

Seed protein and esterase isozyme profile among the accession of lemongrass [*Cymbopogon flexuosus* (Nees ex Steud.) Wats] collected from environmentally different sites

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Summary

Seed protein profile and esterase isoenzyme were studied in ten accessions of lemongrass [*Cymbopogon flexuosus*] collected from northeastern India belonging to two major chemotypes – citral and geraniol rich essential oil. Total four esterase isozyme bands were ob-

served with R_m values in the range of 0.394 to 0.798. Among them one with R_m value 0.747 was unique as it was consistently present in all the accessions irrespective of citral or geraniol rich chemotype. For seed protein, SDS-PAGE analysis of seven accessions (RLJ-TC-1, RLJ-TC-4, RLJ-TC-5, RLJ-TC-8, RLJ-TC-9, RLJ-TC-10 and OD-19) revealed a total of 13 bands among the accessions ranging in the size from 21.5 to 92.0 kd. Six citral rich accessions exhibits very similar seed protein profile with 10 to 11 protein band each. However, the geraniol rich chemo-type RLJ-TC-8 exhibit different profile with only six high molecular proteins. Four seed proteins with molecular weight 92.0, 86.0, 80.4 and 61.6 kd were consistently found in all the chemotypes irrespective of citral or geraniol rich and can be considered as marker for the species. Although esterase isozymes exhibited low polymorphism, yet close similarity of isozyme and seed protein profile can be considered as evidence of genetic homogeneity among the accession of lemongrass.

Key words: chemo-type, *Cymbopogon flexuosus*, esterase isozyme, lemongrass, SDS-PAGE

INTRODUCTION

Lemongrass [*Cymbopogon flexuosus* (Nees ex Steud.) Wats], is a perennial industrial cash crop that grows well and flourish in diverse soils in tropical and sub-tropical agro-climatic condition. The fresh herb of lemongrass upon hydro-distillation yield essential oil, referred to lemongrass oil which is of great value and widely used in the pharmaceutical, perfumery and cosmetic industries and has assured and increasing demand in national and international market. Commercially, lemongrass oil is regularly fractionated to two major constituent chemicals, namely neral and geraniol, which are extensively used for different purposes. The lemongrass oil-yielding grass species (family *Poaceae*) belong to the genus *Cymbopogon* consisting of 140 species that are known and distributed widely in semi-temperate to tropical parts of the world. These species are either indigenous or introduced. Twenty species of *Cymbopogon* are reported to occur in India [1]. The crop is propagated by sub-terranean hardy stem portion referred to as 'slips' although seed propagation of many cultivars is possible. Because of vegetative propagation, the maintenance of genetic homogeneity is assured but due to its out crossing nature it is suggested that genetic heterogeneity prevails among the naturally occurring population although there is no evidence of that effect [2]. Genetics of lemongrass is poorly understood which constraints development of superior genotypes. This in turn has resulted in decline in lemongrass oil production. In 1963–64 India's production was 1700 tons/year which dropped to only 200 tons/year in 2001 [3]. Genetic studies in lemongrass is problematic because most cultivars are demarcated on the basis of herb yield, oil yield and oil quality which are all of quantitative character and highly influenced with many environmental factors particularly seasonal changes [4-6]. Therefore, stability of these traits is difficult while segregation of population following crossing. Moreover, most cultivars are morphologically indistinguishable. Like its related species lemongrass has some variants which differ with respect to

chemical composition and quality of oil. Such variants are referred to chemo-types. Differentiation among the chemo-types is far more problematic since it can be distinguished only with use of sophisticated chemical analysis. Therefore, the study of genetic homogeneity through conventional breeding is an uphill task. Lemon-grass, therefore, presents a case where molecular analysis is effective in studying the genetic homogeneity or heterogeneity. The study of seed protein profile has been established as a reliable and powerful tool for identification of cultivars and genotypes and to work out the degree of similarities and dissimilarities between them [7-9]. Isozyme analysis also provides useful evidence for this purpose [10, 11]. International Rules for Seed Testing (ISTA) have adopted standard SDS-PAGE and IEF (Iso Electric Focusing) methods into international rules for cultivar identification [12]. We have carried out a comparative analysis of the morphological characters, chemical traits and SDS-PAGE analysis of seed protein and esterase isozyme to assess the diversity and relationships among the ten *Cymbopogon flexuosus* accessions.

MATERIALS AND METHODS

Climate conditions

The north eastern region of India has distinct climate variations. High variability of the topography results in climatic variations in short distances. In general, the daily temperature in the plains of Brahmaputra and the Barak Valley as well as in Tripura and in the western portion of Mizo Hills is about 15°C in January, whereas in other parts of the region, the temperature is between 10°C and 15°C. From April it rises and in July except the south-eastern portion of Mizo hills and Shillong, the mean temperature ranges from 25°C to 37.5°C. In October, daily mean temperature in the hilly areas ranges between 20°C and 25°C, whereas in Brahmaputra and Barak Valley, Tripura and the western portion of the Mizo hills it is above 25°C. The lowest temperature is experienced in the upper Himalayas of Arunachal Pradesh which is below freezing point.

Plant material and accessions

The plant material used in this study consisted of ten accessions of *C. flexuosus* namely RLJ-TC-1, RLJ-TC-2, RLJ-TC-3, RLJ-TC-4, RLJ-TC-5, RLJ-TC-6, RLJ-TC-7, RLJ-TC-8, RLJ-TC-9 and RLJ-TC-10 collected from natural habitat of northeastern states of India and was maintained at the experimental field of NEIST, Jorhat. One cultivar OD-19 collected from Kerala and subsequently naturalized in the agro-climatic conditions of Assam was taken as reference. The plant accessions used in this work are listed (tab. 1) with detail of their source, origin and salient morphological characters. Plants were grown in plots of 7.5 m x 3.5 m size in randomized blocks with three replications with the use of standard methods. The phenotypic

information of the accessions is also recorded. The cultivars were assessed for the growth, herbage yield, oil content and quality. Seeds were taken for seed protein profile study and leaves were taken for esterase isoenzyme analysis.

Oil extraction and GLC analysis

Fresh leaves were processed for oil analysis from all field-grown accession and extracted by hydro-distillation, using Clevenger's apparatus. The GLC analysis was performed using a Varion model 3700 gas chromatogram with Flame Ionization Detector (FID). The volatile constituents were separated in column of 2 mm i.d. (internal diameter) x 2 m length, filled with 15% SE 52 on gas chrom Q 80/100 mesh. The column temperature was programmed from 100°C to 210°C at the rate of 3°C/min. after initial loading for 10 min. The major identified compounds and their percentage are presented in table 2.

Electrophoresis of seed protein by SDS-PAGE

For seed protein profile only seven accessions: RLJ-TC-1, RLJ-TC-4, RLJ-TC-5, RLJ-TC-8, RLJ-TC-9, RLJ-TC-10, and OD-19 were used as the others did not produce viable seeds with endosperm. Dehusked seeds in the quantity of 100 mg were grounded in a pre-chilled mortar with 0.2 m Tris (pH 6.5), homogenized and then centrifuged at 10,000 rpm at 40°C and the supernatant was collected and used as seed protein source. Prior to electrophoresis the protein extract was treated with sample buffer containing 10% SDS, 5mM β mercaptoethanol, 3% glycerol and 0.5% bromophenol blue. SDS-PAGE was carried out with 14% separating gel and 4% staking gel. Protein molecular weight marker (PMW-m, Bangalore genei) was co-electrophoresis which allowed to deduce the molecular weight of the individual protein bands for each accession. From the protein profile similarity indices were generated using dendrogram by UPGMA using the software NTSYS pc.V.2.02j.

Esterase isozyme analysis

For esterase isozyme analyses 300 mg of freshly collected 2nd leaf each accession of *C. flexuosus* were grinded in a pre-chilled mortar with 2 ml ice cold extraction buffer (100 mM Phosphate buffer, pH 7.5). The sample was homogenized and centrifuged at 10 000 \times g at 4°C for 15 min. to obtain the supernatant which was used as an enzyme source. Sample was prepared for electrophoresis by mixing 35 μ l extract with 10 μ l tracking dye (0.5% bromophenol blue with 40% sucrose). Native PAGE was done with 7.5% gel at 4°C and subsequently resolved by staining with Fast Blue RR salt and α -naphthyl acetate. The duly stained gels were image captured and analyzed using Diversity Database™ software (pdi, Inc, USA).

Table 1

Salient morphological characters of the accessions with their source of origin

Characters	Accessions													CD(p=0.05)
	RJ-J-TC-1	RJ-J-TC-2	RJ-J-TC-3	RJ-J-TC-4	RJ-J-TC-5	RJ-J-TC-6	RJ-J-TC-7	RJ-J-TC-8	RJ-J-TC-9	RJ-J-TC-10	OD-19			
Habit	erect, straight	erect, straight	pendent	semi pendent	erect, straight	erect, straight	erect, straight	pendant	erect, straight	erect, straight	erect, straight	erect, straight	erect, straight	
Height [cm]	159.3	149.2	116.5	135.2	140.1	167.6	167.6	189.6	178.9	142.3	134.8	134.8	6.99	
	285.8	230.4	221.3	235.7	280.9	258.5	258.5	289.8	280	238.4	240.6	240.6	11.84	
culm	Light brown	Dark red	Reddish purple	Light yellow	Reddish yellow	Reddish brown	Reddish brown	Reddish brown	Reddish brown	Pale yellow	Dark brown	Dark brown		
leaf sheath	Dark brown	Light purple	Reddish brown	Light purple	Light brown	Light green	Light green	Reddish brown	Light brown	Dark brown	Reddish brown	Reddish brown		
Suppress internodes	10-12	23-25	28-31	09-11	11-13	32-35	32-35	36-38	16-18	12-14	15-18	15-18		
R.L	0.18	0.31	0.26	0.2	0.21	0.44	0.44	20	0.24	0.19	0.2	0.2		
ligulae	0.35	0.38	0.58	0.29	0.33	0.47	0.47	0.58	0.3	0.3	0.18	0.18	0.04	
Auricle	0.4	0.27	0.48	0.33	0.32	0.28	0.28	0.24	0.28	0.15	--	--	0.2	
Awn	0.6	0.15	0.15	0.075	0.65	0.15	0.15	0.25	0.7	0.65	0.65	0.65		
L.A (cm ²) avg. of 6 leave	852.7	647.97	976.04	703.01	727.14	243.43	917.75	1111.11	1105.7	771.38	767	767	16.01	
Tiller	No./ bush	49.15	69	52.8	65.6	39.6	39.6	77.9	59.2	69.1	57	57	7.54	
No. of leaves/clump	650.1	1179.6	2070	528	787.2	2047	1306.8	2882.3	1006.4	967.4	969	969	81.977	
Herbage, t/ha	56.48	58.75	70.56	50.79	54.91	30.44	62.31	80.81	60.88	57.33	56.76	56.76	1.722	
Oil %, w/v	0.64	0.52	1.06	0.7	0.56	1.05	0.69	1.17	0.56	0.75	0.65	0.65		
Origin	Bomdilla AP	Barapani ML	Barapani ML	BTAD, Ass	Yaogyimsen NL	Lumla AP	BTAD, Ass	BTAD ASS	Lumla AP	Lumla AP	Ooddakali KR	Ooddakali KR		

* for length with inflorescence. R. L for relative length, AP for Arunachal Pradesh, ML for Meghalaya, Ass for Assam, NL for Nagaland, KR for Kerala

Table 2
Major chemical constituents of oil of lemongrass accessions in different seasons (citral rich)

Accessions	Seasons	Major chemical constituent, %										TIC (%)
		dipentene	linalool	citronellol	citral-b (neral)	geranial	citral-a (geranial)	nerol	neryl acetate	total citral	avg. citral	
OD-19	S1	2.2	0.7	0.3	35.7	2.4	51.0	-	2.5	86.7	79.7	93.8
	S2	1.2	0.5	0.4	25.2	0.2	39.1	6.8	14.5	64.3	87.9	87.9
	S3	1.1	0.6	0.5	38.0	-	50.2	0.4	0.8	88.2	91.6	91.6
RLJ-TC-1	S1	0.4	0.7	0.4	32.3	0.6	53.5	0.8	3.9	85.8	87.0	97.6
	S2	0.2	0.3	0.2	29.5	-	55.7	1.8	5.8	85.2	87.0	92.5
	S3	0.5	0.5	0.4	37.5	-	52.5	0.3	0.3	90.0	92.0	92.0
RLJ-TC-4	S1	0.2	0.8	0.8	31.6	0.2	52.1	0.8	4.7	83.7	87.1	91.2
	S2	-	0.4	-	23.0	-	41.4	6.3	15.7	64.4	87.1	86.8
	S3	0.7	0.7	0.2	34.4	-	51.8	1.3	2.6	86.4	92.0	92.0
RLJ-TC-5	S1	0.1	0.6	0.2	36.5	0.5	56.3	-	1.3	92.8	81.0	95.5
	S2	0.2	0.7	0.6	27.9	-	38.9	6.8	13.2	66.8	81.0	88.3
	S3	0.5	0.8	0.3	35.6	-	47.8	2.5	4.3	83.4	87.1	91.8
RLJ-TC-9	S1	0.2	0.9	0.6	31.7	0.4	59.6	0.4	1.2	91.3	87.0	95.0
	S2	0.1	0.5	0.1	32.3	-	50.4	3.0	7.9	82.7	87.0	94.3
	S3	1.0	0.9	0.2	33.4	-	52.6	0.9	2.2	87.0	91.0	91.0
RLJ-TC-10	S1	0.7	0.8	0.2	36.1	-	52.2	1.8	3.4	88.3	87.1	95.2
	S2	-	0.7	0.2	28.0	-	50.0	4.8	10.0	78.0	87.1	96.7
	S3	0.4	0.5	0.1	34.6	0.2	60.0	0.1	0.8	95.0	87.1	97.1

T for trace Amount. S1 for April to May, S2 for July to August and S3 for November to December, TIC for Total Identified Compounds

Table 2
Major chemical constituents of oil of lemongrass accessions in different seasons (geraniol rich)

Accessions	Seasons	major chemical constituent, %										average citral	T.I.C. (%)
		dipentene	linalool	citronellol	citronellal	citral-b (neral)	geraniol	citral-a	citronellal	citral-b (neral)	gera acetate		
RLJ-TC-2	S1	4.2	0.6	0.2	0.5	6.8	43.5	10.1	1.7	1.9	16.9	69.5	
	S2	0.2	0.8	0.2	0.5	2.2	43.0	7.0	T	2.8	9.2	56.7	
	S3	2.1	0.5	0.2	1.0	5.0	34.8	10.6	1.0	2.3	16.1	57.5	
RLJ-TC-3	S1	1.5	0.8	6.4	11.1	3.8	48.8	11.9	4.1	8.2	15.7	96.6	
	S2	2.4	0.7	5.2	19.5	2.0	43.2	7.3	T	6.7	9.3	87.0	
	S3	2.1	0.5	12.4	16.0	3.8	36.6	9.2	7.5	7.6	13.0	95.6	
RLJ-TC-6	S1	1.8	0.9	8.9	4.6	4.4	45.4	17.4	0.5	2.9	21.8	86.8	
	S2	3.8	0.8	0.4	T	3.9	63.4	9.0	T	6.5	12.9	87.8	
	S3	1.5	0.9	0.4	0.1	3.4	67.7	8.9	0.5	9.5	12.3	92.9	
RLJ-TC-7	S1	0.3	0.9	0.1	0.5	9.9	53.9	17.1	1.6	2.5	27.0	86.8	
	S2	T	0.9	0.2	T	5.6	55.5	13.2	T	4.9	18.8	80.3	
	S3	0.2	0.6	0.1	T	7.9	60.3	15.9	0.1	1.8	23.8	86.9	
RLJ-TC-8	S1	1.6	0.8	3.3	3.7	5.3	62.7	9.2	1.5	7.7	14.5	95.8	
	S2	1.6	0.8	1.8	2.0	4.4	58.9	10.8	1.6	11.9	15.2	93.6	
	S3	1.8	0.8	1.8	4.0	4.8	59.2	9.0	1.9	12.0	13.8	95.3	

'T' for trace Amount. S1 for April to May, S2 for July to August and S3 for November to December, TIC for Total Identified Compounds

RESULTS

Seed protein profile of *C. flexuosus* accessions

Seed protein exhibited considerable polymorphism which is helpful in assessing the similarities or dissimilarities among the accessions. It has been observed that the citral rich accessions have close affinities with respect to seed protein profile. In fact RLJ-TC-1, RLJ-TC-4, OD-19 and RLJ-TC-10 have identical profile with 10 protein bands. The other two citral rich chemo-types (RLJ-TC-5 and RLJ-TC-9) were characterized by the similar 10 protein bands but had one additional band of 91.2 kd each and thus marginally differ (fig. 1). The geraniol rich accession RLJ-TC-8 was distinguished from the citral rich accessions in following points (a) it had only six protein bands, (b) absence of low molecular weight proteins ranging from 51.8 kd to 21.5 kd. (c) Presence of two unique bands of 62.2 kd and 56.5 kd which are absent in citral rich chemo-types. However, four high molecular weight protein viz. 92.0, 86.0, 80.4 and 61.6 kd were found to be common to all irrespective of citral or geraniol chemo-types and hence can be considered as a marker for the species. The similarity matrix of protein band (tab. 3) and UPGMA based dendrogram showing three clusters indicate that genetically the accessions are closely similar, particular the citral rich accessions. Dendrogram analysis (fig. 2) revealed three clusters. However, all the four accession in first cluster are identical. In cluster II, both the two accessions are identical so far as seed protein profile is concerned. Moreover, cluster I and cluster II differ only marginally. However, the accession RLJ-TC-8 significantly differs from others and form a separate cluster. Overall, except one (RLJ-TC-8), all others are remarkably similarly indicate genetic homogeneity among them.

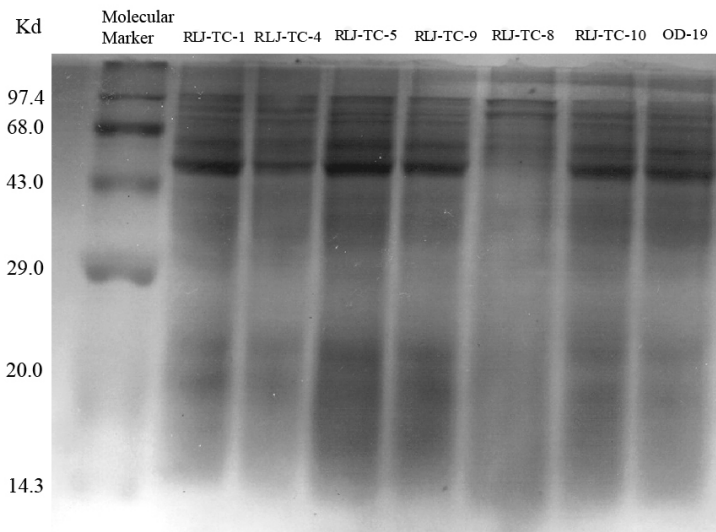


Figure 1.
Photograph of seed protein banding pattern for seven accessions of lemongrass

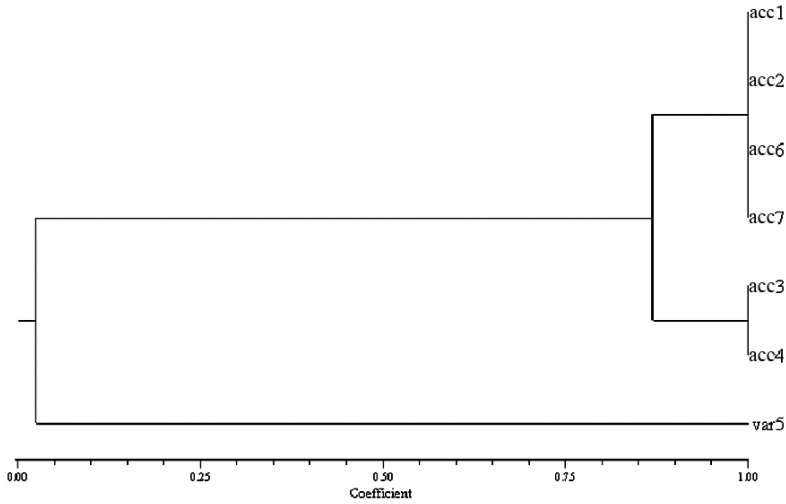


Figure 2. Dendrogram showing the relationship between the 7 strains of lemongrass based on similarity matrix

Table 3
Frequency distribution of seed proteins among the lemongrass accessions with standard cultivar OD-19

Sl. No.	protein band (mol.wt. in kd)	Var-1	Var-II	Var-III	Var-IV	Var-V	Var-VI*	OD-19
1	92.0	P	P	P	P	P	P	P
2	91.2	--	--	P	P	--	--	--
3	86.0	P	P	P	P	P	P	P
4	80.4	P	P	P	P	P	P	P
5	62.2	--	--	--	--	--	P	--
6	61.6	P	P	P	P	P	P	P
7	56.5	--	--	--	--	--	P	--
8	51.8	P	P	P	P	P	--	P
9	41.6	P	P	P	P	P	--	P
10	34.3	P	P	P	P	P	--	P
11	31.5	P	P	P	P	P	--	P
12	22.4	P	P	P	P	P	--	P
13	21.5	P	P	P	P	P	--	P
total bands for individual accessions		10	10	11	11	10	6	10

(Var-I= RLJ-TC-1, Var-II= RLJ-TC-4, Var-III= RLJ-TC-5, Var-IV= RLJ-TC-9, Var-V= RLJ-TC-10, Var-VI= RLJ-TC-8, OD-19).

* RLJ-TC-8 (var VI) which is a different chemo-type being geraniol rich has been found to be different from the rest as revealed by protein profile

Isozyme profiling in *C. flexuosus* accession

All the accessions were characterized by a band of Rm 0.747 showing early evidences of genetic homogeneity (fig. 3). However, two cultivars RLJ-TC-5 and RLJ-TC-9 contain citral exhibiting additional band of Rm 0.798. Similarly RLJ-TC-6 had an additional band of Rm 0.428 and RLJ-TC-2 had an additional band of Rm 0.394 and equally they were geraniol rich. The isozymic profile screened in the present study showing low value of appreciable polymorphism and this will indicate early evidences of genetic homogeneity among the cultivars.

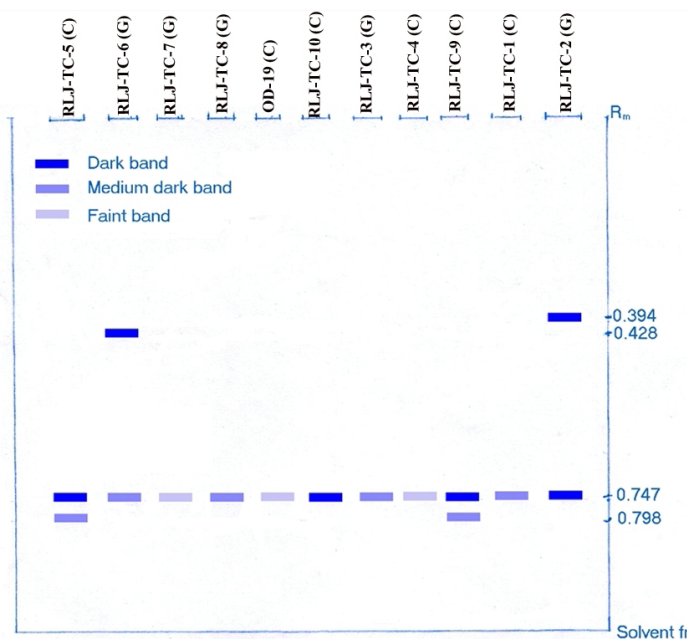


Figure 3. Esterase enzymogram showing the banding pattern of lemongrass accessions. The values of the Rm of different bands are depicted in the right. C – citral rich chemotype, G – geraniol rich chemotype

DISCUSSION

Ascertaining genetic homogeneity in lemongrass by conventional genetic study through crossing is difficult and unreliable due to of wide variability resulting from environmental factors. In this context molecular technique offer effective and highly reliable alternative since the information generated is not influenced by environmental factors, plant age and geographical conditions [7, 13]. A number

of studies have demonstrated that the degree of polymorphism in seed protein profile reflect the degree of genetic diversity as well as inter- and intraspecific relationship [14-16]. In present study, except one, all strains exhibited nearly identical protein profile, indicating lack of diversity at molecular level. This implies genetic homogeneity among the strains. Several workers have shown that different isozyme profile and the degree of its polymorphism can be used to study genetic diversity [12, 17, 18]. In commercial plantation and orchards lemongrasses is propagated asexually or through micropropagation. Hence, such populations are unlikely to be heterozygous. However, among the naturally occurring population, natural insect pollination is possible causing out breeding which generated opinion that such populations are heterozygous. Contrary to that, in present study findings from esterase isozyme complement each other and provide early evidences that high degree of genetic homogeneity prevailed among the accession of lemongrass. Further study is needed in this area to ascertain the exact molecular affinities and to trace out the detail phylogenetic relationships.

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PROFIL BIAŁEK I IZOZYMÓW ESTERAZY W NASIONACH PALCZATKI POGIĘTEJ [*CYMBOPOGON FLEXUOSUS* (NEXX EX STEUD.) WATS] ZEBRANEJ W RÓŻNYCH WARUNKACH ŚRODOWISKOWYCH

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Streszczenie

Badano profil białkowy nasion i izoenzymów esterazy w próbkach palczatki pogiętej [*Cymbopogon flexuosus* (Nexx ex Steud.) Wats] pochodzących z dziesięciu stanowisk, zebranych w północno-wschodniej części Indii, należących do dwóch głównych chemotypów ze względu na zawartości olejków eterycznych: typu cytralowego i geraniolowego. Współczynnik ruchliwości elektroforetycznej R_m badanych prób wahał się w granicach 0,394–0,798. Spośród nich współczynnik R_m o wartości 0,747 wyróżniał się wśród pozostałych i występował na wszystkich analizowanych stanowiskach niezależnie od charakteru chemotypu. Rozdział elektroforetyczny SDS-PAGE profilu białkowego nasion roślin pochodzących z siedmiu stanowisk (RLJ-TC-1, RLJ-TC-4, RLJ-TC-5, RLJ-TC-8, RLJ-TC-9, RLJ-TC-10 i OD+19) charakteryzował specyficzny układ 13 prążków w zakresie wielkości od 21,5 do 92 kD. U roślin należących do chemotypu cytralowego (6 osobników) stwierdzono podobny układ 10–11 prążków. Jednakże w chemotypie bogatym w geraniol RLJ-TC-8 stwierdzono odmienny profil odpowiadający sześciu wysokocząsteczkowym białkom. W przypadku wszystkich badanych chemotypów obserwowano powtarzający się układ 4 prążków dla białek o wielkościach odpowiednio: 92,0, 86,0, 80,4, 61,6 kd, co pozwala uznać go za marker charakterystyczny dla badanych roślin. Pomimo występowania niskiego stopnia polimorfizmu izoenzymów esterazy, przy jednoczesnej wysokiej zgodności pomiędzy wynikami rozdziału elektroforetycznego izoenzymów i profilu białkowego nasion, można wnioskować o genetycznej homogenności badanych stanowisk palczatki pogiętej.

Słowa kluczowe: chemotyp, *Cymbopogon flexuosus*, esteraza, palczatka, SDS-PAGE