurements having CVs of less than 5% were used (Dolezel et al., 2007). Nuclear DNA content was estimated by fluorescence of the propidium iodide stained nuclei relative to that of the internal standard, the diploid *Brachypodium distachyon* line Bd21, which has a 2C value of 600 Mbp (Huo et al., 2006). DNA content in Mbp was converted to pg by using the value 1 pg = 978 Mbp (Dolezel et al., 2003).

Table 1
Chromosome numbers reported for *Ocimum* spp.

Genus *Ocimum*	Chromosome number	Reference
Subgenus *Ocimum*		
Section *Ocimum*		
O. americanum var. *americanum*[1] *(O. canum)*	2n = 24, 26	Pushpangadan and Bradu, 1995
	2n = 26	Mukherjee and Datta, 2006
O. basilicum	2n = 48	Pushpangadan and Bradu, 1995
		Khosla, 1995
	2n = 50, 52, 53, 56, 72, 74	Paton and Putievsky, 1996
O. basilicum var. *crispum*	2n = 52	MuKherjee et al., 2005
O. x citriodorum	2n = 64	Paton and Putievsky, 1996
(O. americanum, citral type*)*	2n = 72	Pushpangadan and Bradu, 1995
(O. basilicum var. *citriodorum)*	2n = 72	Mukherjee et al., 2005
O. kilimandscharicum	2n = 76	Pushpangadan and Bradu, 1995
		Mukherjee and Datta, 2006
Section *Gratissima*		
O. gratissimum	2n = 40	Pushpangadan and Bradu, 1995
		Khosla, 1995
		Mukherjee and Datta, 2006
(O. suave)	2n = 64	Pushpangadan and Bradu, 1995
	2n = 48	Khosla, 1995
(O. viride)	2n = 40	Pushpangadan and Bradu, 1995
		Khosla, 1995
Section *Hiantia*		
O. selloi (O. carnosum)	2n = 48	Pushpangadan and Bradu, 1995
	2n = 64	Khosla, 1995
Subgenus *Gymnocimum*		
Section *Gymnocimum*		
O. campechianum (O. micranthum)	2n = 48	Pushpangadan and Bradu, 1995.
Section *Hierocymum*		
O. tenuiflorum (O. sanctum)	2n = 32	Pushpangadan and Bradu, 1995.
		Khosla, 1995
	2n = 36	Mukherjee and Datta, 2006.
	2n = 76	Paton and Putievsky, 1996.

[1]Recognized taxa within *Ocimum* with synonyms in parentheses (Paton et al., 1999).

The experimental design was fully randomized. Data were analyzed statistically by analysis of variance (ANOVA) followed by the Tukey test, with level of significance set at 0.001.

RESULTS AND DISCUSSION

A statistically significant variation in nuclear DNA content among the eight *Ocimum* spp. was observed (Table 3). The 2C nuclear DNA content ranged from 928 Mbp to 5515 Mbp. Much of the variation between species can be attributed to different ploidy levels. Based on the chromosome numbers, Pushpangadan and Bradu (1995) suggested that *O. basilicum* was an allotetraploid that originated from the interspecific hybridization of the diploid *O. americanum* (referred to as *O. canum*) with an as yet unidentified other *Ocimum* sp. Sobti and Pushpangadan (1982) proposed that interspecific hybridization between the diploid species *O. americanum* (referred to as *O. canum*) and the tetraploid species *O. basilicum* produced the hexaploid species *O. x citriodorum* (referred to as *O. americanum*). Paton et al. (1999) also proposed this hybridization as the origin of *O. x citriodorum*. These species are all

Table 2
Sources of *Ocimum* spp. analyzed for nuclear DNA content

Acc. No.[1]	Species	Germplasm source	Origin	Voucher specimen
Subgenus Ocimum				
Section Ocimum				
1	*O. americanum*	PI 500945[2]	Zambia	A Koroch 27
2	*O. americanum*	PI 652060	Pakistan	A Koroch 28
3	*O. americanum var. americanum*	PI 254352	Iraq	A Koroch 29
4	*O. americanum var. americanum*	PI 652062	Tanzania	A Koroch 37
5	*O. americanum var. pilosum*	PI 500953	Zambia	A Koroch 35
6	*O. americanum var. pilosum*	PI 652052	USA	A Koroch 36
7	*O. basilicum*	PI 170579	Turkey	A Koroch 30
8	*O. basilicum*	PI 211586	Afghanistan	A Koroch 31
9	*O. basilicum* '*Lesbos*'	Commercial[3]	USA	A Koroch 7
10	*O. basilicum* '*Queenette*'	Commercial[3]	USA	A Koroch 8
11	*O. basilicum* '*Cinnamon*'	Commercial[3]	USA	A Koroch 3
12	*O. basilicum* '*Poppy Joe*'	Commercial[4]	USA	A Koroch 43
13	*O. basilicum* '*Perrie*'	N. Dudai	Israel	A Koroch 44
14	*O. basilicum* '*Cardinal*'	N. Dudai	Israel	A Koroch 45
15	*O. x citriodorum* '*Sweet Dani*'	Commercial[3]	USA	A Koroch 22
16	*O. kilimandscharicum*	Commercial[3]	USA	A Koroch 12
Section Gratissima				
17	*O. gratissimum*	PI 652067	Brazil	A Koroch 32
18	*O. gratissimum*	PI 652068	Brazil	A Koroch 33
19	*O. gratisssimum var. gratissimum*	PI 652063	Kenya	A Koroch 40
20	*O. gratissimum var. macrophylla*	PI 652055	Sri Lanka	A Koroch 42
Section Hiantia				
21	*O. selloi*	PI 511865	Uruguay	A Koroch 41
Subgenus Gymnocimum				
Section Gymnocimum				
22	*O. campechianum*	PI 652066	Brazil	A Koroch 38
Section Hierocymum				
23	*O. tenuiflorum*	PI 652059	Maldives	A Koroch 34

[1]Acc. No. = Internal accession number.
[2]PI number = Accession number from USDA collection.
[3]Well Sweep Farm, Port Murray, NJ, USA.
[4]Seeds of Change, Santa Fe, NM, USA.

included in the subgenus *Ocimum*, section *Ocimum*, in the revised taxonomy (Paton et al., 1999). These three species are also grouped together in a molecular phylogenetic analysis based on three plastid DNA regions (Paton et al., 2004). The base chromosome number proposed for these species was 12 (Sobti and Pushpangadan, 1982), although, as summarized in Table 1, some of the reported chromosome numbers for these species are not consistent with a base of 12.

The data presented here on nuclear DNA content is consistent with the proposed ploidy levels of the three species, with some notable exceptions. Two *O. americanum* accessions, 3 and 4, have DNA contents of 1762 and 1906 Mbp, respectively, close to half of the overall mean value of 3070 Mbp for the 8 *O. basilicum* accessions. The DNA content of the *O. x citriodorum* of 4808 Mbp is consistent with the proposed hybridization of diploid *O. americanum* with tetraploid *O. basilicum*, followed by chromosome doubling. However, four of the *O. americanum* accessions had considerably higher DNA content than the two accessions mentioned above. Possible explanations for this are that these may represent populations in which autopolyploidization or past interspecific hybridization has occurred.

O. kilimandscharicum was considered to be a hexaploid, although its origin was not discussed (Sobti and

Table 3
Nuclear DNA content of *Ocimum* spp.

No.[1]	Species	2C (Mbp ± std)	2C (pg)	N[2]	CV range (%)	Significance[3]
Subgenus Ocimum						
Section Ocimum						
1	*O. americanum*	3607 ± 154	3.69	3	1.20–1.51	c
2	*O. americanum*	5515 ± 251	5.64	3	1.35–2.67	a
3	*O. americanum* var. *americanum*	1762 ± 110	1.8	4	2.53–2.91	fg
4	*O. americanum* var. *americanum*	1906 ± 49	1.95	3	2.33–4.90	fg
5	*O. americanum* var. *pilosum*	3195 ± 192	3.27	3	1.54–3.79	cd
6	*O. americanum* var. *pilosum*	4572 ± 283	4.68	3	2.15–2.71	b
7	*O. basilicum*	3307 ± 127	3.38	3	1.36–2.5	cd
8	*O. basilicum*	3394 ± 171	3.47	3	1.34–2.58	cd
9	*O. basilicum 'Lesbos'*	3060 ± 166	3.13	3	1.95–4.33	cd
10	*O. basilicum 'Queenette'*	2879 ± 44	2.94	3	1.55–2.58	de
11	*O. basilicum 'Cinnamon'*	2853 ± 93	2.92	3	1.56–3.16	de
12	*O. basilicum 'Poppy Joe'*	2902 ± 178	2.97	3	1.65–3.16	cd
13	*O. basilicum 'Perrie'*	3190 ± 105	3.26	4	1.45–1.96	cd
14	*O. basilicum 'Cardinal'*	2975 ± 89	3.04	3	1.54–2.71	cd
15	*O. x citriodorum*	4808 ± 322	4.92	3	1.30–2.07	ab
16	*O. kilimandscharicum*	4637 ± 318	4.74	3	2.69–2.91	b
Section Gratissima						
17	*O. gratissimum*	1839 ± 135	1.88	3	2.57–4.80	fg
18	*O. gratissimum*	1690 ± 141	1.73	4	1.61–4.57	fg
19	*O. gratisssimum* var. *gratissimum*	1308 ± 157	1.34	3	1.76–2.72	gh
20	*O. gratissimum* var. *macrophylla*	1422 ± 135	1.45	3	2.08–2.23	gh
Section Hiantia						
21	*O. selloi*	2172 ± 345	2.22	3	2.30–2.99	ef
Subgenus Gymnocimum						
Section Gymnocimum						
22	*O. campechianum*	928 ± 96	0.95	5	1.35–3.53	h
Section Hierocymum						
23	*O. tenuiflorum*	2843 ± 167	2.91	3	1.80–2.75	de

[1]No. = Accession number as listed in Table 2.
[2]N = Number of independent measurements.
[3]Accessions with different letters have significantly different means ($p < 0.001$) according to the Tukey test.

Pushpangadan, 1982). The DNA content of *O. kilimandscharicum* measured in this report was similar to that of the hexaploid *O. x citriodorum*.

Sobti and Pushpangadan (1982) considered that the species *O. gratissimum*, *O. selloi*, and *O. campechianum*, referred to as *O. suave* or *O. viride*, *O. carnosum*, and *O. micranthum*, respectively, had a base chromosome number of 8, although not all the reported chromosome numbers summarized in Table 1 are consistent with this proposal. Because of the wide range of reported chromosome numbers, Khosla and Sobti (1985) suggested four base chromosome numbers, 8, 10, 12, and 16, for these species. The different base chromosome numbers indicated that dysploidy, as well as polyploidy, was an important factor in the evolution of different *Ocimum* spp. (Khosla, 1995).

Different accessions of what is now considered *O. gratissimum* (Paton et al., 1999) were considered to be tetraploids with base chromosome numbers of 10, 12, and 16 (Khosla and Sobti, 1985). The DNA contents of the 4 *O. gratissimum* accessions reported here were similar, with a mean value of 1564 Mbp, similar to two accessions of the diploid species *O. americanum*.

Khosla and Sobti (1985) considered *O. selloi* (referred to as *O. carnosum*), with 2n = 64, to be an octoploid with x = 8 and *O. tenuiflorum* (referred to as

O. sanctum) with 2n = 32, to be a tetraploid with x = 8. Other chromosome numbers have also been reported for both of these species (Table 1). The DNA contents determined here for *O. selloi* and *O. tenuiflorum* were 2172 Mbp and 2843 Mbp, respectively. *O. campechianum* (referred to as *O. micranthum*) was considered to be a hexaploid with 2n = 48, x = 8 (Pushpangadan and Bradu, 1995) although the 2C DNA content measured in this study was the lowest of all the *Ocimum* spp. at 928 Mbp. Overall, some of the nuclear DNA content values measured here are seemingly inconsistent with the reported ploidy levels of some *Ocimum* spp.

Here, we have reported the first analysis of nuclear DNA content of some important *Ocimum* spp. Flow cytometry could be easily incorporated into *Ocimum* breeding programs and would be useful for characterizing the breeding germplasm. The variation in reported chromosome numbers for some of the commercial species may be due to dysploidy in different populations, which could affect the outcome of controlled crosses. Determination of nuclear DNA content would be an easy way of choosing parental plants for optimum compatibility. It would be interesting to determine if the observed variation in crossability among accessions of *O. basilicum* (Nation et al., 1992; Putievsky et al., 1999) is related to differences in DNA content. Also, manipulation of ploidy level is an important tool for plant breeding in several crops (deLaat et al., 1987), and different levels of ploidy can be related to different levels of productivity or fertility. Flow cytometry could be used to screen for desired cytotypes and to control stability of ploidy levels at various steps of a breeding program (Ochatt, 2008). In this study significant variation in nuclear DNA content was observed among accessions of *O. americanum*, which may be due to different ploidy levels of the accessions.

Much remains to be learned about the species relationships within the genus *Ocimum*. In addition to its potential use in breeding programs, flow cytometry could be useful in studies on the genome organization and evolutionary relationships of these interesting and commercially important species.

ACKNOWLEDGMENT

This work was supported by funds provided by the United States–Israel Binational Agricultural Research and Development Fund, project number US-4128-08F.

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