ONTOGENIC VARIABILITY IN ALKALOID SYNTHESIS AND OTHER MORPHOLOGICAL CHARACTERS IN FIVE GENOTYPES OF BELLADONNA[1]

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ABSTRACT.—Research with belladonna often concerns the determination of the stage at which maximum alkaloid content is present in the plant. In the present study, five breeding lines were clonally propagated and analyzed for the alkaloid hyoscyamine and other plant characters. The harvesting was done ten times at regular fortnightly intervals from May 14 to Sept. 26, 1975. Plants assayed for higher alkaloid content at stages 1 and 9. Alkaloid content was significantly lower at stage 3, when the plant was at its peak growth. The sampling dates were set with respect to days after flowering and are suggested to be set as standards for describing sampling dates. The significant differences in alkaloid concentration at different stages of harvest emphasize the desirability of calculating alkaloid content at several growth stages, especially in identifying high-alkaloid strains.

The leaves and roots of belladonna (*Atropa belladonna* L. and *A. acuminata* Royle ex Lindley) contain the anticholinergic alkaloid, hyoscyamine, which has great pharmaceutical utility. The stage of plant growth when the alkaloid synthesis is at its maximum has been studied by different workers. Valayshko and Sova (1), Chopra *et al.* (2), Dalef *et al.* (3), Sethi *et al.* (4) have reported that the alkaloid in the leaves increased up to the beginning of flowering and then decreased, while Brewer and Hiner (5) and Tucakov (6) observed the alkaloid content to be at its peak at blooming time. Another group of workers (7, 8 and 9) reported the highest content at the fruit bearing stage. Kuhn and Schaffer (10) observed the highest alkaloid concentration to occur during spring and thereafter at the blooming stage. While Pawelczyk (11) confirmed the results of Kuhn and Schaffer, he reported that the highest alkaloid concentration occurred at the fruit bearing stage instead of the flowering stage.

These results led us to believe that in belladonna either different stages of growth may be associated with high alkaloid concentration or that no regular trend exists. Further, none of the previous studies stressed genetic uniformity. Belladonna is a cross-pollinating plant and exhibits a tremendous amount of variation in all the traits including alkaloid concentration (12 and 13). Some of divergent results reported above may be a reflection of this variability present in the plant material. Genetically uniform material and standard growth stages have been incorporated into the present study to ascertain precisely the pattern of alkaloid synthesis and/or developmental variation.

MATERIALS AND METHODS

Five belladonna breeding lines, M704, SL7039, M6926, (M6927 X M6926)-6 and SL7062, coded respectively, I,II,III,IV, and V were propagated vegetatively by stem cuttings and were used for this study. The prominent features of these genotypes are given in table 1. The clonally propagated material of the five genotypes was harvested and the leaves sampled for alkaloid concentration (calculated as hyoscyamine) by the B. P. method (14) with a little modification to avoid excessive emulsion formation. A ten g sample of powdered leaves was extracted with methanol in a Soxhlet for 6 hours (till the plant material turned colorless). The extract was concentrated on a water bath and acidified with dil. sulfuric acid and extracted with chloroform (3 times). The acidified layer was basified with 10% ammonia and extracted with chloroform (3 times). The chloroform extract was washed with distilled water and dried over anhydrous sodium sulfate and later at 105° for 3 hrs. Ten ml of N/20 sulfuric acid were added to the dried chloroform extract and titrated with N/20 sodium hydroxide. The alkaloid concentration was determined by the normality equation (1 ml of N/20 H_2SO_4 contained 0.01447 g. alkaloid). The sampling dates in 1975 were May 14 and 29; June 13 and 28; July 13 and 28; August 12 and 27; and September 11 and 26. Plant height, herbage yield, and tillers per plant also were recorded.

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TABLE 1. Description of the five genotypes studied with their code.

Genotype	Code	Prominent characteristics of the genotype
M704	I	High in alkaloid; erect and profusely tillering; fast regeneration and easy to propagate vegetatively; stem green; flowers yellow.
SL7039	II	Medium in alkaloid; erect and moderate in tillering; slow regeneration but easy to propagate vegetatively; stem diffuse purple; flowers purple.
M6926	III	Medium in alkaloid; erect and poor tillering; poor regeneration and difficult to propagate vegetatively; stem purple; flowers purple yellow.
(M6927 X M6926)–6	IV	Medium in alkaloid; erect and moderate in tillering; poor regeration and difficult to propagate vegetatively; stem diffuse purple; flowers light purple.
SL7062	V	Medium in alkaloid; erect and moderate in tillering; good regeneration and fast to propagate vegetatively; stem diffuse purple; flowers purple; has shown exceptionally good field resistance to root rot fungi.

The experiment was laid in a split plot design with the five genotypes as the main plots and ten sampling dates as the subplots. Each treatment consisted of one row plot with two plants replicated four times. Both plant to plant and row to row distance were 1 meter. Diammonium phosphate and muriate of potash were applied to the field at the rate of 100 kg P and 120 kg K per hectare, respectively. Urea was applied at the rate of 200 kg N per hectare in four equal doses at regular intervals.

Statistical analysis of the data was performed according to established methods (15).

RESULTS

The analysis of variance for the four characters revealed that the genotypes and the stages of harvest were significant in all the characters, while the interaction, genotype in relation to the stage of harvest, was significant in the case of alkaloids only (table 2).

TABLE 2. Analysis of variance for different characters in belladonna.

Source of Variation	d.f.	Alkaloid in leaves (%)	Herbage yield (Kg)	Plant height (cm)	Tillers per plant
Replicate	3	0.017	11.108	4639.944*	13.15
Genotype	4	0.295**	48.766**	4664.133**	298.74**
Error (Genotype)	12	0.017	7.962	842.293	16.88
Harvest stages	9	0.156**	70.709**	4896.427**	49.93**
Genotype X harvest stage	36	0.031**	5.478	325.913	10.71
Error (Harvest stage)	135	0.015	4.389	354.867	7.86

*Significant at 5% level.
**Significant at 1% level.

ALKALOID CONCENTRATION

The mean performance of the five genotypes (table 3) revealed that two high levels and one low level of alkaloid occurred during the growing season. At stage 1 the alkaloid concentration was relatively high, 0.55 per cent. The lowest concentration found in the study occurred at stage 3, 0.38 per cent, when about 50 per cent anthesis in the plant had set in. Thereafter the concentration increased again reaching the maximum towards the end of the growing season (stage 9). The alkaloid concentration decreased at stage 10 but never to the extremely low level of stage 3; in fact, it stayed at the same level as it started off at stage 1 (table 3). However, figure 1 revealed that each genotype had a characteristic

TABLE 3. Mean percent alkaloid in leaves of five belladonna genotypes at different stages of harvest.

Genotype	Stages of harvest										Genotype Mean
	1	2	3	4	5	6	7	8	9	10	
I	0.67	0.57	0.42	0.69	0.73	0.93	0.69	0.66	0.78	0.66	0.68
II	0.56	0.44	0.42	0.36	0.33	0.36	0.59	0.54	0.67	0.52	0.48
III	0.45	0.46	0.36	0.40	0.59	0.74	0.64	0.53	0.77	0.56	0.55
IV	0.48	0.36	0.25	0.34	0.41	0.50	0.59	0.66	0.60	0.46	0.46
V	0.59	0.46	0.43	0.48	0.55	0.44	0.56	0.60	0.54	0.61	0.52
Stages mean	0.55	0.46	0.38	0.45	0.52	0.59	0.61	0.60	0.67	0.56	0.54

Critical difference	5%	1%
Two genotype means	0.063	0.088
Two stages of harvest means	0.074	0.097
Two stages of harvest means within the same genotype	0.168	0.221
Two genotype means for the same stage of harvest or different stages of harvest	0.236	0.322

alkaloid synthesis pattern of its own, which in some cases deviated from the mean performance. For example, the highest alkaloid concentration for all the genotypes was at stage 9 except for genotype I for which had it was at stage 6, and for genotype V which had 3 high peaks (stage 1, 8 and 10). In addition, these genotypes differed from one another in the total alkaloid content averaged

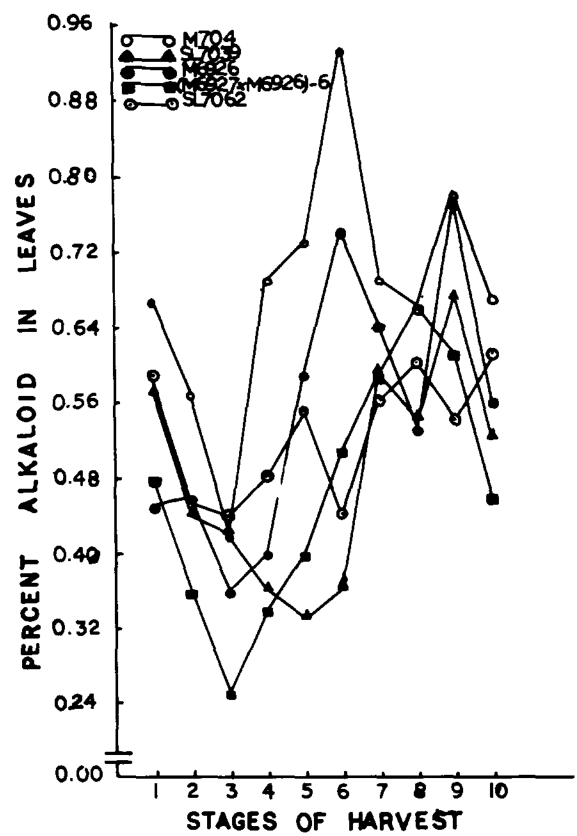

FIG. 1. Alkaloid production in relation to ontogenic development in five genotypes of belladonna.

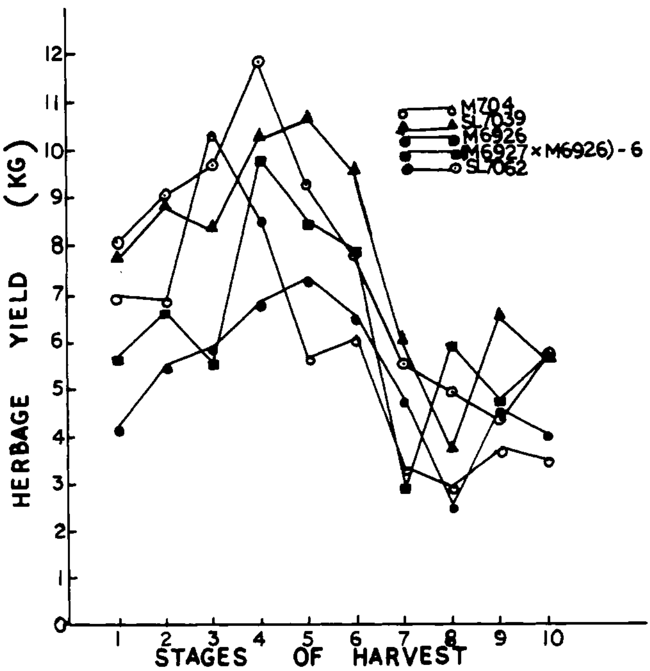

FIG. 2. Fresh herbage yield (kg/plot) of five genotypes of belladonna at different stages of harvest.

over all the 10 harvest stages (table 3). Genotype I had the highest alkaloid content (0.68%), while genotype IV had the lowest (0.46%).

HERBAGE YIELD

Highest herbage yield was observed at stage 4 and the lowest at stage 8 (table 4). The yield seemed not to vary after stage 7 as no statistically significant difference ; were observed. The individual genotypes, though, showed a yield pattern of their own, but it was not as pronounced as for alkaloid content (figure 2).

TABLE 4. Mean fresh herbage yield (kg/plot) for the five genotypes of belladonna at the different stages of harvest.

Genotypes	Stages of harvest										Genotype mean
	1	2	3	4	5	6	7	8	9	10	
I.	7.03	6.91	10.33	8.45	5.60	6.03	3.29	2.95	3.76	3.52	5.79
II.	7.73	8.84	8.35	10.32	10.60	9.50	5.99	3.71	6.55	5.57	7.72
III.	4.20	5.50	5.88	6.89	7.39	6.59	4.79	2.52	4.49	4.05	5.23
IV.	5.77	6.71	5.61	9.84	8.56	7.97	2.98	5.98	4.79	5.66	6.39
V.	8.07	9.02	9.69	11.95	9.28	7.81	5.54	4.96	4.32	5.62	6.63
Stages mean. . .	6.56	7.40	7.97	9.49	8.29	7.58	4.52	4.02	4.79	4.89	6.55

Critical Difference	5%	1%
Two genotype means	1.372	1.924
Two stages of harvest means	1.297	1.705
Two stages of harvest means within the same genotype	ns	ns
Two genotype means for the same stage of harvest or different stages of harvest	ns	ns

ns=not significant

The mean of the ten harvests gave genotypes II and III the highest (7.72 kg) and the lowest (5.23 kg) yields per plant, respectively.

Plant height

Plant height seemed to be unaffected after the commencement of flowering (stage 3) as no significant differences were observed thereafter (table 5). Significant differences were observed between the tallest (V) and the shortest (I) genotypes only, and the rest were more or less of the same height (table 5).

Table 5. Mean plant height (cm) for the five genotypes of belladonna at different stages of harvest.

Genotypes	Stages of harvest										Genotype mean
	1	2	3	4	5	6	7	8	9	10	
I	98.2	120.0	128.2	125.6	128.7	114.7	115.3	125.8	125.8	128.2	121.05
II	96.1	130.7	142.6	142.2	142.8	147.2	150.6	136.2	167.7	155.1	141.12
III	97.2	124.6	148.2	147.2	130.0	154.7	144.6	124.1	160.6	153.6	138.48
IV	97.0	132.6	128.7	149.8	158.6	154.2	143.3	156.8	154.6	160.1	143.57
V	104.1	143.3	148.8	160.1	152.7	151.8	147.1	167.8	152.1	172.2	149.90
Stages mean	98.52	130.04	139.30	144.98	142.56	144.52	140.18	142.14	152.16	153.84	138.82

Critical difference	5%	1%
Two genotype means	14.139	19.823
Two stages of harvest means	11.675	15.345
Two stages of harvest means within the same genotype	ns	ns
Two genotype means for the same stage of harvest or different stages of harvest	ns	ns

ns = not significant.

Tillers per plant

The number of tillers produced at various stages of growth showed significant differences (table 6). Interestingly, more tillers were produced towards the end of the growing season than at the beginning (table 6). At stage 10 nearly 10 tillers per plant were observed as against 5 to 6 in the early stages. The genotypes also expressed marked differences in tillering.

Table 6. Mean number of tillers per plant for the five genotypes of belladonna at the different stages of harvest.

Genotypes	Stages of harvest										Genotype mean
	1	2	3	4	5	6	7	8	9	10	
I	9.7	10.3	12.5	9.1	11.1	14.3	9.6	13.6	12.7	13.2	11.61
II	5.5	4.3	4.5	5.3	5.6	9.6	11.0	9.5	10.8	13.8	7.99
III	3.1	3.2	3.6	4.5	3.1	5.2	5.0	4.2	5.1	4.7	4.17
IV	5.5	4.8	3.3	4.5	6.1	5.3	5.2	9.0	8.0	12.3	6.40
V	7.2	6.2	5.6	7.5	6.2	6.0	7.0	7.2	7.3	8.3	6.85
Stages mean	6.20	5.76	5.90	6.18	6.42	8.08	7.56	8.70	8.78	10.46	7.40

Critical difference	5%	1%
Two genotype means	2.000	2.804
Two stages of harvest means	1.736	2.282
Two stages of harvest means within the same genotype	ns	ns
Two genotype means for the same stage of harvest or different stages of harvest	ns	ns

ns = not significant

DISCUSSION

The greatest ontogenetic range of alkaloid concentration occurred between stage 3 (0.38%), the lowest found, and stage 9 (0.67%). The individual genotypes also exhibited the variability of a similar range. From these results, it appears that the results reported by earlier workers have been actually developmental variation of a heterogeneous and heterozygous source. Further, in the present study, the stages of plant growth have not been arbitrarily fixed but correspond to a definite physiological age, well defined, precise and unambiguous. The data of first flowering was found to be fairly constant from year to year, least affected by varying environmental factors over different years for a particular location. This was taken as base point for calculating the different stages of harvest (table 7).

TABLE 7. Stages of harvest and associated morphological features of belladonna.

Harvest Stage	Time of the year	Days after the appearance of first flower	Morphological features of the plants
1	May 14	10	Flowering at the point of sympodial branching.
2	May 29	25	Beginning of flowering.
3	June 13	40	50% flowering, basal leaves fully mature.
4	June 28	55	80% flowering and appearance of young berries.
5	July 13	70	Predominance of green berries, appearance of axillary laterals.
6	July 28	85	Beginning of purple berry formation with some green berries.
7	August 12	100	Predominance of purple berries with some berries drying.
8	August 27	115	Shoot apices drying and initiation of new foliage at the base.
9	September 11	130	Appearance of new foliage, fresh tillers and flowers.
10	September 26	145	Green berries on the new growth.

From the present study it is evident that alkaloid synthesis followed more than one peak of high and low syntheses during the same growing season. This was contrary to what the majority of other workers had reported, i.e., that alkaloid synthesis was at its peak at only one particular stage, be it before, during or after flowering.

At stage 1 (May 14) the plant was fairly high in alkaloid content, but a gradual decline occurred from stage 1 to stage 2. Thereafter it started building up again reaching the maximum between 5 and 9.

From the data of Phillipson and Handa (16) a similar trend can also be observed. The data for hyoscyamine content as mg/100 g of fresh tissue showed high concentration before flowering in May and at the green fruit stage in July. A decline occurred towards initial flowering and at the time of blue berry formation. Griffin (17) also observed similar differences in the hyoscine content in the leaves of *Datura candida*.

Such an ontogenic variation in secondary metabolites has been observed in other crops also (18–21).

Considering the factors responsible for high and low alkaloid concentration at different stages of development, it must be associated with the metabolic processes of the plant. In belladonna there is evidence to show that young and metabolically active leaves contain more alkaloid than the old leaves (5, 22 and 23).

There are no significant differences in plant height beyond stage 3 (table 5), indicating decreased meristematic activity with the advancement of the growing season. Further beyond stage 3, the photosynthate also gets partitioned into the development of fruit and seed. Leaves also become over mature at stage 3 and tend to turn yellow or fall, giving place to new laterals with new and young foliage (stage 5 to 7) resulting in the rise in the alkaloid concentration. The increased alkaloid concentration during September can be attributed to the presence of more new tillers (table 6) and young foliage (stage 9, table 7). In addition to a prevalent temperature of approximately 20° during this period and ambient for high alkaloid (24), as well as some other factors hitherto unknown for this crop is also indicated.

The decrease in alkaloid content with advanced maturity has been also observed by Hagman, Marten and Hovin (25) in *Phalaris arundinacea* L. and for HCN and P in *Sorghum* by Eck (26).

These results emphasize the desirability of calculating alkaloid percentage at several growth stages for screening high alkaloid genotypes. Further these data indicate that comparing strains for alkaloid concentration, harvested at different times during the growing season may be avoided. In the present study, growth stages have been clearly defined and are suggested to be set as standards to describe the timing of sampling.

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