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A new chemotype of *Ocimum basilicum* var. *purpurascens* Benth. From Madhubani district of Bihar, India and phytochemical investigations of their colchiploids

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Abstract
Of the 160 species of the genus *Ocimum* L. (Lamiaceae), the species *O. basilicum* is medicinally and commercially a very significant one, as it contains a number of aromatic compounds. Various terpenes and phenols found in the species are widely used throughout the world in various ways, namely, in medicines, cosmetic materials, culinary, etc. It occurs in different climatic zones, right from the seashore to the high altitude world over. During a survey of the different aromatic compounds, especially terpenes and phenols, a new chemotype of a variety of the species, *O. basilicum* var. *purpurascens* Benth. was found in the Madhubani district, which possessed three Phytopharmaceuticals important compounds, namely methyl cinnamate (9.61%), linalool (27.88%) and eugenol (42.67%) in leaf and methyl cinnamate (2.87%), linalool (29.35%) and eugenol (48.22%) in inflorescence detected and analyzed with the help of Gas Chromatography. The Chemotype under investigation was named Eugenol>linalool>methyl cinnamate type. Due to the importance of these three in the national economy and the lack of any effort for its genetic improvement led the above authors to carry out chromosome doubling through autopolyploidy to see augmentation of these pharmaceutically important chemicals with other terpenoids if any. Successful autopolyploids were obtained after subjecting the new chemotype under study by colchicine treatment of the shoot apex. Phytochemical, as well, as other investigations, were made upto third polyploid generation. These polyploids achieved considerable cytogenetical normalcy and some terpenoids, such as, linalool, methyl chavicol, geraniol, αβ - terpineole and αβ - caryophyllene were observed to be in higher amounts.

Keywords: *Ocimum basilicum* var. *purpurascens*, phytochemical investigations, colchiploids, eugenol>linalool>methyl cinnamate type, autopolyploidy, gas chromatography, chemotype

Introduction
Of the 160 species of *Ocimum* L. belonging to the mint family, Lamiaceae and distributed throughout the world (WILLIS 1973) [1]. *Ocimum basılicum* var *purpurascens* Benth, a natural tetraploid with 2n=4x =48 chromosomes, is an important herb for medicinal and commercial viewpoints. Its essential oil contains linalool (Sobti *et al.* 1976, Thoppil and Jose 1994, Gupta 1996) [2, 3, 4] Sinha (2006) [8], methyl chavicol (Sobti *et al.*, 1976, Sinha, 2006) [2, 8] and methyl cinnamate (Sobti 1976, Gupta 1996, Sinha, 2006) [2, 4, 8] as main constituents in addition to a number of other terpenoids (citronellal, thujone, cis-ocimene, etc.) and is sold at a high price in the international market. An added advantage with the species under study is that it can grow luxuriantly in any part of the world right from seashore to high altitudes. Taking into consideration its commercial significance and its worldwide distribution, the present investigation was undertaken in order to see improvement if any, in phytochemical constituents and related characters of the species through autopolyploidy. Leaves and inflorescences are the two chief sources of the chemical constituents in the species.

Material and Method
A survey of Lamiaceae herbs had been conducted by Kamat (1992) [5] in the revenue division of Darbhanga of north Bihar (India). During the present investigation, only *O. basilicum* was vigorously searched and two varieties, namely, *O. basilicum* var. *purpurascens* and *O. basilicum* var. *thyrsiflorus* was collected in north Bihar consisting of revenue division of old Kosi (Saharsa) and old Tirhut (Muzaffarpur) in addition to Darbhanga division. Herbaria were kept in the Department of Botany R.K. College (L.N. Mithila University), Madhubani, Bihar.

Identification of the two varieties was confirmed at the National Botanical Research Institute, Lucknow. Of the two varieties, *O. basilicum* var. *purpurascens* was grown without applying any chemical fertilizer for a year in the Experimental Gardens. Seeds were collected & packed in polythene bags and kept in desiccators. Plants raised from these seeds and possessing good qualities were taken for further experimental studies. Fresh leaves and inflorescences from elite diploid and polyploid plants were subjected separately to hydrodistillation in Clevanger's apparatus (Clevanger 1928) [6] to obtain essential oils. Identification and quantification of phytochemical constituents in the oils of leaves and inflorescences of diploid and colchiploids were done with the help of Gas Chromatography (GC) at the Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, in two lots. Oil of leaves and inflorescence of diploid, C_0, and C_1 herbs were analyzed with the help of Perkin Elmer Model 3220B. Gas Chromatograph fitted with a TCD and 2m/3mm ss column packed with 10% FF AP on 80/100 Chromosorp WAW using a temperature programme from 100 to 200 ^{0}C with a rise of 4^0/mm. Injector/detector temperature 200 ^{0}C each. Data were processed on HR 3390A integrator. H_2 was used as a carrier gas. The analyses of the second lot (C_2) were done with Varion GC model CX- 3400 using a capillary column of dimensions 30m x 0.2 mm, temperature programmed from 50 ^{0}C to 220 ^{0}C at the rate of 6% with initial time hold of 2 mins. Injector and detector temperatures of 200 ^{0}C and 225 ^{0}C were used. H_2 was used as a carrier gas at 1ml/min with a split ratio of 1:50. Data processed on Varion 4400 integrator. Identification of compounds is based on running time (RT) of standard compounds. On the basis of phytochemical investigation, the plant under study proved a new chemotype-Eugenol>Linalool>Methyl Cinnamate and presented in (Table-1) along with other chemotypes.

Table 1: Varieties of *O. basilicum* and their chemotypes

S. No.	Varieties	Chemotypes
1.	*O. basilicum* L. var. *basilicum*	
2.	*O. basilicum* var. *crispa*	Methyl chavicol – Linalool type
3.	*O. basilicum* var. Dark opal (var/cultivar)	
4.	*O. basilicum* var. *difforme* Benth.	
5.	*O. basilicum* var. *glabratum* Benth.	Methyl chavicol type and Camphor type
6.	*O. basilicum* var. *majus* Benth.	
7.	*O. basilicum* var *minimum* Danert.	Eugenol type
8.	*O. basilicum* var. *pilosum* Benth.	Geranyl acetate type
9.	*O. basilicum* var. *purpurascens* Benth.	Methyl cinnamate type, Linalool type
10.	*O. basilicum* var. *thyrsiflorus* Benth.	Methyl cinnamate type
11.	*O. basilicum* var. *purpurascens* Benth.	Eugenol>Linalool>Methyl cinnamate type (Understudy)

The actual percentage of each chemical compound was calculated with the help of formulae:

1. Actual area = Total counts/Total area - Acetone counts (used as solvent).
2. Percentage of compounds = Area of the peak of individual compound/Actual area.

Observation and Discussions: Oils from leaves and inflorescences of the 'elite plants' understudy and their colchiploids were extracted separately and analyzed with the help of Gas Chromatography (GC). The total number of compounds, the name of the chemical compounds and their percentages, the total percentage of identified compounds in the leaves and inflorescences of the diploid 'elite plant' under study and their colchiploids are shown in a comparative Table-2.

Table 2: Comparative phytochemical constituents in diploid (2n=4x=48) and polyploids (2n=8x=96)

S.N.	Name of the chemical compound	Leaves %				Inflorescences %				+/- (comparison between diploid & C_2	
1		Diploid	C_0	C_1	C_2	Diploid	C_0	C_1	C_2	Leaves	Infl.
1	α-pinene	0.28	solvent	1.09	0.21	0.23	0.21	0.06	0.06	-	-
2	Camphene	0.20	---	---	0.07	0.10	0.16	0.16	0.05	-	-
3	β-Pinene	0.06	0.82	---	---	0.08	0.41	0.14	0.04	-	-
4	Myrcene	0.10	2.19	---	0.26	0.06	0.12	0.02	0.03	+	-
5	1,8-cineole	3.15	3.63	---	0.11	1.90	1.02	1.89	0.36	-	-
6	Limonene	1.42	3.67	2.42	0.61	0.75	1.89	2.05	---	-	+
7	p-cymene	0.62	0.06	0.02	0.05	0.18	0.07	0.04	0.12	-	-
8	Terpinolene	0.89	0.24	0.30	0.09	0.34	0.07	0.04	---	-	-
9	Linalool	27.88	16.29	16.03	21.29	29.35	24.38	29.26	36.85	-	+
10	Linalyl acetate	0.86	0.16	0.37	0.11	1.99	1.42	2.19	0.11	-	-
11	β-Caryophyllene	1.30	1.18	1.07	0.24	0.70	1.59	1.59	1.86	-	+
12	Terpineole	1.07	0.91	0.71	0.95	2.70	1.68	2.39	1.93	-	-
13	Geranyl acetate	2.05	0.16	0.16	0.89	0.26	1.30	0.33	0.08	-	-
14	Geraniol	3.82	1.93	1.02	0.49	3.76	2.17	3.29	3.56	-	-
15	Methyl cinnamate	9.61	9.61	8.71	10.11	2.87	3.26	2.58	2.37	+	-
16	Eugenol	42.67	56.61	62.28	44.60	48.22	46.24	48.31	43.19	+	-
17	Methyl chavicol (Estragole)	---	---	---	---	---	---	---	1.65	-	+
Total % of identified compounds		95.98	97.46	94.18	80.08	94.30	85.99	94.34	92.26		
No. of compounds present/Compounds identified		25/16	19/14	21/12	57/15	27/16	26/16	28/16	48/15		

Table 3: Classification of terpenoid compounds found in Diploid and Colchiploids of *O. basilicum* var. *purpurasens.* Terpenoid

Monoterpenoid		Sesquiterpenoid	
1. Acyclic Linalool Linalyl acetate Myreene Geraniol and Geranyl acetate 2. Cyclic A. Monocycle 1,8- Cineole α-Terpineole Terpenolene Limonene and p-Cymene B. Bicyclic α- Pinene β-Pinene and Camphene	*Diterpenoid	Bicyclic β-caryophyllene (Unsaturated cyclic hydrocarbon)	Triterpenoid*

*Not found in *O. basilicum* var. *purpurascens*

Table 4: Classification of Chemical compounds found in Diploid and Colchiploids of *O. basilicum* var. *purpurascens*

Chemical compounds			
Alkenes α-Pinene β- pinene Camphene Myreene Limonene p-Cymene Terpinolene and β-caryophyllene	Esters Linalyl acetate Geranyl acetate and Methyl cinnamate (Phenolic)	Alcohols 1,8 - Cineole Linalool Terpineole and Geraniol	Phenol Eugenol

Table 5: Classification of chemical compounds on the basis of presence and absence of oxygen in their structures found in Diploid and Colchiploids of *O. basilicum* var. *purpurascens*

Chemical Compounds	
Oxygenated compounds (Terpenoids) Linalool Geraniol α- Terpineole 1,8- Cineole Linalyl acetate Geranyl acetate Eugenol Methyl cinnamate and Methyl chavicol	Non- oxygenated Compounds (Terpenes) Myreene Limonene α-Pinene Camphene β-caryophyllene β-Pinene and Terpinolene

Classification of chemical compounds on the basis of presence and absence of oxygen in their structures found in Diploid and Colchiploids of *O. basilicum* var. *purpurascens.*

Gas Chromatographic analyses revealed the presence of the different compounds, including turbines, terpenoids and phenols in the oils of the leaves and inflorescence of the source elite plant and its three polyploid generations. Of the various compounds detected and mentioned in the comparative Table No.-02 with the help of GC in the source elite plant, 8 were alkenes (α -pinene, β- pinene, camphene, myrcene, limonene, p-cymene, terpinolene, β-caryophyllene), 4 were alcohols (1, 8- cineole, linalool, terpineole, geraniol), 3 esters (linalyl acetate, geranyl acetate, and methyl cinnamate, the latter also being a phenolic compound) and one phenol (eugenol). The above compounds (Table No. -04), if grouped on the basis of the presence and absence of oxygen in their structures, those containing oxygen called oxygenated compounds were linalool, geraniol, α-terpineole, 1, 8-cineole, linalyl acetate, geranyl acetate, eugenol and methyl cinnamate and those devoid of oxygen called non-oxygenated compounds were myrcene, limonene, α-pinene, β-pinene, camphene, β-caryophyllene and terpinolene (Table No.- 05).

The dominating compound present in the leaf of the parent plant was linalool (27.88%) and eugenol (42.67%), the former being an acyclic monoterpenoid and the latter a phenol (Table No.-03). Its inflorescence contained the same two compounds - linalool and eugenol- as the major compounds, but their contents were higher than those of the leaf. The percentage of the former was 29.35, while that of the latter was 48.22. In addition to the above methyl cinnamate was the only major compound (more than 5%) in the leaf of the elite source plant. Since in both the parts of the parent, the three were the principal compounds and eugenol was higher than linalool, the chemotype *of O. basılicum* var. p*urpurascens*, growing in this area, was named eugenol>linalool>methyl cinnamate type. Other compounds in the leaf and inflorescence were the minor ones whose percentage varied from 0.06 (β-pinene in leaf and myrcene in inflorescence) to 3.82 (geraniol in leaf). Altogether 25 and 27 peaks were obtained during GC analyses in leaf and inflorescence, respectively. But of these only 16 in both the plant parts could be identified, leaving 9 and 11 remaining compounds unidentified in them. Since the total percentage of the identified compounds in them were 95.98 and 94.30, the remaining unidentified ones constituted minor percentages of the oil, that is, 4.02 and 5.70, respectively, all attaining a status of minor compounds (Table-2).

Thoppil and Jose (1994) [3] reported linalool (41.6%), citronellal (20.2%) and thujone (5.1%) in *O. basilicum* var. p*urpurascens* Benth. collected from Cochin, India. On the other hand, Gupta (1996) [4] reported this variety to be a methyl cinnamate type. Ravid *et al.* (1997) [7], working on linalool contents and its chemistry of various varieties of *O. basilicum*, found its 0.3% only in O. basilicum var. *purpurascens*. This variety, growing in Israel, was a methyl chavicol type. In other variety, a hybrid of *O. basilicum* var. *basilicum* x *O. basilicum* var. *purpurascens* collected from Switzerland and grown in Israel, the authors reported 46.5% of linalool. This hybrid, however, was linalool and transmethyl cinnamate type. Working further on enantiomeric composition of linalool in the oils of the two herbs, they revealed the acyclic monoterpenoid to be (R) (-) and optically pure. Though the authors are silent on the appearance of linalool in the aforesaid hybrid, it appears quite possible that the gene responsible for linalool biosynthesis might have come from linalool rich variety, *O. basilicum* var. *basilicum.*

As far as the content of linalool in the leaf was concerned, it was decreased in the first colchiploids generation (C_0). It further decreased, though little, in C_1 plants, but an improvement (21.29%) was observed in the third generation (C_2) in comparison to C_0 (16.29%) and C_1 (16.03%). However, the percentage of the compound in C_2 was lesser than that observed for elite diploid source plants.

The inflorescence of the parent was a better source of linalool, constituting 29.35% of the total oil. Hence, the inflorescence

of this variety of *O. basilicum* may be suggested as its main source of the linalool. It may be recalled that inflorescence was taken for isolation of oil just before flowering. Like the leaf, its content fell in C_0 (24.38%), but exhibited a sign of remarkable recovery and improvement in C_1 (29.26%) achieving 36.85% in the C_2 herbs. Inflorescence of colchiploids may safely be suggested as the chief source of linalool.

On the other hand, eugenol contents in leaf and inflorescence of the parent were 42.67% and 48.22%, respectively. But, the leaf and inflorescence of its cholchiploids behaved differently as far as their content was concerned. In the leaf, there was a considerable leap (56.61%) in C_0 and C_1 (62.28%) plants which came down to 44.60% in the C_2. Though the quantity of eugenol in C_2 was lesser than those of C_1 and C_0, it was higher than the source plant by 2.53%. The inflorescence behaved differently from the leaf. The C_0 exhibited a decline (46. 24%) but C_1 showed an increasing trend by containing its 48.31% only to come down 43.19% in C_2. Hence, as far as, eugenol content was concerned, the only leaf of the colchiploid can be suggested to be the main source and not the inflorescence. However, the fluctuating and unstable behaviour of eugenol production in the leaf and inflorescence suggested that more cholchiploid generation should be chased in order to come to a final conclusion. Also, analyses of oil should be undertaken at various stages of growth of the stable polyploid to suggest either of the two parts as the main source of the two major compounds.

The third major compound, that is methyl cinnamate was considerably higher in quality in leaf, thus, the latter being the obvious principal source of the compound. The inflorescence of the source plant possessed only 2.87% of methyl cinnamate. Colchiploid generations of the plant showed leaf and inflorescence behaving differently. The leaf of C_0 has a similar quantity (9.61%), but its content decreased to 8.71% in C_1, finally attaining a percentage of 10.11 in C_2. Polyploid generations, on the other hand, exhibited a gradual decrease, except C_0 which exhibited a quantity of 3.26%. A comparative Table of major phytochemical constituents found in the diploid source plant and their colchiploids of *O. basilicum* var. *purpurascens* are also given in Table-2.

Sobti (1976) [2] analyzed major components of essential oil of its growth and observed linalool, methyl cinnamate and methyl chavicol, the latter either present in minor quantities before flowering (1.94%) and during flowering (2.10%) or not at all detected after flowering. It was, therefore, clear that the present experimental plant, *O. basilicum* var. *purpurascens* Benth. was a new chemotype- Eugenol>Linalool>Methyl cinnamate type.

Like the various species of *Ocimum*, *O. basilicum* in general, and the variety *O. basilicum* var. *purpurascens* in particular, had a preference for simple terpenes, that is, monoterpenes (Table No. 03). Among the latter, leaf and inflorescence of the variety understudy preferred acyclic and monocyclic monoterpenes more than bicyclic ones. The source elite plant contained 5 acyclic (linalool, linalyl acetate, myrcene, geraniol, and geranyl acetate), 5 monocyclic (1, 8-cineole, α-terpineole, terpinolene, limonene, and p- cymene) and only 3 bicyclic monoterpenes (α-pinene, β-pinene, and camphene). Besides these, β-caryophyllene, a sesquiterpene was also found in the source plant.

In addition to the above 14 terpenes, both leaf and inflorescence of the parent possessed a phenol, eugenol and an ester methyl cinnamate.

As far as C_0 was concerned, its leaf contained all the terpenes except bicyclic camphene. In the C_1 herbs, altogether 4 terpenes - one acyclic (myrcene), one monocyclic (1, 8-cineole) and two bicyclic (β-pinene and camphene) were eliminated, while from the C_2 plants only β-pinene (a bicyclic monoterpene) was absent. On the other hand, the inflorescence of the first (C_0) and the second (C_1) polyploid generations inherited all the 14 terpenes from the parent. But, the C_2 colchiploids showed the absence of two monocyclic terpenes, namely, terpinolene and 1, 8 - cineole.

On the basis of above, it appeared obvious that the source plant and its colchiploids had a preference for the monoterpenes, especially those of simple chemical structure, that is, cyclic and noncyclic once. It was also evident that if some terpenes were eliminated in the colchiploids, those were cyclic ones and more preferably bicyclic ones. In the leaves of C_0, C_1, and C_2, one, three and again one cyclic monoterpene were found to be absent. Myrcene was the single acyclic monoterpene to have been eliminated in the leaf of C_1 herbs. Similar studies were made in the inflorescence of the colchiploids as well. They retained all the five acyclic monoterpenes (with a simple chemical structure). It was also interesting to observe that elimination of the above compounds in the inflorescence of the colchiploids was at a minimum. Inflorescence of C_2 only showed the absence of terpenes of monocyclic nature. The elimination of a higher number of monoterpenes in the leaves and retention of many of them in the inflorescence of the colchiploids suggested that with the advancing growth processes from vegetative to reproductive one, the plant developed the capability of retention. It may also be suggested that the absence and the presence of a compound in a colchiploid depended also on the physiology of the parts along with the genetic make-up of the plant.

As far as, the phenols were concerned, eugenol was present in the leaves and inflorescences of all the colchiploids. On the other hand, methyl chavicol or estragole, another phenol, was absent from the source plant and from all the colchiploids, except inflorescence of the C_2 generation. Its sudden presence in these plants was an interesting finding.

Conclusions

On the basis of the above observation and discussion, the variety under study was a new chemotype and was designated as a eugenol>linalool >methyl cinnamate type as the 3 compounds were the major component in the diploid source Plant. Inflorescence of C_2 herbs was a better source of linalool than leaf - the two parts containing 36.85% and 21.29%, respectively. Eugenol was augmented in the leaf of the induced autopolyploids going to the level of 62.28% in C_1 herbs from 42.67% of a diploid parent. Leaf of C_2 plants proved to be the best source of methyl cinnamate (10.11%). The source plants and its colchiploids of the chemotype under investigation, had a preference for monoterpenes, especially acyclic and cyclic ones, all of which were chemically simple structures.

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