CHARACTERIZATION OF THE GRANDIFLORA-MULTIFLORA GENOTYPES IN PETUNIA HYBRIDA VILM. BY SEED GERMINATION, GROWTH STUDIES AND BIOCHEMICAL CONSTITUENTS

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ABSTRACT

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By

Linda Lee Knowlton

Nine lines of P. hybrida Vilm., divided into three sets, each including a homozygous multiflora gg, homozygous grandiflora GG and hybrid Gg were used for this study. The relationship of seed germination, seedling growth rate, leaf elemental composition, total chlorophyll, sugar and starch content as well as quantitative sugar differences in vegetative leaf tissue to genotype was investigated. Seed germination was consistantly high for the hybrid Gg (92%), intermediate for gg (77%), and low for GG (45%). The fresh and dry weight of 28 day old seedlings was inconsistant but, after 49 days the Gg hybrid was the most vigorous followed by the gg and GG genotypes. No differences were observed in N, P, K, Na, Mn, Fe, Cu, Zn, or Al in alternate leaf tissue of the three genotypes. Differences in Ca, Mg, and B occurred but they were not uniform with respect to genotype or to genotypes within a set. Calcium and Mg were

generally highest in gg and lowest in GG. Boron in one of two experiments showed the same pattern. These differences suggested that there might also be a difference in chlorophyll content since each of these elements is involved in the production of chlorophyll.

The amount of total chlorophyll was determined in mature leaf tissue and in all three experiments it was not specifically correlated to the grandiflora-multiflora genotype. Similarly, sugar and starch content was examined but again there were no significant differences in the three genotypes. Qualitative differences of extractable sugar compounds were observed using thin-layer chromatography. A band, R_f = 0.52-0.56, was resolved in extracts obtained from vegetable tissue of Gg and gg genotypic plants. The extracts obtained from GG plants did not have this band. This band did not co-chromatograph with any of the simple sugar standards used including mannose, arabinose, glucose, galactose, sucrose, and maltose. Gas-liquid chromatography was attempted to further characterize the unknown compound but the samples were insufficient for definitive results.

CHARACTERIZATION OF THE GRANDIFLORA-MULTIFLORA GENOTYPES IN *PETUNIA HYBRIDA* VILM. BY SEED GERMINATION, GROWTH STUDIES AND BIOCHEMICAL CONSTITUENTS

By

Linda Lee Knowlton

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And to my father, I dedicate this and all my other accomplishments past and future. He and my family have given me the moral support I have needed to continue and finally finish my education.

TABLE OF CONTENTS

	Page
LIST OF TABLES	iv
LIST OF FIGURES	vi
LITERATURE REVIEW	1
STATEMENT OF PROBLEM	5
INFLUENCE OF THE GRANDIFLORA-MULTIFLORA GENOTYPES OF PETUNIA ON SEED GERMINATION, SEEDLING GROWTH AND ELEMENTAL FOLIAR COMPOSITION	
INTRODUCTION	7
MATERIALS AND METHODS	10
RESULTS AND CONCLUSIONS	14
INFLUENCE OF THE GRANDIFLORA-MULTIFLORA GENOTYPES OF PETUNIA ON TOTAL CHLOROPHYLL, STARCH, AND SUGAR CONTENT AND ON THE QUALITATIVE SUGAR CONTENT IN VEGETATIVE TISSUE	
INTRODUCTION	30
MATERIALS AND METHODS	33
RESULTS	40
DISCUSSION	51
CONCLUSIONS AND RECOMMENDATIONS	55
LITERATURE CITED	57

LIST OF TABLES

Table		Page
1.	*Petunia hybrida* Vilm. breeding and hybrid lines	11
2.	Seedling fresh and dry wts at 28 and 49 days and plant ht at 49 days for grandiflora and multiflora genotypes in experiment 1.	17
3.	Seedling fresh and dry wts at 28 days and 49 days and plant ht at 49 days for grandiflora and multiflora genotypes in experiment 2	18
4.	Elemental analysis of 49 day old seedlings of grandiflora and multiflora genotypes in experiment 1.	21
5.	Elemental analysis of 49 day old seedlings of grandiflora and multiflora genotypes in experiment 2.	24
6.	*Petunia hybrida* lines utilized in this study.	34
7.	Total chlorophyll, starch, and sugar content of vegetative leaf tissue of plants in experiment 1.	41
8.	Total chlorophyll, starch, and sugar content of leaf tissue of plants in experiment 2 on a fresh-weight basis	42
9.	Total chlorophyll, starch, and sugar content of leaf tissue of plants in experiment 2 on a leaf area basis	43
10.	Total chlorophyll, starch, and sugar content of leaf tissue in experiment 3.	44

Table Page

11. Total chlorophyll, starch, and sugar content of leaf tissue in experiment 3 on a leaf area basis 45

12. R_f values and color reactions of standard sugars and extracts from grandiflora-multiflora petunia vegetative tissue . . . 47

LIST OF FIGURES

Figure		Page
1.	Percent seed germination in relationship to the grandiflora and multiflora genotypes in petunia	16
2.	Relationship of Boron, Magnesium and Calcium content in vegetative tissue and grandiflora and multiflora genotypes of petunia in experiment 1	23
3.	Relationship of Boron, Magnesium and Calcium composition in vegetative tissue and grandiflora and multiflora genotypes in petunia in experiment 2	26
4.	Extraction technique for leaf tissue for quantitative and qualitative analysis of chlorophyll, sugar and starch.	36
5.	Composite thin-layer chromatogram of sugar standards and extracts from grandiflora and multiflora petunia vegetative tissue . . .	49

LITERATURE REVIEW

Petunia hybrida Vilm. is one of the most popular hybrid bedding plants in the United States and the present varieties are the product of a long process of careful breeding and selection from an initial cross between P. axillaris which was introduced in Europe in 1793 by Lamarch and P. violaceae which was also introduced about the same time (Ferguson and Ottley, 1932). Since the original hybridization of the two species, P. hybrida has emerged as an extremely polymorphic species. Mutations have occurred such as the double flower (Scott, 1937), grandiflora (Reimann-Philipp, 1968; Bianchi, 1959) and the apetalous characteristic (Sink, 1973). Although the inheritance of these traits has been well established, little research has been conducted on the biochemical and physiological effect of the grandiflora-multiflora gene locus on petunia growth and development.

Multiflora and grandiflora petunias have been differentiated by their growth habits and flowering characteristics (Ewart, 1963). Multiflora petunias generally have numerous, small flowers having small

calyxes with long, narrow sepals and slender, delicate filaments, whereas grandiflora petunias have a small number of large flowers with large calyxes, short, broad sepals and short, thick anther filaments. Also, grandiflora plants usually have a lighter green foliage than multiflora ones.

The inheritance of the grandiflora-multiflora trait has been investigated by several researchers. Bianchi (1959) concluded from his work with the variety "WeiBe Wolke" that grandiflora and multiflora characteristics were determined by alleles of a single gene, G and g respectively. He also found that grandiflora homozygotes exhibited various degrees of sub-lethality caused perhaps by the apparently reduced chlorophyll content. However, this sub-lethality could not explain deviations in the expected monohybrid segregation. Thus from further back-cross studies, he concluded that this pronounced certation effect arose from a linkage of self-sterility alleles with the alleles determining flower size.

Reimann-Philipp (1962) confirmed Bianchi's findings but found no linkage between the self-sterility alleles and those for flower size. He abscribed the lower number of seeds to the action of a zygotic lethal factor (l). Many individuals of the variety "WeiBe Wolke" showed a linkage between G and l.

Seidel (1962) demonstrated that the G locus in superbissima petunias (tetraploids) and in diploid

grandifloras are the same. Ewart (1963) confirmed the single gene mode of inheritance for the grandiflora-multiflora characteristic and also concluded that lethal and sub-lethal alleles may be closely linked with the G allele resulting in a weak class of homozygoous dominant petunias.

Chlebowski (1967a) found that the gene determining flower size in P. hybrida Vilm. and in P. axillaris, one of its ancestors (Ferguson and Ottley, 1932 and Natarella, 1974), were in fact the same. Later, Chlebowski (1967b) demonstrated that fimbriate borders which is dominant over smooth edges and green petal margins were both weakly linked to the grandiflora trait. However, these linkages like those involving lethality are not universal to the species and are found only in certain lines. Kline (1972) stated that the linkage between G and the lethal gene(s) has been broken in inbred breeding lines used for hybrid seed production.

Because of the inherent weakness of the homozygous dominant (GG) plants, they can be inbred only to a limited degree and Gg and GG genotypes are difficult to distinguish phenotypically. Thus, a method for determining between the homozygous (GG) and heterozygous (Gg) grandiflora plants would facilitate breeding procedures. Testcrossing of F_2 progeny is time consuming and costly; therefore, if a method of determining the genotype could be developed, breeding time could be shortened considerably.

Determining the effect of the grandiflora-multiflora gene on petunia plant growth and development would lead to a better understanding of the physiological action of this gene locus. Using several of the methods used by Natharella and Sink (1973) in determining the physiological effect of a monogenic trait (double-single), an attempt was made to determine such differences due to the grandiflora-multiflora gene and their possible relationship to the phenotypic characteristics of these genotypes.

STATEMENT OF PROBLEM

In the past few years there has been an increased emphasis on genetic research of higher plants aimed at elucidating the manner in which a specific gene mediates a phenotypic trait through changes in biochemical processes.

Studies have been made on the inheritance and phenotypic anatomical differences of the multiflora-grandiflora character in Petunia hybrida Vilm. However the relationship of genotype and plant morphology, nutritional levels and physiology needs to be determined.

THE INFLUENCE OF THE GRANDIFLORA-MULTIFLORA GENOTYPES OF PETUNIA ON SEED GERMINATION, SEEDLING GROWTH, AND ELEMENTAL FOLIAR COMPOSITION

INTRODUCTION

Petunia hybrida Vilm. varieties may be divided into grandifloras and multifloras by their growth habits and flowering characteristics. Multiflora plants generally have dark green foliage and a large number of small flowers with small calyxes and long, narrow sepals and slender filaments; the grandifloras generally have lighter foliage, smaller numbers of flowers with calyxes with short, broad sepals and short, thick anther filaments (Ewart, 1963).

Several studies (Bianchi, 1959; Chlebowski, 1967a; Ewart, 1963; Ferguson and Ottley, 1932) indicated that gradiflora and multiflora characteristics were determined by a single gene, *G* and *g* alleles respectively, and that homozygous *GG* showed degrees of sub-lethality perhaps due to lower chlorophyll content. Bianchi (1959) observed a certation effect in addition to the sub-lethality. He concluded certation arose by linkage of self-sterility alleles with alleles determining flower size.

Reimann-Philipp (1962) found no linkage between the self-sterility alleles and flower size and abscribed the reduced number of seeds to a zygotic letha factor *l* (normal

allele L) which often reduced fertilization by pollen tubes which carried it, so that its function could also be explained as certation. He concluded that the low number of grandiflora homozygotes was due to the sub-lethality of the genotype GG caused by the chlorophyll defect and the linked zygotic lethal factor.

Ewart (1963) also concluded that lethal and sub-lethal alleles may be closely linked with the dominant G resulting in a class of weak homozygous dominant petunias and further suggested that alleles of gene(s) controlling vigor may interact with the large flower-viability gene linkage.

Seidel (1962) showed that the G locus determining large floweredness in superbissima petunias (tetraploids) and in diploid grandifloras was the same. The genes determining flower size in P. hybrida grandifloras and in P. axillaris were found to be the same locus by Chlebowski (1967a).

Green margins of petals in P. hybrida grandiflora and P. hybrida vulgaris (multiflora) also appeared to be linked with the grandiflora character (Chlebowski, 1967b). This linkage, like that involving lethality and fimbriate borders (Chlebowski, 1967b), is not universal to the species but is found only in certain genetic lines. Kline (1972) stated that the linkage between G and the lethal gene(s) has been broken in breeding lines.

Hence, the grandiflora gene is a monogenic inherited characteristic and it controls some as yet unknown physiological action, the type of growth and flowering of petunia plants. The research reported herein was conducted in an effort to better understand the influence of this gene locus on the physiological aspects of seed germination, seedling growth, and elemental content of the vegetative leaves.

MATERIALS AND METHODS

Three sets of P. hybrida Vilm. each composed of a homozygous multiflora (gg), homozygous grandiflora (GG), and their hybrid (Gg), were obtained from Harris Seed Co. (Table 1). Seed germination was determined by placing 50 seeds on Whatmann No. 1 filter paper moistened with 5 ml of deionized-distilled water in a 10 cm petri dish. The dishes were placed in a chamber at 16 hrs light: 8 hrs dark at 26°C and 22°C respectively. The number of germinated seedlings was recorded daily beginning the second day, continued for 8 days and finally at 14 days. In this study, the standard deviation for each line was computed and the means of the three lines of each genotype were compared using the Studentized t test (Steele, and Torrie, 1960).

In the remaining experiments, the nine lines were considered as treatments and then partitioned into three subclasses: genotypes, sets (each comprised of the three genotypes) and the set by genotype interaction. In cases where the genotypes were also significant in the AOV, the mean separation for the entire genotype (the three lines)

Table 1.--*Petunia hybrida* Vilm. breeding and hybrid lines.

MSU Line	Genotype and Set	Description
750	gg-1*	Salmon Perfection
751	GG-1	FII for Salmon Perfection, very weak
752	Gg-1	Hybrid, Salmon Perfection, fringed, glowing salmon
753	gg-2	Ace of Hearts
754	GG-2	FII for Ace of Hearts weak
755	Gg-2	Hybrid, Ace of Hearts, bright red
756	GG-3	FII for Roulette, weak
757	gg-3	Roulette
758	Gg-3	Hybrid, Roulette, red and white bicolor

*gg--multiflora

G---grandiflora

were compared. Tukey's hsd test at the 0.01 level was used for mean separation (Steele and Torrie, 1960).

Plants for experimental purposes were obtained by sowing the seeds on dampened peat-lite mixed with terra-lite. After 2-3 true leaves appeared, 15-21 days from sowing, the seedlings were transplanted into 2.5 X 2.5 cm plastic pots filled with dampened peat-lite mixed with terra-lite. These flats (32 pots/flat) were watered on alternate days with 10 ml per pot of half-strengh Hoagland's nutrient solution (Hoagland and Arnon, 1950) and water. The plants were grown in a greenhouse at 22-27°C and with supplemental light when necessary to extend the day length to 16 hrs to induce flowering.

For growth data, seed was sown at two different times; experiment 1 on 12 April 1972 and experiment 2 on 3 May 1972. The fresh and dry wts and no. of leaves were determined at 28 days from sowing and at 49 days the same parameters and the plant ht were recorded. In experiment 1, 4 plants were sampled at 28 days and 2 at 49 days for each line studied; whereas, in experiment 2, 8 plants were sampled at 28 days and 5 at 49 days. Each plant was considered a replicate.

Elemental analysis was conducted for the vegetative foliage of mature plants according to the procedure of Kenworthy (1960). Petunia seedlings grow in two stages, the vegetative leaves are alternate and when flowering begins, opposite leaves are produced. The alternate

leaves are usually larger than the opposite leaves and at the stage sampled, they are also more abundant; therefore, these were used for analysis. Three replicates when obtainable were analysed for 10 major and minor elements.

RESULTS AND DISCUSSION

The seed germination study comparing the multiflora and grandiflora genotypes appears in Fig. 1. These results are representative of 5 sets, each consisting of the three genotypes, which were observed in a preliminary study. At three days after sowing, the percent of seeds germinated in lines having the same genotype were not statistically different from one another and were thus combined. However, the percent germination in the three genotypes were significantly different from each other; the Gg genotypes had higher rates of germination than either GG or gg. Maximum germination occurred eight days after sowing and the gg genotype was 78.4% of the hybrid Gg while GG was only 46.0% of the hybrid (Fig. 1). These data indicated that inbred multiflora and grandiflora breeding lines lacked the vigor of their respective hybrids. Seed germination of the hybrid appeared to be primarily due to heterosis in contrast to the inherent weakness of the inbred lines.

In both experiments on seedling growth (Tables 2 and 3) lines or treatments were significantly different but not genotypes for 28 days. This variance was due to set

Figure 1. Percent seed germination in relationship to the grandiflora and multiflora genotypes in petunia.

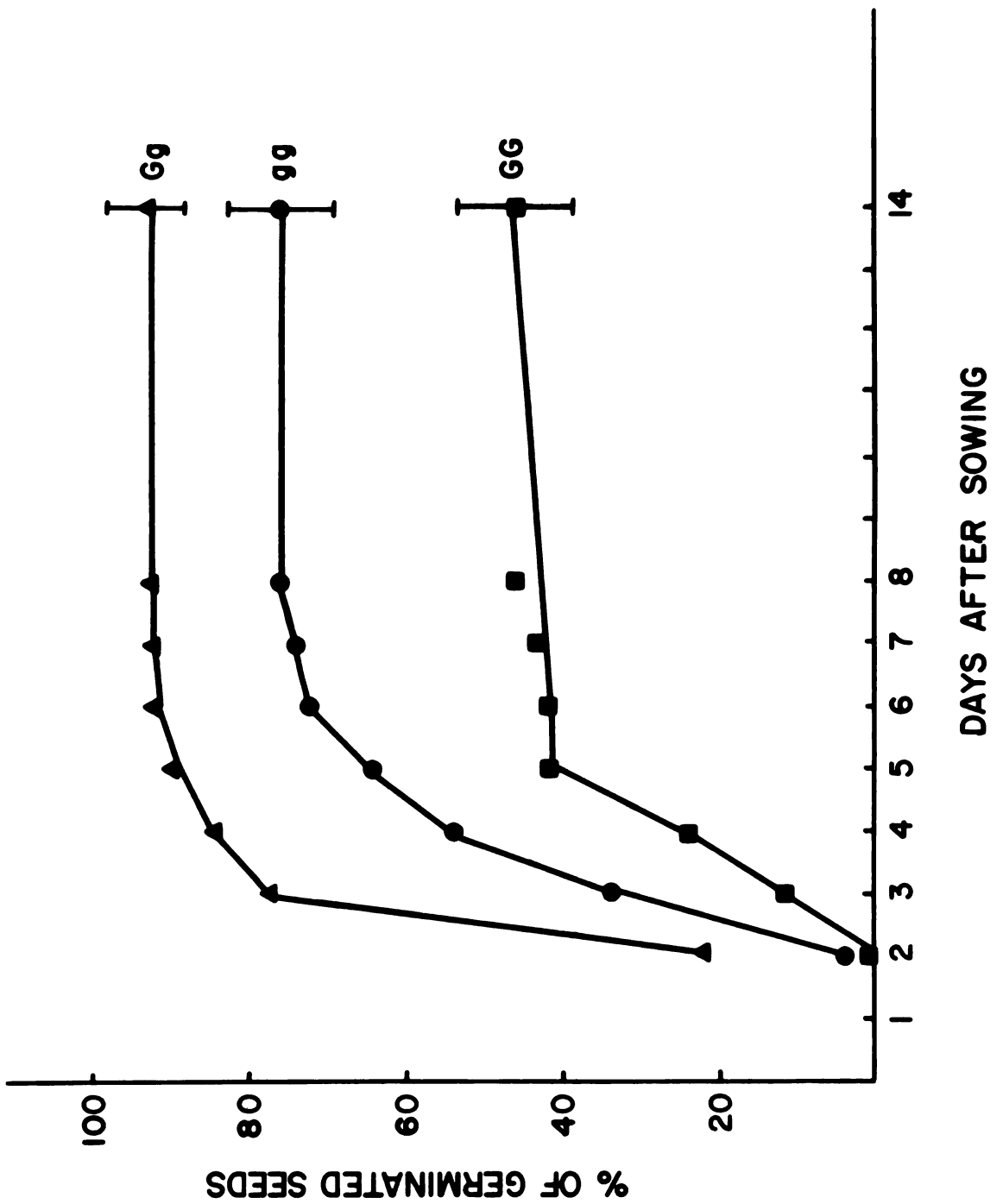

Gg
gg
GG
100
80
60
40
20
% OF GERMINATED SEEDS
1
2
3
4
5
6
7
8
14
DAYS AFTER SOWING

Table 2.--Seedling fresh and dry wts at 28 and 49 days and plant ht at 49 days for grandiflora and multiflora genotypes in experiment 1.

Genotype and Set	Fresh wt (grams/seedling)		Dry wt (grams/seedling)		Plant ht at 49 days** in cms
	28 days*	49 days**	28 days*	49 days**	
GG-1	0.0345a***	2.7340	0.026a	0.1700	4.65a
GG-2	0.1051ab	7.2320	0.0075ab	0.3955	3.25a
GG-3	0.0272a	3.4285 4.4650a	0.0014a	0.1255 0.2300a	5.15ab
gg-1	0.2813 c	9.9455	0.0166 c	0.6345	11.00 d
gg-2	0.1872 bc	9.2130	0.0143 bc	0.6345	5.65ab
gg-3	0.1256ab	7.985 8.9895 b	0.0090abc	0.5240 0.6200 b	7.55 c
Gg-1	0.2174 bc	19.8480	0.0149 bc	1,3725	11.80 d
Gg-2	0.1375ab	15.8790	0.0126 bc	1.2125	11.35 d
Gg-3	0.1881 bc	17.6845 17.8040 c	0.0114 bc	1.2985 1.2900 c	8.40 c
HSD 0.01	0.1603	4.454	0.00095	0.3290	1.83

*Means of 4 plants.
**Means of 2 plants.
***Means having the same letters are not significantly different at the 0.01 level-Tukey's HSD.

Table 3.--Seedling fresh and dry wts at 28 days and 49 days and plant ht at 49 days for grandiflora and multiflora genotypes in experiment 2.

Genotype and Set	Fresh wt (grams/seedling)		Dry wt (grams/seedling)		Plant ht at 49 days** in cms
	28 days*	49 days**	28 days*	49 days**	
GG-1	0.1477a***	6.093a	0.0124a	0.4146a	3.58a
GG-2	0.2105ab	8.494ab	0.0123a	0.5540 bcd	3.22a
GG-3	0.2083ab	5.787a	0.0110a	0.3602abc	5.92 b
gg-1	0.4567 bc	13,936 c	0.0305ab	0.9518 e	12.55 d
gg-2	0.2397abc	12.195 bc	0.0157a	0.6918 bcde	5.62 b
gg-3	0.2125ab	8.149ab	0.0127a	0.5018a	9.44 c
Gg-1	0.4054abc	11.894 bc	0.0299ab	0.7644 cde	13.94 d
Gg-2	0.4881 bc	12.165 bc	0.0335 b	0.7768 de	10.54 c
Gg-3	0.5635 c	12.156 bc	0.0379 b	0.8108 de	13.34 d
HSD 0.01	0.2947	4.230	0.0221	0.2437	1.56

*Means of 8 plants.

**Means of 5 plants.

***Means having the same letters are not significantly different at the 0.01 level-Tukey's HSD.

and/or set by genotype interactions, independent of the multiflora-grandiflora genotype influence. At 49 days, the fresh and dry wts for each genotype were significantly different. The GG genotype was lowest in both parameters followed by the gg genotype which was intermediate and the hybrid (Gg) had the highest fresh and dry wts (Table 2).

In experiment 2, lines were significantly different from one another but this was not due to significance of the genotypes. Thus, the means of the three lines of the same genotype were not combined as in experiment 1 (Table 3). However, in considering each set independently, gg and GG genotypes were higher than the GG lines for fresh and dry wts at 49 days. Plant ht followed the same trends as in experiment 1, Gg being the tallest followed by gg and lastly GG lines (Table 3).

The differences in vigor as indicated by this growth data are related to genotype with F_1 hybrids accumulating up to twice as much fresh and dry wt as either of the parents at 49 days. Since there was no interaction between genotype and set, whether this increase is the result of a more efficient genotype related metabolism or other factors is unknown. The grandiflora inbreds, genotype GG, were the slowest growing as indicated by their reduced rate of increase in fresh and dry wts, due perhaps to the weakness of their genotype by lowered chlorophyll and/or elemental content.

Although significant differences were observed between various lines used in the study for the other elements, only those elements which had significant differences at the 0.01 level for genotypes as well as lines were investigated in further detail. Multiflora and grandiflora genotypes indicated significant differences in the % Mg and Ca in vegetative leaves of mature plants (Table 4). In all sets, GG was lower in Ca than gg and in all but the second set from Gg lines. The % Mg in sets 2 and 3 for the GG genotypes was intermediate between the Gg and gg lines but not statistically different. The Gg and gg lines in sets 2 and 3 were different with gg having the highest Mg content. In set 1, the 3 genotypes did not differ in % Mg (Table 4 and Fig. 2).

When the elemental analysis was repeated with plants grown at a later date, significant differences were observed for the elements Ca and Mg and also for B. In sets 1 and 3, the Gg lines had a greater % Ca than the GG lines; the gg lines had the highest % Ca of the three genotypes and was greater than Gg lines for sets 1 and 2. For % Mg, the gg lines had a higher quantity than Gg lines in sets 2 and 3 and the GG line in set 3. In set 1, there were no differences in % Mg. In sets 1 and 3, B (ppm) was higher in gg than in GG and Gg of set 1 was greater than any GG genotype. There were no differences in set 2 in B content (Table 5 and Fig. 3).

Table 4.--Elemental analysis of 49 day old seedlings of grandiflora and multiflora genotypes in experiment 1.

Genotype and Set	Percent of element on dry wt basis					ppm						
	N	K	P	Ca	Mg	Na	Mn	Fe	Cu	B	Zn	Al
GG-1	4.10	6.51	0.545	2.06a*	1.10ab	3892	80	72	22.6	48.7	77	88
GG-2	3.69	6.92	0.472	2.54 bcd	1.20abcd	6385	118	69	16.7	43.5	75	118
GG-3	4.05	6.64	0.474	2.33ab	1.23 bcd	4943	110	80	18.8	49.8	64	117
gg-1	2.91	4.84	0.361	3.17 ef	1.20abcd	4977	118	61	17.2	60.9	80	98
gg-2	4.44	7.60	0.786	2.94 e	1.30 cd	4211	132	100	25.1	48.4	75	100
gg-3	3.67	7.87	0.606	3.46 f	1.41 d	4950	99	68	26.0	44.4	85	116
Gg-1	2.76	5.77	0.390	2.80 cde	0.98a	3911	95	62	14.9	64.1	75	97
Gg-2	2.96	7.20	0.462	2.47 bc	1.04ab	4605	80	59	15.1	55.1	80	97
Gg-3	3.00	6.99	0.418	2.87 de	1.10abc	3753	87	60	19.0	50.0	82	103
HSD 0.01	n.s.	n.s.	n.s.	0.37	0.23	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

*Means having the same letter are not significantly different.

Figure 2. Relationship of Boron, Magnesium and Calcium content in vegetative tissue and grandiflora and multiflora genotypes of petunia in experiment 1.

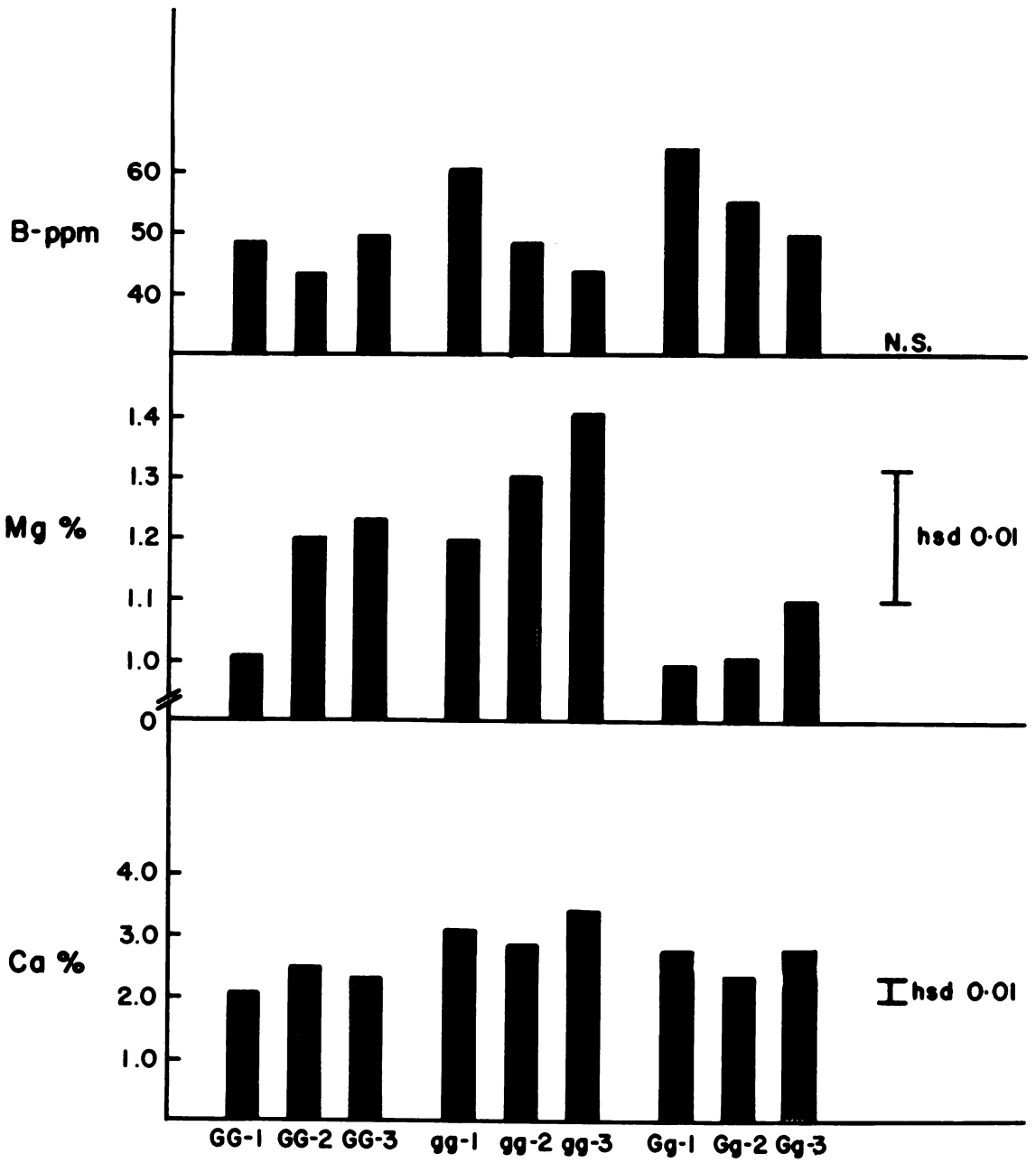
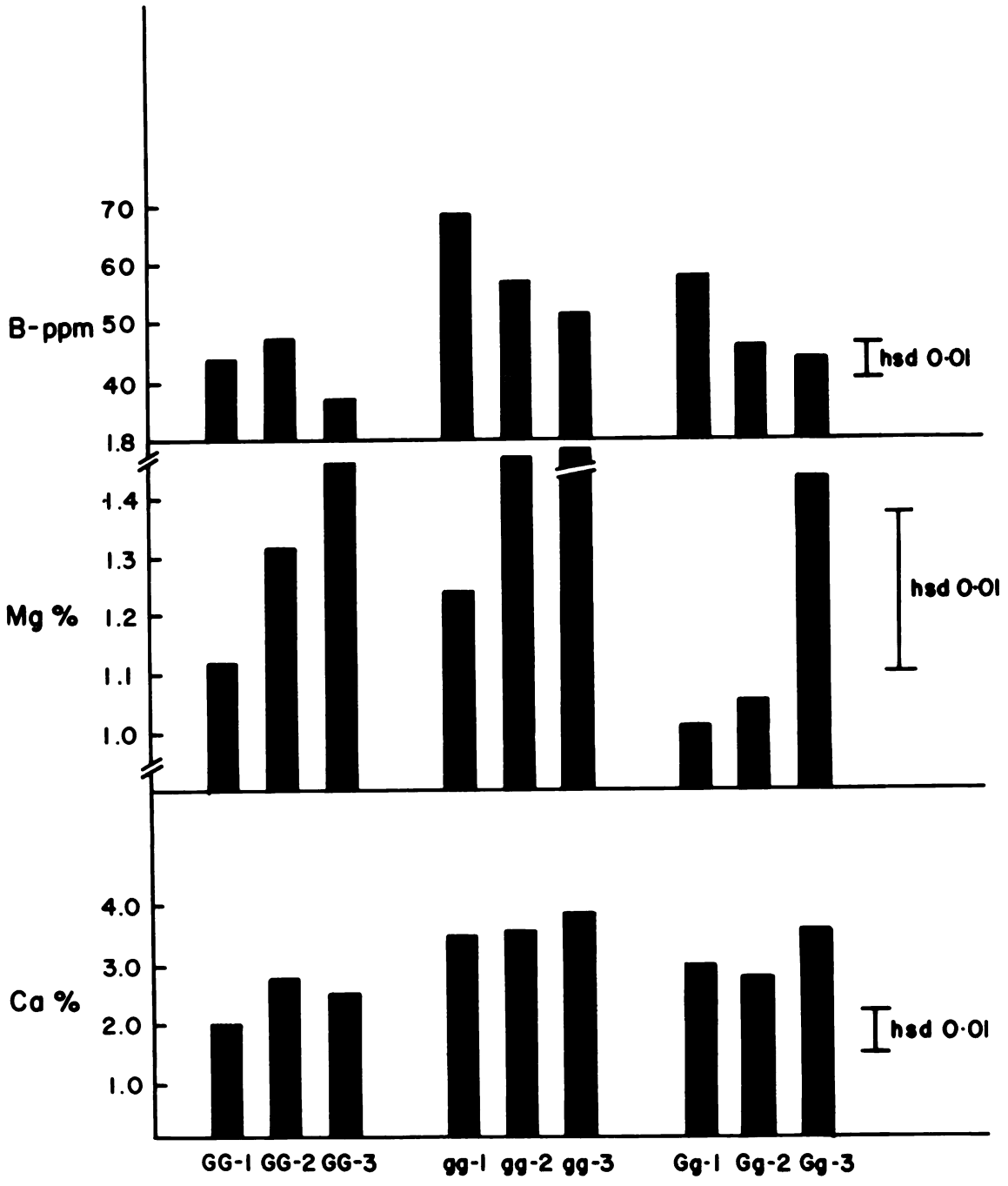

B-ppm
60
50
40
N.S.
Mg %
1.4
1.3
1.2
1.1
1.0
0
hsd 0·01
Ca %
4.0
3.0
2.0
1.0
hsd 0·01
GG-1 GG-2 GG-3 gg-1 gg-2 gg-3 Gg-1 Gg-2 Gg-3

Table 5.--Elemental analysis of 49 day old seedlings of grandiflora and multiflora genotypes in experiment 2.

Genotype and Set	Percent of element on dry wt basis					ppm						
	N	K	P	Ca	Mg	Na	Mn	Fe	Cu	B	Zn	Al
GG-1	3.92	11.72	0.424	2.00a*	1.12ab	3140	75	76	5.0	44.2ab	68	120
GG-2	4.10	9.93	0.265	2.83 b	1.32 bc	5634	73	67	7.0	47.3abc	114	126
GG-3	2.40	5.24	0.291	2.52ab	1.46 c	3738	74	91	2.2	37.3a	108	164
gg-1	3.43	7.11	0.172	3.54 cd	1.24abc	4134	90	63	3.0	68.8 d	90	130
gg-2	2.87	8.34	0.298	3.58 cd	1.47 c	3405	104	69	14.0	57.9 bcd	136	130
gg-3	2.01	5.94	0.246	3.89 d	1.78 d	3669	97	57	1.0	52.3 bc	143	126
Gg-1	2.57	5.08	0.217	3.00 bc	1.01a	2392	73	65	4.5	58.9 cd	122	131
Gg-2	3.93	12.08	0.223	2.77 b	1.15ab	4259	74	63	5.0	46.6abc	157	114
Gg-3	2.24	6.23	0.293	3.62 cd	1.44 c	3120	102	76	9.5	44.8ab	193	202
HSD 0.01	n.s.	n.s.	n.s.	0.68	0.27	n.s.	n.s.	n.s.	n.s.	13.7	n.s.	n.s.

*Means having the same letter are not significantly different at the 0.01 level-Tukey's HSD.

Figure 3. Relationship of Boron, Magnesium and Calcium composition in vegetative tissue and grandiflora and multiflora genotypes in petunia in experiment 2.


B-ppm
70
60
50
40
1.8
hsd 0·01
Mg %
1.4
1.3
1.2
1.1
1.0
hsd 0·01
Ca %
4.0
3.0
2.0
1.0
hsd 0·01
GG-1 GG-2 GG-3
gg-1 gg-2 gg-3
Gg-1 Gg-2 Gg-3

Ca was generally highest in lines with the gg genotype, lowest in GG lines while Mg also followed this pattern. Ca is involved in the maintenance of cell membranes and functions in ion transport especially in chloroplasts and mitochondria. In fact, these organelles are sites of Ca accumulation along with Mg (Epstein, 1972; Larkum, 1968; Noble, 1967; Stocking and Ongun, 1962). Magnesium is a primary constituent of chlorophyll as well as being active as a co-factor in numerous biochemical pathways, especially those involving phosphate transfer. Unlike Ca, this is a moble ion, readily translocated from older tissues to young meristematic regions (Epstein, 1972). Boron is involved in the translocation of sugars from leaves and especially in switching the degradation of glucose to either glucolysis or the pentose shunt (Epstein, 1972). Whether or not any of these differences in ion concn is of physiological importance cannot be determined from the above data alone since in most cases, these elements are ordinarily present in super-optimal concn in the plant.

Thus, differences in Ca, Mg, and B may be related to chlorophyll content and/or metabolic differences. Since in most cases, Mg is especially high in the gg lines, this may indicate that these plants do in fact have a higher concn of chlorophyll than do the GG lines. Whether these differences are the result of the grandiflora-multiflora locus and a direct effect of these alleles, cannot be definitely determined by this study. Studies dealing with

the actual chlorophyll content of these genotypes may partially answer this question and will be pursued in future studies. However, it must also be recognized that these differences may be the result of other genes common to the three sets and which may mask or act synergistically with this particular gene locus especially since these differences in elemental content were not consistent in all three sets. The elemental values obtained for the hybrids do closely resemble those reported by Carlson and Carpenter (1972) for the grandiflora hybrid "Pink Magic" as optimum, and thus the fact that similarities did appear within all of the gg, GG, and Gg lines indicates that regardless of their genetic differences, the basic physiology was very similar.

Although multiflora petunias appear to have greater vigor than grandiflora inbreds as shown, both the germination and growth studies, the hybrids surpassed these in terms of seed germination and early seedling growth. Except for this difference in viability and vigor however, all of the three genotypes appeared to have generally the same basic physiology as indicated by the levels of elements.

INFLUENCE OF THE GRANDIFLORA-MULTIFLORA GENOTYPES OF PETUNIA ON TOTAL CHLOROPHYLL, STARCH, AND SUGAR CONTENT AND ON THE QUALITATIVE SUGAR CONTENT IN VEGETATIVE TISSUE

INTRODUCTION

In the past few years there has been an increased emphasis on genetic research of higher plants aimed at elucidating the manner in which a specific gene mediates a phenotypic trait through changes in biochemical processes. In these studies, there are two aspects to be considered: (1) determination of the inheritance of a particular phenotypic character to be investigated and (2) determine physiological or biochemical processes which correlate with the character.

In Petunia hybrida Vilm., multiflora and grandiflora cultivars, can be differentiated by their growth habit and flowering characteristics. Multiflora petunias generally have numerous, small flowers and calyxes, with long, narrow sepals and slender, delicate anther filaments; whereas, grandiflora petunias generally have a small number of large flowers with large calyxes and short broad sepals and short, thick anther filaments. Also, the grandiflora genotypes generally have lighter green foliage than multifloras.

Inheritance of the grandiflora trait has been investigated by several researchers. Bianchi (1959)

concluded that grandiflora and multiflora plant characteristics were determined by alleles of a single gene, G and g respectively. He also found that grandiflora homozygotes show varying degrees of sub-lethality caused perhaps by the apparently reduced chlorophyll content they exhibited. This sub-lethality could not explain deviations in the expected monohybrid segregation thus, from further backcrossing studies, he concluded that this pronounced certation effect arose by linkage of self-sterility alleles with alleles determining flower size. Reimann-Philipp (1962) confirmed Bianchi's findings but found no linkage between self-sterility alleles and flower size and abscribed the lower number of seeds produced in grandiflora homozygotes to the action of a zygotic lethal factor (l). Ewart (1963) confirmed the mode of inheritance and also concluded that lethal and sub-lethal alleles may be closely linked with the G allele resulting in weak homozygous dominant grandiflora petunias.

Chlebowski (1967) demonstrated that fimbriate borders and green petal margins were both weakly linked to the grandiflora trait. However, these linkages that involve lethality were not universal to the species and were found only in certain lines. Kline (1972) stated that the linkage between G and the lethal gene(s) has been broken in breeding lines. Thus, the mode of inheritance of the grandiflora condition has been well established by these investigations. In a previous study, Knowlton and

Sink (1975) found that the basic physiology of grandiflora and multiflora genetypes was similar based on the levels of elements in vegetative tissue and seed germination studies. This paper will present the results of biochemical studies conducted to determine total chlorophyll composition and total free sugar and starch as they relate to the grandiflora-multiflora genotypes and qualitative evaluation of soluble sugars by thin-layer chromatographic analysis.

MATERIALS AND METHODS

Nine lines of P. hybrida Vilm., divided into three sets, each including a homozygous multiflora gg, homozygous grandiflora GG and hybrid Gg were used for this study (Table 6). The seeds were sown on the surface of moistened peat-lite mixed with terra-lite (Jiffy Mix). After 2-3 leaves appeared (15-21 days from sowing), 16-32 seedlings per line were transplanted into 2.5 X 2.5 cm plastic pots filled with peat-lite mixed with terra-lite. The plants were watered with half-strength Hoagland's nutrient solution (Hoagland and Arnon, 1950) and at all other times with tap water. The plants were grown in the greenhouse with supplemental light used, when necessary, to provide a 16 hour photoperiod to promote flowering. Three groups of seed were sown at different times of the year; experiment 1 on January 23, experiment 2 on May 4, and experiment 3 on September 15. Vegetative leaf tissue was taken after flowering had begun (75-100 days from sowing).

The chlorophyll, sugar and starch extraction technique was modified from that of McCready, et al. (1956). Alternate vegetative leaves were selected at

Table 6.--*Petunia hybrida* lines utilized in this study.

MSU Line	Genotype and Set	Description
750	gg-1*	Salmon Perfection inbred
751	GG-1	Inbred Salmon Perfection, very weak
752	Gg-1	Hybrid, Salmon Perfection, Fringed, glowing salmon
753	gg-2	Ace of Hearts inbred
754	GG-2	Inbred Ace of Hearts, weak
755	Gg-2	Hybrid, Ace of Hearts, Bright red
756	GG-3	Inbred Roulette, weak
757	gg-3	Roulette inbred
758	Gg-3	Hybrid, Roulette, red and white bicolor

*gg--multiflora

G--grandiflora

random from plants of each line and placed on ice. In experiment 1, approximately 1 gram of tissue was used whereas in experiments 2 and 3, 20-1.2 cm. diameter discs were punched out of the leaves and weighed. Three samples were analyzed (Fig. 4) in each experiment for each of nine lines. The tissue was placed in centrifuge tubes containing 10 ml of warm (50-55°C) 80% ethanol. Each sample was ground in a Tembroek homogenizer for 3-5 minutes and returned to the centrifuge tube, the mortar and pestle rinsed into the tube, 2X with 3-5 ml aliquots of 80% ethanol. The tubes were placed in a 50-55°C water bath for 5-10 minutes, centrifuged for 10 minutes at 10,000 X g and the supernatant decanted into a 50 ml test tube. Ten ml of hot 80% ethanol was added and the pellet, suspended and centrifuged as above. The ethanol washing was repeated and the combined supernatants stored under N_2 gas at 2-4°C until the following day.

Five ml of distilled water and 6.5 ml of 52% perchloric acid were added to the pellet in the tube, stirred occasionally for 20 minutes, then 10 ml H_2O was added and centrifugation done at 10,000 X g for 10 minutes. The supernatant was decanted and the residue treated again. After 20 minutes, the residue and solution was washed into the 50 ml tube and the combined sample passed through Whatmann #1 filter paper on a Buchner funnel. The liquid was brought to 50 ml volume, stoppered and stored in the cold (2-4°C) until analyzed for total starch.

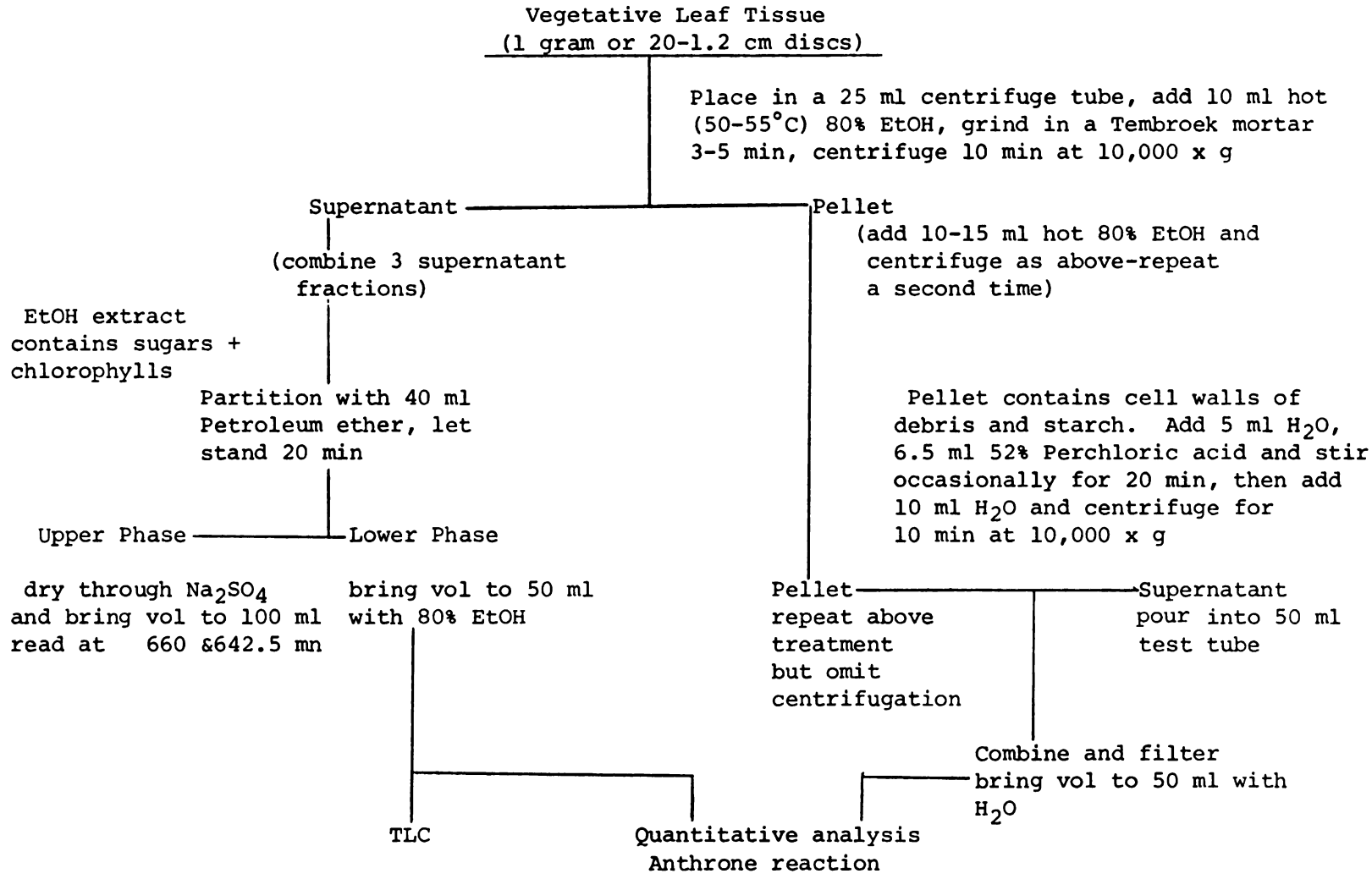


Figure 4. Sampling and extraction procedures used to obtain extracts for anthrone reaction, TLC, and quantitative analysis of chlorophyll, starch, and sugars in vegetative tissue of Petunia hybrida.

To remove the chlorophylls, the 80% ethanol extract was partitioned against 25 ml of petroleum ether. After 20-40 minutes, the lower phase was removed and washed again with 25 ml of petroleum ether. The ether phases were combined, filtered through dry Na_2SO_4, brought to 50 ml, and read on the spectrophotometer at 660 and 642.5 nm to determine the amount of total chlorophyll using a standard formula (Association of Official Agricultural Chemistry, 1965).

The ethanol sugar fraction was brought to 50 ml with 80% ethanol and 10 ml removed for thin-layer chromatography (TLC). The remaining 40 ml were evaporated, brought to 50 ml with distilled water and placed under N_2 in the cold (2-4°C) until assayed.

The anthrone reaction was used to determine sugar and starch content in the ethanol and perchloric acid fractions, respectively (McCready et al., 1950 and Clegg, 1956). In both cases, 1 gram anthrone was dissolved in 500 ml concentrated H_2SO_4. Duplicate aliquots (0.2-0.5 ml) were used for each sample and 5 ml of the anthrone-sulfuric acid reagent was added to each sample aliquot, stirred and placed immediately in ice to stop the reaction. The samples and standard aliquots were heated in a boiling water bath for 7.5 minutes, cooled in ice and the % transmission read at 630 nm on a Bausch-Lomb colorimeter. The standard curve was computed from glucose standards using linear regression equation and least squares

techniques after converting % transmission to O.D. using a conversion table solution of this equation for the X variable by the method of linear calibration where

X = µg glucose equivalents/aliquot and y = O.D.

of the sample or standard was computed (Snedecor and Cochran, 1967).

Thin-layer plates covered with 250 nm of silica gel G (Uniplate) were divided into 12 1.2 cm sections. The sugar standards were prepared as 1 ug/ul concentrations in 95% EtOH. The extract samples were reduced to dryness and brought up to 5 ml with 95% ethanol. Fifty ul of each reduced extract of each line was streaked onto the plates as well as 25-50 ul of the standards. The plates were run ascending in a solvent system of Acetone: Water: Chloroform: Methanol (75: 5: 10: 10) to about 15 cm (Lewis and Smith, 1969). After air drying, the plates were sprayed with a phenol-sulfuric acid reagent (Lewis and Smith, 1969) and heated 10-15 minutes at 100°C. The chromatograms were also observed under long and short UV light and the color and position of each spot recorded.

A difference in TLC patterns for the gg and Gg lines versus GG in the center (R_f .49-0.53) of the plates was identified; new plates were run in each experiment and the middle third (R_f 0.33-0.67) of these plates scraped off and eluted several times with 80% MeOH. These samples were then dried and the residue treated with TMS

(trimethylsilyl) to prepare ethers for gas-liquid chromatography (GLC). In all cases, duplicate GLC runs were made (Farshtchi and Moss, 1969). The column used was U-shaped glass 6 ft X 15 mm in diameter, packed with 2% OV-1 on 80-120 mesh Chromosorb. Temperatures were: Detector 225°C, injector 225°C, and column 160°C. Nitrogen inlet pressure was adjusted to 40 ml/min and was used as the carrier gas. Hydrogen was adjusted to 40 ml/min and compressed air (O_2) to 400 ml/min.

The compound that appeared on the TLC plates in the gg and Gg genotypes was also delineated in the GLC data but the amount of compound in the tissue extracts was not sufficient to quantitize the unknown compound.

RESULTS

In experiment 1, total chlorophyll content was significantly higher in leaf tissue in the Gg genotype lines than in the gg or GG genotype lines (1.20 mg/g fresh weight vs. 0.98 and 0.77 mg/g fresh weight), respectively (Table 7). However, in the remaining experiments, no significant differences in chlorophyll content were found (Tables 8-11) as determined on either a fresh weight or leaf area basis.

Total free sugar on a leaf area basis was not significantly different among the three genotypes in any experiments. There were, however, significant differences in sugar content on a per fresh weight basis in experiments 2 and 3. In experiment 2, the sugar content in lines with the GG genotype (9.22 mg/g fresh weight) was significantly higher than that in lines with the gg genotypes (6.38 mg.g fresh weight) while that of the Gg genotype was not significantly different from either of the other two genotypes (Table 8). In contrast, in experiment 3, Gg lines had the highest sugar content of the three genotypes and was greater than that of the gg lines (10.95 mg/g fresh weight

Table 7.--Total chlorophyll, starch, and sugar content of vegetative leaf tissue of plants in experiment 1.

Genotype and Set	Line	Chlorophyll mg/g Fresh Weight	Starch mg Equilivants glucose/g Fresh Weight	Sugar mg Equilivants glucose/g Fresh Weight
gg-1	750	1.01 abcd[a]	13.19 bc	7.46 ab
gg-2	753	1.11 cd	10.14 b	8.12 abc
gg-3	757	0.82 ab	10.51 b	9.27 bc
		0.98 y'[b]	11.27 y'	8.28
GG-1	751	0.74 a	8.82 a	5.97 a
GG-2	754	0.90 abc	8.88 a	9.57 bc
GG-3	756	0.68 a	7.60 a	7.33 ab
		0.78 x'	8.43 x'	7.62
Gg-1	752	1.038 bcd	11.13 b	7.86 abc
Gg-2	755	1.36 de	12.92 bc	10.00 c
Gg-3	758	1.20 e	12.00 bc	7.40 ab
		1.20 z'	12.02 z'	8.42
HSD 0.01		0.24	1.53	2.54
HSD 0.01 (means)		0.18	0.73	n.s.

[a]Column means followed by the same letter (a-e) are not significantly different at the 1% level Tukey's HSD.

[b]Column means followed by the same letter (x, y, z) are not significantly different at the 1% level Tukey's HSD.

n.s. = no significant difference.

Table 8.--Total chlorophyll, starch, and sugar content of leaf tissue of plants in experiment 2 on a fresh-weight basis.

Genotype and Set	Line	Chlorophyll mg/g Fresh Weight	Starch mg Equilivants glucose/g Fresh Weight	Sugar mg Equilivants glucose/g Fresh Weight
gg-1	750	1.20	5.77	9.03 bcd[a]
gg-2	753	0.74	8.91	5.39 a
gg-3	757	0.44	4.43	4.72 a
		0.79	6.37	6.38 x'[b]
GG-1	751	0.81	7.12	11.70 d
GG-2	754	0.95	11.62	6.48 ab
GG-3	756	0.75	6.77	9.50 bcd
		0.82	8.50	9.22 y'
Gg-1	752	1.15	5.06	10.59 cd
Gg-2	755	1.09	7.99	7.45 abcd
Gg-3	758	0.67	10.64	7.16 abc
		0.97	7.90	8.40 x'y'
HSD 0.01		n.s.	n.s.	3.54
HSD 0.01'		n.s.	n.s.	2.64

[a]Column means followed by the same letter (a-e) are not significantly different at the 1% level Tukey's HSD.

[b]Column means followed by the same letter (x,y,) are not significantly different at the 1% level Tukey's HSD.

n.s. = no significant difference.

Table 9.--Total chlorophyll, starch, and sugar content of leaf tissue of plants in experiment 2 on a leaf area basis.

Genotype and Set	Line	Chlorophyll mg/AREA	Starch mg Equilivants glucose/AREA	Sugar mg Equilivants glucose/AREA
gg-1	750	0.73 c[a]	8.78	5.60
gg-2	753	0.46 ab	13.46	3.54
gg-3	757	0.32 a	5.81	3.40
		0.50	9.35	4.18
GG-1	751	0.40 a	14.29	5.84
GG-2	754	0.63 c	16.90	4.42
GG-3	756	0.40 a	11.65	5.70
		0.48	14.27	5.32
Gg-1	752	0.62 c	6.96	5.77
Gg-2	755	0.60 bc	14.41	4.14
Gg-3	758	0.43 a	16.33	4.62
		0.55	12.57	5.14
HSD 0.01		0.18	n.s.	n.s.
HSD 0.01'		n.s.	n.s.	n.s.

[a]Column means followed by the same letter (a-e) are not significantly different at the 1% level Tukey's HSD.

n.s. = no significant difference.

Table 10.--Total chlorophyll, starch, and sugar content of leaf tissue in experiment 3.

Genotype and Set	Chlorophyll mg/g Fresh Weight	Sugar mg/Fresh Weight
gg-1	0.91	11.44 bc[a]
2	0.82	5.44 a
3	0.76	5.34 a
	0.83	7.40 x'[b]
GG-1	0.92	11.89 bc
2	0.99	8.20 ab
3	0.68	7.89 a
	0.86	9.327 x'y'
Gg-1	0.83	14.24 c
2	0.93	12.11 c
		6.50 a
	0.76	10.95 y'
$HSD_{0.01}$	n.s.	3.89
$HSD_{0.01}$	n.s.	2.907

[a]Column means followed by the same letter (a-c) are not significantly different at the 1% level Tukey's HSD.

[b]Column means followed by the same letter (x, y) are not significantly different at the 1% level Tukey's HSD.

n.s. = no significant difference.

Table 11.--Total chlorophyll, starch, and sugar content of leaf tissue in experiment 3 on a leaf area basis.

Genotype and Set	Chlorophyll mg/AREA	Starch mg/AREA	Sugar mg/AREA
gg-1	0.63 ab	12.46	7.97 bc[a]
2	0.54 ab	3.15	3.62 a
3	0.62 ab	6.89	4.41 a
	0.60	6.89	5.33
GG-1	0.51 ab	4.72	6.63 bc
2	0.74 b	6.43	6.06 ab
3	0.44 a	8.63	5.09 a
	0.56	6.59	5.93
Gg-1	0.43 a	8.12	7.57 bc
2	0.50 ab	6.59	6.84 bc
3	0.39 a	3.98	3.34 a
	0.44	6.23	5.92
HSD 0.01	0.29	n.s.	1.85
HSD 0.01	n.s.	n.s.	n.s.

[a]Column means followed by the same letter (a-c) are not significantly different at the 1% level Tukey's HSD.

n.s. = no significant difference.

vs. 7.40 mg/g fresh weight) (Table 10). In this experiment, the GG lines were intermediate between the Gg and gg lines and were not significantly different from these two genotypes (9.33 mg/g fresh weight) (Table 10).

Starch content also varied among genotypes in the three experiments, in experiment 1, the total starch content was greater in the gg and Gg lines than in the GG lines, while the Gg lines were also significantly greater than gg lines in starch content (Table 7). Another pattern emerged in experiment 2, GG lines were again highest in starch content followed by the Gg lines and lastly by the gg lines. The GG lines were higher in starch level than the other two genotypes and the Gg lines were higher in starch level than the gg lines (Table 9). However, when the starch content was placed on a fresh weight basis, no significant differences were observed between the three genotypes (Table 8). In experiment 3, there were no differences in starch content among the three genotypes on either a fresh weight or leaf area basis (Tables 10 and 11).

The R_f values for the sugar extract developed on TLC plates showed that the gg and Gg lines resolved an additional band approximately R_f 0.52-0.56 and was absent in the aliquots obtained from the GG lines (Table 12 and Figure 5). The color of the spots and the standard sugars are recorded on Table 12. The most intense spot for each line was at R_f 0.33-0.37 and corresponded to the glucose standards sugar, R_f .34.

Table 12.--R_f values and color reactions of standard sugars and extracts from grandiflora-multiflora petunia vegetative tissue.

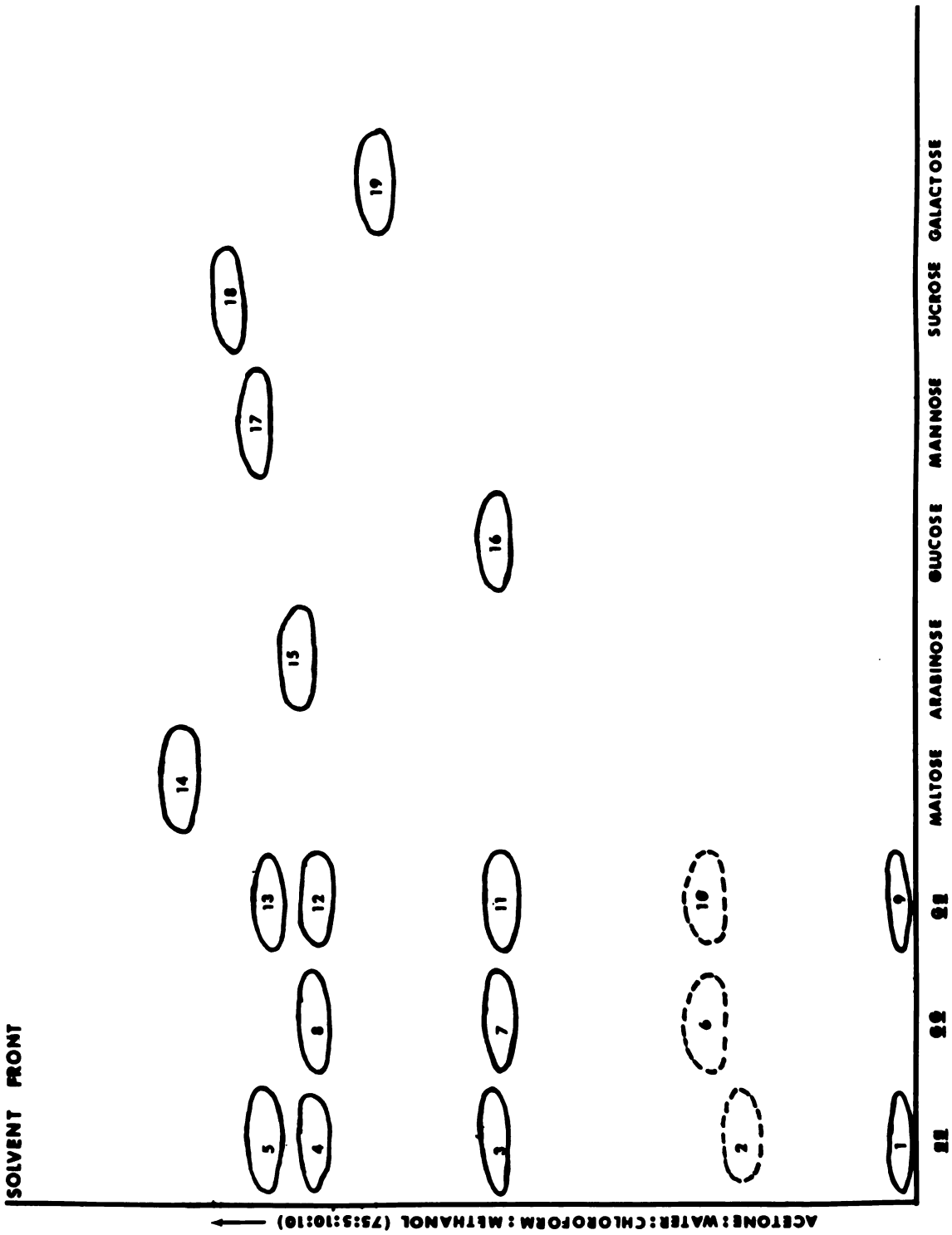
Compound Number	R_f	UV	Spray (see text)	Source[a]
1	0.01	green	--	gg
2	0.16	green	--	gg-1
3	0.36	--	yellow	gg
4	0.50	--	green	gg
5	0.54	--	green	gg
6	0.20	green	--	GG-1
7	0.35	--	yellow	GG
8	0.50	--	green	GG
9	0.01	green	--	Gg
10	0.18	green	--	Gg-1
11	0.35	--	yellow	Gg
12	0.50	--	green	Gg
13	0.53	--	green	Gg
14	0.60	grey	--	Maltose[b]
15	0.49	grey	--	Arabinose
16	0.34	--	yellow	Glucose
17	0.54	green	--	Mannose
18	0.57	grey	--	Sucrose
19	0.44	red	pink	Galactose

[a]Average of three lines of each genotype and duplicate plates.

[b]Standard sugar solutions 1 ug/ul ethanol.

Note: -- indicates no reaction under these conditions.

Figure 5. Composite thin-layer chromatogram of sugar standards and extracts from grandiflora and multiflora petunia vegetative tissue.


SOLVENT FRONT
ACETONE : WATER : CHLOROFORM : METHANOL (75:5:10:10)
MALTOSE
ARABINOSE
GLUCOSE
MANNOSE
SUCROSE
GALACTOSE
1
2
3
4
5
6
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19

The GLC data indicated a peak in the fractions from gg and Gg lines but not in GG lines. This peak was found in extracts from all three experiments and ranged in retention times from 6.2 to 8.4 minutes depending on when the samples were run. The samples were too small however to characterize the unknown by running it with standard sugar samples.

Figure 5. Composite thin-layer chromatogram of sugar standards and estracts from grandiflora and multiflora petunia vegetative tissue.

DISCUSSION

In a previous study, it was observed that differences found in Ca, Mg, and B may be related to chlorophyll content and/or metabolic differences (Knowlton and Sink, 1975). Also, apparent differences in the color of the foliage tend to indicate that the G locus in petunia may, in fact, affect the metabolism of chlorophyll. Thus, it appeared that determining the chlorophyll content of the leaves would be a logical place to begin since the leaves of the multiflora genotype petunias generally appear to be much darker green in color than the homozygous or heterozygous grandiflora lines. However, the results in all three experiments (Tables 7-11) did not support these visual observations and inferences from previous research. In experiment 1, the chlorophyll content was highest in the hybrid lines on a fresh weight basis and not the multiflora lines. This may be explained by heterosis of the hybrid lines or interference by other compounds such as carotenoids. However, in the other two experiments, differences between genotypes either on a fresh weight or leaf area basis were observed. This can be best explained

by consideration of the relationships of chlorophyll concentrations in the cells of the various genotypes. In multiflora leaves, the chlorophyll concentration may be high and thus the leaves appear deep green while in the grandiflora genotype, the cells are larger and even though there is a similar amount of chlorophyll, the leaves appear lighter because the relative concentration is much lower due to the larger volume of the individual cells. The weight of these cells could very easily be similar in the various genotypes if the change in volume were due not to a larger number of oganelles but to a less dense cell sap. The discrepancy observed in experiment 1, might be explained by the more favorable environmental conditions under which those plants were grown, especially favoring the hybrid genotype.

Carbohydrate content was considered since it may influence water retention of the cells and with the larger leaves and flowers found in grandiflora lines, more energy substrates might be required as increased levels of sugar and starch. The starch content of the lines was different in experiment 1 on a fresh weight basis and in experiment 2 on a leaf area basis but the results of these analysis were inconclusive since the patterns were different in both cases (Tables 7 and 9). The amount of starch accumulated in cell walls is probably not necessarily correlated to genotypes but more likely influenced by environmental conditions and their complex interaction with the

grandiflora-multiflora genotypes. This integration is regulated not by one gene but by many which may or may not be linked with the grandiflora-multiflora locus.

Lastly, the sugar content in the three genotypes was on the whole not different. And, again, different patterns arose in the three experiments (Tables 7-11). This also indicates that environmental effects are probably responsible for these differences and that there is an unknown optimum carbohydrate level of available sugars.

Even though there appeared to be no quantitative differences in total chlorophyll, starch, or sugar content that could be attributed to the presence of the G or g alleles, a qualitative difference was observed in the form of a sugar compound found both by TLC and GLC techniques. This extra band was evident whenever the recessive g allele was present, i.e., in the multiflora gg and grandiflora heterozygous Gg lines. Thus, it appears that the presence this allele in some way modifies the presence of endogenous substrates of the cells in the vegetative leaves in these genotypes. The gene-enzyme-substrate mechanism for gene action might also be the place of modification.

Further work on identifying and isolating the unknown sugar should be carried out using GLC with larger samples and standard sugars, mass spectroscopy would also be of value as indicated by the reaction of the unknown to the anthrone reagent. It is relatively certain from

these results that the unknown substance is a carbohydrate of low molecular weight and may possible be used in identifying heterozygous grandiflora plants for breeding purposes.

CONCLUSIONS AND RECOMMENDATIONS

As shown by the results of elemental analysis and determination of total sugar and starch content of vegetative tissue, grandiflora-multiflora breeding lines and hybrids of petunia are physiologically similar. While it appears that multiflora plants have darker foliage than grandiflora plants, the amount of chlorophyll extracted from the various genotypes was not significantly different possible indicating that the density of the chlorophyll varies in the cells of the two phenotypes rather than the total amount present or could be modified by the presence of other pigments. The unknown compound found by TLC separation seems to be present only in sugar extracts from genotypes carrying the recessive g allele. This allele may code for a different amino acid than the G allele and thus result in a different protein structure that in turn contributes to the production of a specific sugar. Since the molecular weight was not determined nor a positive identification made using standard sugars, the relationship to the plant's metabolism is unknown. It is also possible that this unknown is, in fact, present in the GG genotype

but in amounts undetectable by the techniques used in this study and that this characteristic is quantitative rather than qualitative. The unknown may be an indirect factor affecting cell division and density discussed above and thus in turn could regulate the growth rate and germination and vigor of the various genotypes.

Further studies on characterizing the unknown could be conducted using paper chromatography techniques, GLC and mass spectroscopy to further determine the compound's structure. The role of the unknown in the plant's metabolisms should also be investigated by using other biochemical tests, assays, and histochemical techniques. Cytological studies on leaf and other tissues that appear to be larger in the grandiflora phenotypes than in the multifloras could also aide in determining whether the G locus does in fact affect cell division and density.

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