ENVIRONMENTAL AND GROWTH REGULATOR EFFECTS ON THE MORPHOLOGY OF DOUBLE FLOWERING PETUNIA HYBRIDA HORT.

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ABSTRACT

ENVIRONMENTAL AND GROWTH REGULATOR EFFECTS ON THE MORPHOLOGY OF DOUBLE FLOWERING *PETUNIA HYBRIDA* HORT.

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Double flowering in *Petunia hybrida* Hort, is monogenic dominant to the single flower type. The double genotypes *DD* and *Dd* are generally female sterile whereas single types *dd* are always fertile. Natarella (1968) has shown that the sterility in the double genotypes is a result of physical hindrance of carpel development by the numerous petal and stamen initials which occur on the receptacle during initiation and early differentiation. It is desirable to restore female fertility in double genotypes for breeding and genetic purposes and doubleness is also a character which can be analyzed for gene action in the control of floral morphology. Thus, these studies were conducted to determine the influence of growth regulator substances and environmental factors on the *D* gene which determines floral doubleness.

Preliminary tests were conducted with triiodobenzoic acid (TIBA), trichlorobenzoic acid (TCBA), indoleacetic acid (IAA), naphthaleneacetic acid (NAA), estrogen, 5-fluorouracil, phenylboric acid and ethrel. Further studies were conducted mainly with TIBA and IAA; the former at 80, 100, 120 or 140 ppm was found to produce single flower phenocopies in the heterozygous double *Dd* but complete reversion and restoration of female fertility in the homozygous *DD* was not observed. Indoleacetic acid at 25 ppm and above inhibited growth and flowering and when growth resumed no change in flower morphology was observed.

The effect of temperature on pistil formation was studied both in the greenhouse and in growth chambers. In the summer months, the percent of *DD* and *Dd* flowers with reduced and/or malformed pistils was significantly increased. The presence of pistillate structures in *DD* was markedly influenced by the temperature in the growth chamber study; again, high temperature favored pistil development. Several possible modes of action of TIBA and auxin interrelationships on the *D* gene are presented.

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I. INTRODUCTION

Petunia hybrida Hort. is an economically important member of the Solanaceae. The double-flowered character in this species is monogenically inherited, DD and Dd, and is dominant to the single-flowered type dd.

The morphogenetic effect of this gene has been studied by Natarella (1968). He observed that in the single-flowered genotype dd the initiation of floral parts is acropetal, whereas in the double genotypes Dd and DD, the initiation pattern is altered by a proliferation of primordia which differentiate into petals and stamens. In the case of the heterozygous Dd, a few normal pistils are observed in some flowers but none are found in the homozygous DD flowers.

Induction of fertile pistils in the female sterile homozygous double genotype would be a method of maintaining homozygous double inbreds from seed. This is desirable since this genotype, the only one which can be used as a pollen parent for hybrid seed production, is currently maintained by asexual means and infection by tobacco mosiac virus (TMV) leads to a degeneration and loss of valuable pollen production stock.

Modifacation of sex-expression in flowering plants by environmental and hormonal agencies has become a common approach to solve these problems of development of floral organs.

It was the purpose of this study to investigate environmental and hormonal effects on pistil development and flower morphology of the homozygous and heterozygous double, and the single flowered *Petunia hybrida* Hort.

The objectives were to observe changes in flower morphology and pistil formation as affected by greenhouse environment, controlled temperatures, and various growth regulators including triiodobenzoic acid, trichlorobenzoic acid, indoleacetic acid, naphthalaleneacetic acid and 2-chloroethylphosphonic acid, 5-fluorouracil and phenylboric acid.

II. LITERATURE REVIEW

Heslop-Harrison (1963a) proposed a scheme for the ontogeny of a hermaphrodite flower which consists of four stages:

(i)		(ii)		(iii)		(iv)
Apex vegetative	$\rightarrow$	Sequential initiation of primordium	$\rightarrow$	Determination of lateral primordium	$\rightarrow$	Outgrowth of lateral organs

Stages (ii) and (iv), which are the objects of this study, involve the actual initiation and ontogeny of the individual floral organs. It is this stage (iv) of flower development that may be influenced by exogenously applied growth substances such as auxins. He stated that two stages, (i) to (ii) and (iv), of flowering are affected by auxin level. A high auxin level is required for (i), the onset of floral initiation and secondly the auxin concentration promoting stamen growth is lower than that for pistil growth. In some plants this level is susceptible to chemical and environmental control through the auxin balance of the plant. Both IAA and NAA, applied exogenously, are effective in promoting pistil development (Heslop-Harrison, 1957).

He also suggested that: in hermaphroditic species, unisexual flower bearing rudiments of the missing sex, pistil, are a result of differential suppression (stage iv) by the intrusion of the additional stamen primordia before the pistil primordia. Therefore, such unisexual flowers are not committed to a predetermined path of development but may be made to develop characteristics of both staminate and/or pistillate flowers.

The action of IAA on sex-expression in cucumber was studied by Galun, 1962; Ito and Saito, 1956; Laibach and Kribben, 1950; Nitsch et al., 1952; and many others.

Laibach and Kribben (1950) and Nitsch et al. (1952) found that IAA and NAA can induce the formation of pistillate flowers and suppress male flowers in cucumber whereas NAA was more effective than IAA. Ito and Saito (1956) gave a similar report. Galun (1962) reported that floral buds, according to the position on the plant, should be male, but when detached and cultured in the presence of IAA, they developed into female flowers. Smith (1967) reported that auxin induces femaleness in Carex.

Leopold (1961) stated that the most remarkable feature of auxin transport in plants is its polarity. There are many other physiological implications of auxin transport, such as, tropistic movements, apical dominance, and root initiation. These can be useful tools for studying the effect of auxin on the genetics and physiology of flowering and flower morphology. Growth inhibitors and auxin transport inhibitors thus can be the major agents for studying the control of flower development. These effects can be correlated with physiological responses which characterize auxin transport.

Triiodobenzoic acid (TIBA) is readily mobile in plant tissue, accumulates both in the growing tips of roots and shoots and is extremely stable in plants.

Almost all the morphological responses of vegetative plants to TIBA, such as loss of apical dominance (Kuse, 1953), also of polarity of tissue (Keitt, 1966; Niedergang and Skoog, 1956), seem to indicate that auxin levels are altered in the plant (Galston, 1947). TIBA inhibits the secretion of IAA out of the tissue (Hertel and Leopold, 1963; Keitt and Baker, 1967). Kays (1968) applied 100 ppm TIBA weekly on the single-flowered, grandiflora petunia White Cascade and observed a random deletion of stamens, elongation and fusion of petal lobes and elongated pistils. Pollination with pollen from untreated plants resulted in good seed set and these seeds germinated and grew normally. It is suggested that TIBA behaves like a weak auxin, probably by its inhibition of transport of endogenous or added auxin (Keitt and Baker, 1966; Kuse, 1953; Niedergang and Skoog, 1956).

The growth regulating effect of 2,3,6-trichlorobenzoic acid (TCBA) closely resembles TIBA. It is reported that TCBA treatment causes epinasty, malformed and reduced size leaves, distortion of inflorescence, small but regularly shaped fruits and overall reduction in growth of plants (Way, 1964; Cock, Taylor and Jubb, 1965).

2-chloroethylphosphonic acid (Ethrel) has been actively used as a growth regulating chemical in cucumber and many other plants. Its ethylene releasing property in plant tissue has been confirmed (Cooke and Randall, 1968; Warner and Leopold, 1969). Promotion of femaleness in Ethrel treated cucumber plants has recently been reported by Miller, Lower and McMurray, 1969; Putnam, 1969; Robinson, Shannon and Manuel, 1969; Rudich et al., 1969; and Sims, 1969. Other Ethrel responses reported are reduced internode (number and lengths) and permanent or temporary stunting, (Miller, Lower and McMurray, 1969; Robinson et al., 1969), induction of flowering (Cooke and Randall, 1968) and flower bud abortion at high Ethrel levels (Robinson et al., 1969).

Opposing effects of gibberellin and ethylene on plant growth were reported by Bukovac and Wittwer, (1961); Mitchell and Wittwer, (1962); Scott and Leopold, (1967). Gibberellins are known as promoter of maleness in flowering plants (Bukovac and Wittwer, 1961; Peterson et al., 1960). Burg, 1962 and Burg and Burg, 1966, reported that the ethylene responses in plant growth and development are strikingly similar to auxin responses and they suggested that auxin effects may be due to auxin induced ethylene production (Zimmerman and Wilcoxon, 1935). Galun, et al. (1965) reported that auxin content was higher in hermaphroditic cucumber plants than in andromonecious plants, whereas Atsom, et al. (1968) determined that GA is higher in monoecious plants.

5-fluorouracil is a nucleic acid synthesis inhibitor which has been reported by Zeevaart (1962) to affect floral differentiation by blocking DNA multiplication. Galston et al. (1969) suggested that 5-fluorouracil curtailed RNA synthesis without affecting auxin induced growth.

Phenylboric acid has been used as a tool for studying the action of the lanceolate gene in tomato (Mathan, 1965). This chemical stimulates the gene action through induction of an increase in oxidative enzyme activities. Haccius and Messfeller (1961) found that phenylboric acid caused a reduction of petals in Kalanchoe blossfeldiana.

TIBA enhanced the rate of IAA uptake, presumably by inhibiting basipetal transport (Keitt, 1967). Atsom et al. (1967) reported that TIBA inhibits the synthesis of gibberellin (GA).

Love and Love (1945), by applying mammalian female hormones, the estrogen group, in lanolin paste to the leaf axils of male plants, found that this hormone caused a partial suppression of the stamens and promotion of pistil formation. Unfortunately, further investigations along this line were discontinued.

The mode of action of auxin is not known; it seems that the enzymatic controlling mechanism is through a general increase of production rather than a triggering of the production of a particular protein (Galston et al, 1969). One evidence is that TIBA, a sulfhydryl inhibitor, might act as an inhibitor of the enzymatic destruction of IAA (Pilet, 1963). Auxin inhibition is more specific than through the general respiratory scheme (Neidergang and Leopold, 1967).

According to Stebbins and Yagil (1966), morphological expression may be the result of a complex physiological chain reactions triggered by a single gene. They studied the awned and hooded lemma in barley which is known to be controlled by a single gene, hood KK which is temperature sensitive. This gene gives rise to a hooded lemma which bears one or two extra florets on the lemma, the proximal one being inverted morphologically. Probably the hooded gene initiated another differentiation cycle by altering the mitotic rhythm. The first accountable evidence of gene action was the accelerated synthesis of DNA and RNA during the earliest development of the hood. They postulated that the synthesized nucleic acids speed up mitosis, causing decreased cell size and resulting in a change in the orientation of the spindles from lemma axis-parallel to a three dimensional direction. Inverted polarity, they believe, is a hormonal induced action which occurs at the later stage of hood development, and could be the primary action of the hooded genes. Cold temperature ($4.5^{o}C$) and short days applied prior and during the earliest stage of hood differentiation weakened or cancelled the hood expression.

The fact that high temperature lowers the effectiveness of apical dominance may be a result of inhibited auxin transport (Gregory and Hancock, 1955). This evidence may fit into the scheme hypothesized by Heslop-Harrison (1963a), that flower development may be influenced by auxins through environmental factors.

III. MATERIAL AND METHODS

A. Plant Material

Three genetic lines were selected from the fourth inbred generation obtained by self-pollinating a heterozygote in each generation starting with the double-flowering multifora variety Cherry Tart. These lines are MSU-500 single-flowered genotype dd, MSU-499 homozygous double-flowered genotype DD, and MSU-503 heterozygous double-flowered genotype Dd. Plants for research purposes were obtained by vegetative propagation of cuttings from stock plants of these three genotypes.

B. Cultural Procedures

Stem cuttings were treated with Hormodin No. 2 rooting compound, then placed in sterilized sand and watered twice daily. A glass cover and/or bottom heat were used to encourage rooting during the winter months; the cuttings usually were well rooted in two to three weeks.

The rooted cuttings were then transplanted into a 1:1:1 mixture of soil, peat and sand in 2 1/4 inch square peat pots. In two weeks the root system was well developed and the plants were then potted into 4-inch clay pots with the same soil moisture and were automatically watered.

The fertility was maintained by weekly application of N-P-K 20-20-20 fertilizer and 12% EDTA at 5 ppm. Greenhouse temperatures were a minimum of $70^{o}F$ day and $65^{o}F$ night; they varied with the prevailing outdoor temperature. During the winter months, supplemental illumination by 100 watt incandescent bulbs 3 feet apart and 3 feet above the plants was provided from 10:00 p.m.

to 2:00 a.m. to maintain flowering.

C. Experimental Design

Experiments were designed either as a randomized block design or a completely randomized design, of three or four replications and were conducted either in the greenhouse or in growth chambers.

Flowers at anthesis were collected starting four weeks after the first growth regulator application, and the morphology of the pistils was examined. The pistils were classified as follows: (1) normal appearing, (2) malformed and/or reduced, and (3) pistil not present; and were recorded on a per treatment per replication basis (Figure 1A).

D. Growth Regulators

Eight growth regulators were used in different experiments (Table 1). These included 2,3,5-triiodobenzoic acid (TIBA) from Eastman Organic Chemicals; Floratone, the commercial product of the salt form containing TIBA active ingredient from Amchem Products, Inc.; 2,3,6-trichlorobenzoic acid (TCBA); 3,indoleacetic acid (IAA) and 1-naphthaleneacetic acid (NAA) from Eastman Organic Chemicals; 1,3,5(10)-Estratrien-3ol-17-one (Estrogen) from Mann Research Laboratories; 2-chloroethylphosphonic acid (Ethrel) from Amchem Products, Inc.; 5-fluorouracil from Calbiochem; and phenylboric acid from Aldrich.

Growth regulators were applied either by foliar spray or by droplet application. Air pressure sprayers were used for the foliar spray. The

Figure 1. Pistil categories and the effect of chloroethylphosphonic acid on flowering of MSU-499 homozygous double-flowered Petunia.

A. Pistil completely absent in a flower of MSU-499.

B. A pistil of reduced size in a flower of MSU-499.

C. A malformed pistil in a flower of MSU-503.

D. A normal appearing pistil in a flower of MSU-500.

E. Effect of 200 and 1,000 ppm 2-chloroethylphosphonic acid (Ethrel) on flowering plants of MSU-499 and an untreated plant.

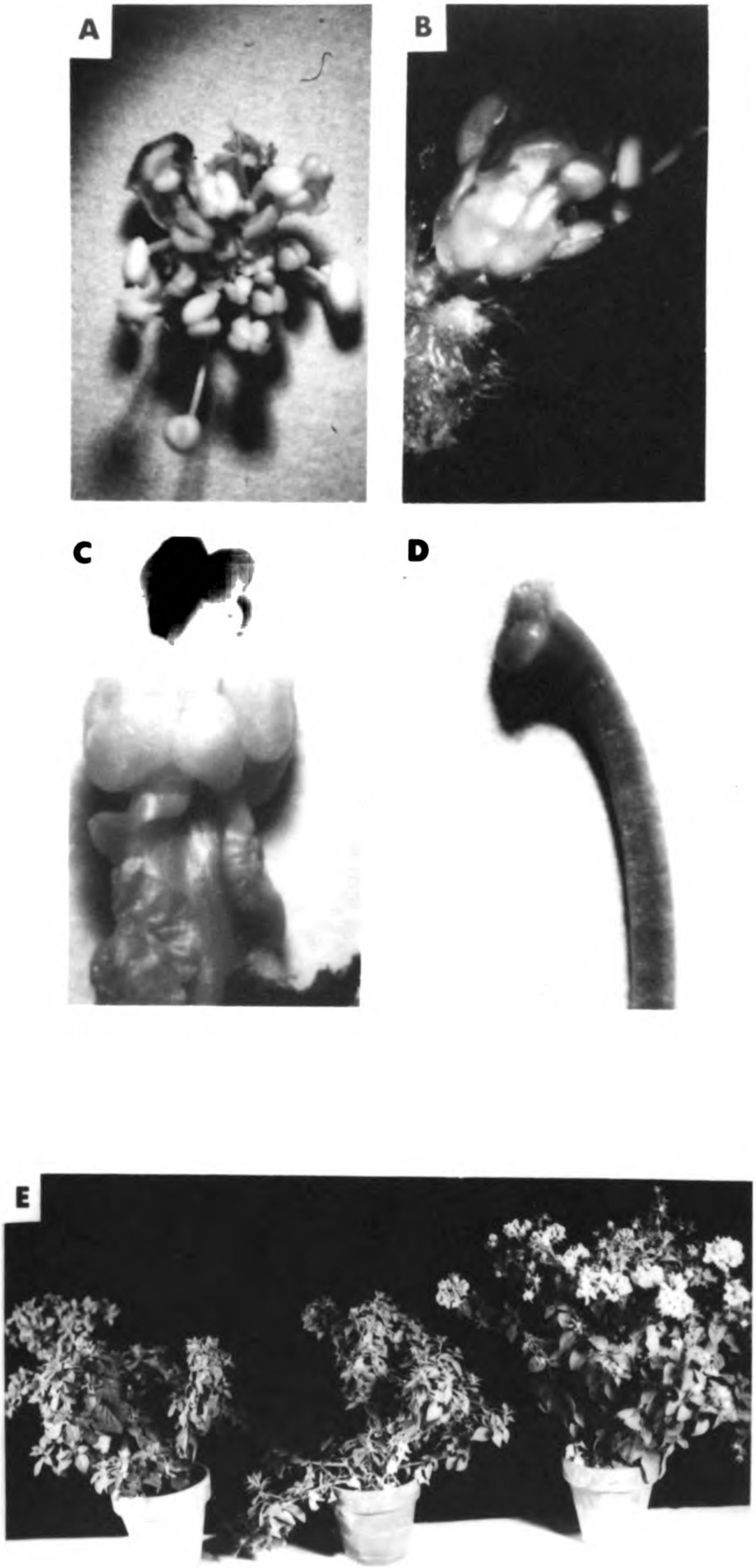
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Table 1. A summary of the material and methods of the growth regulator studies.

Growth regulator	Plant material	ppm	Method	Dates	Reps	Plants/ rep	Flowers collected
TIBA	499, 503, 500	50, 100	spraying	weekly, 10/10-11/20, 1968	3	2	weekly, 11/11-12/10, 1968
TIBA	499, 503, 500	100, 120, 140	spraying	weekly, 1/29-2/26, 1969	3	2	weekly, 2/19-3/20, 1969
TIBA (Floratone)	499	100, 120, 140 200	spraying	weekly, 3/21-4/15, 1969	3	2	weekly, 4/15-5/27, 1969
TCBA	499	50, 100	spraying	weekly, 10/10-11/20, 1968	3	2	weekly, 11/11-12/10, 1968
IAA	499, 503, 500	10, 20, 40	spraying	weekly, 1/29-2/26, 1969	2	2	weekly, 2/19-3/20, 1969
IAA	499, 503, 500	25, 50, 100	spraying	weekly, 3/24-4/15, 1969	2	2	----------
NAA	499, 503, 500	25, 50, 100	spraying	weekly, 4/2-4/15, 1969	3	2	----------
Estrogen	499	10, 20	dropping	every 3 days 11/1 -12/3, 1968	3	2	weekly, 11/11-12/10, 1968
Ethrel	499	200, 1,000	spraying	8/5, 8/12, 1969	3	2	9/20, 1969
5-fluorouracil	499	25, 50, 100	dropping	every 3 days 4/2 -4/20, 1969	3	2	weekly, 4/30-5/26, 1969
phenylboric acid	499	200, 400	spraying	weekly, 7/19-7/30, 1969	3	4	----------

entire plant was thoroughly and evenly sprayed until the run-off point was reached. Or, alternatively, three drops of the growth regulator solution were placed on the meristem.

E. Greenhouse Environment Study

This study was conducted to determine the influence of temperature, and aging on pistil formation.

The study was made from March through August, 1968 and was repeated again in 1969. All three genotypes, MSU-500, MSU-499 and MSU-503, were examined. A randomized block design with 4 replications of 4 plants each was used each year.

F. Controlled Environment Study

The temperatures used in these studies were 80^{o}F DT, 75^{o}F NT and 62^{o}F DT, 57^{o}F NT in two growth chambers, respectively. Fourteen hours of light was maintained in both chambers. Twenty-five foot candle (980 erg/sq cm) light was maintained by 8 incandescent lights, 25 watts each and 6 cool white-influorescent light tubes. There were six plants each of MSU-499 and MSU-503 placed at random in each growth chamber, and they were watered daily and fertilized weekly with 600 ppm N using 20-20-20. The experiment was conducted from November 26, 1968 until February 26, 1969. Later, it was found that growth of the plants in the high temperature growth chamber was suppressed due to rapid transpiration. Therefore, starting on February 5, the temperatures were modified to 75°F DT, 70°F NT and 60°F DT, 55°F NT, respectively. Also, the plants in the high temperature chamber were watered twice daily. As a result, growth of the plants in the high temperature chamber returned to normal.

Another temperature study was conducted with further modifications. Only one growth chamber was used, with ten MSU-499 plants. Throughout the 9-week experimental period, the plants were provided with fourteen hours of light and the light intensity was the same as with the previous study. In the first 3 weeks, the plants were grown under 85°F DT and 70° NT; the second 3 weeks, 65°F DT and 50°F NT; and the last 3 weeks, 85°F DT and 70°F NT, again.

G. Low Temperature Study

A 35°F temperature environment was provided to the MSU-499 and MSU-503 plants to observe the effect of low temperature on pistil formation. There were three replications of two plants of each double-flowered genotype in each treatment. The plants were kept in the 35°F growth chamber during the treatment period and then returned to the greenhouse. The experiment was commenced on February 28, 1969, and was repeated in April with the duration of the low temperature treatment being modified to 5 days and 10 days, respectively.

IV. RESULTS

A. Growth Regulator Studies

Triiodobenzoic Acid:

Preliminary studies with triiodobenzoic acid (TIBA) were conducted in the fall of 1968. In all three genotypes, vegetative growth was retarded and flower size reduced. Lateral branching and flower number were increased as shown in Table 2. Plant height and leaf size were decreased. In some flowers, the inner whorls of petals were modified to petalloid anthers, either in original color or green. In other flowers, the corolla completely resembled that of the MSU-500 single genotype and in the extreme case, only portions of the single whorl of petals appeared (Figure 2A). No concentration effect was observed. A reduction in the number of stamens was also observed for all concentrations of TIBA-treated MSU-499 and MSU-503. Each control flower always had about twenty stamens, whereas in many TIBA treated plants, most of the flowers had only a few stamens in each; some of the stamens in MSU-503 were shortened. In some flowers of MSU-499, the stamens were completely deleted and the central part of the flowers were clustered with greenish petaloid structures. It was observed that the complete or partial disappearance of the stamens was always correlated with a reduction of the petals. Flowers of distorted shape were occasionally found in the TIBA treated plants (Figure 2B). Some of the TIBA treated MSU-500 bore flowers of distorted shape, or had

Table 2. The effect of TIBA treatments on flowering and pistil formation of the MSU-500 single-flowered and MSU-499 and MSU-503 double-flowered Petunia.

	Treatment	Total no. of flowers	% of flowers with reduced and/or malformed pistils	% of flowers with normal appearing pistils
MSU-499	Control	134	1.6	--
	100 ppm	205	8.4	--
	120 ppm	170	12.5	--
	140 ppm	103	6.0	--
MSU-503	Control	52	15.0	--
	100 ppm	103	54.0	3.0
	120 ppm	110	74.5	5.5
	140 ppm	126	43.0	23.0
MSU-500	Control	108	--	100.0
	100 ppm	182	2.7	97.3
	120 ppm	233	5.0	95.0
	140 ppm	132	0.7	99.3

folded corollas (Figure 2A).

Table 2 shows the total number of flowers, percent of flowers with reduced and/or malformed pistils or with normal appearing pistils. In all three TIBA concentrations applied, the highest percent of reduced and/or malformed pistils was found in the plants of MSU-499 and MSU-503 treated with 120 ppm TIBA. In the MSU-503 plants, the greatest promotion of normal appearing pistil development was achieved with the 140 ppm TIBA treatment.

Figure 2. The effect of TIBA treatment on flower morphology of *Petunia hybrida* Hort.

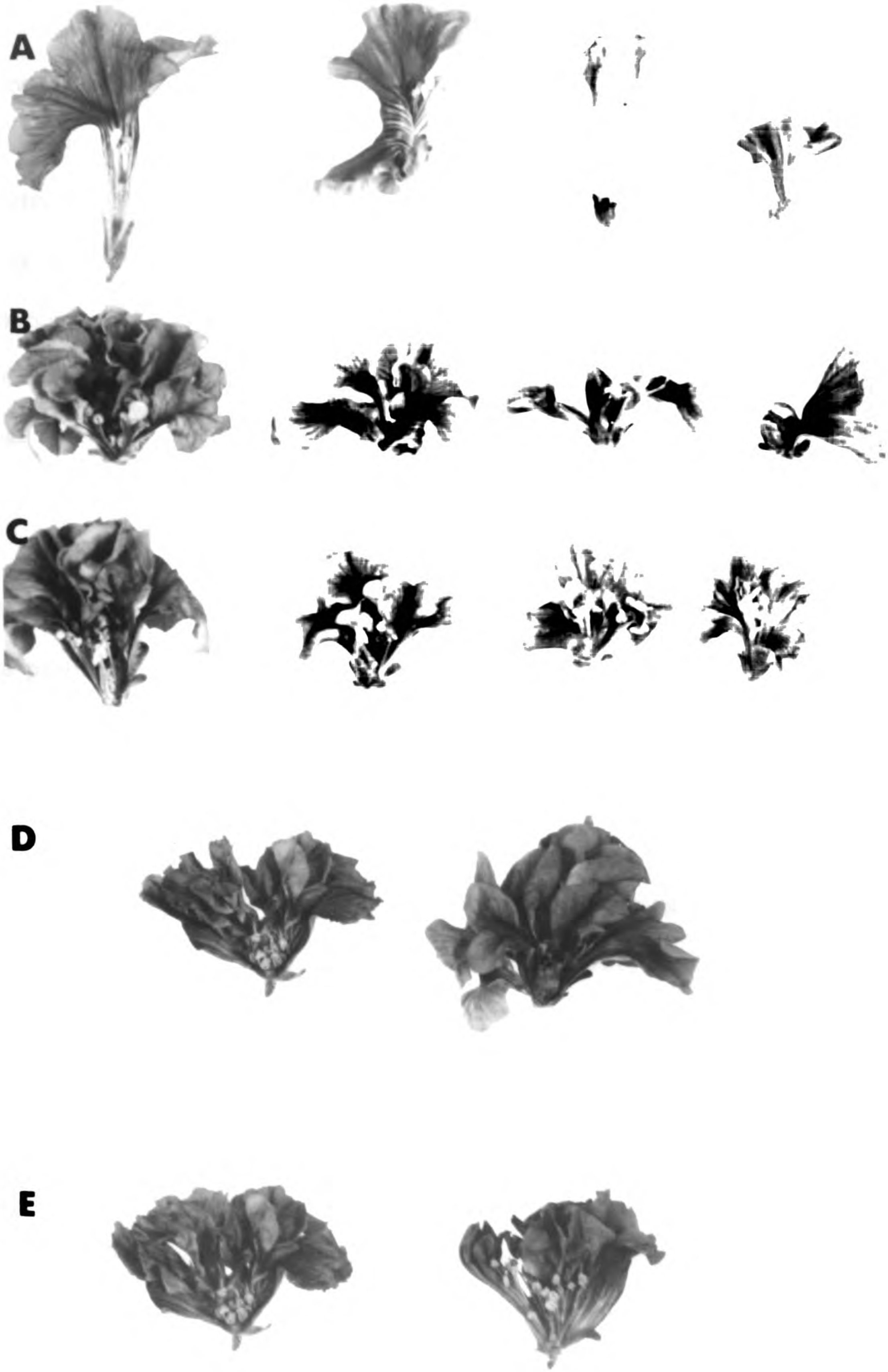
A. A flower from an untreated plant and 3 flowers from 100 ppm TIBA treated plants of MSU-500 showing distortion of flower shape and a reduction in flower size.

B. A flower from an untreated plant and 3 flowers from 100 ppm TIBA treated plants of MSU-499 showing distortion of flower shape, reduction in flower size and floral doubleness.

C. A flower from an untreated plant and 3 flowers from 100 ppm TIBA treated plants of MSU-503 showing distortion of flower shape, reduction in flower size and floral doubleness.

D. A flower from an untreated plant, without pistil, and a flower from 140 ppm TIBA treated plant of MSU-499 showing a thick, short, malformed pistil.

E. A flower from an untreated plant, without pistil, and a flower from 140 ppm TIBA treated plant of MSU-499 showing a reduced size pistil.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Twenty-three percent of the flowers developed normal appearing pistils, whereas only 5.5 and 3.0 percent in the 120 and 100 ppm treatments, respectively. In MSU-500, development of normal appearing pistils was slightly inhibited in the TIBA treatments as compared with the control with 100 percent normal appearing pistils. Again, the greatest effect was found in the 120 ppm TIBA treatment in which only 95 percent of the flowers had normal appearing pistils, whereas there were 97.3 and 99.3 percent in the 100 and 140 ppm TIBA treatments, respectively.

In the second TIBA treatment experiment with MSU-499 homozygous double, a change from double to single flower form was observed as it was in the preliminary study (Figure 3). In some flowers, the number of stamens was reduced and they were modified as such with long, thin and straight filaments instead of the typical short, malformed type commonly observed in control flowers of MSU-499.

In the 200 ppm TIBA treatment, growth and flowering were completely inhibited in replications II and III. All flower buds were aborted.

However, flowering was only partially inhibited in the first replication of which the plant material was older than the other two replications. The percent of flowers with reduced size or malformed pistils in this replication was almost identical with the control plants.

Table 3 shows that there appeared to be a concentration effect on pistil formation in the 120 and 140 ppm TIBA treatment. There were 5.5 and 20

Figure 3. Flowers from plants of MSU-499 treated with TIBA.

A. A flower from an untreated plant and two flowers from 100 ppm TIBA treated plants showing a reduction in flower size and floral doubleness.

B. Two flowers from 120 ppm TIBA treated plants showing a reduction in flower size and floral doubleness, and partial deletion of stamens as compared to a flower from an untreated plant.

C. A flower from 140 ppm TIBA treated plant showing a reduction in flower size and floral doubleness as compared to a flower from an untreated plant.

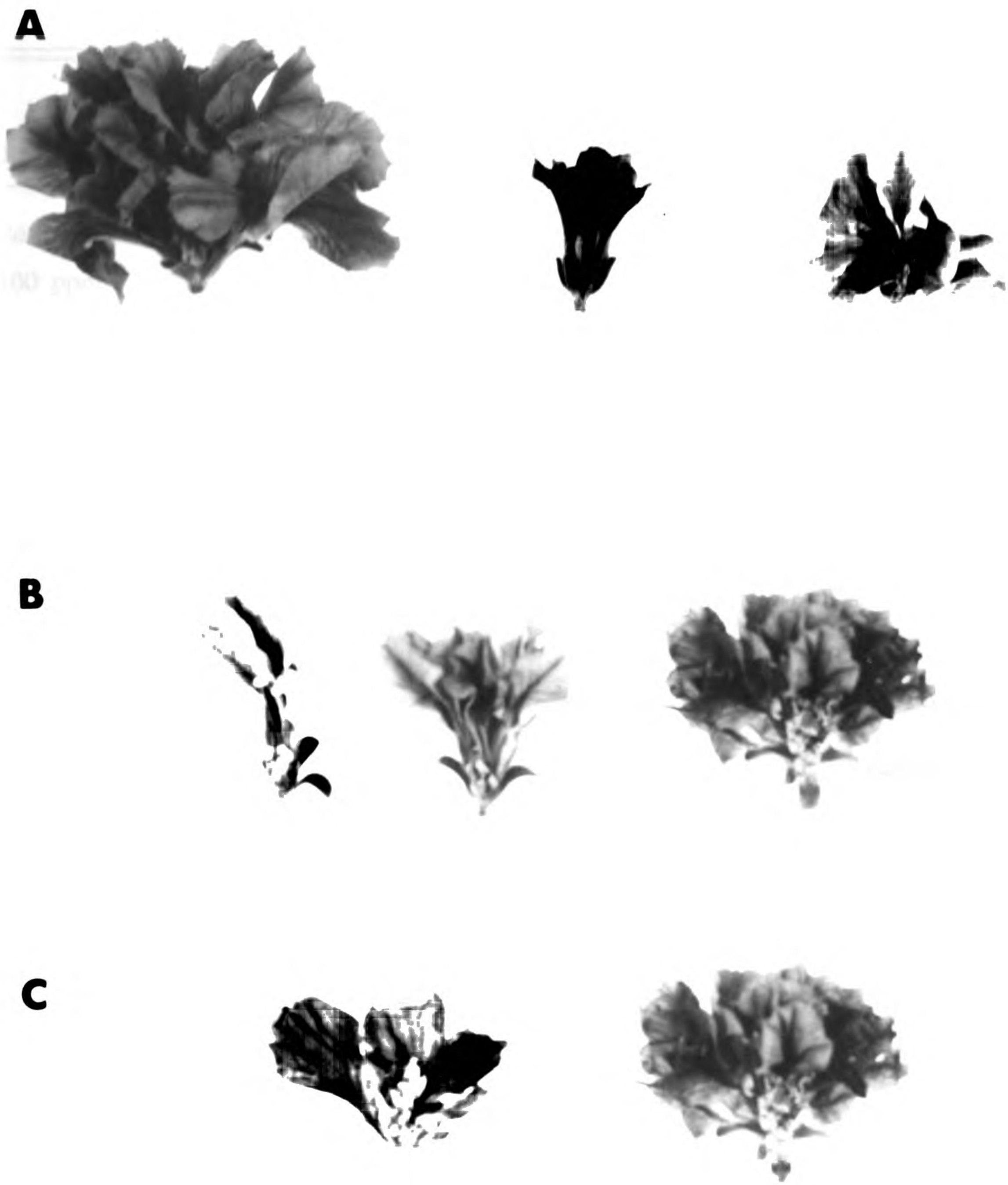

A
B
C

Table 3. Effect of TIBA treatment on pistil formation of MSU-499 homozygous double flowered Petunia.

Treatment	Number of flowers								% of flowers showing reduced and/or malformed pistils
	Rep. I*		Rep. II		Rep. III		Total		
	no**	P	no	P	no	P	no	P	
Control	125	5	72	6	46	1	243	12	4.7
100 ppm	155	3	40	2	24	1	249	6	3.7
120 ppm	93	5	32	1	29	3	154	9	5.5
140 ppm	58	19	14	0	9	1	81	20	20.0
200 ppm	73	3	--	-	--	-	73	3	4.0

* Plants in the first replication were older.

** no = without pistils

percent of flowers developed reduced and/or malformed pistils in these two treatments, respectively. However, the 100 and 200 ppm TIBA treatment showed no effect on pistil formation when compared with the control.

Trichlorobenzoic Acid:

The effects observed in trichlorobenzoic acid (TCBA) treated MSU-499 and MSU-503 plants were similar to the TIBA treatments. Lateral branching and flower number were increased; petal doubleness, flower size and plant height were reduced.

Indoleacetic Acid:

In all three genotypes observed, flowering was suppressed in the 40 ppm and higher IAA treatments. Flowering in plants treated with 20 ppm IAA was delayed three weeks.

In MSU-499, 7 percent of the flowers had reduced and/or malformed pistils at 20 ppm, and also at 40 ppm IAA, which was high when compared with the control, 1.6 percent. Plants treated at 10 ppm IAA resulted in only 2 percent of flowers with reduced and/or malformed pistils.

In MSU-503, there were 39.4, 39.6 and 16.8 percent of reduced and/or malformed pistils in the plants treated at 10, 20 and 40 ppm IAA, respectively; a significant increase compared to the control, 15 percent. A higher percentage of normal appearing pistils developed in the plants treated at 20 and 40 ppm IAA; 3.4 and 7.2 percent, respectively, compared to the 10 ppm treatment. No normal appearing pistils were observed in the control.

Pistil development in all IAA treated MSU-500 plants was not affected.

In the second study with 25, 50 and 100 ppm IAA, flowering was severely inhibited in 25 ppm, and was completely inhibited in the 50 and 100 ppm treatments. Flowering was resumed at a period of four weeks after the last application of IAA. There was no observable morphological change in those flowers.

Table 4. The effect of IAA treatment on flowering and pistil formation of MSU-500 single-flowered and MSU-499 and MSU-503 double-flowered Petunia.

	Treatment	Total no. of flowers	% of flowers with reduced and/or malformed pistils	% of flowers with normal appearing pistils
MSU-499	Control	134	1.6	--
	10 ppm	167	2.0	--
	20 ppm	160	7.0	--
	40 ppm	115	7.0	--
MSU-503	Control	52	15.0	--
	10 ppm	87	39.4	0.6
	20 ppm	73	39.6	3.4
	40 ppm	52	16.8	7.2
MSU-500	Control	108	--	100.0
	10 ppm	247	--	100.0
	20 ppm	113	--	100.0
	40 ppm	121	--	100.0

Naphthaleneacetic Acid:

Normal vegetative growth was severely suppressed in the plants treated at 25, 50 and 100 ppm naphthaleneacetic acid (NAA) and anthesis was completely inhibited. Leaves were large and thick and the plants were dwarfed. Vegetative growth gradually resumed after the NAA treatment was stopped, and anthesis was observed in four-weeks time. The observed growth suppressing effect in NAA treated plants was greater than in the IAA treated plants.

1,3,5(10)-Estratrien-3 ol-17-one:

In the preliminary study of 1,3,5(10)-Estratrien-3ol-17-one (Estrogen), there was no significant morphological change observed in the treated plants.

2-Chloroethylphosphonic Acid:

Preliminary study of 2-chloroethylphosphonic acid (Ethrel) showed that two sprays of 200 or 1,000 ppm Ethrel inhibited growth and flowering completely (Figure 1E). Flowering was resumed five weeks after the second treatment, no morphological change was observed in the flowers.

Other Growth Regulators:

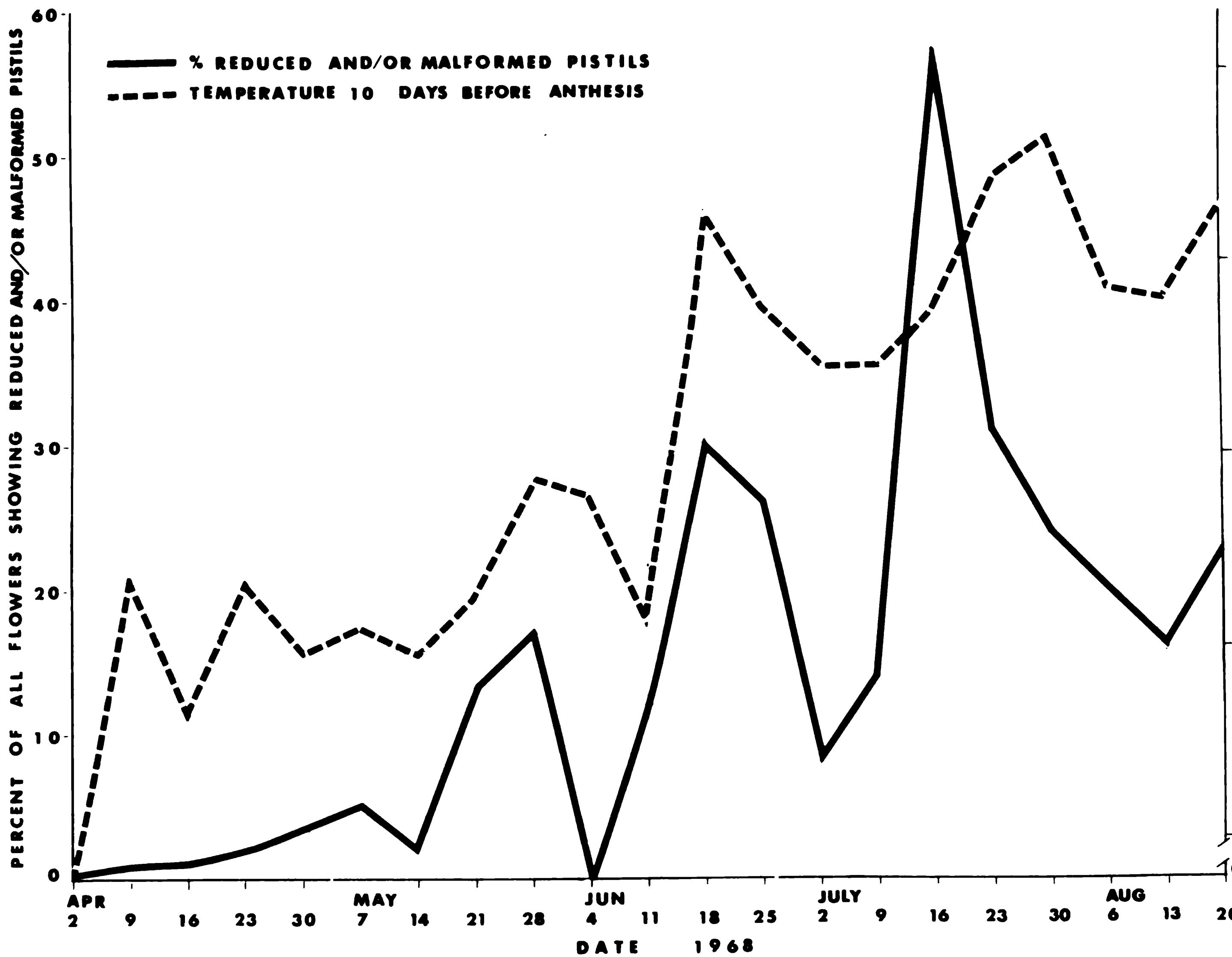
No morphological response was observed in the preliminary studies on 5-fluorouracil and phenylboric acid.

B. Greenhouse Environment Study

The influence of environment on pistil formation of the three genotypes of *Petunia hybrida* Hort. was studied from March through August in 1968 and 1969. The results were plotted as the weekly occurrence in percent for each category of pistil structure of all flowers: with reduced and/or malformed, or normal appearing pistils. The number of flowers without pistil structure was also recorded. No pistils of category 1 were observed in flowers from MSU-499 (Figure 1). The weekly average of the local daily maximum temperature at ten-day intervals before anthesis was also plotted to show the relationship of temperature with pistil formation (Figures 4-7). The weekly total number of flowers collected and the corresponding weekly mean high temperature are listed in Table 5.

In the 1968 study, the percent of all flowers showing reduced and/or malformed pistils in MSU-499 showed a tendency to increase from March to July and then to level off in August. In Figure 4, it can be observed that pistil development was closely related with the charted weekly mean high temperature ten days prior to anthesis. There were four noticeable peaks at which pistil development was expressed as percent of flowers with reduced and/or malformed pistils: 5 percent, 17 percent, 30 percent and 57 percent on May 7, May 28, June 18 and July 16, respectively, and this corresponds to when the temperature ten days before anthesis was also high. The

Figure 4. The influence of greenhouse environment on pistil formation in homozygous double-flowered Petunia, MSU-499, showing percent of flowers with reduced and/or malformed pistils from April to August 1968.



% REDUCED AND/OR MALFORMED PISTILS
TEMPERATURE 10 DAYS BEFORE ANTHESIS
PERCENT OF ALL FLOWERS SHOWING REDUCED AND/OR MALFORMED PISTILS
0
10
20
30
40
50
60
TEMPERATURE °F
0
50
60
70
80
90
APR 2
9
16
23
30
MAY 7
14
21
28
JUN 4
11
18
25
JULY 2
9
16
23
30
AUG 6
13
20
DATE 1968

Figure 5. The influence of greenhouse environment on pistil formation in heterozygous double-flowered Petunia, MSU-503, showing percent of flowers with reduced and/or malformed or normal appearing pistils from April to August 1968.

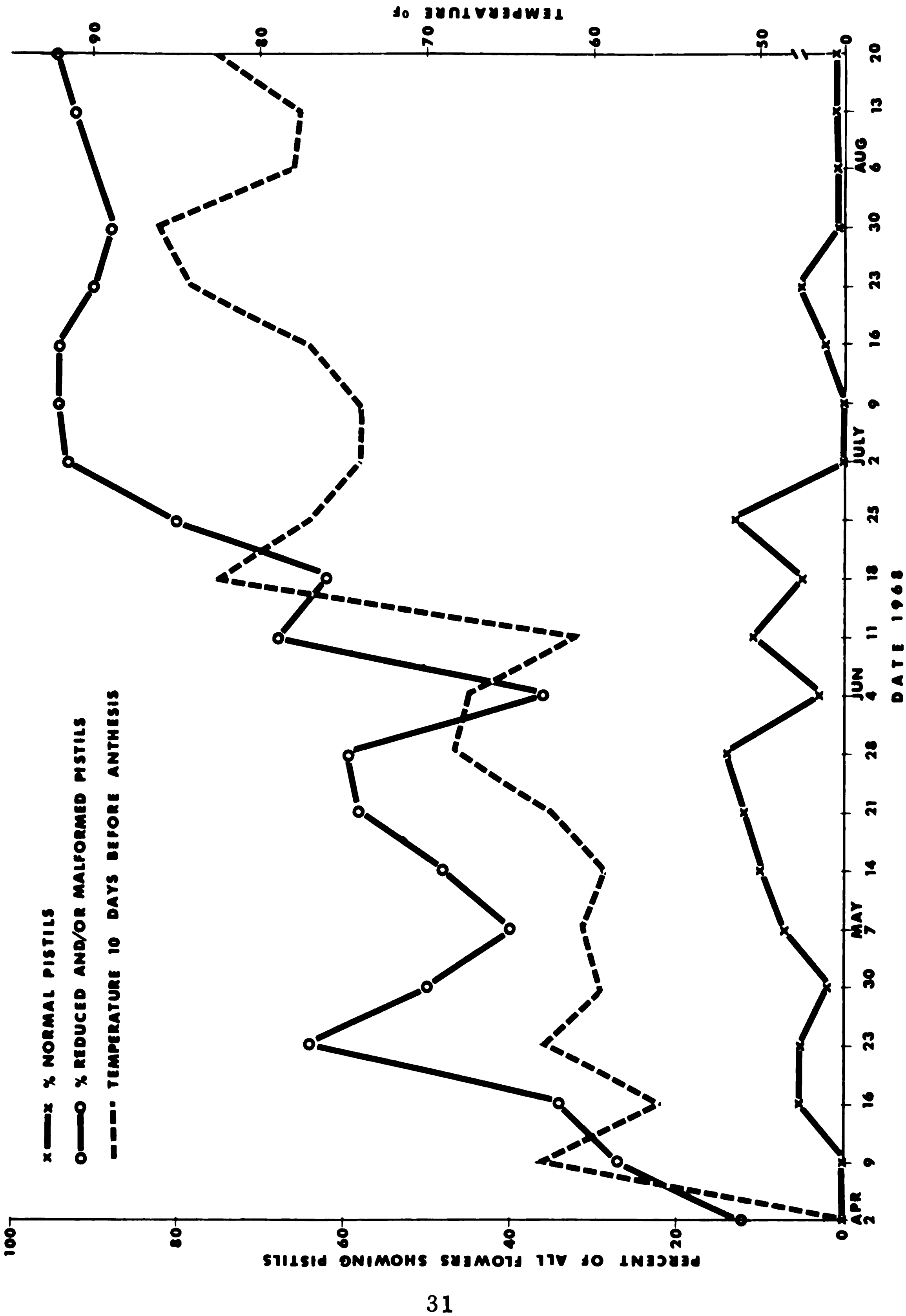
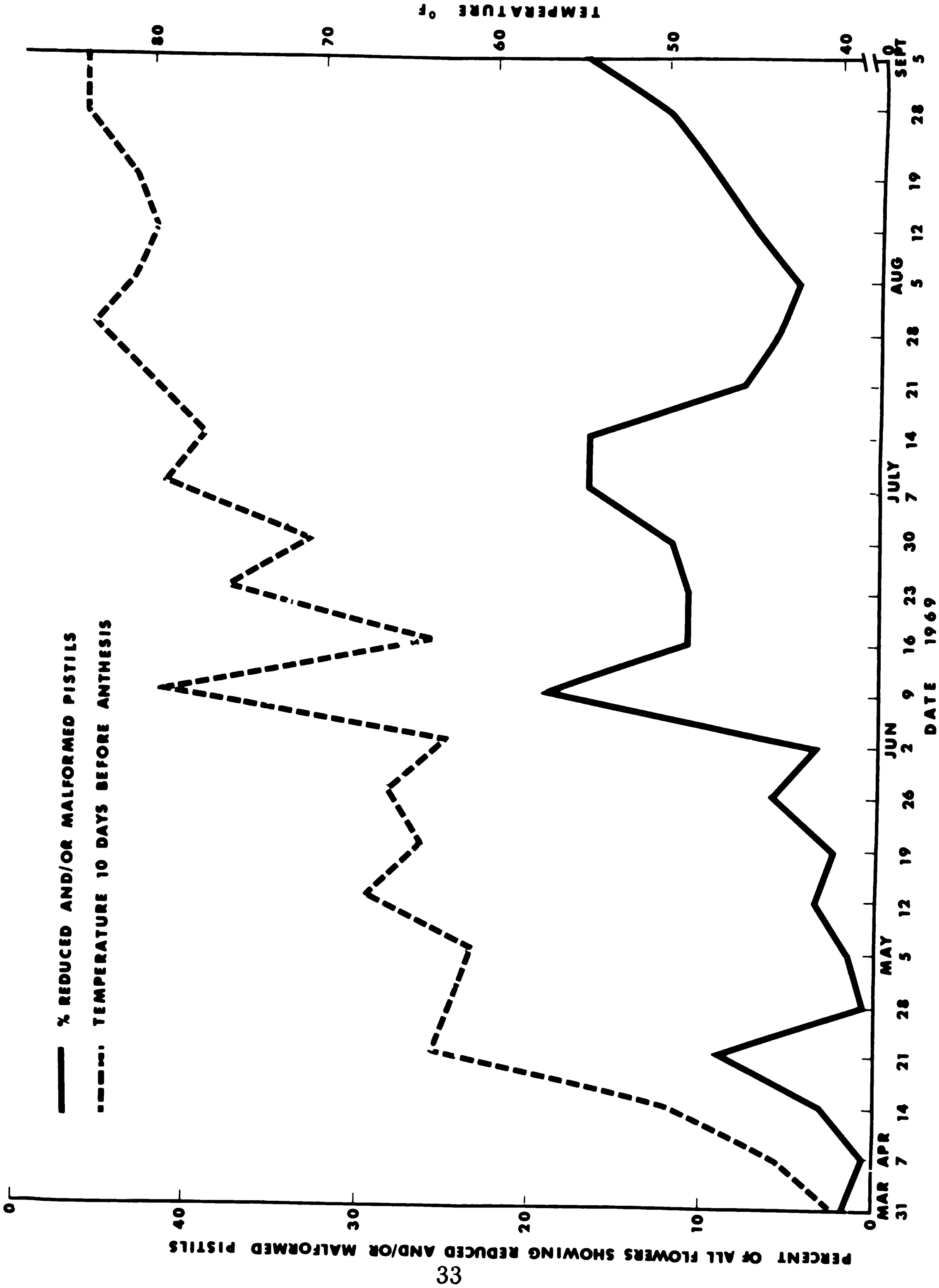

% NORMAL PISTILS
% REDUCED AND/OR MALFORMED PISTILS
TEMPERATURE 10 DAYS BEFORE ANTHESIS
PERCENT OF ALL FLOWERS SHOWING PISTILS
0
20
40
60
80
100
TEMPERATURE °F
50
60
70
80
90
DATE 1968
APR
2
9
16
23
30
MAY
7
14
21
28
JUN
4
11
18
25
JULY
2
9
16
23
30
AUG
6
13
20

Figure 6. The influence of greenhouse environment on pistil formation in homozygous double-flowered petunia, MSU-499, showing percent of flowers with reduced and/or malformed pistils from April to August 1969.



% REDUCED AND/OR MALFORMED PISTILS
TEMPERATURE 10 DAYS BEFORE ANTHESIS
PERCENT OF ALL FLOWERS SHOWING REDUCED AND/OR MALFORMED PISTILS
TEMPERATURE °F
DATE 1969
0
10
20
30
40
50
60
70
80
MAR 31
APR 7
14
21
28
MAY 5
12
19
26
JUN 2
9
16
23
30
JULY 7
14
21
28
AUG 5
12
19
28
SEPT 5

Figure 7. The influence of greenhouse environment on pistil formation in heterozygous double-flowered *Petunia*, MSU-503, showing percent of flowers with reduced and/or malformed, or normal pistils from March to September 1969.

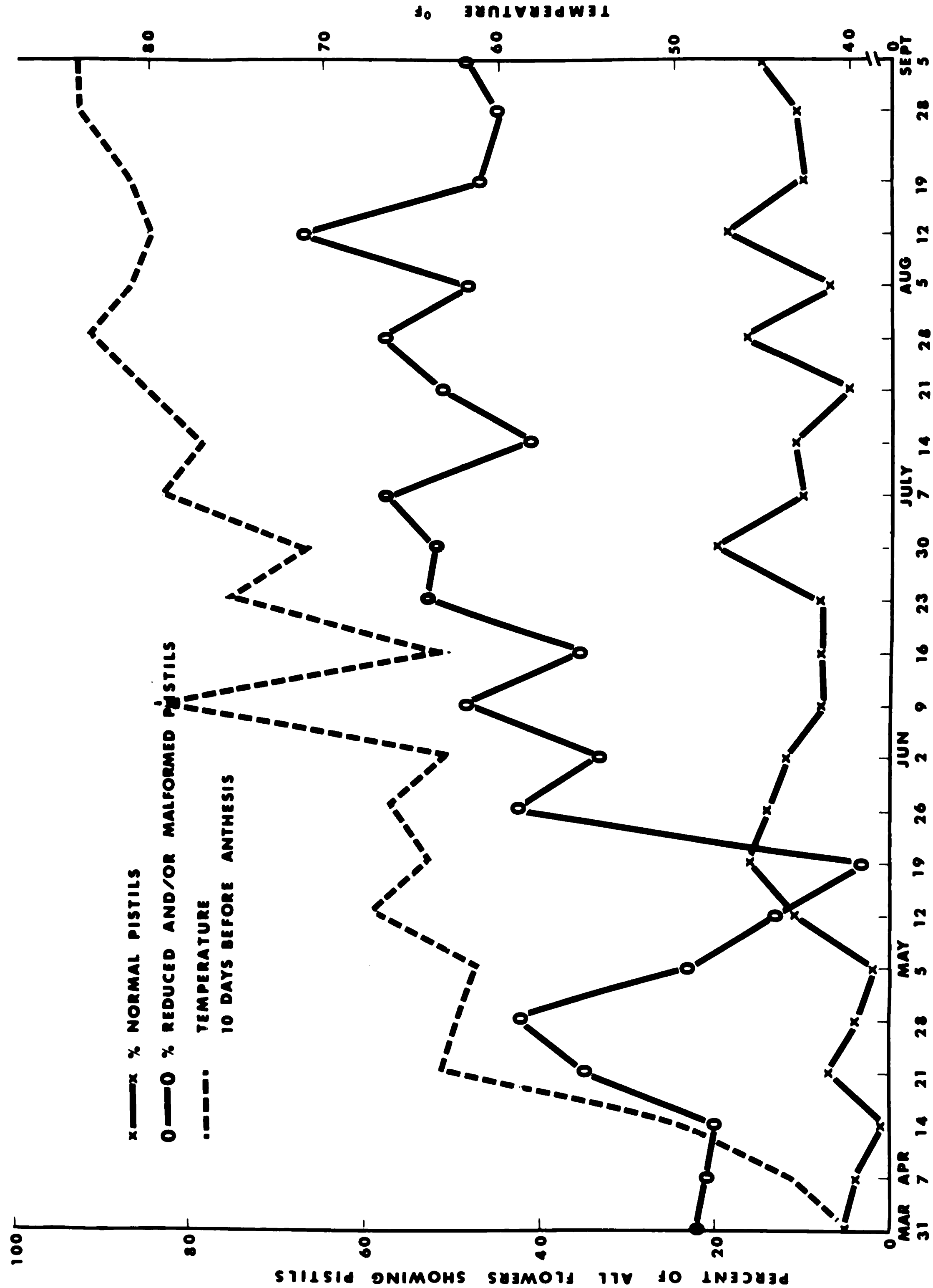

% NORMAL PISTILS
% REDUCED AND/OR MALFORMED PISTILS
TEMPERATURE
10 DAYS BEFORE ANTHESIS
PERCENT OF ALL FLOWERS SHOWING PISTILS
0
20
40
60
80
100
TEMPERATURE °F
40
50
60
70
80
MAR 31
APR 7
14
21
28
MAY 5
12
19
26
JUN 2
9
16
23
30
JULY 7
14
21
28
AUG 5
12
19
28
SEPT 5
DATE 1969

Table 5. The weekly total number of flowers collected and the corresponding weekly mean high temperature in the greenhouse environment study, 1968.

Date	Weekly mean high temperature	MSU-500	MSU-499	MSU-503
April 2	62	295	131	158
9	60	141	90	74
16	65	196	163	184
23	64	254	125	151
30	60	230	103	98
May 7	58	238	195	164
14	69	200	111	129
21	67	200	134	133
28	65	196	194	153
June 4	70	180	151	136
11	87	112	91	74
18	72	168	163	139
25	80	210	248	232
July 2	75	206	99	55
9	79	170	71	16
16	86	208	167	180
23	84	280	115	20
30	78	300	142	180
Aug. 6	81	---	---	---
13	81	170	97	95
20	84	113	118	101

heterozygous double MSU-503, Figure 3, had an increase in the percent of flowers with reduced and/or malformed pistils from March to August, whereas the percent of normal appearing pistils (Category 2) remained low, within the 12 percent, throughout the experimental period, and showed a decline after July. There was an average of 64 percent reduced and/or malformed pistils and 5 percent normal appearing pistils found throughout the period of study. From April to June, the rise and decline of the reduced and/or malformed pistils and of temperature were roughly parallel. In Figure 5 there are three major peaks of reduced and/or malformed pistils: 62, 60 and 69 percent on April 23, May 28 and June 11, respectively. The corresponding temperature ten days before was correlated with these major peaks except for June 11. In July and August the percentage of reduced and/or malformed pistils was high and relatively constant at about 90-95 percent. The weekly mean high temperature of these two months was between 75-87^{o}F, which could be correlated with the high percentage of reduced and/or malformed pistils.

Throughout the experimental period, MSU-503 had a higher percent of flowers with reduced and/or malformed pistils when compared with MSU-499. There was no observed change in the floral morphology of MSU-500 during these studies.

The weekly increase and decrease in flower number of all three genotypes seemed to be parallel with each other. MSU-500 always had a higher flower number than MSU-499 and MSU-503. Flowering markedly declined from July 30 on in MSU-499 and in MSU-500, whereas the decline of flowering in MSU-503 was more gradual. The total number of flowers of the three genotypes seemed to be in contrast with the rise and fall of the temperature at or a few days before anthesis (Table 5).

In the 1969 study, in MSU-499, the percent of flowers with reduced pistils in general was higher in the later months, but not as great as in 1968. Pistil development again was associated with the daily maximum temperature ten days before anthesis, showing four peaks at April 21, June 9, July 7-14 and September 9, successively (Figure 6). In MSU-503, 40 percent of the flowers had reduced and/or malformed pistils and 10 percent had normal appearing pistils. The former figure was lower than 1968, and the latter higher. However, as with MSU-499, the increase in percent of flowers with pistils was not as sharp as in 1968, the highest being 78 percent, but still appeared to parallel the rise and fall of temperature. Development of normal appearing pistils was steady and little affected by temperature (Figure 7).

Similar to the 1968 study, the weekly increase and decrease in flower number of all three genotypes seemed to be parallel with each other. From July 21 on, flower number markedly declined in the two double types while the single type remained steady throughout the period of study. Again, flower abortion and reduction in flower size was observed in July and August when temperature was high (Table 6).

Table 6. The weekly total number of flowers collected and the corresponding weekly mean high temperature in the greenhouse environment study, 1969.

Date	Weekly mean high temperature	MSU-500	MSU-499	MSU-503
March 31	--	186	80	77
April 7	40	264	135	102
14	46	256	138	117
21	63	213	45	126
28	62	264	218	138
May 5	61	375	209	111
12	69	--	142	187
19	65	507	284	125
26	68	425	170	240
June 2	63	552	186	335
9	80	715	327	319
16	62	715	327	319
23	76	621	280	306
30	72	820	324	389
July 7	80	782	153	212
14	79	1113	651	703
21	81	946	217	291
28	83	1008	345	119
Aug 5	82	1136	494	133
12	81	1016	144	122
18	82	1120	237	74
28	85	1210	376	190
Sept 4	85	1130	95	236

C. Controlled Environment Study

MSU-499 plants in the $60^{\circ}F$ growth chamber had 6.2% flowers with reduced and/or malformed pistils, whereas those in the $75^{\circ}F$ DT chamber had 12.4 (Table 7). The percentage of flowers of MSU-503 with reduced and/or malformed pistils in the $60^{\circ}F$ DT chamber and $75^{\circ}F$ chamber were 25% and 57%, respectively. There were 4.3% of the MSU-503 flowers with normal appearing pistils in the $60^{\circ}F$ DT chamber and 10.9% in the $75^{\circ}F$ DT chamber. The results indicated that in $75^{\circ}F$ DT and $70^{\circ}F$ NT, pistil development was increased twofold when compared to the $60^{\circ}F$ DT and $55^{\circ}F$ NT chamber.

Table 7. The effect of controlled environment temperature on pistil formation of double-flowered Petunia.

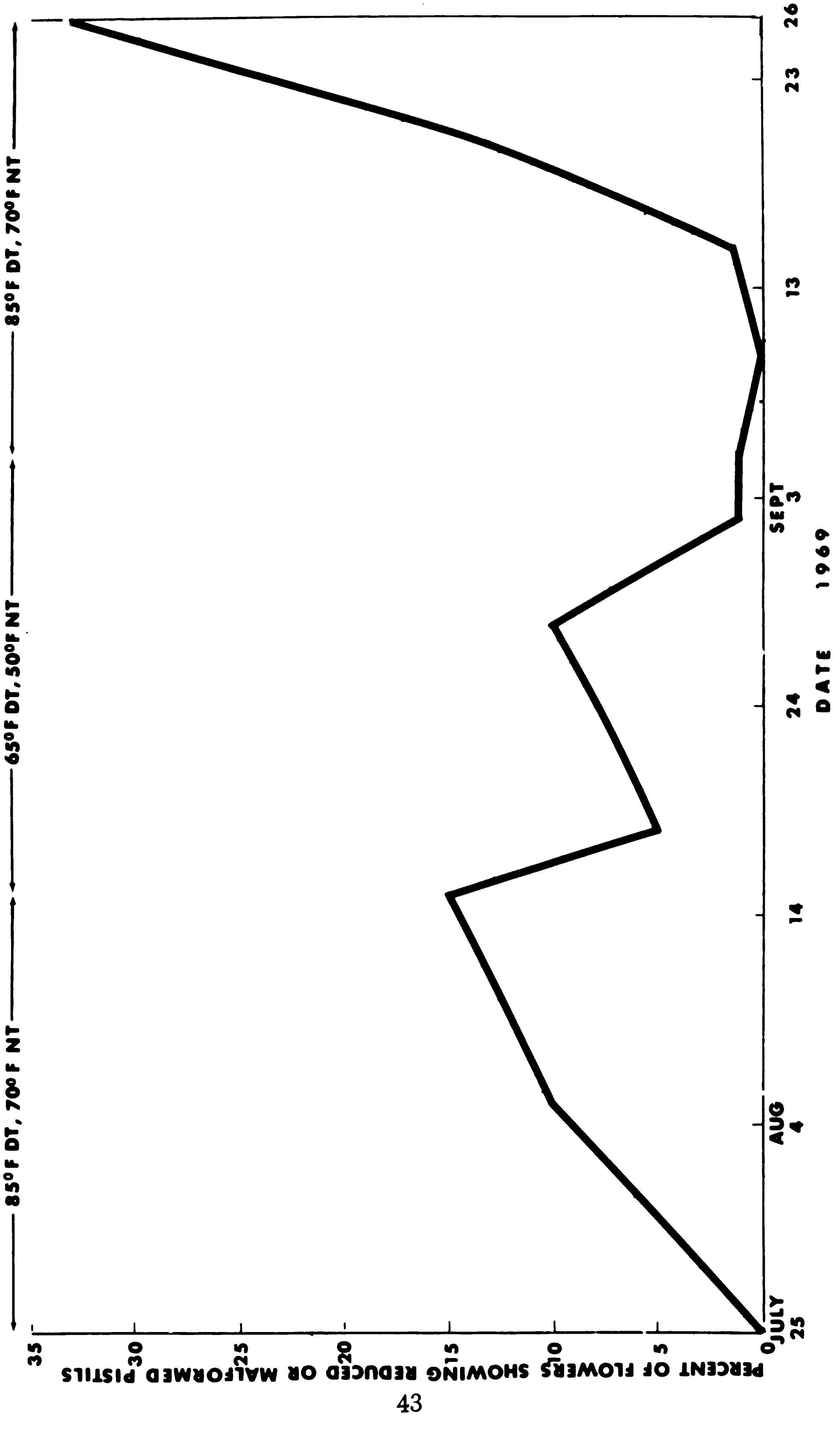
	Temperature	Total no. of flowers	% of flowers with reduced and/or malformed pistils	% of flowers with normal appearing pistils
MSU-499	$60^{\circ}F$ DT, $55^{\circ}F$ NT	113	6.2	--
	$75^{\circ}F$ DT, $70^{\circ}F$ NT	161	12.4	--
MSU-503	$60^{\circ}F$ DT, $55^{\circ}F$ NT	140	25.0	4.3
	$75^{\circ}F$ DT, $70^{\circ}F$ NT	73	57.0	10.9

In the second growth chamber temperature study, flower collecting was started 10 days after the first high temperature treatment and then at the end of the third week. Flowers were collected three times during the second three weeks (low temperature). In the remainder of the study period, flowers were collected at five day intervals. The results are expressed as percent of flowers with reduced and/or malformed pistils and are shown in Figure 8. It shows that pistil development was enhanced starting from the tenth day of high temperature treatment and markedly decreased at the low temperature period, dropped to zero percent at the end of that treatment, then increased at the third stage when temperature was high again, starting at the tenth day of high temperature treatment and increased.

D. Low Temperature Study

The effect of temperature, below the range for optimal growth, on pistil development of MSU-499 and MSU-503 was studied. There was no observable change in flower morphology in this study.

Figure 8. The effect of controlled environment temperature on pistil formation in homozygous double-flowered Petunia, MSU-499, showing percent of flowers with reduced and/or malformed pistils over a period of 9 weeks.


85°F DT, 70°F NT
65°F DT, 50°F NT
85°F DT, 70°F NT
PERCENT OF FLOWERS SHOWING REDUCED OR MALFORMED PISTILS
0
5
10
15
20
25
30
35
JULY 25
AUG 4
14
24
SEPT 3
13
23
26
DATE 1969

V. DISCUSSION

Numerous biochemical and morphological changes occur in the transition of a vegetative apex to a mature flower. Heslop-Harrison (1963a) in a review of sex expression in plants, has defined four stages of development in floral morphogenesis.

(i)		(ii)		(iii)		(iv)
Apex, vegetative	⟶	Sequential initiation of primordia	⟶	Determination of lateral primordia	⟶	Outgrowth of lateral organs

In step (i) the transition from vegetative to floral initiation occurs most likely through some hormonal agent which has not yet been identified. In stage (ii) the apex passes through an orderly sequential initiation of primordia. Stage (iii) is included for diclinous flowers in which unisexuality occurs. In step (iv) the actual ontogeny of the floral organs including differentiation of stamens, petals and carpel occurs. Petunia flowers are hermaphroditic and therefore in this discussion stage (iii) as proposed by Heslop-Harrison (1963a) will not be included. The double-flowering gene D in Petunia has been morphogenetically studied by Natarella (1968). He observed that in the double-flowering genotypes DD and Dd carpel development was physically hindered by the petal and stamen growth that D affects on the receptacle. Since the carpel is centrally located it is actually encased by the petals and stamens during differentiation. Rarely, a normal pistil capable of seed set is observed in Dd genotype flowers, but one has not been observed in DD types. Thus, the production of pistil organs in Petunia is actually not a type of sex expression in the classical sense that the plant is a hermaphrodite, dioecious or monoecious. Rather, a gene D is active during initiation and it has a secondary influence on pistil presence and function.

Control of floral ontogeny by environment and growth substances is usually operative at stage (ii) or (iv) and as Heslop-Harrison (1957, 1963a, 1963b) mentions, with regard to the former stage, the initiation will proceed unless the "treatment is so severe as to arrest growth altogether." Stage (iv) is also readily influenced by growth substances and environmental conditions.

There are numerous reports of auxin influences on floral ontogeny and sex-expression (Galun, 1962; Heslop-Harrison, 1957, 1963b; Ito and Saito, 1956; Laibach, 1952; Nitsch et al., 1952: and Smith, 1967). In this study, of all the growth substances tested, the auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA) and 2,3,6-trichlorobenzoic acid (TCBA) were the only ones that had an effect on flower morphology and pistil development. The former compound was used more extensively since TCBA was more phytotoxic to petunia plants when applied as a foliar spray. TIBA can act as a weak auxin (Niedergang and Leopold, 1957); an SH reagent, will overcome apical dominance (Galston, 1947; Galston et al., 1968), reduce auxin concentration and polarity in callus formation and influence auxin movement in isolated sections, and reduce the IAA content in pea roots (Hertel and Leopold, 1963; Keitt and Baker, 1966; Kuse, 1953, Niedergang and Leopold, 1957; Neidergang and Skoog, 1956;

Thimann et al., 1948; and Winter, 1967). Kiermayer (1961) reported that TIBA simulated auxins in supressing stamen development while not affecting pistil growth in Solanaceous plants and the action was greater when NAA was included. The exact mode of action of TIBA does not at this time appear to be precisely known but a reduction in IAA, increased ratio of immobile to mobile IAA and decreased polar transport all may apply to the results of this study. Certainly the appearance of TIBA treated plants indicated an auxin response. There was a loss of apical dominance, and increase in the number of flowers and an obvious retardation of plant growth. There was also a differential in genotypic response to TIBA.

Single flower phenocopies were observed on heterozygous Dd plants but DD plants did not express a complete reversion to single flowers. The TIBA did not exhibit specific action on double flower morphogenesis. All degrees of flower doubleness from complete expression to single phenocopies were observed and malformed and distorted flowers occurred on single dd genotype plants. Thus, TIBA appeared to act on both stage (ii) and (iv) in floral morphogenesis of Petunia, and possibly only stage (iv) was involved since anatomical examination was not done to confirm if initiation, stage (ii), was inhibited. This finding supports the suggestion that TIBA may act as an auxin transport inhibitor since auxin is known to influence both stages in floral morphogenesis (Heslop-Harrison, 1957). It is noteworthy that TIBA will effectively inhibit the action of D in some cases without causing an inhibition of carpel initiation and development which occur later on in the differentiating receptacle.

A preliminary experiment applying TIBA at 100 ppm and including IAA at 40 ppm in the same solution gave a typical TIBA response. This may be evidence for polar transport inhibition of IAA (Galston et al, 1969; Heslop-Harrison, 1967; Kuse, 1953; Hertel and Leopold, 1963; Thimann and Bonner, 1948; Winter, 1967) but extensive studies need to be undertaken to verify this supposition.

The IAA and NAA studies did not result in a change in flower morphology mainly because vegetative growth and flowering were completely inhibited by the exogenous auxin treatments employed. Here the problem may be one of absorption and/or concentration and frequency in applying the exogenous auxin. A lower concentration and higher frequency of application might be desirable.

Plants with 5-fluorouracil showed no morphological response, probably because this RNA synthesis inhibitor does not interact with the auxin affected system (Galston et al., 1969 and Zeevaart, 1962).

Phenylboric acid has been reported (Mathan, 1965) to induce an increase in activity of oxidative enzymes which induce a change in leaf form to resemble the effect of the lanceolate gene in tomato. This chemical did not affect the morphological expression of the D gene.

Perhaps the sequence of biochemical events that D affects were not altered by phenylboric acid, oxidative enzymes were not involved and also the D gene is active only during differentiation, whereas the lanceolate charactei is one that is manifest in total plant growth and development.

An increased percentage of reduced and malformed pistils in both double types is found in the summer months of the greenhouse study and high temperature condition of the growth chamber studies. Such high temperature effect might be a consequence of cellular division and enlargement, thus increasing growth rate overcomes the physical interference of pistil development.

Gregory and Hancock (1955) noted that high temperatures reduced apical dominance and suggested that auxin transport is inhibited. Strasburger (1900) and Schaffner (1920) have generalized from their extensive experiments that environmental conditions which favor flowering also enhance pistil formation. Since the results of this study indicated that summer conditions favor pistil formation in Petunia, in both double genotypes, therefore, pistil formation may be correlated with the rise of auxin level due to high temperature, either increasing auxin synthesis of inhibiting degredation. The mode of auxin action on pistil formation is not known. According to Natarella (1968), there is no morphological difference in the floral initiation of homozygous and heterozygous flower buds. It is thus suggested that floral development, rather than initiation is sensitive to environmental factors such as temperature through changes in endogenous hormonal levels.

It is known that floral initiation begins approximately three weeks before anthesis in Petunia (Cathey, 1969). From the environmental study, the percent of total flowers with malformed and/or reduced, or normal appearing pistils seemed to be positively correlated with the temperature ten days before anthesis. This is therefore an affect on development and not initiation. The controlled temperature studies in the growth chamber further confirmed this relationship. It is reasoned that since a high temperature environment ten days before anthesis was able to enhance pistil development that a stage of development after initiation was influenced. This major step, most probably determination, may not be as easily affected by environment and/or exogenous growth regulators, as the later developmental stage of the pistil.

The development of normal pistils in the single-flowered type, dd was not sensitive to exogenously applied TIBA or to temperature. Malformed and distorted flowers resulted but no gross differences in the presence or absence of individual parts were observed. Also, the percent of normal pistils in the heterozygous double type was unaffected by temperature, whereas the percentage of reduced and/or malformed pistils in both double types was greatly enhanced by TIBA treatment or high temperature. This suggests that development of a normal pistil involves a different development mechanism which is not affected by the temperature or growth regulator treatments used in this study.

VI. CONCLUSION AND SUMMARY

Growth regulator substances and environmental factors were studied in relation to female sterility which is commonly observed in double-flowered petunias. Of the numerous growth substances tested, triiodobenzoic acid (TIBA) induced single-flowered phenocopies. Complete reversion of double to single types occurred with the heterozygous genotypes Dd, whereas it was only partial on the homozygote DD. Intensive studies previously reported indicated that TIBA can overcome apical dominance, influence auxin movement and simulate auxin suppressing stamen development. The possible action of TIBA is that it acts as an auxin transport inhibitor and thereby causes an increase of auxin level at the synthetic site, the apex. The balance of endogenous auxin level is believed to influence floral organ development; higher auxin levels suppress the action of D in producing double flowers and thus female fertility is restored since the carpel can develop normally.

Summer greenhouse conditions and high temperature growth chamber treatment were effective in inducing a higher percentage of flowers with reduced or malformed pistils in the double types, whereas only very few double flowers in the winter months or at a lower temperature in the growth chamber produce pistillate structures. The results of this study further indicate that a high temperature environment ten days before anthesis enhanced pistil development and thus supports the hypothesis that floral organ differentiation is influenced. The high temperature effect may be related to a change of endogenous auxin level since it has been reported that high temperatures lower apical dominance, thus suggesting that auxin transport is inhibited.

Further work could be developed in several aspects: first, to obtain more detailed and precise knowledge of the endogenous auxin level in relation to the expression of flower types as conditioned by D and d; secondly, the improvement of the methods of applying exogenous growth regulators. A breakdown of each weekly application into seven diluted daily applications is suggested (Cathey, 1969), with the assumption of creating a more steady and homogenous hormonal environment throughout the treatment period. Thirdly, to find out if there are significant interactions between high temperature and TIBA treatment. Fourth, to observe the hormonal control of floral organ morphogenesis by bud culture so as to eliminate at least partly the complicated interaction of the site of floral organ development with hormones from other plant parts, and fifth, to determine whether fertile pollen is produced and seed set is possible on TIBA treated plants.

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