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Intra-specific differentiation, genetic variability and their prospect for exploitation in medicinally important plant Black henbane (*Hyoscyamus niger* L.)

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ABSTRACT

Black henbane (*Hyoscyamus niger* L.) is frequently cultivated as a rich source of tropane alkaloids particularly hyoscyamine and hyoscine (Scopolamine) which are extracted from its whole aerial herb including dried leaves and flowering tops. These valuable natural compounds are widely used in modern medicinal preparations as well as pharmaceutical industries in India and around the world. In the present study, genetic divergence among twenty-nine accessions of *Hyoscyamus niger* L. was evaluated for the nature along with amount of genetic diversity for eleven economic traits. Accordingly, all the twenty-nine accessions were grouped into eight diverse clusters (cluster I-VIII). The enormous diversity was also indicated by the wide range of D^2-values (4.032 to 544.535). A number of unique accessions HN-30, HN-31, HN-15, HN-22-Y, and HN-29-Y were identified that may be interesting genotypes for the genetic improvement of the different morphometric and tropane alkaloid traits. Therefore, these accessions can be further exploited in future hybridization programs for the development of a new variety/cultivar for commercial productions.

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1. Introduction

Black henbane (*H. niger* L.) having diploid chromosome number (2n=34 belongs to the family 'Solanaceae' (Srivastava and Lavania, 1987). *H. niger* L. is usually an annual/biennial plant (Schläppi, 2011), having purple to yellow colored flowers with dark purple veins, alternate toothed leaves and a branched root, with poisonous effects having characteristic foul odor (Whitson et al., 2000). There are about eleven species of the genus *Hyoscyamus* known, namely *H. niger* L., *H. muticus* L., *H. albus* L., *H. aureus* L., *H. desertorum* L., *H. flaccidus* L., *H. pussilus* L., *H. kurdicus* Bornm., *H. squarrosus* Griff., *H. reticularis* L. and *H. arahnoideus* Pojark. (Yang et al., 2002). These are distributed from the Canary Islands over Europe and Northern Africa to Asia, Pakistan and in hilly areas of Jammu and Kashmir in India (Panda, 2002; Sreeramu, 2004). The diversity of ethnobotanical, biological, phytochemical and pharmacological properties of a wide spectrum of medicinal plants all over the world determines their increasing use in different fields of pharmacy, biotechnology, traditional and folk medicines (Ganesan and Xu, 2017; Mohammadhosseini, 2017; Mohammadhosseini et al., 2017; Nunes and Miguel, 2017). *H. niger* L. is highly valued for tropane alkaloids which are mainly present in leaves, flowering tops and roots of the plant (Anonymous, 1959; Camilo et al., 2017; Sobha et al., 2018; Venditti et al., 2018). The alkaloid content in leaves increases with maturity and reaches the maximum at the time of flowering stage after which it decreases. Unripe fruits contain more alkaloids than ripe ones. The principal tropane alkaloids present in the various parts of *H. niger* L. are hyoscyamine ($C_{17}H_{23}O_3N$) and hyoscine or scopolamine ($C_{17}H_{21}O_4N$) in smaller quantity and trace amounts of tropine and scopoline.

Hyoscyamine and hyoscine are widely used for their psychoactive properties (including visual hallucinations and a sensation of flight), mydriatic, antispasmodic, anti-cholinergic, analgesic and sedative

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Table 1
Geographical distribution of twenty nine genetic stocks/ their codes and origin of *Hyoscyamus niger*.

Sr. no.	Accession code	Origin/Places of collections
1.	HN -1	CSIR-CIMAP, Lucknow, U.P. (India)
2.	HN -2	Jammu, Jammu & Kashmir, (India)
3.	HN -3	Kashmir, Jammu & Kashmir, (India)
4.	HN -4	Local, Lucknow, U.P. (India)
5.	HN -5	CSIR-CIMAP, Lucknow, U.P. (India)
6.	HN -6	Chamoli, Uttarakhand, (India)
7.	HN -7	Bageshwar, Uttarakhand, (India)
8.	HN -8	Lucknow, U.P., (India)
9.	HN -9	Lucknow, U.P., (India)
10.	HN -10	CSIR-CIMAP, Lucknow, U.P., (India)
11.	HN -11	CSIR-CIMAP, Lucknow, U.P., (India)
12.	HN -12	CSIR-CIMAP, Lucknow, U.P., (India)
13.	HN -13	Kosesse, sitovalvia republic
14.	HN -14	Katra, Jammu & Kashmir, (India)
15.	HN -15	CIMAP, Lucknow, U.P., (India)
16.	HN -16	Palampur, Himachal Pradesh, (India)
17.	HN -17	U.S.A.
18.	HN -18	Pantnagar, Uttarakhand, (India)
19.	HN -19	Austria
20.	HN -20	Austria
21.	HN -21	Yugoslavia
22.	HN -22	Poland
23.	HN -22-Y	Poland
24.	HN -28	Germany
25.	HN -28-A	CSIR-CIMAP, Lucknow, (India)
26.	HN -29	Poland
27.	HN -29-Y	Germany
28.	HN -30	CSIR-CIMAP Lucknow, (India)
29.	HN -31	CSIR-CIMAP Lucknow, (India)

properties (Supria et al., 2004). According to Begum et al. (2010), lignans, courmarinolignans, withanolides, lignanamides, glycerides, saponins, flavonoids and their glucosides are found in the seeds of *H. niger* L. used as an anti-inflammatory drug. Black henbane in different formulations has been recommended by herbalists for reducing painful swellings, treating gout, headaches, aching joints and in particular in the treatment of toothache. In more recent times, it has been a part of treatment for ulcers, various respiratory diseases including a whooping cough and asthma, insomnia and rheumatism. It has sometimes been used as an alternative to opium poppy (*Papaver somniferum*). To see above medicinal importance and high demand of the tropane alkaloids at national and international levels, distinctive high crude tropane alkaloid containing genotypes are needed (Lal et al., 2000; Lal et al., 2005). Recently, various methods have been applied in order to increase tropane alkaloids like the use of iron oxide nano-particles as abiotic elicitor in hairy root culture for enhancement of tropane alkaloids (Moharrami et al., 2017) and application of 3 to 5% nitrogen nano-chelate fertilizers (Banani et al., 2017). Since, inheritance of tropane alkaloids and biomass in *H. niger* L. is controlled through the genes (Sharma et al., 1992; Sharma et al., 2001), the objective of our present study is to evolve or identify genotypes accumulating high amount of crude alkaloids, including hyoscyamine and hyoscine in the herbage of *H. niger* L. accessions.

2. Experimental

2.1. Plant material and edapho-climatic conditions of experimental location

More than hundred accessions were collected from different places of India and abroad. Removing duplicates, twenty-nine accessions of *H. niger* L. included in this study (see Table 1). The accessions were grown in the randomized block design (RBD) with three replications during the first week of November and harvested at the end of April. All the twenty-nine accessions were evaluated for the two consecutive years 2013-2014 and 2014-2015 at the research farm of the CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow, 226015, U.P. (India). Each treatment consisted of single row 5.00 m long and 1.50 m apart (The plant to plant distance was maintained at 60 cm). The experimental location at the institute research farm was located at 26.50 N latitude and 80.500 E longitude, and 120 m above the mean sea level. All the treatments in the experiment were received normal intercultural operations and irrigations. Since the nutrient requirement of the crop is high, 100 kg of N_2O, 85 kg of P2O5 and 80 kg of K_2O h^{-1} were applied during field preparation as basal and top dressing.

2.2. Data collection

In order to know the range of genetic divergence, the morphometric and alkaloid content data were recorded on twenty nine accessions of *Hyoscyamus niger* L., for the eleven economic important traits, namely days to 50% flowering, plant height (cm) at full maturity, number of primary branches/plant, inflorescence length (cm), days to maturity, fresh herb yield (g/plant), dry herb yield (g/plant), seed yield (g/plot), total crude tropane alkaloid content (%), hyoscyamine (%) and hyoscine (%) for two years.

2.3. Extraction of total crude tropane alkaloids, hyoscyamine and hyoscine

Crude tropane alkaloids, hyoscyamine and hyoscine were determined in each sample by the high performance liquid chromatography (HPLC) procedure. For extraction of the crude drug, hyoscyamine and hyoscine, the fresh biomass in each material was properly chopped into small pieces and shade dried. After complete drying, samples were finely powdered and extracted using a mixture of chloroform-methanol (25%) ammonium hydroxide 15:5:1 (V/V/V). HPLC separation was performed on Eurospher C18 reversed phase column.

Table 2

Intra- and inter cluster divergence ($\bar{D}^2$) among eight clusters of *Hyoscyamus niger* accessions.

Clusters	I	II	III	IV	V	VI	VII	VIII	D^2
I	**15.432 (3.93)**	29.697 (5.45)	31.974 (5.65)	28.353 (5.32)	25.387 (5.04)	46.694 (6.83)	398.859 (19.97)	115.723 (10.76)	94.632
II		**20.719 (4.55)**	62.626 (7.91)	60.277 (7.76)	34.463 (5.87)	56.642 (7.53)	395.136 (19.88)	102.218 (10.11)	93.979
III			**16.437 (4.05)**	26.439 (5.14)	35.332 (5.94)	83.29 (9.13)	527.143 (22.96)	176.755 (13.29)	119.999
IV				**22.03 (4.69)**	52.213 (7.23)	55.688 (7.46)	421.555 (20.53)	143.309 (11.97)	101.233
V					**17.487 (4.18)**	82.994 (9.11)	522.888 (22.87)	160.226 (12.66)	116.374
VI						**0 (0)**	231.644 (15.22)	27.897 (5.28)	71.863
VII							**0 (0)**	178.153 (13.35)	***334.422***
VIII								0 (0)	113.035

D^2-Average of inter cluster D^2 values; D values ($\sqrt{D^2}$) are in parenthesis; intra- cluster values in bold fonts; Highest value=Italics; Lowest value=Italics.

An isocratic mixture of triethylammonium phosphate buffer (30 Mm, pH=6.2) and acetonitrile (75:25) was used as eluent. Hyoscyamine and scopolamine were determined by the external standard method at 210 nm. The crude drug, hyoscyamine and hyoscine (alkaloid content) analysis was carried out by the procedure as was previously described (Cromwell, 1955; Kamada et al., 1986; Gupta et al., 1999; Popova et al., 2018).

2.4. Statistical analysis

The pooled mean values of the two years were statistically analyzed for the mean ($\bar{X}$), range, standard error etc and subjected to D^2-statistics and canonical analysis according to Mahalanobis (1936) and Rao (1952) methods by the statistical software CSIR-CIMAP 0.3 version, available in the Department of Genetics and Plant Breeding at CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow, U.P.(India) based on the Chaudhary and Singh (1985) as well as Panse et al. (1967) approaches.

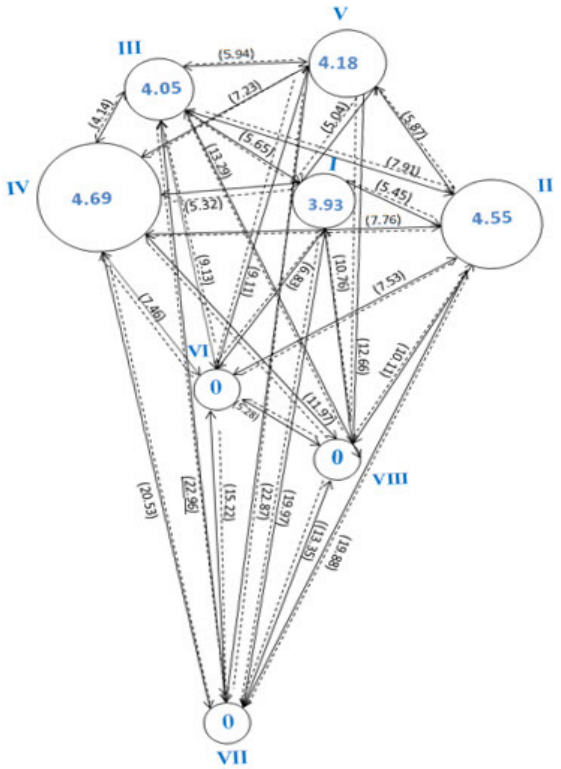


Fig. 1. Cluster diagram with their genetic distance of twenty-nine accessions of *Hyoscyamus niger* accessions.

3. Results and Discussion

3.1. Estimation of genetic divergence in terms of D^2-values and canonical analysis

The nature, amount and magnitude of genetic variation among the twenty-nine accessions were evaluated by Mahalanobis (1936) method. D^2-statastics wherein, the divergence between any pair of populations was quantified in terms of D^2-values. Individual D^2-values for all the n(n-1)/2=406 were calculated, where n=29 accounts for the number of accession pairs. The all twenty-nine accessions were grouped into eight diverse clusters such that the accessions within the clusters had smaller D^2-values among themselves than those between clusters. Thus, intra-cluster distance for each of the clusters was observed to be lesser than the inter cluster distances in all cases. The highest intra-cluster distance was observed (22.03) for the cluster IV was invariably smaller than the lowest intercluster divergence between cluster I and V (25.387), thus authenticating the clustering pattern formed in this study (Table 2 and Fig. 1). The intra-cluster divergence ranged from 0.000 to 22.03, whereas, the intercluster divergence ranged from 25.387 to 522.888 between clusters (I and V) and (V and VII), as also depicted in Fig. 1. The average intercluster divergence was the highest for cluster VII ($\bar{D}^2$=334.422) followed by cluster III ($\bar{D}^2$=119.999). It was the least for cluster VI ($\bar{D}^2$=71.863); other clusters were moderately divergent with an average $\bar{D}^2$ values of 116.274-93.979.

Table 3

Distribution of *Hyoscyamus niger* accessions in different clusters according to their geographic origins/locations.

Clusters	Number of accessions in clusters	Geographic locations and accessions	Accessions
I	11	CSIR-CIMAP, Lucknow, U.P. (India) Jammu, Jammu & Kashmir, (India) Kosice, Slovakia Republic, U.S.A. Pantnagar, Uttarakhand, (India) Austria Germany Poland	HN-1, HN-2, HN-6, HN-10, HN-11, HN-13, HN-17, HN-18, HN-20, HN-28, HN-29
II	07	CSIR-CIMAP, Lucknow, U.P. (India) Austria Yugoslavia Poland Germany	HN-5, HN-15, HN-19, HN-21, HN-22, HN-22-Y, HN-29-Y
III	04	Lucknow, U.P., (India) Katra, Jammu & Kashmir, (India) Palampur, Himachal Pradesh, (India)	HN-8, HN-9, HN-14, HN-16
IV	02	Kashmir, Jammu & Kashmir, (India), Bageshwar, Uttarakhand, (India)	HN-3, HN-7
V	02	Lucknow local, CSIR-CIMAP, Lucknow, U. P. (India)	HN-4, HN-12
VI	01	CSIR-CIMAP, Lucknow, U. P. (India)	HN-28-A
VII	01	CSIR-CIMAP, Lucknow, U. P. (India)	HN-30
VIII	01	CSIR-CIMAP, Lucknow, U. P. (India)	HN-31
Total cluster = 8	29		29

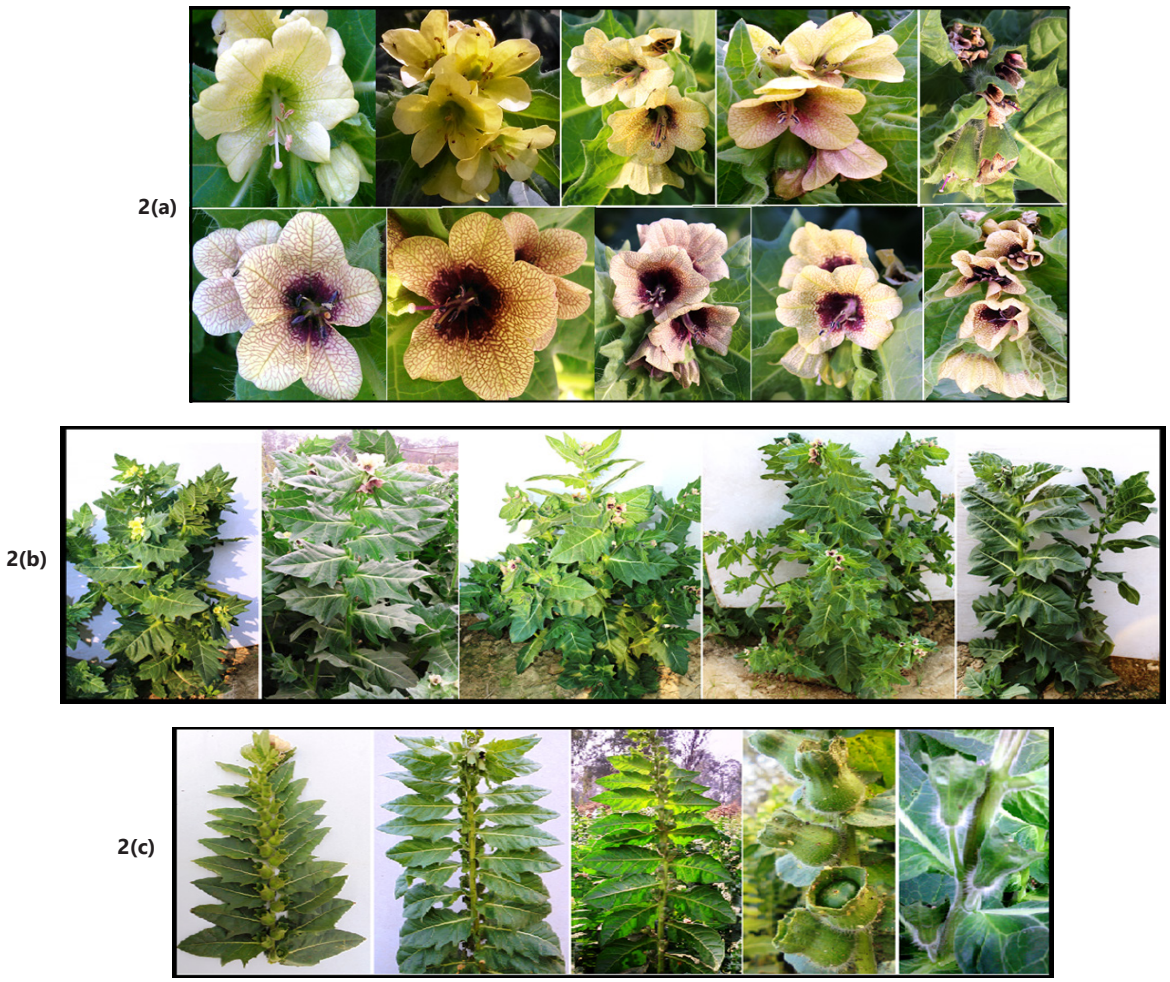


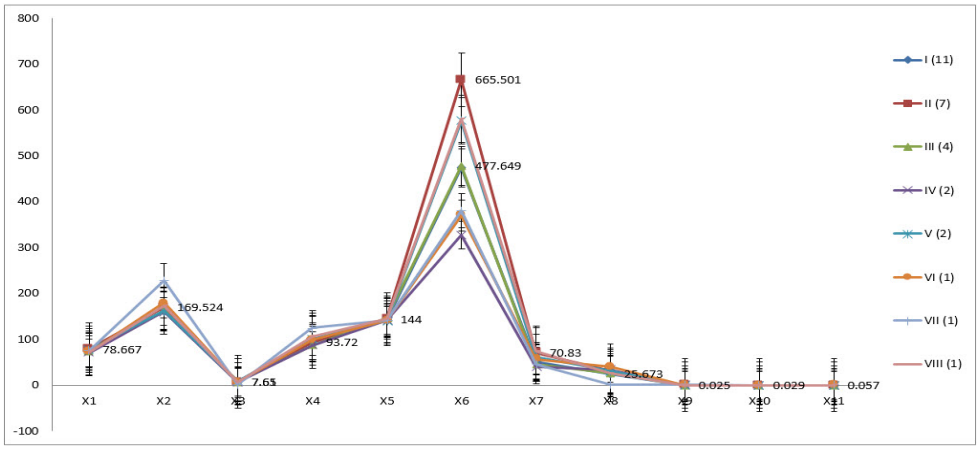
Fig. 2. (a) Genetic diversity in floral morphology and color in *Hyoscyamus* niger. (b) Genetic diversity of plant morphology in *Hyoscyamus niger*. (c) Genetic variability in arrangement and structure of capsule in *Hyoscyamus niger*.

Table 4

Cluster means of the eleven economic traits in *Hyoscyamus niger* accessions.

Clusters and Number of Accessions	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}
I (11)	76.734	160.056	7.167	93.794	141.685	470.169	50.383	23.74	0.1007	0.03	0.054
II (7)	78.667	169.524	7.650	93.720	144.000	665.501	70.830	31.814	0.143	0.027	0.0514
III (4)	72.875	162.583	7.610	86.30	146.083	477.649	45.95	25.673	0.025	0.029	0.057
IV (2)	70.750	159.950	7.225	87.08	141.75	326.863	40.74	33.948	0.09	0.022	0.046
V (2)	77.835	162.000	8.610	100.406	140.335	575.310	61.425	30.587	0.046	0.031	0.055
VI (1)	73.000	179.890	6.660	98.166	141.00	370.37	56.793	40.093	0.202	0.070	0.094
VII (1)	76.000	227.560	1.220	124.96	141.67	380.39	45.503	1.323	0.487	0.081	0.108
VIII (1)	72.830	173.000	7.110	106.296	144.00	580.360	74.200	26.58	0.309	0.095	0.118

Where, X_1=Days to 50% flowering; X_2=Plant height (cm); X_3=Number of primary branches/plant; X_4=Inflorescence length (cm); X_5=Days to maturity; X_6=Fresh herb yield (g/plant); X_7=Dry herb yield (g/plant); X_8=Seed yield (g/plot); X_9=Crude alkaloid content (%); X_{10}=Hyoscyamine (%); X_{11}=Hyoscine (%).



Where, X_1=Days to 50% flowering; X_2=Plant height at full maturity (cm); X_3=Number of primary branches/plant; X_4=Inflorescence length (cm); X_5=Days to maturity; X_6=Fresh herb yield (g/plant); X_7=Dry herb yield (g/plant); X_8=Seed yield (g/plot); X_9=Total crude alkaloid content (%); X_{10}=Hyoscyamine (%); X_{11}=Hyoscine (%).

Fig. 3. Graphical representation of the cluster means of eleven traits in *Hyoscyamus niger* accessions.

3.2. Estimation of divergence in clusters, cluster means and average performance of clusters

Genetic diversity among the 29 accessions was relatively large; although 37.93% accessions fall within cluster I and 24.14% within cluster II followed by cluster III (13.79%). Cluster IV and cluster V contributed 6.90% (each). The remaining three single clusters, i.e. cluster VI, VII and VIII (each contributed 3.45%) were isolated and distantly placed from the rest of the other clusters (Table 3). Seed yield/plot had the maximum character contribution ranks first (22.14%) towards genetic divergence followed by hyoscyamine content (15.94%) and hyoscine content in percent (15.82%), while the least character contribution towards the genetic divergence was made by the character days to maturity (1.05%). Enormous variations in color, shape and size of the flower, inflorescence, and leaves were also observed in the *H. niger* L. accessions (Fig. 2a, 2b and 2c). In addition, no parallelism was observed between genetic diversity and geographical diversity. Therefore, the present results support the findings in *Plantago*, *Vetiver* and *Capsicum* species (Lal et al., 2000; Thul et al., 2009; Kumar et al., 2018; Lal et al., 2018a; Lal et al., 2018b). This level of divergence suggests that genetic forces, particularly selection and genetic drift may have created variations within the same ecological zone to a considerable extent.

The mean performances of each cluster for the studied traits indicated considerable differences between them for all the characters and the corresponding results are presented in Table 4. As seen, cluster II had the highest mean for the days to 50% flowering (78.667), i.e. late maturing which is desirable trait for *H. niger* L. and cluster III had the lowest mean for day to 50% flowering (72.83). Cluster VII and cluster IV had the highest mean (227.56) and the lowest mean (159.95) for plant height, cluster V had maximum number of primary branches (8.61), while minimum number of primary branches (1.22) was recorded for cluster VII. Cluster VII had the highest mean for inflorescence length (124.94) and cluster III had the lowest mean for inflorescence length (86.30). Cluster III had late maturity (146.083), while cluster V was early maturing (665.501). The highest cluster mean

Table 5

Mean performances of five economic trait specific promising accessions of *Hyoscyamus niger*.

Sr. No.	Accession code	Dry herb yield (g/plant)	Seed yield (g/plot)	Total crude tropane alkaloids (%)	Hyoscyamine (%)	Hyoscine (%)
1.	HN-9	54.89	38.136	0.0349	0.0247	0.0585
2	HN-11	45.743	26.083	0.0647	0.0441	0.0703
3	HN-12	67.513	40.476	0.0480	0.0395	0.0535
4	HN-14	38.636	25.84	0.0172	0.0345	0.0780
5	HN-15	86.730	34.136	0.1338	0.0321	0.0533
6	HN-16	54.193	23.853	0.0155	0.0299	0.0235
7	HN-17	56.033	20.82	0.1536	0.0349	0.0775
8	HN-21	76.096	25.953	0.1435	0.0258	0.0463
9	HN-22	75.586	41.59	0.1106	0.0314	0.0562
10	HN-22-Y	79.586	24.016	0.1376	0.0297	0.0526
11	HN-28-A	56.793	40.093	0.2021	0.0704	0.0938
12	HN -29-Y	62.66	42.406	0.1860	0.0177	0.0229
13	HN-30	45.503	1.323	0.4871	0.0810	0.1080
14	HN-31	74.200	26.58	0.3093	0.0945	0.1180

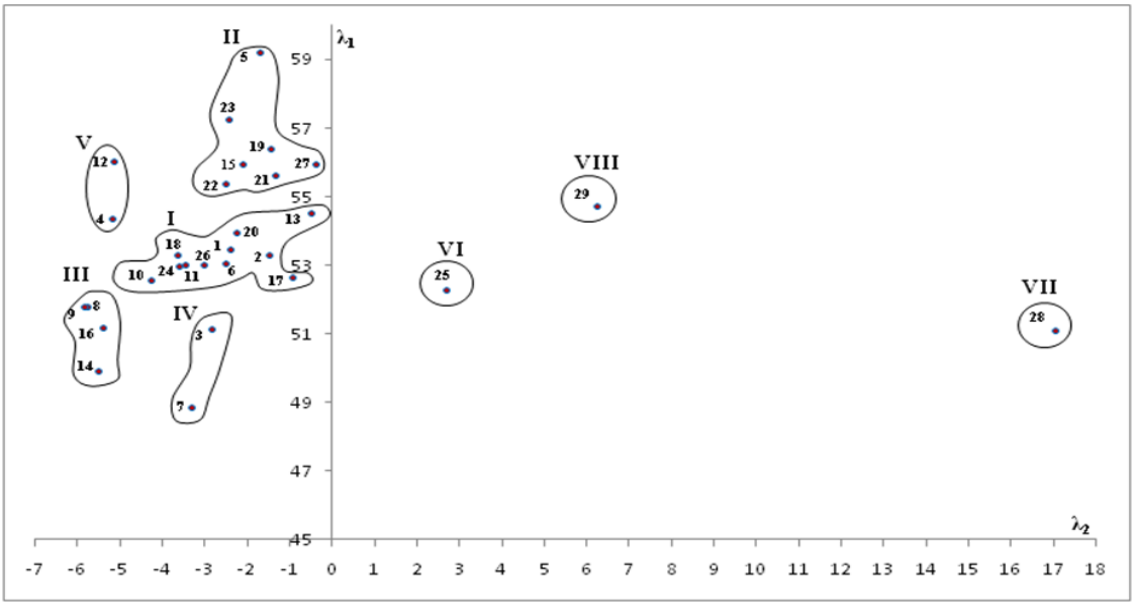


Fig. 4. Spatial distribution of twenty-nine genetic stocks of *Hyoscyamus niger* in λ1- λ2 chart.

(665.501) for fresh herb yield/plant (g) was recorded in cluster II and the lowest in cluster IV. Cluster VIII had the highest dry herb yield (74.20), whereas cluster IV had the lowest cluster mean for dry herb yield. Cluster VI had the highest seed yield/plot (40.093) and the lowest total crude tropane alkaloids (0.202), while cluster VII had the highest total crude tropane alkaloids (0.487) in percent and the lowest seed yield/plot (g) (1.323). Cluster VIII had the highest cluster mean values for hyoscyamine (0.095) and hyoscine (0.118) content in percent whereas, cluster IV had the lowest mean values for hyoscyamine (0.022) and hyoscine (0.046) content in percent. From the perusal of estimates presented in Table 4 and Fig. 3, it is apparent that considerable diversity existed for all characters in various studied genotypes. Clusters II, VII and VIII recorded high mean value for majority of the important traits like fresh herb yield (g/plant), dry herb yield (g/plant), total crude tropane alkaloid (%), and hyoscine content in percent. The genotypes exhibited random pattern of distribution for various clusters revealing that genetic and geographical diversity were not related to each other. An enormous diversity among the accessions was indicated by the high range of D^2-values from 4.032 to 544.535.

An analysis of cluster composition revealed that the largest cluster (Cluster I) comprised of eleven accessions collected from CSIR-CIMAP, Lucknow, U.P. (India) (3), Jammu, Jammu and Kashmir, India (1), Kosice Slovakia Republic (1), U.S.A. (1) Uttarakhand (India) (2), Austria (1), Germany (1), Poland (1), whereas cluster II had seven accessions collected from CSIR-CIMAP, Lucknow, U.P. (India) (2); Austria (1); Yugoslavia (1); Poland (2) and Germany (1). Cluster III consists of four accessions of which two were collected from Lucknow, U.P., (India), one from Katra, Jammu and Kashmir, (India) and one from Palampur, Himachal Pradesh, (India). Cluster IV and V having two accessions of which one collected from Kashmir, Jammu and Kashmir, (India), one from Bageshwar, Uttarakhand, (India), one from Lucknow local, CSIR-CIMAP and one from Lucknow, U. P. (India).

On the other hand, three solitary clusters VI, VII and VIII represented accessions collected from the same place, i.e. CSIR-CIMAP, Lucknow, U.P. (India) (Table 3).

The genetic divergence among twenty-nine accessions of *H. niger* L. was measured and confirmed in terms of spatial distribution as well as through the canonical analysis. Since the proportionate contribution by the first two canonical roots λ1 (60.26%) and λ2 (16.042%) was more than 76.31%, the two dimensional representations (λ1 and λ2 chart) were found to be adequate and the conducted experiment was significant. The distribution of each individual against λ1 and λ2 coordinating axes are presented in Fig. 4. A very high genetic divergence was noted among the twenty-nine accessions of *H. niger* L. as represented by the spatial distribution in λ1 and λ2 chart. From the above study, five best accessions, namely HN-30, HN-31, HN-15, HN-22-Y and HN-29-Y were selected on the basis of genetic diversity and the performance of high dry herb yield/plant hyoscyamine and hyoscine and total crude alkaloids content in percent (Table 5).

4. Concluding remarks

In nutshell, thirteen accessions were chosen for the best dry herb yield (g/plant), seed yield (g/plot), total crude tropane alkaloid content, hyoscyamine and hyoscine percent out of which, accessions, namely HN-15 followed by HN-22-Y were the best for dry herb yield (g/plant), accession HN-29-Y followed by HN-22 had maximum seed yield (g/plot) and accession HN-30 and HN-31 had maximum total crude alkaloids content, hyoscyamine and hyoscine percent. All these accessions could be recommended for further commercial exploitation selection of genotype for hybridization. Selection of the genotypes should be based on genetic diversity rather than geographical diversity. Considerable genetic divergence was available in the evaluated accessions of *Hyoscyamus niger* L. Therefore, the development of hybrids between the promising accessions and genotypes of different clusters appears practicable, making improvement in *H. niger* L. possible.

Conflict of interest

The authors declare that there is no conflict of interest.

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