PHENOTYPIC VARIABILITY AND THE INFLUENCE OF TEMPERATURE
ON SOMATIC INSTABILITY IN CULTURES DERIVED FROM HYBRIDS
BETWEEN NICOTIANA LANGSDORFFII AND N. SANDERAE¹

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PLANT cultures showing variegation for flower color have originated, as described by SMITH and SAND (1957), following the interspecific hybridization of *Nicotiana langsdorffii* and *N. sanderae*. Genetic studies conducted with one of these derived cultures, variegated-1, have shown that the variegated phenotypes and *N. sanderae*, which is nonvariegated, differ from each other in the alleles present at a single, major locus. Solid-colored *N. sanderae* has the constitution V/V . The principal variegated-1 phenotypes are speckled, v_s/v_s ; sectorial, v_s/v_s ; and rare sectorial, v_s/v_s . The genetic data are interpreted to indicate that v_s and v_s are unstable alleles which may undergo reversible changes between their alternative forms in the lineage of certain germ cells thus modifying the genetic ratios among progeny. Also, these events occurring in the cells of the developing petal epidermis give rise to color variegation of the mature flowers. Differences in frequency and developmental timing of these events lead to different phenotypes of variegated flowers. This variability has both heritable and environmental components. The studies reported here were designed to estimate the magnitude of several causes of this phenotypic variability and to investigate the effect of temperature during development as a contributing factor.

MATERIALS

In these studies three major phenotypes of variegated plants were employed: the speckled and sectorial phenotypes of a variegated-1 culture and the flecked phenotype of a variegated-3 culture. Both cultures have been derived by selection and backcrossing to *Nicotiana sanderae* following interspecific hybridization with *N. langsdorffii*.

The variegation phenomenon concerns the distribution of anthocyanin pigmentation in the epidermal cells of the petals. The corolla of nonvariegated *N. sanderae* is uniformly solid red in color. The speckled phenotype has scattered, small clusters of red cells on a background of colorless cells. The sectorial phenotype has frequent, clearly defined sectors of speckled tissue on a background of solid red tissue. This sectoring of variegated-1 may occur over a wide range of time during plant or flower development. Thus a speckled sector may involve only a few petal cells or it may include a large portion of the petal epidermis of a flower, branch or plant. The variegated-3 (flecked) phenotype resembles the variegated-1 (sectorial) phenotype in

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possessing sectors of speckled tissue on a background of solid red tissue. However, variegated-3 shows a dramatic difference in the timing of the sectoring events. In this line the sectors are frequent but medium to small in size, giving a flecked appearance to the petals. Thus large sectors are extremely rare in the flecked phenotype, presumably because sectoring events occurring early in development are rare in this line.

OBSERVATIONS

Phenotypic variability

Variation in speckling-intensity phenotype among sister plants. It has been found possible to score flowers for speckling intensity by estimating the frequency of pigmented epidermal cells. A corolla lobe of a fresh flower is viewed at high-dry power through a microscope employing a green filter. It is possible to count the number of pigmented cells per microscope field of view. With the microscope and magnification used, one field of view encompassed approximately 200 epidermal cells.

During a period of six days in the summer of 1952 20 speckled plants (progeny from self pollinating one speckled plant) were scored in the above manner. These results are reported by SMITH and SAND (1957).

The data are not considered exhaustive enough to permit detailed analysis of differences among individual plants. It is indicated, however, that the variegated-1 culture contains speckled plants whose flowers vary in their average frequency of pigmented epidermal cells. Other studies have shown that at least a portion of this variability has a genetic basis.

Variation in speckling-intensity phenotype with temperature during development. A clone of speckled plants was established in the fall of 1952. When beginning to flower two members of the clone were placed in each of three different constant temperature greenhouses at 55°F, 70°F, and 80°F. Scoring was begun four days after initiating the constant temperature treatments. During the ensuing 12 days three flowers were scored from each of the six plants, employing the technique described above for estimating speckling intensity.

Analysis of variance has demonstrated highly significant differences in speckling intensity among the three temperature treatment means. The linear regression on temperature is highly significant and deviations from regression are not significant. It is concluded that the speckling intensity of flowers of the tested clone is temperature sensitive. The response is linear within the temperature range employed. An increase in temperature is accompanied by a decrease in the frequency of pigmented cells in the petal epidermis (fig. 2). Other speckled plants of the variegated-1 culture have not been tested under controlled conditions, but field observations give the impression that the temperature response is a general phenomenon.

Variation in speckling-intensity phenotype with age of flower. Members of two clones of speckled plants were used in the spring of 1954 to provide material for an estimate of the change in speckling intensity that occurs as a mature flower grows older on the branch.

Five plants were employed from each clone and two branches from each plant. From each selected branch the three most terminal blooms were harvested on one day. These blooms were designated as flowers 1, 2, and 3 in order from the branch

FIGURE 1.—Flowers of different ages from two clones of variegated *Nicotiana*. Speckling intensity increases from top to bottom with increasing age of flower.

FIGURE 2.—Flowers from a clone of variegated *Nicotiana* cultured at different controlled temperatures. Speckling intensity decreases with increasing temperature.

FIGURE 3.—Flowers from clone S5230-5 of variegated *Nicotiana* cultured at different controlled temperatures. Sectoring frequency is highest at 80°F.

apex. The most apical flower is the youngest. Thus ten flowers of each of three age-classes were harvested from each clone.

The Hunter Color Difference Meter calibrated to a white standard was used to measure the total reflectance of a 1.5 square centimeter area on each corolla. Total reflectance measurements were taken also on flowers of solid red and solid white phenotypes.

For each clone the mean total reflectance values were calculated for each flower age. Total reflectance was interpreted as an inverse measure of speckling intensity: the greater the reflectance the lighter the speckling.

The results show that speckling intensity increases with increasing flower age within each clone (fig. 1). The oldest blooms of the light-speckled clone are darker on the average than the youngest blooms of the dark-speckled clone. Thus a valid comparison of speckling intensity between plants requires the use of flowers of the same age.

Variation in sectoring-frequency phenotype among plants. In the summer of 1952, 127 flowers from nine sectorial plants of the variegated-1 culture were scored for their sectoring frequency. For this purpose the sectors were divided into two size categories: (1) small sectors, the points of origin of which can be seen in the petal lobe, and (2) large sectors, which originate at points that are not visible in the petal lobe, i.e., in regions of earlier development of the flower or branch.

Both small and large sectors were counted on the adaxial surface of each lobe of

TABLE 1
Sectoring-frequency variation among sectorial plants of variegated-1

Plant pedigree number	(n) Number flowers scored	(A) Mean large sector degrees per flower	(B) Mean small sector number per flower	(C) Adjusted† mean small sector number per flower	($s_{\bar{x}}$)‡ Standard error of adjusted mean
*S527-3	3	3.7	329	332	23.7
*S527-15	12	84.7	221	288	11.8
*S527-25	15	3.2	281	283	10.6
S526-11	11	79.1	216	277	12.4
*S527-36	30	54.8	226	266	7.5
S529-1	9	12.9	236	245	13.7
*S527-27	30	77.3	191	243	7.5
*S527-22	8	23.9	212	227	14.5
*S527-34	9	2.3	197	198	13.7
Total	127	49.1	223	258	3.7

* = Seven full-sister, sectorial plants.

† = Calculated using: $C = 360 B / (360 - A)$.

‡ = Error variance for adjusted sector number = $s^2 = 1,684$ (117 degrees of freedom).

$s_{\bar{x}} = \sqrt{s^2/n}$ used in calculation.

every flower. When the lobes of a flower are seen at full face view, the large sectors take the form of wedges of tissue emanating from the center of the flower. Thus such large sectors may vary in angular size from a few degrees to 360 degrees when they include the whole flower. For each large sector the angular width in degrees and the position of its limiting radii on a 360 degree reference circle were recorded.

When large sectors occur, the count of small sectors will be reduced in proportion to the area in large sectors. Thus in order to compare the small sector frequencies of the different plants their small sector averages have been adjusted to the values that would have been expected had no large sectors been present. A summary of this portion of the data is presented in table 1.

It is considered that the unequal and relatively small numbers of flowers scored from different plants prevent appraisal of precise differences in sectoring frequency between specific plants. Nevertheless, the variability is large among the adjusted plant averages. These data indicate that certain sectorial plants of the variegated-1 culture differ from each other in their frequencies of small sectors.

Other studies support this conclusion of heterogeneity of the population of sectorial plants. In results reported by SMITH and SAND (1957) genetic tests of two sister sectorial plants ($v_s v_s$) have shown correlation between the frequency of sectors of speckled somatic tissue ($v_s v_s$) and the frequency of speckled progeny ($v_s v_s$) obtained from self pollinating the respective plants.

Large-sector size distribution. As discussed in the previous section, large sectors have been defined as those not entirely included within the area of a mature petal lobe. Since these large sectors take the form of wedges of tissue, the angular width in degrees was used as a measure of the size of each large sector.

Of 131 flowers scored, 51 possessed at least one large sector. The size of these sec-

FIGURE 4.—Size (angular width) distribution of large sectors on petals of variegated *Nicotiana*. The distribution is discontinuous.

tors ranged from a few degrees to 360 degrees; more than half of the 61 sectors were less than 40 degrees in angular width. While the data are a minimum, a pattern of discontinuous distribution emerges (fig. 4). There exist deficiencies in the distribution representing sector sizes which are not as frequently produced as others. These deficiencies are clear in the regions centered at 80 and 160 degrees, and extend over a range of about 40 degrees in each case. In addition no sectors between 210 and 360 degrees in size were found on the flowers scored. Although it is known that sectors in this large size range do occur, they are relatively infrequent. In conjunction with the gaps in the distribution there are clusters with sector modes near the sizes 60, 120, and 190 degrees.

It is proposed that such a distribution lends support to a concept that the sectors delineate ontogenetic cell lineages. These data suggest that at a very early stage in floral development there exists a definite number of primordial cells whose descendants will constitute all of the epidermis of the mature corolla. These cells would have to be sought, near the time of initiation of the petal primordia, at a stage in development earlier than was studied by AVERY (1933) for the tobacco leaf.

For example, we may consider the existence of six initials, (or of six primordial cells derived from a single initial) each destined under normal conditions to produce one sixth of the mature corolla epidermis. Then a sectoring event occurring in one of these cells should result at floral maturity in a sector occupying one sixth of the corolla or 60 degrees of petal epidermis. If two adjacent initials were of the altered type (probably due to a sectoring event in a common ancestral cell) then a 120 degree sector should result. Under such circumstances it would be expected that sectors between 60 and 120 degrees would be observed at frequencies much less than those

for the modal sizes. The data approximate this condition. Thus the discontinuous distribution of sector size favors the hypothesis of a definite number of corolla epidermis initials in these plants. It is also indicated that these initials may be no more than six in number, and that an orderly pattern of cell lineage connects these initials with their mitotic descendants in the epidermis of the mature corolla.

Large-sector position distribution. The position of a large sector on the mature corolla can be defined in terms of the locations of its limiting radii or marginal limits. The locations of 94 such marginal limits were recorded by noting whether they occurred (1) in one of the five petal lobes but not along a mid-lobe line, (2) along one of the five sinus lines between lobes, or (3) along one of the five mid-lobe lines. This position distribution of sector limits is summarized below:

	Lobe 1	Lobe 2	Lobe 3	Lobe 4	Lobe 5	Sinus line	Mid-lobe line
Origin	6	6	8	7	9	9	2
Termination	5	8	9	7	7	9	2

The data indicate that each of the five lobes of the corolla is equally likely to contain the margin of a large sector. However, a large sector is much more likely to have a limit along one of the five sinus lines between lobes than along one of the five mid-lobe lines. In fact, despite the much smaller area involved, as many sectors have origin or termination radii in a sinus as within any one entire lobe.

It is not thought that this observation indicates any different predisposition toward sectoring of sinus versus lobe lineage cells. Rather, it is considered to reflect a different pattern and frequency of cell division in the two lineages, which function to generate the five-lobed form of the mature corolla. Apparently there are fewer late, intussusceptive divisions of cells near the sinus lines than there are near the mid-lobe lines. If such divisions were to occur near the sinus lines they would be expected to alter the five-lobed morphology of the corolla, and to disperse the sector limits from their observed concentration along these sinus lines. The tendency for sector limits to coincide with petal lobe limits may indicate relatively independent cell lineages for the five petals soon after corolla initiation. This observation can be interpreted as evidence that these sympetalous flowers are morphogenetically polypetalous.

Temperature effect on sectoring

Materials and methods

As described above, two cultures of sectorially variegated *Nicotiana* plants are available, each of which contains individuals whose petals possess sectors of speckled epidermal tissue on a background of solid red tissue. Sectorial plants of the variegated-1 culture show the presence of some very large sectors as well as small sectors. On the other hand the sectors are much more limited in size range in the variegated-3 culture. Most of the latter sectors are small enough to be entirely included within the petal lobe in which they occur. This fact permits the petal lobe to be the sampling unit for measuring the frequency of sectors in the case of variegated-3 plants. Be-

cause of this convenience a clone of variegated-3 was used in the following study, summarized previously (SAND 1956).

Cuttings were taken in October, 1953 from a member of the clone S5230-5. These cuttings were rooted, potted individually, and kept together in the greenhouse until the cuttings began to flower. On January 1, 1954 two members of the clone, selected at random, were placed under each of four different constant temperature greenhouse conditions: 50°F, 60°F, 70°F, and 80°F. Two additional cuttings were continued under the previous greenhouse conditions in which the temperature was not automatically regulated. Flowers were harvested from these ten plants as they bloomed, and data were taken from each flower. Harvesting was continued until at least 21 flowers had been obtained from each plant. Other flowers were self-pollinated on each plant.

Each of three petal lobes per flower were scored for: (1) lobe width, (2) number of proximal points of speckled sectors in one square centimeter, and (3) number of epidermal cells per 100 ocular micrometer divisions.

The technique employed was to dissect the petal lobe and place it on a 4" x 5" glass plate. It was carefully covered with a microscope slide and briefly pressed by a lead weight. After removing the weight, the lobe width at its maximum point was measured to the nearest tenth of a centimeter. This temporary mount was then viewed by reflected light at 30X under a binocular dissecting microscope. A square, approximately one centimeter on a side, etched on the microscope slide, framed a corresponding area on the petal beneath it. This permitted the number of speckled sectors in 1.09 square centimeters near the center of the lobe to be counted. Only sectors containing eight or more cells were counted. The petal was then remounted on a glass slide under a cover glass. This preparation was viewed by transmitted light at high dry power under a microscope supplied with an ocular micrometer. The micrometer was rotated until its line was parallel to the long axis of the epidermal cells in view, whereupon the number of cells included in 50 divisions was counted. The micrometer then was rotated 90 degrees and the count was repeated. Record was kept for each lobe of its position on the flower, and for each flower of its date of harvest and of the plant and branch from which it was removed.

Results of temperature effect studies

General results. Several facts of general interest became obvious during the course of the experiment. Primary among these were the following: (1.) The variation of speckled-sector frequency within temperature treatments was large and showed trends rather than random variation during the course of the experiment. (2.) A larger flower size resulted at the lowest temperature. (3.) A near absence of pollen existed after about forty days of treatment at 50°F. (4.) A markedly different rate of bloom was correlated with temperature differences. At 80°F a branch would produce a mature flower on an average of every 1.4 days, whereas at 50°F an average of 3.2 days elapsed between blooms on a branch.

The experiment was discontinued upon obtaining 21 flowers from each of the 50°F plants. The data analyzed are for the first 21 flowers scored from each plant, although additional flowers were measured from the more rapidly blooming treatments. The

TABLE 2

Temperature treatment means calculated from data for three petal lobes from each of 42 flowers per treatment

Temperature treatments F°	Speckled sectors per cm ²	Epidermal cells per 10 ⁻⁴ cm ²	(Petal width) ² cm ²	Speckled* sectors per 10 ⁴ epidermal cells
50	8.0	7.52	3.1	0.98
60	10.2	8.33	2.9	1.18
70	10.1	9.45	2.6	1.11
80	11.4	9.90	2.7	1.23
Average	9.91	8.802	2.81	1.126†

* = Adjusted to the average number of epidermal cells per unit petal area by using the average error regression line.

† = A minimum estimate of somatic mutation rate per 10⁴ cells is obtained by multiplying the tabulated value by 8.

TABLE 3

Summary of mean squares and significance levels obtained from analyses of variance of data for three variates from controlled temperature experiment

Source	d.f.	Speckled sector frequency	Epidermal cell population density	Petal lobe width
Temperature treatments	4	774.2†	1,932.0†	0.47†
Manual vs. automatic	1	2,197.1†	41.9 n.s.	0.01 n.s.
Among 4 automatic	3	299.9†	2,562.0†	0.62†
Linear regression on temp.	1	769.5†	7,498.6†	1.48†
Deviations from regression	2	65.1 n.s.	93.7*	0.20†
Petal lobes	2	326.0†	10.9 n.s.	0.32†
Lobe 1 vs. lobes 2 + 5	1	651.5†	—	0.64†
Lobe 2 vs. lobe 5	1	0.5 n.s.	—	0.00 n.s.
Harvest orders	20	32.8 n.s.	119.9†	0.022†
Treatments × orders	80	104.4†	78.0†	0.026†
Remainder	523	—	25.6	0.0087
Treatments × lobes	8	27.6 n.s.	—	—
Lobes × orders	40	12.7 n.s.	—	—
Treatments × lobes × orders	160	10.1 n.s.	—	—
Residual	315	40.1	—	—

* = Significant at five percent level.

† = Significant at one percent level.

n.s. = Not significant at five percent level.

21 orders represent order of harvest and have the significance of flower blooming sequence or relative node position. Each datum is classified therefore for lobe position, order of bloom, plant number, and temperature treatment.

Figure 3 shows flowers of variegated-3 harvested from the four constant temperature treatments 52 days after beginning treatment of the clonal cuttings. The high frequency of sectoring in the flower from the 80°F treatment is evident. Figure 5 shows a dissected petal lobe of a variegated-3 flower.

TABLE 4

Summary of mean squares and significance levels obtained from two analyses of covariance of data from controlled temperature experiment

Source	d.f.	Speckled sector freq. adj. for covariance with cell population density	Lobe width adjusted for covariance with cell population density
Temperature treatments	4	—	0.1600†
Among 4 automatic	3	115.2†	—
Linear regression on temp.	1	186.9*	—
Deviations from regression	2	73.1 n.s.	—
Petal lobes	2	323.1†	0.3150†
Harvest orders	20	34.6 n.s.	0.0120 n.s.
Remainder + treatments $\times$ orders	602	—	0.0100
Treatments $\times$ orders	80	103.0†	—
Remainder	522	28.3	—
Regression treatment means <i>vs.</i> average error regression	1	161.4*	0.45†

* = Significant at five percent level.

† = Significant at 1 percent level.

n.s. = Not significant at 5 percent level.

The data are summarized in tables 2, 3 and 4, and in figures 6, 7, 8, 9 and 10. These data indicate an effect of temperature upon the frequency of sectoring. This sectoring variable is of primary interest, however its interpretation depends upon simultaneous consideration of variation in epidermal cell size and petal size. These analyses are presented in the following sections and their interrelationships are considered further in the later interpretation.

Temperature treatment effects. Figure 6 shows the regressions on temperature of the three variates: (1) speckled sectors per unit petal area, (2) epidermal cells per unit petal area, and (3) petal area. In addition is shown the regression of speckled sector frequency on temperature after adjusting to the mean population density of epidermal cells. The ordinate values are represented as a percent of the experiment mean for each variate in order to facilitate comparison among the trends. Sectoring frequency and cell population density increase with temperature, whereas petal size decreases.

The temperature treatment means are presented in table 2. The values for sector frequency are shown in units of sectors per square centimeter. Cell size or number of cells per unit length was used to give an estimate of epidermal cell population density expressed as cells per unit petal area. This was accomplished by multiplying together the number of cells per 50 ocular micrometer divisions measured along a line *parallel* to the cells' long axes (cell lengths per 50 ocular micrometer divisions) and the number of cells per 50 ocular micrometer divisions measured along a line *perpendicular* to the cells' long axes (cell-widths per 50 ocular micrometer divisions). This product estimates the number of cells per 2500 square ocular micrometer divisions, or cells per 0.042 square millimeters. The experiment estimates an average of 8.8×10^4 epidermal cells per square centimeter. Measurements for the third variate, lobe width, were squared to give items which should be a function of lobe area. The

FIGURE 5.—Dissected petal lobe of a flower from clone S5230-5 of variegated *Nicotiana*. The smaller sectors are more frequent near the petal margins.

last column of table 2 contains values for speckled sector frequency adjusted for variation in epidermal cell size.

Table 3 summarizes the results of analyses of variance. Speckled sector frequency shows a highly significant linear regression on temperature with nonsignificant deviations from regression. Strong linear trends correlated with temperature treatments also are evident for epidermal cell population density and petal lobe width. These trends are generally descriptive of the temperature effects despite significant departures from linearity for the latter two variates.

The variation in epidermal cell size may be conceived as capable of affecting both petal lobe width and the frequency of sectors scored per unit petal area. Therefore, the analyses of covariance summarized in table 4 were performed. There exists a positive correlation between the linear regression of speckled sector frequency on temperature and the linear regression of cell population density on temperature. Nevertheless, after adjustment of the former for covariance with cell population density, there still remains a statistically significant linear regression of sector frequency on temperature. Adjusted deviations from this linear regression are not significant. Table 4 also demonstrates that the temperature treatment means' regres-

FIGURE 6.—Regressions on temperature of the three variates measured. The dotted line and the points shown are for the regression of speckled sector frequency on temperature after correcting for variation of cell number in the petal areas sampled.

sion of sector frequency on cell population density is significantly greater than the corresponding average error regression. This relationship is shown in figure 7. Apparently temperature has an effect on sectoring frequency greater than the net effect due to the uncontrolled factors in the experiment which influence cell population density. Similar considerations from table 4 indicate that the temperature effect on lobe width cannot be entirely accounted for by correlated changes in cell population density.

Interaction effects of temperature treatments and orders of harvest. The different orders or periods of harvest do not show significant variation in sector frequency when averaged for all the temperature treatments. This is shown in table 3 which indicates, however, that the corresponding order effects for cell size and lobe width are statistically significant. These latter effects are correlated with each other: Table 4 shows that the harvest order means for lobe width do not differ significantly after being adjusted for covariance with cell population density.

Table 3 shows that sector frequency, cell population density, and lobe width all give a significant interaction of temperature treatments and orders of harvest. These interactions indicate that differences among the temperature treatment responses change during successive periods of harvest. The most pertinent of these interactions, that for speckled sector frequency, is shown in table 4 to remain statistically significant after adjustment for covariance with cell population density.

Figure 8 graphically represents the increase in the regression of speckled sector frequency on temperature during progress of the experiment. For purposes of clarity

FIGURE 7.—Covariance regressions. The regression of temperature treatment means is significantly greater than the average error regression.

FIGURE 8.—Increase in the regression of speckled sector frequency on temperature during progress of the experiment. The points shown are for the third harvest period.

the 21 orders were divided into three successive harvest periods of seven orders each, and regressions were calculated for each of the three order series. The correlation between higher sectoring frequency and higher temperatures increases in successively later times of harvest.

Figure 9 by comparison shows that there is no distinguishable alteration in the

FIGURE 9.—Constancy of the regression of cell population density on temperature during progress of the experiment. The points shown are for the third harvest period. Figures 8 and 9 together indicate an effect of temperature on sector frequency independent of its effect on cell size.

FIGURE 10.—Variation among petal lobes in the average values obtained for the three variates measured.

FIGURE 11.—Influence of the temperature at which the parental clone is cultured upon the frequency of speckled progeny obtained from these clonal parents.

regression of cell population density on temperature during progress of the experiment. This is also true for petal lobe width.

Further consideration of these results is given in the interpretation.

Effects of petal lobe position. The sketch accompanying the graphs of figure 10 shows the convention which was used in designating petal lobes 1, 2, and 5. The graphs display the variation among the different lobe means for speckled sector frequency, cell population density, and petal area. There are more speckled sectors per unit area in lobe 1 than in lobes 2 or 5. Lobe 1 is smaller than lobes 2 or 5. However, the three lobes are not greatly different in their average number of epidermal cells per unit area. The statistical significance of the foregoing observations is shown in table 3. Their implications are discussed in a following section.

Effect of temperature on progeny segregation. While the data were being taken on the somatic sectoring of members of the clone, other flowers of the same plants were self-pollinated to produce seed at each temperature. Seed from several capsules was bulked for each plant, and these seed samples and the resulting seedlings were treated alike. Progeny families from two plants at each temperature were scored for their frequency of speckled individuals. The results for 342 total offspring are summarized in figure 11. The progeny segregations are different from seed set at the different temperatures, giving higher frequencies of speckled offspring from the higher temperature treatments.

Interpretation of temperature effect studies

No estimate of a possible plant by treatment interaction is provided because each cutting was subjected only to one temperature treatment within the time period of the experiment. Thus the interpretation is based upon the assumption that plant randomization was adequately successful in distributing any plant by treatment interactions without order over the different treatments.

Joint consideration of treatment and order effects is essential because of the clear pattern of interaction between these two effects on speckled sector frequency. Figure 8 dramatizes the pattern of this interaction and tables 3 and 4 demonstrate its statistical significance. It is clear that the temperature effect on speckled sectors per square centimeter increases with the passage of time following introduction of the plant to the different temperature treatments. It ranges in an orderly manner from an average *decrease* of 5.7 percent of the mean per three orders of harvest at 50°F through *decreases* of 4.8 and 3.2 percent at 60 and 70 degrees to an average *increase* of 16.4 percent of the mean per three orders at 80°F.

There are two effects confounded in orders: time order of harvest and order of bloom or position on the branch. The analysis would be clear if it could be assumed that the orders of harvest would have been homogeneous in the absence of different temperature treatments. However, the data favor an increase in sector frequency as the rate of growth of the plant increases. This is indicated for the early period of growth of each branch and is suggested also by the increased sector frequency accompanying increased rate of bloom at higher temperatures. If there were such a correlation between sector frequency and flower position on the branch, it would appear that this increase had been overcome and thrown into a net decrease with the passage of time by larger temperature induced decreases at 50, 60, and 70 degrees F. Whereas one would interpret that this node-position increase had been augmented by culture at 80°F, thus accounting for the divergence noted between the 70 and 80 degree regressions cited above.

However, it should be emphasized that despite the compound nature of the order effect, the pattern of its interaction with temperature treatments favors the interpretation that temperature affects the number of sectors scored per square centimeter.

But since the temperature of culture also affects the size of the epidermal cells and thus the number of cells in one square centimeter (cell population density) of petal area, it is important to determine if the number of sectors counted in these areas could be different merely because the areas contain different numbers of cells.

Figure 9 shows that the effect of temperature on cell population density is present very early in the experiment and that the slope of the regression does not differ greatly from one percent of the mean per F degree throughout the experiment. Apparently cell size responds quickly to temperature whereas sectors per square centimeter respond more slowly, as would be expected if the sectors represent cell lineages. It is evident that the temperature effect on speckled sectors per square centimeter reaches a value during the last third of the experiment which cannot be accounted for by the nearly constant regression on temperature of cell population density. This conclusion is supported by table 4, which demonstrates that speckled

sector frequency adjusted to the mean cell population density continues to show a regression on temperature.

Another approach is to compare the average error regression with the regression of treatment means, both of which are graphed in figure 7. The statistical significance of the difference between these two slopes is shown in table 4. Any factor which affects cell size is expected also to affect the number of sectors counted per square centimeter because of the correlated alteration of the number of cells sampled in one square centimeter of petal area. The magnitude of this source of technical correlation is estimated by the average error regression coefficient: a measure of the variation in sector frequency attributable to variation in cell size. A principal source of this variation in cell size within treatments is likely the variation in degree of maturity of bloom at harvest. If temperature were causing a variation in scored sector frequency merely because of an effect upon cell size, then the regression of treatment means should be the same as the average error regression. Only a controlled factor which affects sectoring frequency independently of cell size should give the observed regression differences. This is evidence in favor of a more primary effect of temperature upon sectoring frequency. Reservations are made, however, because the present studies have not eliminated the possibility that the detected temperature effect may be due to differential competition (dependent upon temperature) between epidermal cells following the primary mutational events.

Also, if increased temperature caused the mutational events to occur earlier in development, it could simulate increased frequency of somatic sectoring in these data because a lower size limit was put on the sectors scored. However, the progeny segregations (fig. 11) for which the sampling procedure does not eliminate products of late mutational events, indicate a temperature effect on frequency independent of timing. But this conclusion is justified only if it is assumed that the different segregations result primarily from temperature influence on gamete proportions of the treated clone, rather than from influence during embryo development of the seed progenies on the treated clone.

It is interesting to note from figure 6 the negative correlation between cell population density and petal area or lobe width. Table 4 demonstrates, however, that all of the increase in petal width accompanying decrease in temperature cannot be accounted for by the correlated increase in cell size. Apparently the longer period of floral development at lower temperatures results not only in larger individual cells but also in more cell divisions to give a larger total number of epidermal cells. Both mechanisms contribute to the larger flower size at 50°F than at the higher temperatures.

Figure 10 and table 3 show that lobe 1 gives a significantly higher speckled sector frequency than lobes 2 or 5. But this cannot be due to differences in cell population density for table 3 demonstrates that the petal lobes do not differ significantly in the latter variate. It should be noted, however, that petal area or width is less for lobe 1 than for lobes 2 or 5, as is shown in figure 10 and table 3. Thus it would appear to follow either: (1) that the intrinsic sectoring frequency of lobe 1 is greater than that of lobes 2 or 5, or (2) that a sampling problem results in more sectors being counted on the smaller lobe. The writer prefers the latter interpretation. Small sectors are more concentrated near the petal margins (see fig. 5) and the square centimeter scoring

area includes more of these marginal sectors when superposed on a small petal than it does when superposed on a large petal.

A similar interpretation cannot be invoked, however, to explain as illusory the measured temperature effect on sector frequency. In such a case the sector frequency response to temperature should be present as soon after initiating treatment as is the petal size response to temperature. The more delayed response observed for sector frequency indicates a component of the response that is influenced by temperature independently of petal size.

It should be noticed also that since the smaller size of lobe 1 is not accompanied by the presence of smaller cells it must be that it is due to fewer cells being present. Thus this inherent size difference in lobes is mediated by control of the number of cell divisions, whereas the temperature-induced size difference in petals has both a cell size and cell number component.

DISCUSSION

Genetic studies of the variegated-1 culture of *Nicotiana* plants (SMITH and SAND 1957) demonstrate that a single major locus, *V*, controls inheritance of the variegation system. These studies also favor the interpretation of segregation for two alleles, v_s and $v_{\bar{s}}$, which undergo relatively frequent changes similar to reversible mutations. The reversals resemble temporary inhibition and release from inhibition of a locus critical to pigment synthesis.

Reversible changes in somatic tissue, with that from v_s to $v_{\bar{s}}$ occurring earlier in development than the reverse change, are consistent also with the variegated phenotypes. Thus, by this interpretation solid-colored tissue results from the presence of the locus in its uninhibited condition, v_s . A change at the locus to its inhibited condition, $v_{\bar{s}}$, in certain cells during somatic development originates sectors of potentially colorless tissue. Such sectoring events occurring at an early stage in development result in larger sectors at maturity than do similar events occurring later in development. However, these sectors are not completely colorless, but contain clusters (and rarely small subsectors) of pigmented cells. This speckled condition is considered to be due to a reverse change at the locus from $v_{\bar{s}}$ to the uninhibited condition, v_s , in certain cells of the sector very late in development. Thus it is proposed that a study of phenotypic variability in these variegated flowers is a study of variation in the frequency and developmental timing of reversible genetic events which alter the function of the *v* locus in somatic tissue. These functional alterations may resemble the "local state" changes at the *H₂* locus in *Salmonella* (LEDERBERG and IINO 1956).

CATCHESIDE (1947) has reported a variegated type position effect on sepal and petal color in *Oenothera*. Translocated dominant loci express themselves during development alternatively as normal dominant and lower grade mutant alleles. Although there is presently no direct evidence for the involvement of a translocation in the *Nicotiana* material, the alternating phenotypic expression in these variegated cultures resembles that in the *Oenothera* case.

DEMEREK (1931) studied the rates of mutation of two genes of *Delphinium ajacis*. In the case of the rose-alpha gene he found the rate of mutation to purple to be relatively constant in somatic tissue during different stages of sepal and raceme develop-

ment. Lavender-alpha had a high mutability early and late, and a low mutability in mid-stages of ontogeny. The variegated-1 (sectorial) phenotype resembles rose-alpha: the sector size and position distributions suggest that the sectors represent ontogenetic cell lineages and that the frequency of the change v_s to v_s is not greatly different during successive stages of development. Alternatively in the variegated-3 (flecked) phenotype the change v_s to v_s is largely confined to relatively late stages of petal development. This difference in developmental timing of the sectoring event is heritable, but its genetic basis is not yet clear.

In this connection it should be observed that McCLINTOCK (1951) has shown a genetic control of the timing of mutational events in maize endosperm. In her *Ds-Ac* system an increase in dosage of *Ac* results in later developmental timing of *Ds* events.

Variegation in the *Nicotiana* cultures appears to result from reversible changes in the functional condition of a genetic locus. The developmental timing and the frequency of these changes are subject to genetic control, but are also critically influenced by environmental temperature.

Environmental influence on variegation in maize has been shown by EYSTER (1926) and by VAN SCHAIK (1955). LEWIS (1953) has demonstrated effects of environmental factors including temperature upon the frequency of rogue tomatoes. Specific temperature effects on *Drosophila* mosaicism have been shown by PROKOFYEVA-BELGOVSKAYA (1947) and by HOUSE (1955). And MULLER (1928) detected a temperature effect upon the frequency of lethals in *Drosophila*. In maize possessing the gene *Dt*, RHOADES (1941) demonstrated a temperature effect upon the frequency of mutations from *a* to *A*. PETERSON (1952) has reported a temperature influence upon a mutable pale green locus in maize, and FABERGÉ and BEALE (1942) found an effect of temperature on an unstable gene in *Portulaca*. The direction of the effect has varied with the material employed in these cases of temperature influence on genic expression or mutation. Similarly in the *Nicotiana* material, the effect of higher temperature is to increase the rate of change from v_s to v_s but to decrease the reverse change.

SUMMARY

Nicotiana cultures are employed in which variegation of flower color is interpreted in terms of reversible "mutations" between the allelic forms v_s and v_s . Differences in frequency and developmental timing of these events have both heritable and environmental components. Several causes of the resulting phenotypic variability are evaluated.

Although speckling intensity increases with age of the flower, different speckled plants are shown to vary in their average frequencies of pigmented epidermal cells. This reflects differences in their rates of somatic change from v_s to v_s , which are consistent with their different breeding behaviors. Similarly, different sectorial plants vary phenotypically in their average frequencies of sectors, thus showing different rates of somatic change from v_s to v_s , which are correlated with different breeding behaviors.

Data are presented which indicate that higher temperatures decrease somatic

speckling but increase sectoring. And for the latter phenomenon a resulting effect on progeny segregation has been detected.

Higher temperatures during development also result in a decreased epidermal cell size and a decreased petal size. Consideration of these variables shows that the temperature treatments give different sectoring frequencies per unit cell population. In addition these data indicate that the temperature induced differences in petal size have both a cell size and a cell number component, whereas the inherent difference in size between particular petal lobes of the same flower probably is due only to cell number.

The distribution of sector size favors that the sectors delineate ontogenetic cell lineages for the petal epidermis.

The distribution of sector position is evidence that these sympetalous *Nicotiana* flowers are developmentally polypetalous.

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