This is to certify that the

thesis entitled

In vitro Nutrient requirements and chlorophyll precursor analysis of wild-type, nuclear and cytoplasmic albino mutants of Petunia inflata.

presented by

Mr.Chumpol Borkird

has been accepted towards fulfillment of the requirements for

Master's degree in Horticulture

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Major professor

Date 12-10-80

IN VITRO REQUIREMENTS AND CHLOROPHYLL PRECURSOR ANALYSIS OF WILD-TYPE, NUCLEAR AND CYTOPLASMIC ALBINO MUTANTS OF *PETUNIA INFLATA*

By

Chumpol Borkird

A THESIS

Submitted to
Michigan State University
in Partial Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

Department of Horticulture

1980

ABSTRACT

IN VITRO NUTRIENT REQUIREMENTS AND

CHLOROPHYLL PRECURSOR ANALYSIS OF WILD-TYPE, NUCLEAR

AND CYTOPLASMIC ALBINO MUTANTS OF PETUNIA INFLATA

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The optimum in vitro nutrient requirements of three genetic strains of Petunia inflata, the wild-type, nuclear and cytoplasmic albinos were determined. Tests were done individually on iron, sucrose, thiamine-HCl, indoleacetid acid (IAA), Kinetin (K), benzylaminopurine (BAP), variable pH from 4-7 and complex grwoth promoting substances including coconut milk, casein hydrolysate and plant extract added to Murashige and Skoog (1962) medium. Optimum concentration of each compound were combined to devise a new medium for each genetic strain. The chlorophyll precursor analysis was analyzed from ammoniacal aqueous acetone extracts of plants grown in different light regime with and without feeding with δ-Amino-leuvulinic acid. Millimolar extinction coefficient and Arnon's equation were used to calculate the quantities of chlorophyll and it's precursor from the absorption spectra. Both albino mutants accumulate Protoporphyrin IX and the nuclear albino is more unstable.

ACKNOWLEDGEMENTS

I wish to extend my appreciation to D. K. C. Sink, for his guidance during the course of this study. I also wish to thank the other members of my guidance committee, Dr. M. J. Bukovac and Dr. W. Tai, for valuable discussions, technical assistance and kindly making available equipment necessary to perform experiments.

A special note of appreciation is given to my parents and my grandmother for their encouragement and constant concern for my education. To them, this and all future accomplishments are dedicated.

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In Vitro Growth Requirements of Normal Green, Cytoplasmic and Nuclear Albinos of *Petunia inflata*.

INTRODUCTION

Plant protoplast cultures and their fusion to produce somatic hybrid cell lines and plasts are two areas of tissue culture that currently are receiving considerable research attention because of their usefulness in genetic modification studies and potential for plant improvement (Carlson and Pollacco 1975, Carlson 1973, Bajaj 1974). A major obstacle in protoplast fusion studies is the availability of a sound selection system universally applicable to all plant species. Light sensitivity, nitrate reductase deficient and drug sensitive mutants all have been successfully used (Power, et. al., 1977, Melchers and Labib 1974, Glimelius, et. al., 1978). However, the most universally applied selection system thus far has been the use of one or both fusion parents as albino or chlorophyll deficient mutants (Gleba, et. al., 1975, Krumbiegel and Schieder 1979, Schieder 1977, Belliard et. al., 1978, Cocking, et. al., 1977, Dudits, et. al., 1977, Maliga, et. al., 1977). Up to the present time, specific nutrient requirements and phenotypic stability of chlorophyll deficient mutants of higher plants employed in these protoplast studies have not been extensively studied.

In general, researchers have used either the basal medium formulated by Murashige and Skoog (1962), Linsmaier and Skoog (1965), Gamborg et. al. (1968), Schenk and Hildebrandt (1971), Eriksson (1965), or Ohira et. al. (1973). Although there appears to be a high degree of constancy in the major and minor salt requirements, in contrast the requirements for

organic constituents are quite variable (Murashige, 1973). In addition, almost all previous studies on nutritional requirements used callus or suspension cultures to test medium components (previous references) but our experiments involved shoot tips.

Since albinos are auxotrophic, the experiments were designed to test only the basic nutritional requirements of carbon source, iron as $Na_2FeEDTA$, pH, selected growth regulators and complex growth promoting substances such as casein hydrolysate (CH), coconut milk (CM) and plant extract (PE) and even for wild type plants, the growth increased significantly in the modified medium as will be presented.

MATERIALS AND METHODS

Three genetic strains of Petunia inflata, the wild-type and two mutants, a cytoplasmic and a nuclear-controlled albino, were used as test organisms. The cytoplasmic albino originated as a mutant callus culture derived from a wild type protoplast culture (J. B. Power - personal communication) and the nuclear albino by gamma irradiation of seeds (K. C. Sink - personal communication). These three genetic strains were propagated in vitro by terminal and internode cuttings and subcultured monthly in modified Murashige and Skoog (1962) medium containing MS salts and iron with 0.8 mg/l Thiamine-HCl, 100 mg/l myo-inositol, 3 mg/l IAA, 0.3 mg/l K and 3% sucrose. It was solidified with 9.0 qm/l agar and the pH was adjusted to 5.8 with 0.1 HCl or 0.2 N NaOH prior to autoclaving at 15 psi for 20 minutes. This medium is herein referred to as MS-C medium. IAA was initially filter sterilized but further experiments indicated no effect of autoclaving IAA on the in vitro responses of the P. inflata genetic strains.

The explants used for experiments were shoot tips approximately 2 cm. long with 3 - 4 expanding leaves, taken from one month old stock plants in culture on MS-C. The shoot tops were weighed prior to and after culture in the experimental medium for one month.

The experimental media were modified Murashige and Skoog (1962) containing MS salts, 100 mg/l myoinositol and with various concentrations of iron, sucrose, thiamine-HCl, idoleacetatic acid (IAA), kinetin (K),

benzylaminopurine (BAP); also, variable pH adjusted with 0.1 N HCl or 0.2 N NaOH for the range between 4.0 to 7.0 was tested. Complex growth promoting substances tested included coconut milk (CM), plant extract (PE), and casein hydrolysate (CH) at different concentrations.

The coconut milk was obtained from the liquid endosperm of fresh ripe coconuts. It was extracted and filtered through several layers of cheesecloth. The pH was adjusted to 10.0 with 2.0 N NaOH and held overnight at 4°C., and then the pH was readjusted to 7.0 with 5.0 N HCl and stored at -20°C (H. Murakishi - personal communication).

The plant extract from wild-type P. inflata was prepared from leaves of vegetative seedlings grown in the greenhouse. They were homogenized in a commercial blender, the homogenate was filtered through several layers of cheesecloth, heated to 70 - 80°C for 15 minutes and refiltered. The extract was stored frozen at -20°C.

Each compound was tested individually and the optimum concentrations of each test substance were combined to devise a new medium. The optimum concentrations of growth regulators were bracketed and all possible combinations were tested since the interaction of auxin and cytokinin in plant tissue culture is well-established.

The concentration of tested compounds used singly in experiments is indicated in Table I and for the combination tests in Tables II, III and IV for the wild-type, cytoplasmic and nuclear albinos, respectively.

The stock plants as well as experimental plants were grown under fluorescent light (Cool White G.E. F 40CW. RS. WM) at 19 $Em^{-2}s^{-1}$ 16-hour photoperiod, 25± 1°C. Each treatment was replicated 5 times

and most were repeated at least once. The initial fresh weight of the shoot explants was recorded and again 30 days later when an experiment was terminated. The mean fresh weight of initial explants, final shoot weights and increases in weight were calculated together with the standard error.

Table 1. Concentrations of complex substances, growth regulators, vitamin, iron, sucrose and pH tested for growth promotion.

Chemicals	Concentrations							
Complex substances								
CM (%)	0	5	10	15	20	25	30	35
CH (g/l)	0	0.5	1.0	1.5	2.0	2.5	3.0	3.5
PE (%)	0	1	3	5	8	10	15	20
Growth regulators								
BAP (mg/l)	0	0.5	1	1.5	2	2.5	3	3.5
IAA (mg/l)	0	1	2	3	4	5	6	7
K (mg/l)	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7
K re-test	0	0.2	0.4	0.6	0.8	1.0	1.2	1.4
Vitamin								
Thiamine HCl	0	0.2	0.4	0.6	0.8	1.0	1.2	1.4
(re-test)	0	0.4	0.8	1.2	1.6	2.0	2.4	2.8
$Na_2FeEDTA$ (mM)	0	0.025	0.05	0.075	0.1	0.125	0.15	0.175
Sucrose (%)	0	1	2	2.5	3	3.5	4	5
pH	4	4.5	5	5.5	5.8	6	6.5	7

Note: Iron supplied as $Na_2FeEDTA$ at 0.1 mM is the same concentration as in MS medium (1962).

Table 2. Combination tests for normal green wild-type Petunia inflata.

Media Code	CM ml/l	CH gm/l	IAA mg/l	K mg/l	Th-HCl mg/l	pH	Sucrose gm/l	Fe mM	PE ml/l
G-1	-	-	1	0.2	0.6	5	40	0.1	-
G-2	-	-	1	0.2	-	5	40	0.1	-
G-3	-	-	1	0	0.6	5	40	0.1	-
G-4	-	-	1	0.3	0.6	5	40	0.1	-
G-5	-	-	2	0	0.6	5	40	0.1	-
G-6	-	-	2	0.2	0.6	5	40	0.1	-
G-7	-	-	2	0.3	0.6	5	40	0.1	-
G-8	-	-	3	0	0.6	5	40	0.1	-
G-9	-	-	3	0.2	0.6	5	40	0.1	-
G-10	-	-	3	0.3	0.6	5	40	0.1	-
MS-C	-	-	3	0.3	0.8	5.8	30	0.1	-

G = Wild type Petunia inflata

Table 3. Combination tests for the nuclear albino Petunia inflata

Media Code	CM ml/l	CH gm/l	IAA mg/l	K mg/l	Th-HCl mg/l	pH	Sucrose gm/l	Fe mM	PE ml/l
N-1	20	-	5	0.1	1.5	6	3.5	0.1	1
N-2	-	-	5	0.1	1.5	6	3.5	0.1	1
N-3	20	-	5	0.1	1.5	6	3.5	0.1	-
N-4	20	-	5	0.1	1.5	4	3.5	0.1	1
N-5	20	-	5	0.3	1.5	6	3.5	0.1	1
N-6	20	-	5	0.7	1.5	6	3.5	0.1	1
N-7	20	-	3	0.1	1.5	6	3.5	0.1	1
N-8	20	-	3	0.3	1.5	6	3.5	0.1	1
N-9	20	-	3	0.7	1.5	6	3.5	0.1	1
N-10	20	-	0	0.1	1.5	6	3.5	0.1	1
N-11	20	-	0	0.3	1.5	6	3.5	0.1	1
N-12	20	-	0	0.7	1.5	6	3.5	0.1	1
MS-C	-	-	3	0.3	0.8	5.8	3	0.1	-

N = nuclear albino Petunia inflata

Table 4. Combination tests for the cytoplasmic albino Petunia inflata

Media Code	CM ml/l	CH gm/l	IAA mg/l	K mg/l	Th-HCl mg/l	pH	Sucrose gm/l	Fe mM	PE ml/l
C-1	20	-	2	1	0.4	5	4	0.1	-
C-2	-	-	2	1	0.4	5	4	0.1	-
C-3	20	0.5	2	1	0.4	5	4	0.1	-
C-4	20	-	2	1	0.4	5	4	0.1	3
C-5	20	-	2	0.5	0.4	5	4	0.1	-
C-6	20	-	2	2.5	0.4	5	4	0.1	-
C-7	20	-	1	0.5	0.4	5	4	0.1	-
C-8	20	-	1	1	0.4	5	4	0.1	-
C-9	20	-	1	1.5	0.4	5	4	0.1	-
C-10	20	-	3	0.5	0.4	5	4	0.1	-
C-11	20	-	3	1.5	0.4	5	4	0.1	-
C-12	20	-	3	1.5	0.4	5	4	0.1	-
MS-C	-	-	3	0.3	0.8	5.8	3	0.1	-

C = cytoplasmic albino Petunia inflata

RESULTS

Effect of BAP on Multiple Shoot Formation

En MS-C medium, multiple shoot formation occurred sporadically for the normal green and cytoplasmic albino plants and did not occur in the nuclear albino. The multiple shoots originated from extensive adventitious shoot formation on callus produced at the base of the shoot tip cuttings of the three genetic strains of P. inflata when grown in MS medium with BAP from 0.5 - 5.0 mg/liter. The numerous, spindly shoots were not suitable to use as explants for further experimentation. Also, the growth of the main shoot seemed to be inhibited even at 0.5 mg/l BAP. Therefore, medium MS-C was used to supply all explants and serve as the control medium.

Green sectors were occasionally observed on adventitious shoots of the cytoplasmic albino when grown in MS medium + BAP.

Effect of Complex Compounds on Fresh Weight Increase

Each genetic strain of P. inflata had different growth factor requirements. The wild-type plants did not respond to CM, CH or PE. In contrast, some of the compounds inhibited the growth of normal green plants even at the lowest concentration tested, 5% CM and 0.5 gm/l CH (Appendix I, II and Table V). PE was inhibitory at 5% and above and was without effect at lower concentrations (Table V). CH even at 0.5 gm/l

to 3.5 gm/l inhibited the growth of all three genetic strains (Appendix II). Even though the fresh weight of the cytoplasmic albino in medium with 0.5 gm/l CH is higher than the control medium, the fresh weight increase was due mostly to callus formed at the base of cuttings.

The cytoplasmic albino grown on media with CH changed color from pale yellow to almost white. This pigment change was consistently observed. CH seems, therefore, to inhibit chlorophyll synthesis in the cytoplasmic albino; however, no effect was observed in the normal green or the nuclear albino.

Even though CM inhibited the growth of normal green plants, it had some stimulatory effect on the nuclear and cytoplasmic albinos at 20% whereas higher concentrations were inhibitory and lower concentrations were not adequate (Appendix I).

PE did not have an obvious promotive effect on the growth of the normal green plants at 3% or below and was inhibitory at higher concentrations. A similar trend was found for cytoplasmic albino plants where 5% PE was inhibitory and lower concentrations had no effect. In contrast, low level of PE at 1 - 3% stimulated the growth of the nuclear albino (Table V). The best concentration tested for fresh weight increase was 1%. Higher concentrations inhibited growth and also appeared to inhibit chlorophyll formation especially at levels above 10%. The nuclear albino is unstable and plants showed considerable chlorophyll formation which is in contrast to the cytoplasmic albino which was quite stable in the MS-C medium. Addition of PE above 10% resulted in less chlorophyll formation in the nuclear albino which may be the consequence of growth inhibition.

Effect of Growth Regulators on Fresh Weight Increase

The three genetic strains of P. inflata had a lower IAA requirement for growth than the level in MS-C (3 mg/l). The nuclear albino also grew in MS basal medium without IAA, which produced the highest fresh weight after one month in culture, but the increase was primarily from extensive callusing at the base of cuttings. The nuclear albino also had the highest fresh weight at the highest level of IAA (5 and 7 mg/l). Therefore, 0, 3.0 and 5.0 mg/l IAA were used in combination tests with different concentrations of K at all combinations. The best IAA concentration tested for the normal green and cytoplasmic albino was 1.0 and 2.0 mg/l respectively (Appendix III).

There were no differences between autoclave and filter sterilized IAA in growth of nuclear or cytoplasmic albinos but autoclaved IAA was superior for the normal green plants at 3.0 mg/l (Appendix IV).

The optimum concentrations of K for normal green and nuclear albino plants were 0.2 and 0.1 mg/l, respectively, which is lower than the 0.3 mg/l used in MS-C. The cytoplasmic albino responded quite differently from the other two genetic strains. Maximum fresh weight growth was obtained at 10-fold higher concentration than for the nuclear albino and 5-fold higher than for the normal green plants (Appendix V and VI).

Moreover, K also had a similar stimulatory effect on green sector formation as did BAP on the cytoplasmic albino. Green sectors on leaves were formed using basal medium with K from 0.4 to 1.0 mg/l. No green sectoring was observed below 0.4 or higher than 1.2 mg/l K. Actually, green sectors were sometimes noticed in leaves of adventitious shoots

of the cytoplasmic albino stock cultures but they occurred very infrequently. The high levels of cytokinins, BAP and K showed obvious effects on promoting green sectors and they occurred consistently and were repeatable.

Effect of Thiamine-HCl on Fresh Green Weight Increase

Thiamine-HCl appeared to be the only vitamin requirement for the three genetic strains of P. inflata, since the other vitamins in MS medium did not show any promotive effect when compared to MS-C, which contained only thiamine-HCl. The optimum levels of thiamine-HCl for the normal green and cytoplasmic albino plants were found to be 9.6 and 0.4 mg/l, whereas that for the nuclear albino was higher. The greatest fresh weight obtained from the nuclear albino was in MS + 1.4 thiamine-HCl and was still increasing, suggesting that the optimum was higher (Appendix VII). Therefore, experiments with higher concentrations of thiamine-HCl were conducted and the greater fresh weight obtained was at 0.8 mg/l (Appendix VIII). However, of 1.2 and 1.6 mg/l thiamine-HCl, the 1.6 level resulted in the best overall growth. The intermediate concentration of 1.5 mg/l was selected for use in the combination test.

Effect of Iron, Sucrose and pH on Fresh Weight Increase

Iron supplied at 0.1 mM as in MS medium proved to be optimum for maximum growth of all genetic strains of P. inflata (Appendix IX).

The three genetic strains all had a rather high requirement for sucrose when provided as the sole carbon source. A level of 3.5% and

4.0% were optimal for the nuclear albino and both the normal green and the cytoplasmic albino, respectively (Appendix X). All plants grown in media with low sucrose (1%) or in the absence of sucrose had very low fresh weight and many died.

With respect to optimum pH, the three genetic strains preferred low pH medium. Routinely the pH of media were adjusted to 5.6 - 6.0 but pH 5.0 was found optimum for the maximum growth of the nuclear albino. Of more interest, the highest fresh weight of the nuclear albino occurred at pH 4.0. Also, the growth of the other genetic strains was not inhibited at pH 4.0 while above pH 6.0, growth inhibition of the three genetic strains occurred (Appendix XI).

Combination Tests

The new formulated media yielded 2, 2.5 and 4-fold increases in the final fresh weight when compared to the original MS-C medium for the nuclear albino, wild-type and the cytoplasmic albino plants (Appendices XII, XIII, XIV). The media composed of the optimum concentrations observed from the individual tests of each substance did not result in the highest fresh weight and some modification is required for the best growth of each genetic strain. CM was not necessary when the hormone level is optimum (media N-2 and C-2, Tables 3 and 4, Appendices XIII, XIV). In contrast, 3% PE resulted in the greatest fresh weight of the cytoplasmic albino (medium C-4, Table 4, Appendix XIV). However, it was not essential for the nuclear albino (medium N-3, Table 3, Appendix XIII). CH and thiamine-HCl were also found to be unnecessary for the cytoplasmic

albino and the normal green plants (media G-2, C-3, Tables 2 and 4, Appendices XII, XIV). Interestingly, the nuclear albino had the second best growth in medium N-4 which had pH 4.0 (Table 3, Appendix XIII).

From the results on different hormonal combinations, it can be concluded that K is necessary for growth of the normal green plants and the optimum concentration in the combination test was 0.3 mg/l, whereas the optimum concentration of IAA was 1.0 mg/l, which is lower than the MS-C medium by three-fold (media G-3 through G-10, Table 3, Appendix XIII). Both K and IAA were also found necessary for the growth of the nuclear albino, especially at high concentrations, 0.7 and 5 mg/l, respectively (media N-5 through N-12, Table 3, Appendix XIII). For the cytoplasmic albino, the fresh weight increase seems to depend upon the concentrations of K more than that of IAA (media C-5, C-1, C-6; C-7, C-8, C-9; and C-10, C-11, C-12, Table 4, Appendix XIV). However, the optimum concentrations are 2.0 mg/l IAA and 1.0 mg/l K.

SE (G) - Standard error of the final shoot weight of the normal green P. inflata

SE (C) - Standard error of the final shoot weight of the cytoplasmic albino

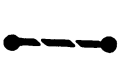
SE (N) - Standard error of the final shoot weight of the nuclear albino

OSW - Original shoot weight

SE (OSW) - Standard error of the original shoot weight

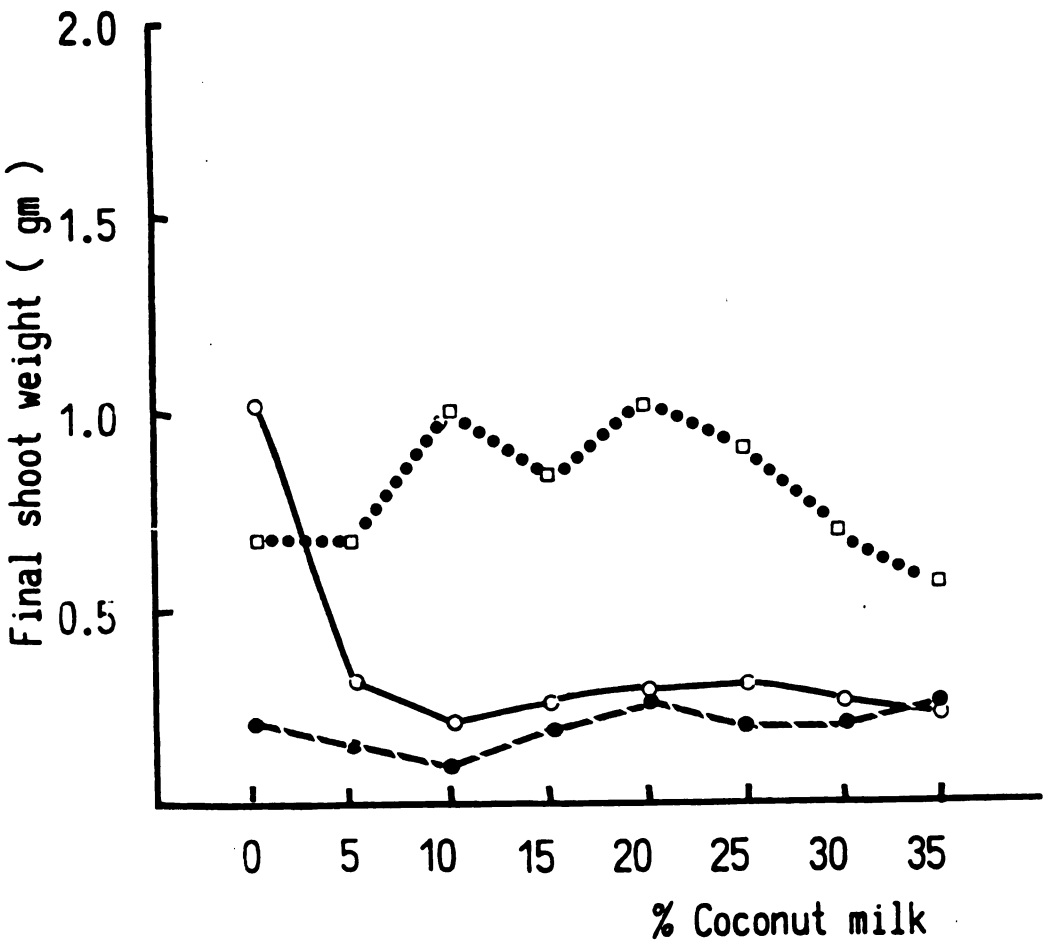
Normal green, wild-type P. inflata

Cytoplasmic albino P. inflata



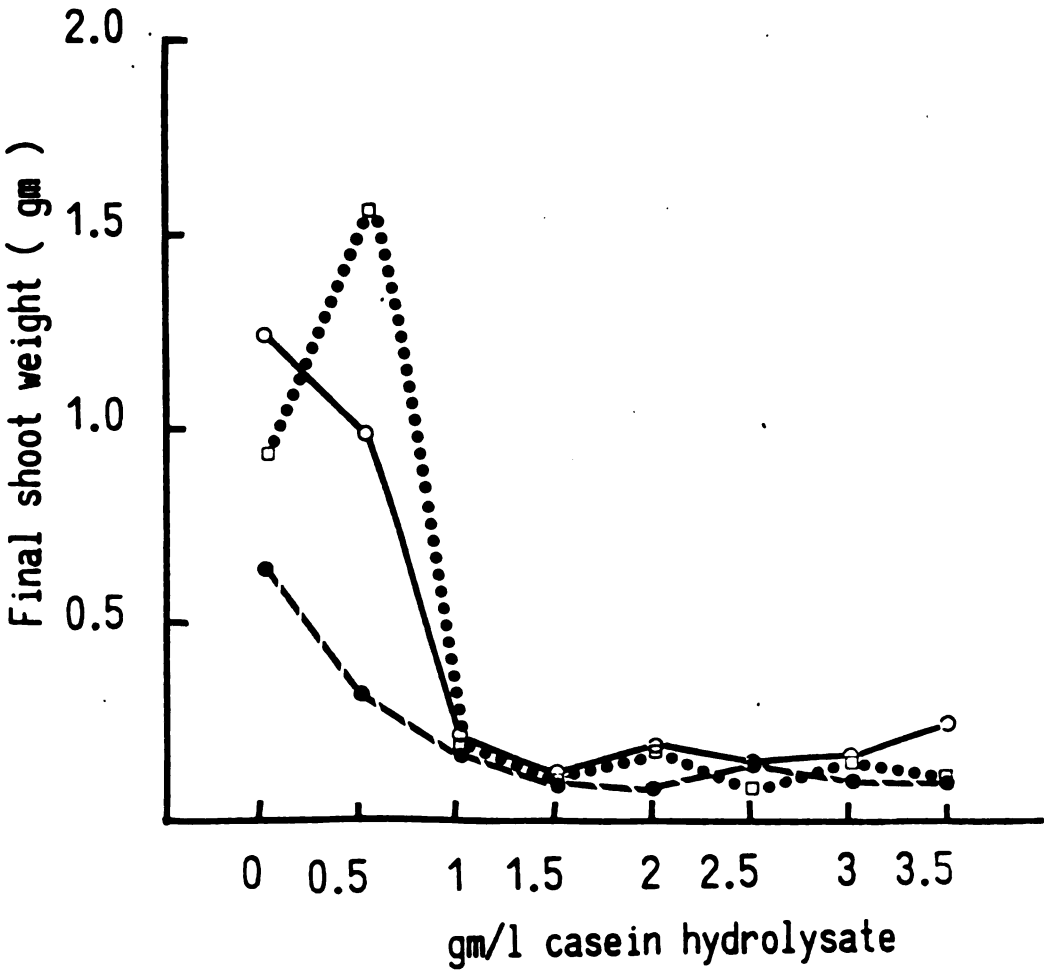
Nuclear albino P. inflata

Figure 1. Effect of coconut milk on fresh weight increase of the wild-type, nuclear and cytoplasmic albino of Petunia inflata.



SE (G)E	.442	.086	.0844	.2041	.1477	.1344	.0841	.0789
OSW	.0147	.0095	.0102	.0091	.0114	.0115	.0165	.0139
SE (OSW)	.008	.0064	.005	.0057	.007	.0013	.0081	.0055
SE (C)	.1566	.1645	.2885	.1799	.1593	.3481	.2142	.1543
OSW	.0223	.02	.0364	.0487	.023	.0184	.0151	.0111
SE (OSW)	.0051	.0181	.0153	.0408	.0125	.0076	.0076	.0019
SE (N)	.0666	.0354	.0545	.1039	.1781	.0819	.0555	.1065
OSW	.0146	.0102	.0141	.0125	.0142	.0134	.0113	.0152
SE (OSW)	.0071	.0034	.007	.0033	.0036	.0034	.0031	.0107

Figure 2. Effect of casein hydrolysate on fresh weight increase of the wild-type, nuclear and cytoplasmic albino of Petunia inflata.



SE (G)	.3752	.4667	.1431	.0815	.0888	.0344	.1291	.1005
OSW	.0259	.0148	.021	.0159	.0171	.0202	.0225	.0228
SE (OSW)	.0102	.006	.0157	.0117	.0129	.0079	.0076	.0093
SE (C)	.0527	.4551	.0711	.0566	.0979	.0986	.0266	.1254
OSW	.0279	.0209	.0195	.0231	.0231	.0333	.0371	.0171
SE (OSW)	.0147	.01	.0142	.0143	.0078	.011	.0137	.006
SE (N)	.092	.1481	.1587	.0358	.0337	.0814	.0686	.0526
OSW	.0135	.0121	.0141	.0143	.0181	.021	.0241	.0163
SE (OSW)	.0052	.0029	.0038	.0025	.0115	.0054	.0119	.0038

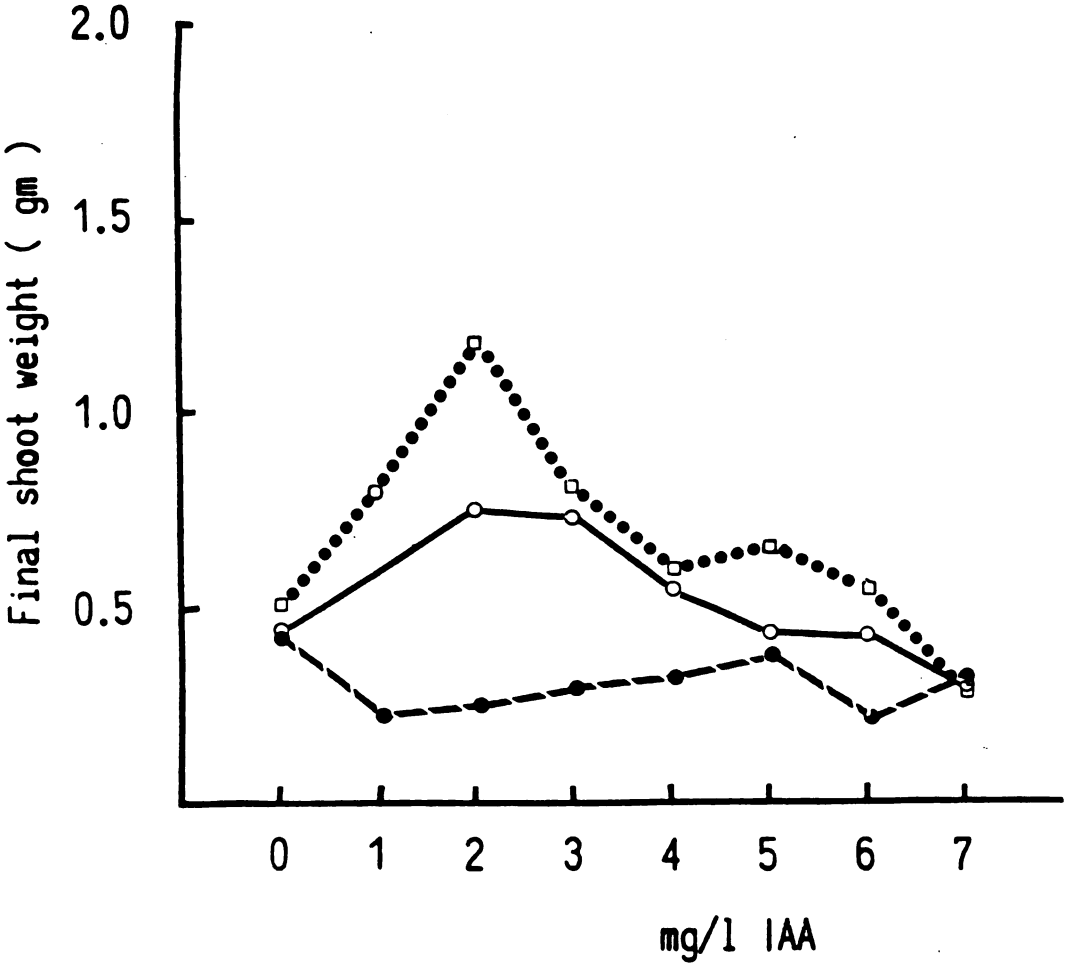
Table 5. Effect of Plant Extract on Fresh Weight Increase of the Normal Green, Nuclear and Cytoplasmic albino of *Petunia inflata*.

% Extract	Genetic Strains		
	Normal green	Nuclear albino	Cytoplasmic albino
0	++++	+	++++
1	++++	++++	++++
3	++++	+++	++++
5	++	++	++++
8	++	++	+++
10	++	+	+++
15	++	+	++
20	++	+	++

Key:

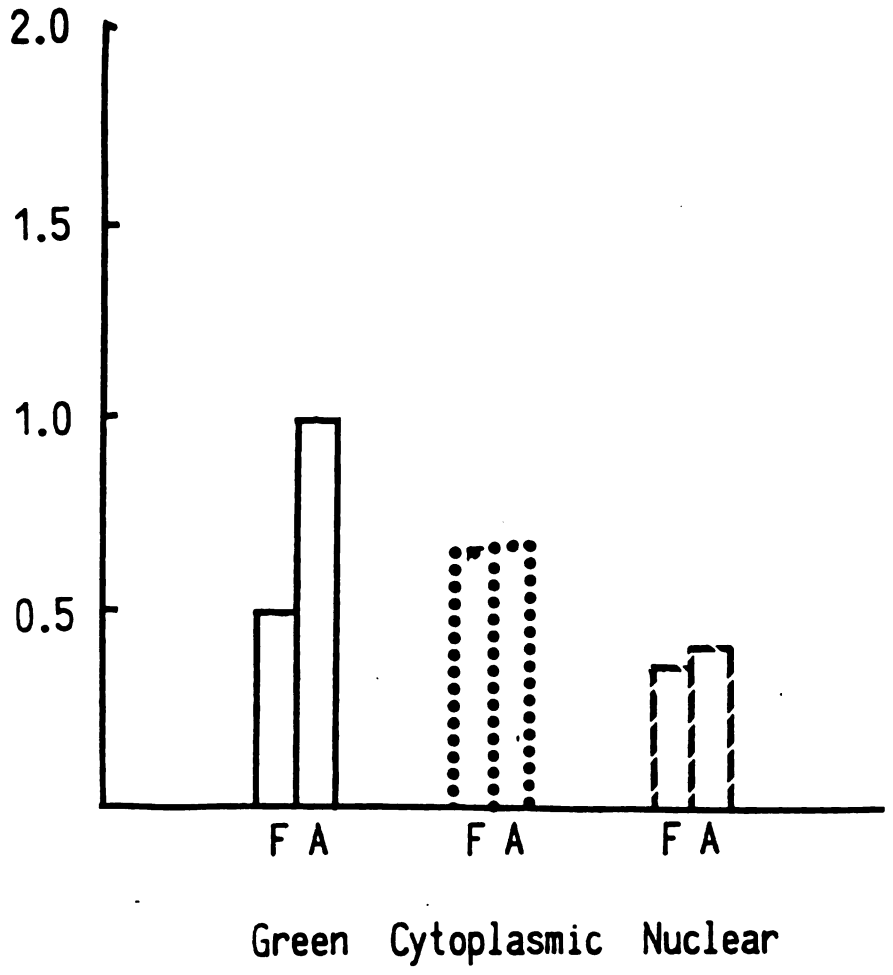
\+ = Poor
++ = Fairly good
+++ = Good
++++ = Excellent

Figure 3. Effect of IAA on fresh weight increase of the wild-type, nuclear and cytoplasmic albino of Petunia inflata .



SE (G)	.1995	.2814	.1098	.2276	.1954	.2054	.1025	.1125
OSW	.0232	.0206	.0182	.0259	.0269	.0264	.0226	.0326
SE (OSW)	.0077	.0046	.0062	.0113	.0102	.0071	.0095	.0084
SE (C)	.1006	.3193	.3245	.0619	.2238	.1541	.1286	.2704
OSW	.024	.0128	.019	.0184	.0184	.0301	.0252	.0217
SE (OSW)	.01	.0073	.0146	.0161	.0101	.012	.0151	.0196
SE (N)	.0854	.0613	.1167	.1487	.1821	.1857	.1611	.1857
OSW	.0168	.0173	.0163	.0227	.0198	.0223	.0222	.0218
SE (OSW)	.0105	.0089	.0142	.0086	.0062	.0025	.006	.0085

Figure 4. Effect of autoclaved versus filter-sterilized IAA on fresh weight increase of the wild-type, nuclear and cytoplasmic albino of Petunia inflata.

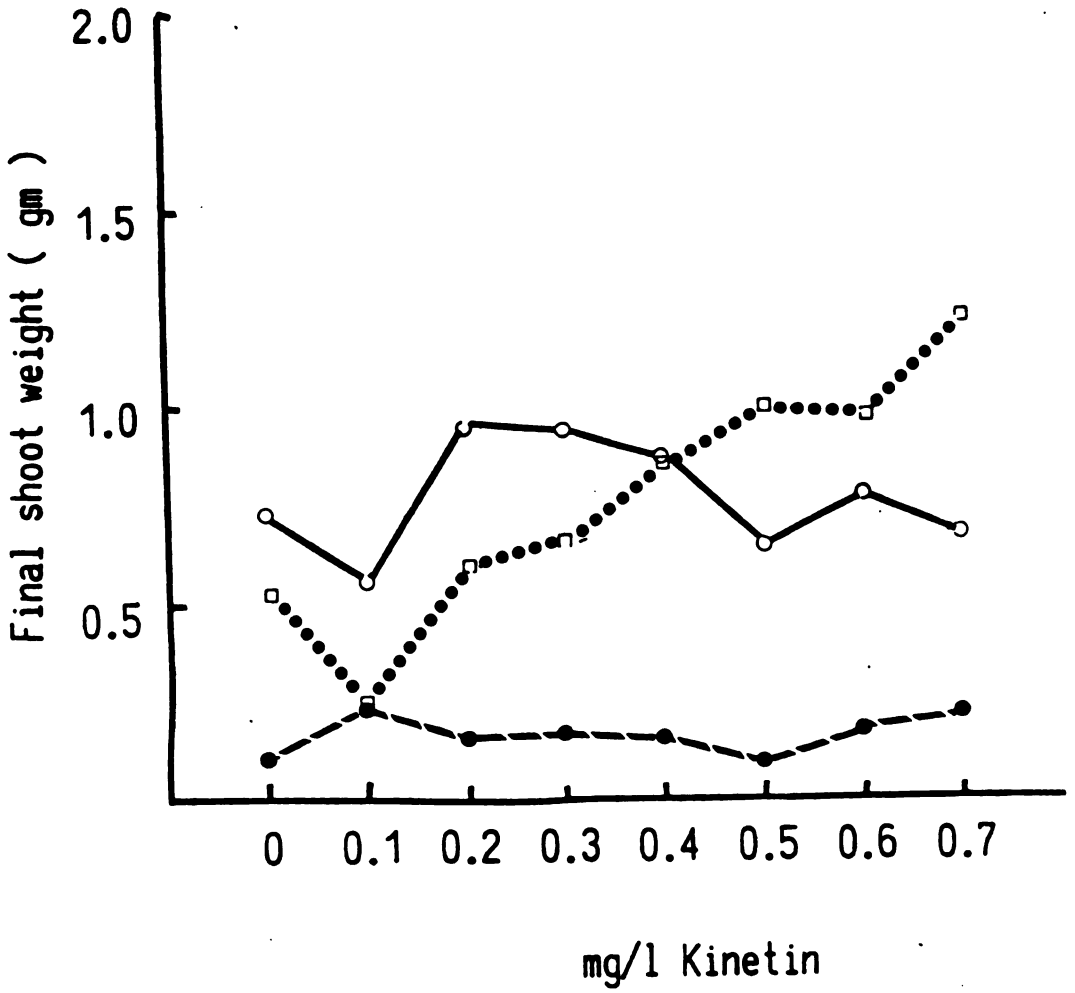


SE	.442	.2161	.1566	.1081	.1199	.0152
OSW	.0147	.0115	.0223	.0218	.0118	.0145
SE (OSW)	.008	.0067	.0051	.0079	.0019	.0029

A - Autoclaved

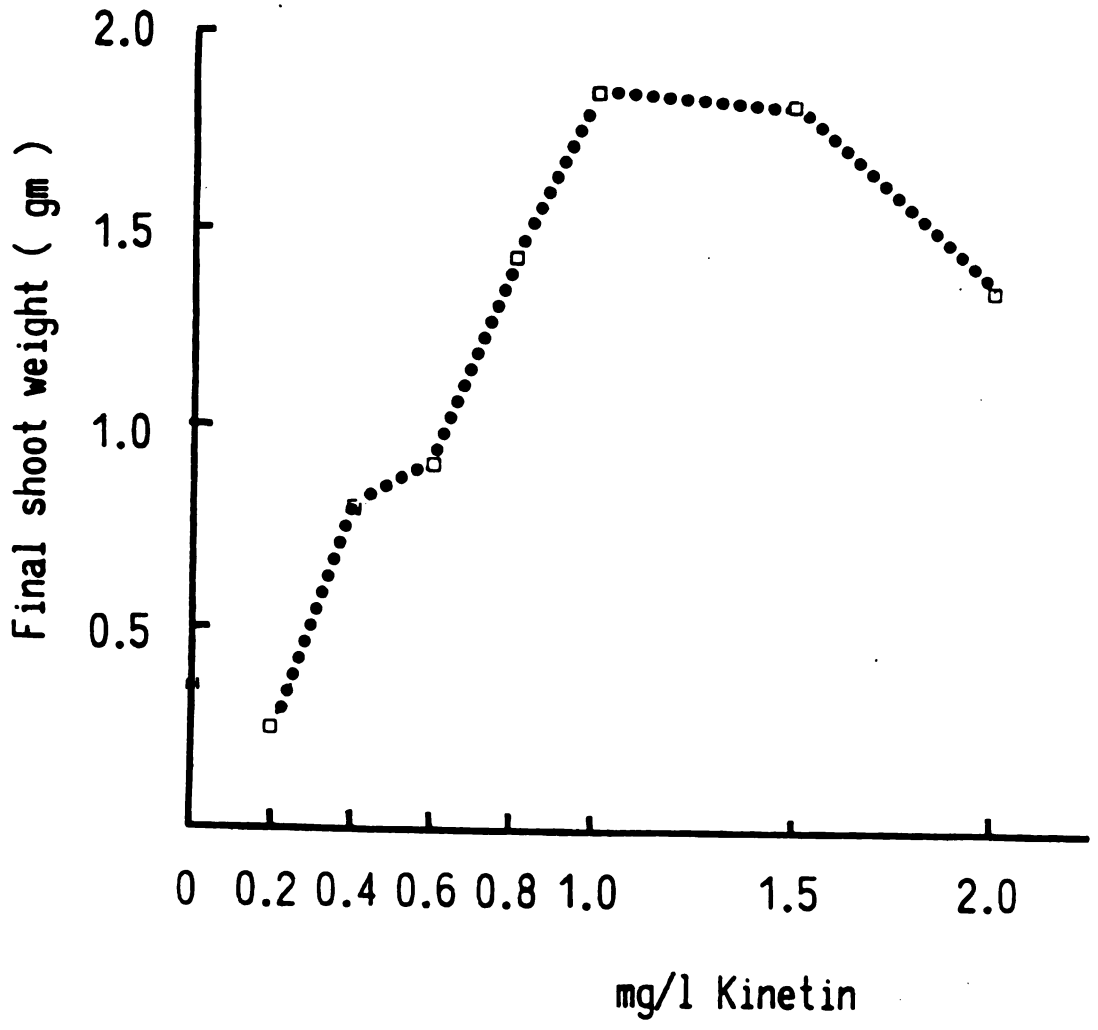
F - Filter-sterilized

Figure 5. Effect of kinetin on fresh weight increase of the wild-type nuclear and cytoplasmic albino of Petunia inflata.



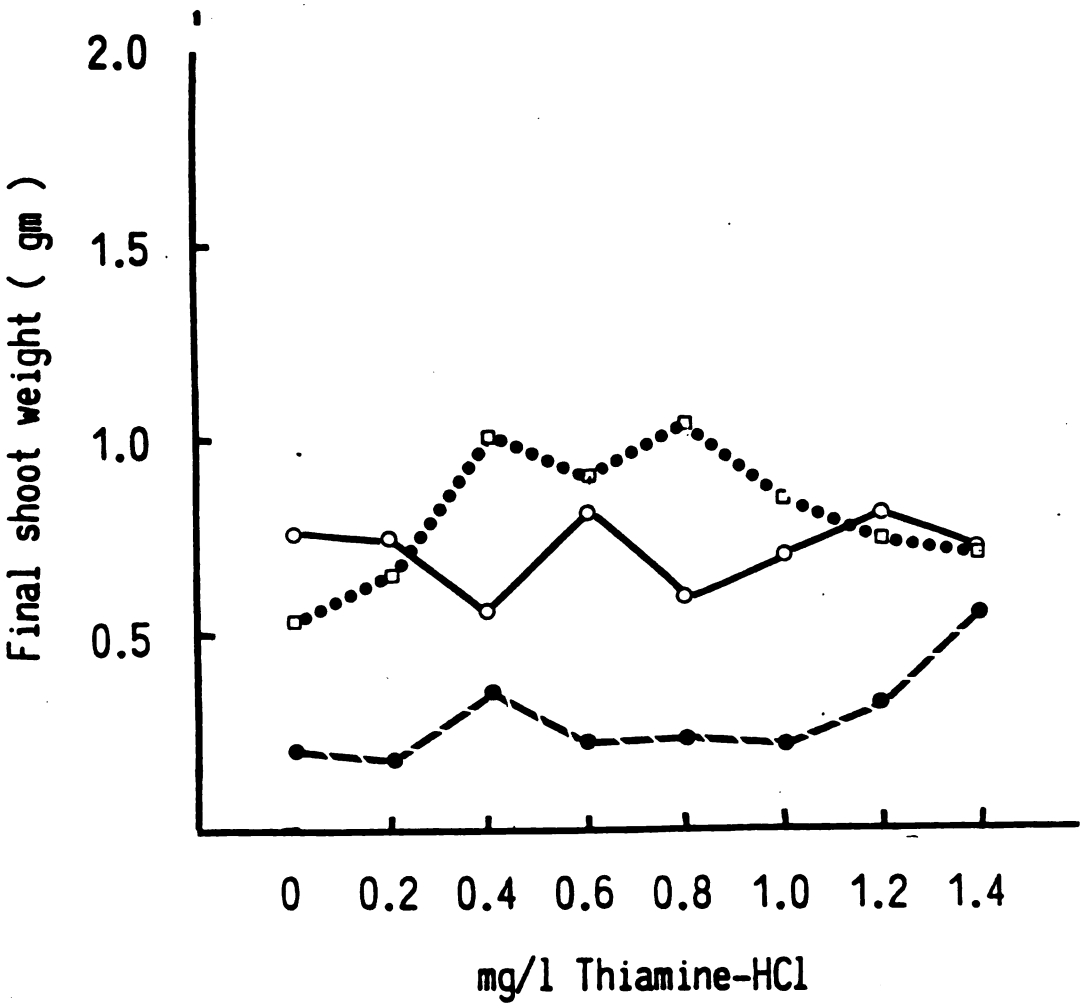
SE (G)	.1753	.1322	.2643	.4599	.3201	.1458	.2287	.2514
OSW	.0191	.0182	.0275	.0213	.0184	.0201	.0222	.0185
SE (OSW)	.0042	.0085	.0116	.0085	.0053	.0057	.0083	.0113
SE (C)	.1884	.1827	.1521	.3415	.1524	.3156	.18	.3068
OSW	.0253	.0263	.0183	.0301	.0256	.0247	.0322	.0233
SE (OSW)	.0109	.0094	.0061	.0078	.0088	.004	.0074	.0082
SE (N)	.0271	.1093	.0577	.1111	.1545	.0179	.0433	.1075
OSW	.0109	.0103	.0114	.0139	.0098	.0127	.013	.0115
SE (OSW)	.005	.0035	.0018	.0086	.0048	.0073	.0041	.0039

Figure 6. Retest of kinetin for fresh weight increase of the cytoplasmic albino of Petunia inflata.



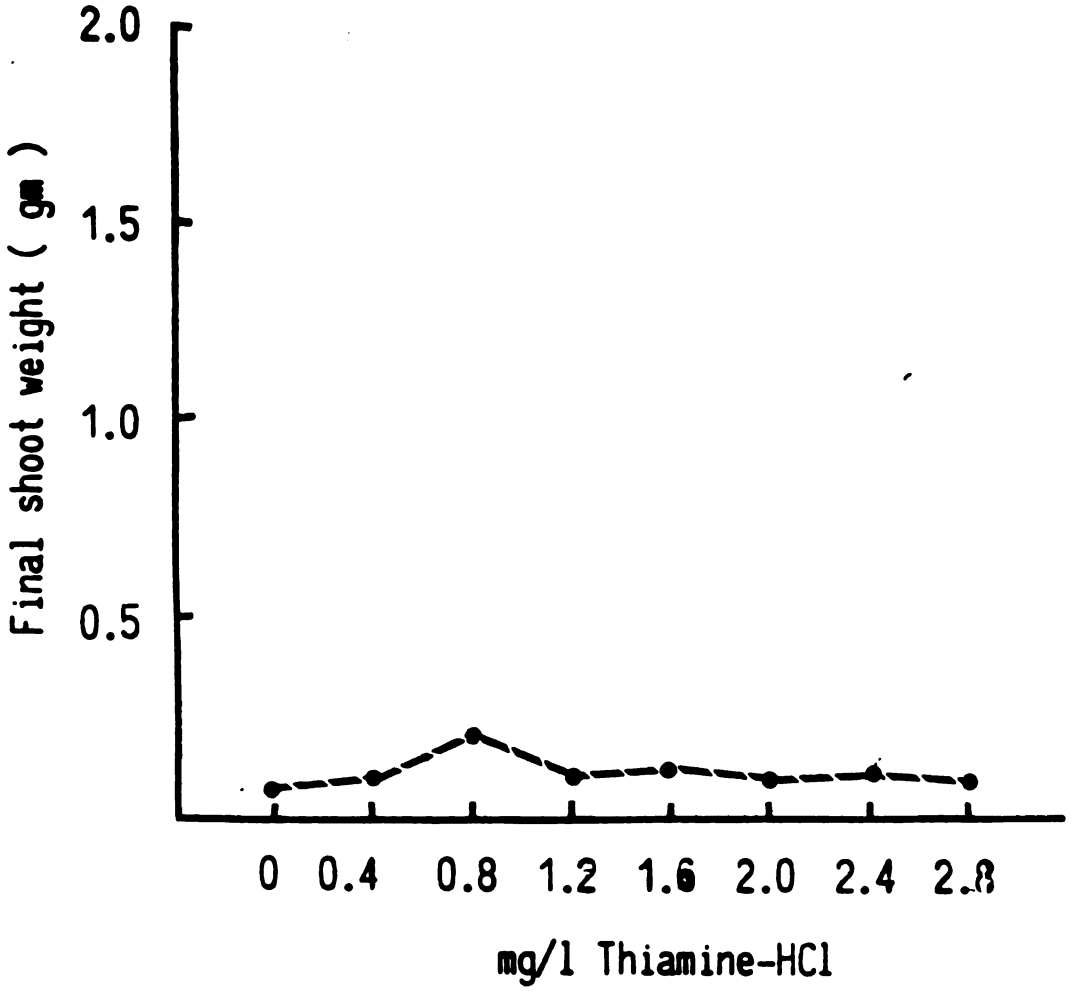
SE (C)	.1574	.2221	.3679	.4925	1.0028	.6964	.9896	.2343
OSW	.116	.0107	.0098	.0123	.011	.0156	.0103	.004
SE (OSW)	.0066	.0021	.0028	.0102	.0026	.0121	.0036	.001

Figure 7. Effect of thiamine-HCl on fresh weight increase of the wild-type, nuclear and cytoplasmic albino of Petunia inflata.



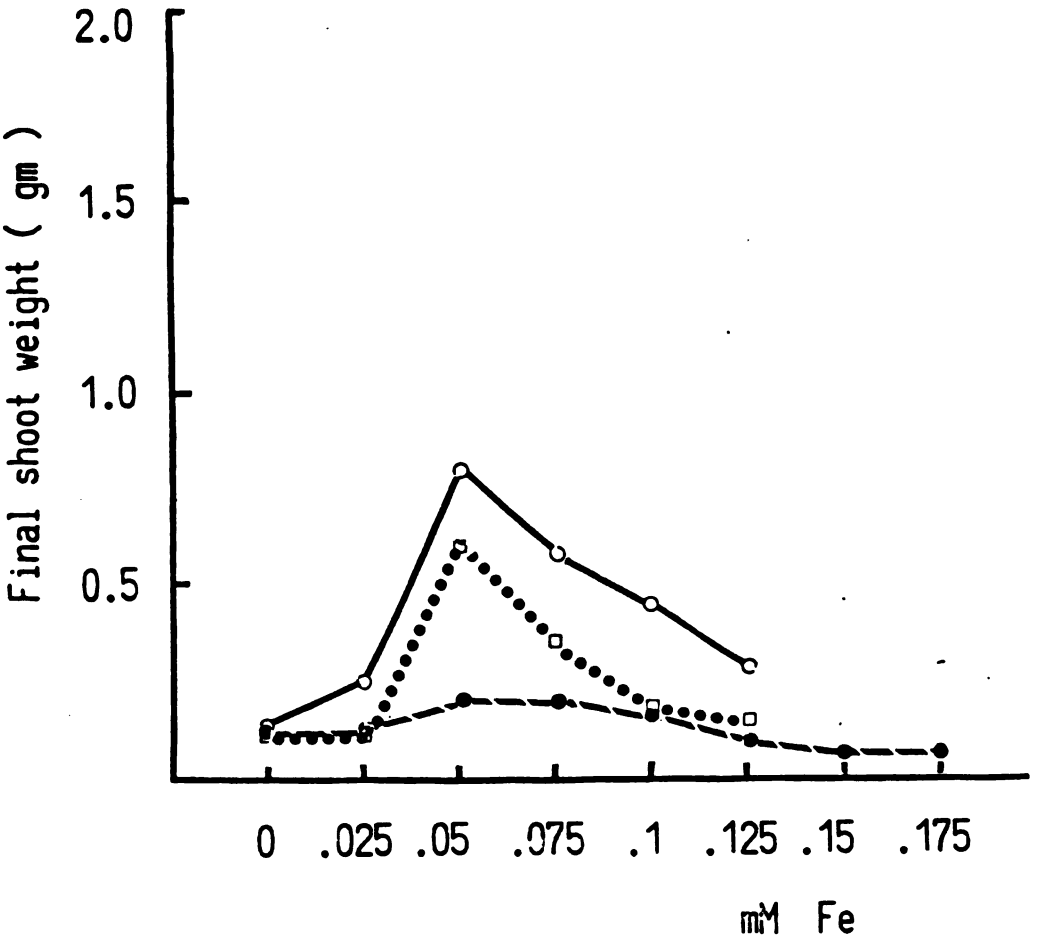
SE (G)	.2317	.2058	.1486	.4381	.1621	.3751	.2378	.4385
OSW	.0118	.0099	.0154	.0136	.0119	.0142	.0175	.0216
SE (OSW)	.0054	.0028	.0111	.0027	.0034	.0139	.0062	.024
SE (C)	.2291	.3539	.3524	.4015	.2194	.4997	.1662	.2641
OSW	.0155	.0244	.0338	.0196	.0171	.016	.0259	.0257
SE (OSW)	.0044	.0152	.0201	.0074	.0122	.0076	.0198	.0129
SE (N)	.0617	.089	,1386	.0669	.0765	.0866	.3326	.2157
OSW	.0214	.0109	.0154	.0133	.0174	.0147	.0117	.0196
SE (OSW)	.0052	.0074	.0076	.007	.0088	.0055	.0041	.0084

Figure 8. Retest of thiamine-HCl for fresh weight increase of the nuclear albino of Petunia inflata.



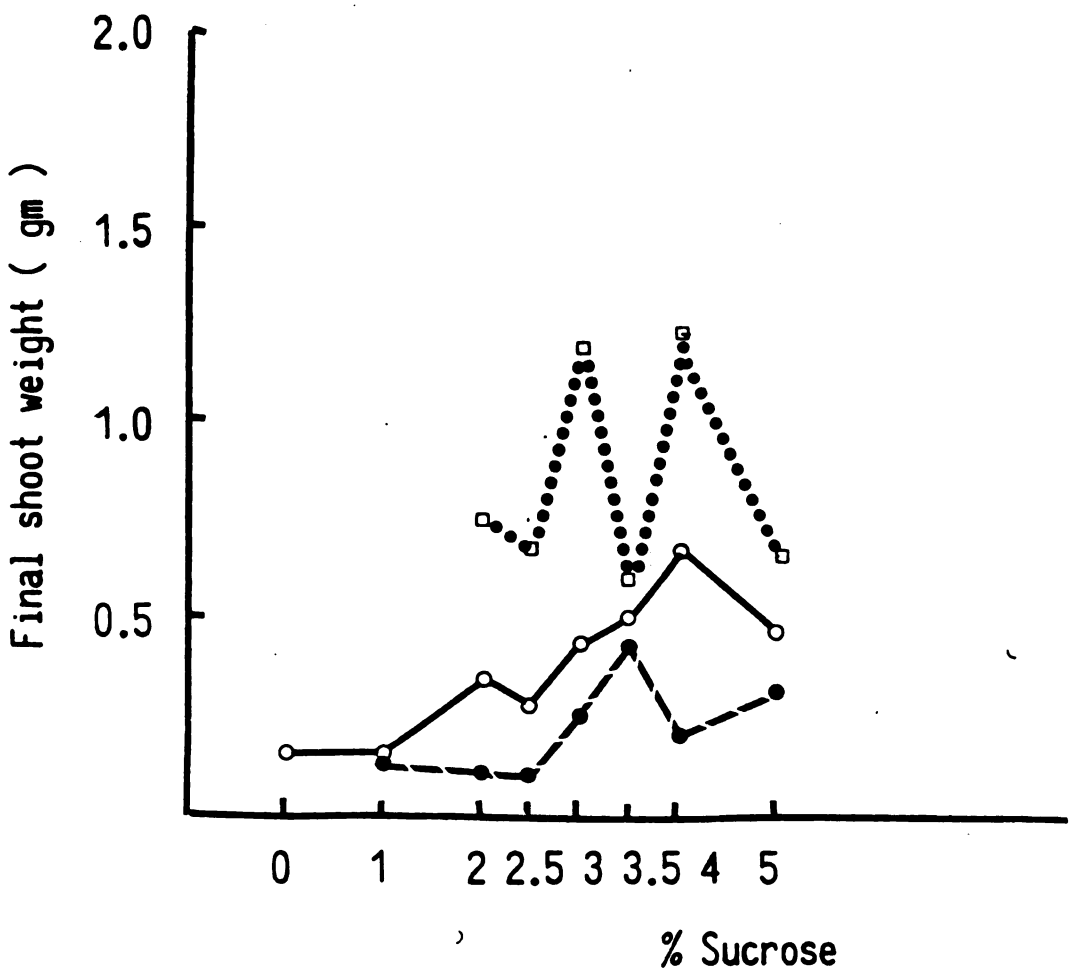
SE (N)	.0154	.0265	.1862	.055	.092	.0412	.0653	.0365
OSW	.014	.0097	.0164	.0142	.0117	.0122	.0119	.0101
SE (OSW)	.0056	.0052	.0032	.0037	.0033	.0024	.0038	.0058

Figure 9. Effect of iron on fresh weight increase of the wild-type nuclear and cytoplasmic albino of Petunia inflata.



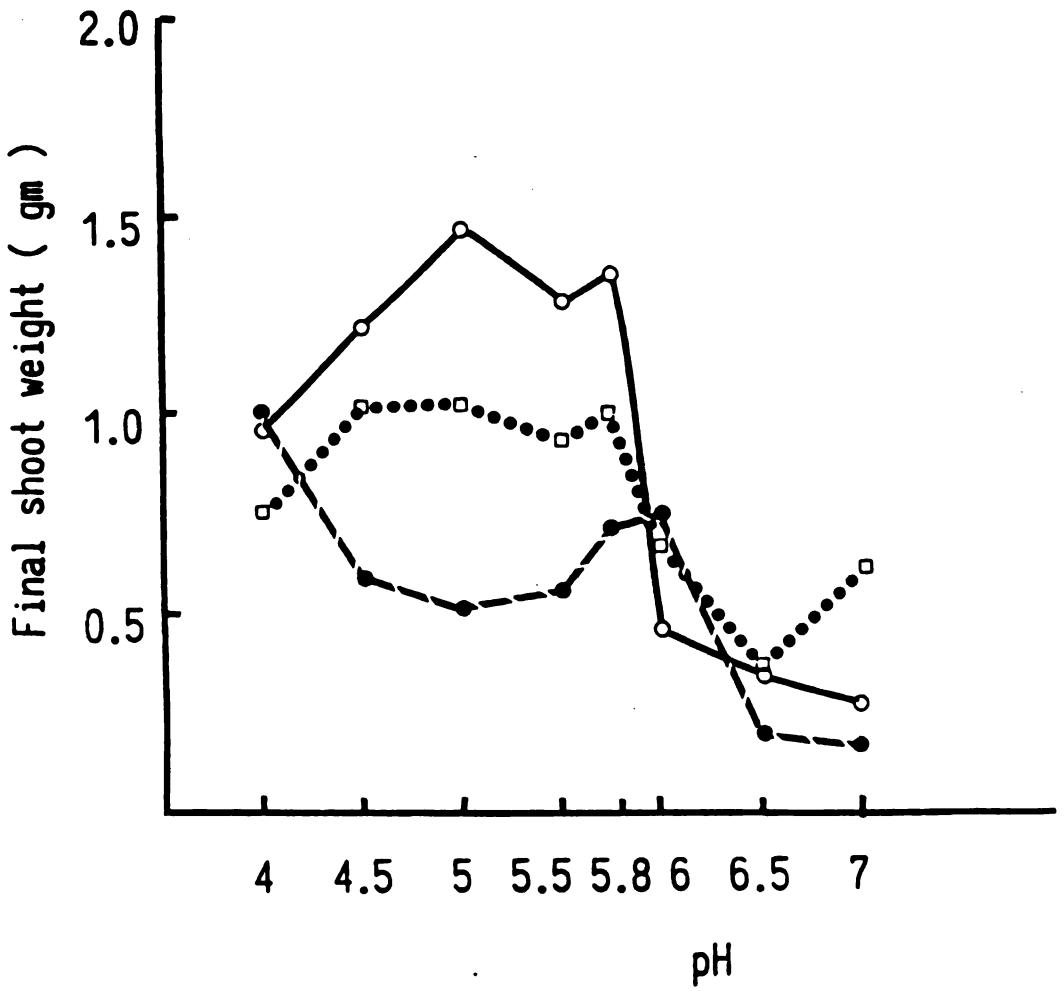
SE (G)	.0387	.0246	.2704	.2782	.1196	.0383	-	-
OSW	.0221	.0238	.0262	.0102	.0208	.0202	-	-
SE (OSW)	.0124	.0102	.007	.0058	.0057	.015	-	-
SE (C)	.0279	.0102	.4038	.2414	.1757	.0642	-	-
OSW	.0153	.0102	.0143	.0122	.0137	.0122	-	-
SE (OSW)	.0063	.0032	.0033	.0034	.0016	.0058	-	-
SE (N)	.0548	.0668	.0954	.182	.1195	.1151	.0233	.0545
OSW	.0102	.0114	.0119	.0125	.0152	.0176	.0133	.0134
SE (OSW)	.0047	.0047	.0044	.0035	.0065	.0071	.003	.0104

Figure 10. Effect of sucrose on fresh weight increase of the wild-type nuclear and cytoplasmic albino of Petunia inflata.



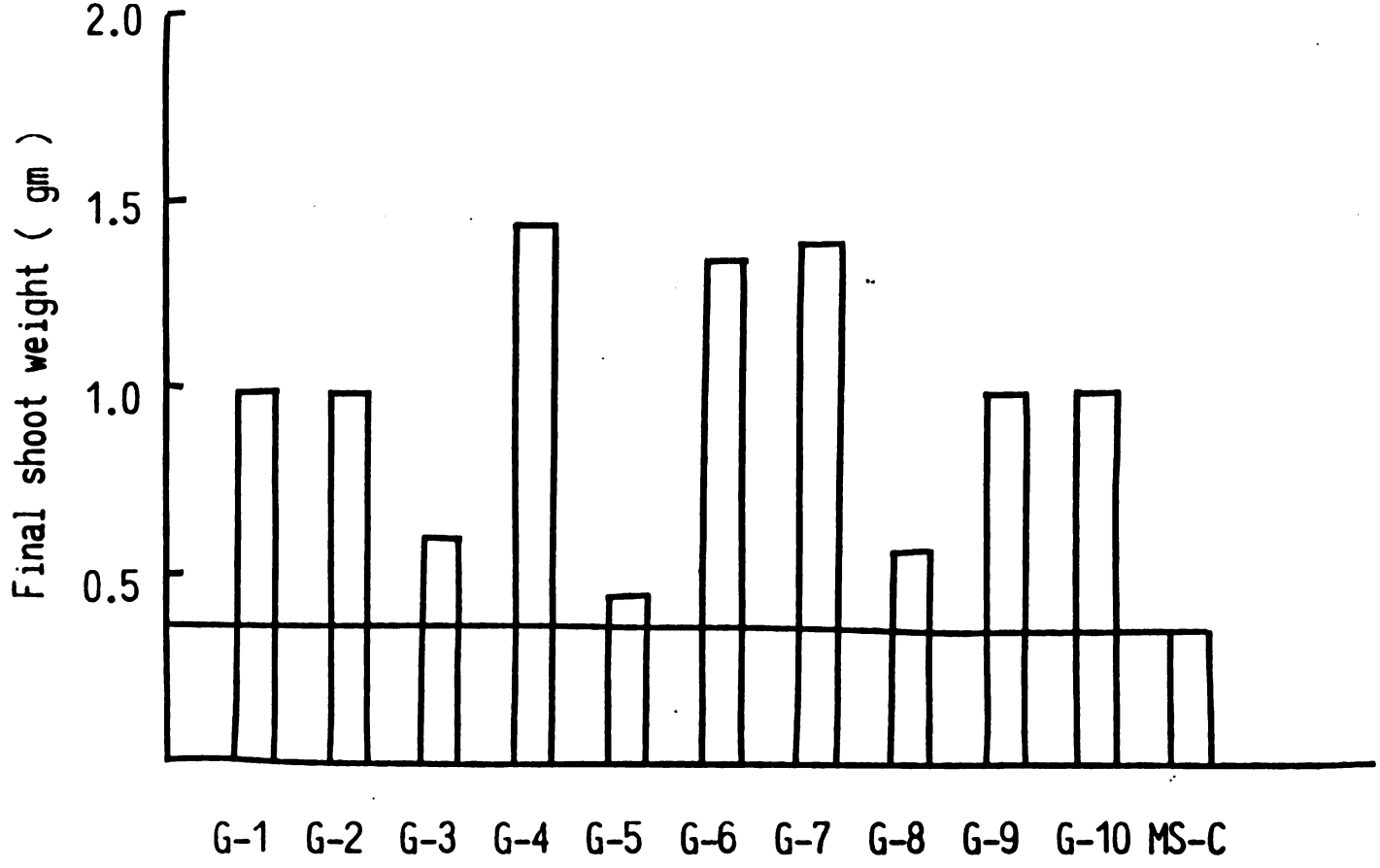
SE (G)	-	.0473	.229	.0378	.1667	.3095	.3164	.2402
OSW	-	.0228	.0248	.0306	.0245	.025	.0258	.024
SF (OSW)	-	.0096	.0116	.0137	.0066	.0105	.0088	.0098
SE (C)	-	-	.2085	.1986	.2273	.5865	.2584	.1536
OSW	-	-	.03	.037	.0344	.0348	.029	,0314
SE (OSW)	-	-	.0042	.01	.0061	.0142	.0097	.0089
SE (N)	-	.0372	.0526	.0624	.1787	.3476	.1693	.2894
OSW	-	.015	.0212	.0212	.0228	.0246	.021	.0298
SE (OSW)	-	.0014	.0067	.0115	.0082	.0144	.0057	.0126

Figure 11. Effect of pH on fresh weight increase of the wild-type, nuclear and cytoplasmic albino of Petunia. inflata.



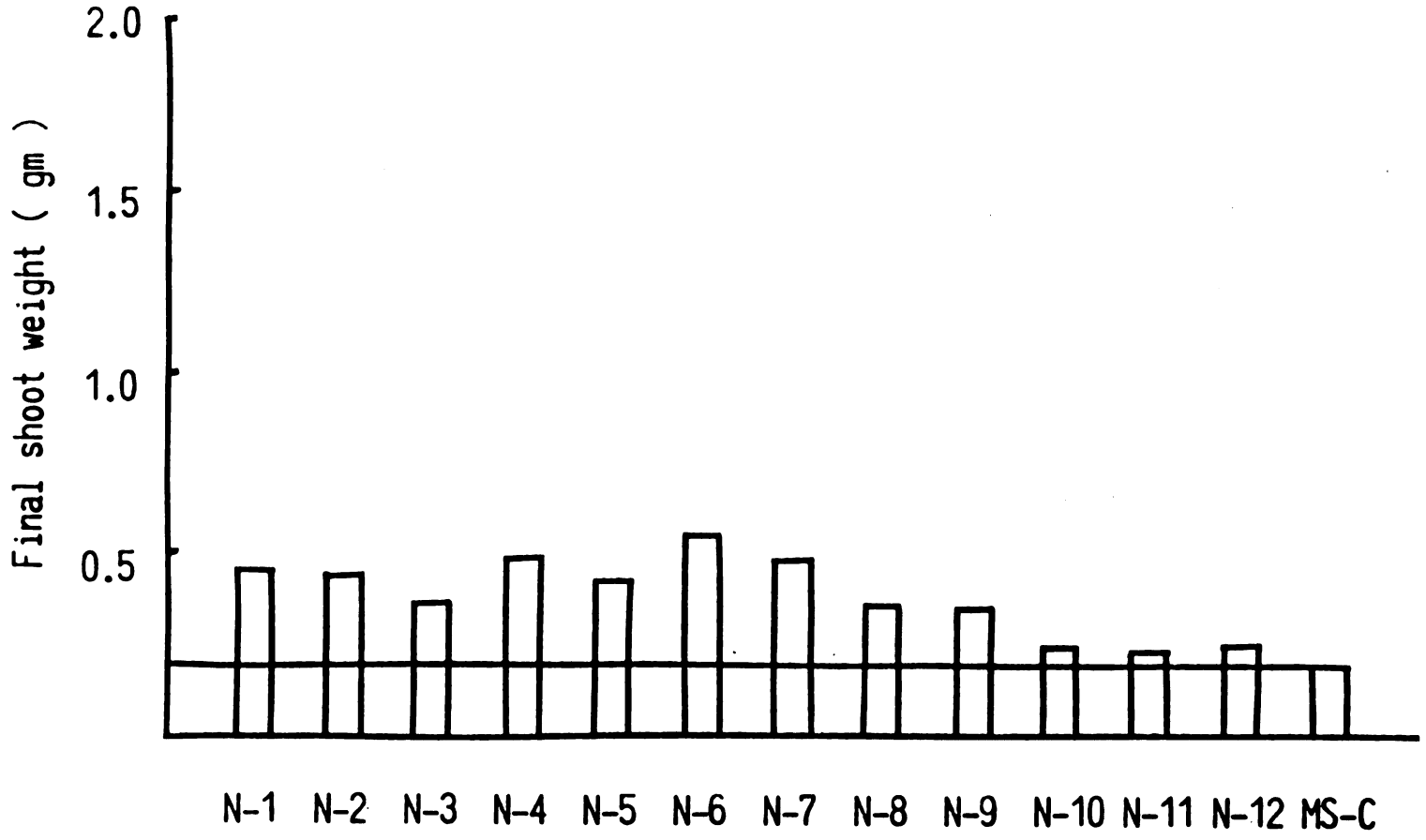
SF (G)	.4557	.3878	.3008	.3327	.6045	.1229	.2149	.0923
OSW	.021	.0268	.0222	.0316	.0234	.026	.0328	.0263
SE (OSW)	.0143	.0056	.0091	.0062	.0027	.0066	.0052	.0087
SE (C)	.6168	.2883	.4051	.5081	.5766	.4609	.3082	.1562
OSW	.0079	.0127	.0168	.0165	.0135	.0141	.0131	.0096
SE (OSW)	.0044	.0028	.0023	.0046	.0043	.0099	.0097	.0065
SE (N)	.0078	.2175	.171	.1566	.0651	.3055	.1723	.071
OSW	.0111	.0148	.0092	.0176	.021	.0204	.0135	.014
SE (OSW)	.004	.0098	.0025	.0076	.0041	.011	.005	.0025

Figure 12. Combination tests for normal green Petunia inflata .



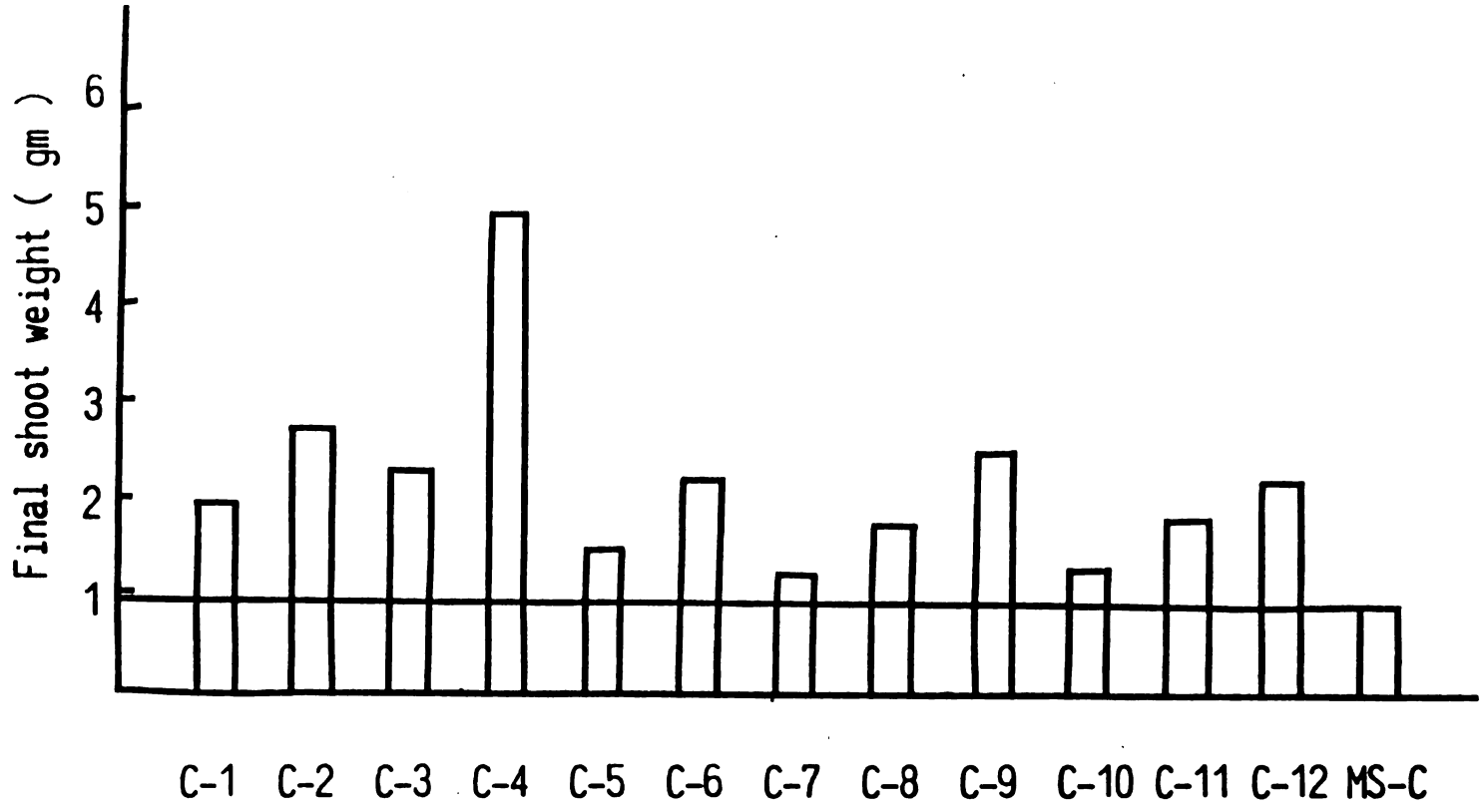
SE	.7001	.3781	.2715	.2784	.2473	.5006	.4502	.2073	.3381	.4166	.142
OSW	.0374	.032	.0355	.0315	.0282	.0304	.0346	.0306	.0296	.0336	.0298
SE (OSW)	.02	.0116	.0082	.0053	.0079	.0141	.0062	.0121	.0115	.0154	.0076

Figure 13. Combination tests for the nuclear albino of Petunia inflata.



SE	.3356	.1749	.1558	.153	.2117	.3177	.3171	.1594	.1389	.0786	.0785	.0852	.0866
OSW	.0244	.0272	.0223	.0172	.028	.02	.0143	.0207	.0163	.0183	.019	.018	.021
SE (OSW)	.0106	.0171	.0049	.0077	.0074	.008	.0074	.0168	.0095	.007	.001	.0064	.0082

Figure 14. Combination tests for cytoplasmic albino of Petunia inflata.



SE	.892	.2533	.8056	.848	.3328	.9399	.2271	.5927	1.3678	.6389	.547	1.48	.3095
OSW	.0208	.0166	.0192	.026	.025	.0226	.0228	.0226	.0264	.0195	.0236	.0238	.0244
SE (OSW)	.0137	.005	.0068	.0052	.0069	.0087	.0091	.003	.0036	.0099	.0094	.0048	.0013

DISCUSSION

Marked increases in fresh weight of three genetic strains of P. inflata can be achieved by modification of a few components in MS medium without any change in the mineral composition. The fresh weight increases are significant, from 2 up to 4 times higher than in the previous standard medium, MS-C. The requirements for growth factors such as CM and PE are optional but still helpful for the nuclear and cytoplasmic albinos whereas CH was found to be inhibitory. The role and composition of these complex factors have been suggested by Tulecke 1961, Steward and Caplin 1952, but up to now they are still obscure. In this research, growth regulators in the complex substances might not be the major contributors to the fresh weight increase since they were also tested both individually and in combinations and did not stimulate such increases. However, the growth regulators are still required for optimum growth. The hormonal requirements of albino plants were studied in the tobacco chlorophyll-deficient mutant by Schaeffer and Menser (1975) and high IAA (up to 10 mg/l) was found beneficial for growth which is in contrast to our work, in which high levels of IAA were inhibitory. All genetic strains of P. inflata in these studies required a rather high concentration of sucrose, even for the normal green plants, and they did not survive at the low level of sucrose (1%) although the green plants were autotrophic. The sucrose requirement is probably due to the lower light intensity which is inadequate for the

normal green plant to photosynthesize properly as compared to the greenhouse growing conditions. This high sucrose might also serve as a carbon source and partly as an osmotic stabilizer as proposed by Brown, et. al, 1979, and Coffin, et. al., 1976. Also, the optimum pH for maximum growth was found to be lower than in other media proposed (Murashige and Skoog 1962, Linsmaier and Skoog 1965, Gamborg et. al., 1968, Schenk and Hildebrandt 1972, Eriksson 1965, Ohira, et. al., 1973).

It is obvious that new media formulations with different salt concentrations and combinations might not be necessary to obtain optimum growth of chlorophyll deficient mutants. Actually, minor changes in the concentrations of the ingredients previously used in MS-C were found to increase the growth of three genetic strains of P. inflata from two up to four-fold. Apart from that, the quality and manner of incorporating the ingredients into media, e.g., autoclaved versus filter sterilized, were also found to be important, as had been suggested by Romberger and Tabor (1971). In order to obtain optimal growth, experiments have to be run systematically since interaction of ingredients, especially growth regulators, might be the key factor that regulates growth and differentiation as has been proposed in numerous publications. However, the minor point as stated above about integrating the ingredients and their quality must not be overlooked.

Tissue culture has great potential in industrial uses, agriculture and scientific studies. (Murashige 1974, Puhan, et. al., 1971, Reinert and Bajaj 1977, Thorpe 1978). Especially in plant propagation, the maximum growth in vitro is desirable to reduce operating and high labor cost.

This probably can be accomplished by simply some minor changes in the media ingredients or concentrations which can be tested quite easily in commercial laboratories. For scientific research, albinos interest scientists working in photosynthesis and also plant protoplast culture and somatic hybridization. However, very little work is done with albinos, especially the cytoplasmic albino because of the unavailability of plant materials. Optimum growth in vitro is required to overcome this problem. Regularly, somatic hybridization using albino or albino complementation systems utilize albino suspension cultures as cell sources of protoplasts. It is well-established that genetic instability exists in the calls state of tissue culture and also occurs in albino tissue culture in our experiments as well as regreening in albino in suspension culture of P. hybrida (Ad Kool - personal communication). The use of axenic culture of albino plants might have the advantage over the conventional suspension culture. Also, the regeneration capacity can also be retained.

SUMMARY

1. BAP was found non-beneficial for multiple shoot induction of three genetic strains of P. inflata. Green sectors were formed on leaves of cytoplasmic albino when grown in MS medium with BAP or K.

2. Growth factors, CM and PE stimulate growth in the albinos but inhibit in normal green plants. CH is inhibitory to all genetic strains tested as well as inhibiting chlorophyll formation in the cytoplasmic albino.

3. Growth regulators, IAA and K, are required for maximum growth and an antagonistic effect of auxin and cytokinin was observed.

4. Thiamine-HCl is required for the nuclear and cytoplasmic albino but not for the normal green plants.

5. Iron at 0.1 mM as in MS medium is optimum whereas higher sucrose and low pH are required for all genetic strains of P. inflata. The nuclear albino can perform quite well at pH 4.

6. Combination tests are necessary to identify the best combination for maximum growth because of the antagonistic effect of each ingredient.

ABSTRACT

CHLOROPHYLL PRECURSOR ANALYSIS AND THEIR MODIFICATION BY CHEMICALS IN CYTOPLASMIC AND NUCLEAR ALBINO MUTANTS OF PETUNIA INFLATA

by

Chumpol Borkird

A nuclear albino and a cytoplasmic albino of Petunia inflata were analyzed to determine the site of blockage in the chlorophyll biosynthetic pathway. Precursor accumulation was studied by ammoniacal aqueous acetone extracts of plants grown in different light regimes with and without feeding of δ- aminoleuvulinic acid. Millimolar extinction coefficient and Arnon's equation were used to calculate the quantity of chlorophyll and its precursors from absorption spectra. Wild-type plants were used as control. Total chlorophyll of wild-type plant is higher than either the nuclear or cytoplasmic albinos. The Chlorophyll a/b ratio increased when the normal green plants were grown in the dark and decreased in the albinos. The chlorophyll biosynthetic pathway of both albinos was blocked at Protoporphyrin IX. The nuclear albino can be considered less stable than the cytoplasmic albino. Plant extract and casein hydrolysate were found to inhibit chlorophyll synthesis in the nuclear and cytoplasmic albinos respectively. $FeCl_3$ partially overcame albinism in both the nuclear and cytoplasmic albinos whereas $FeSO_4$ and $Na_2FeEDTA$ only maintained traces of chlorophyll. Anthocyanin pigmentation was observed after incubating leaves in sucrose solutions containing iron.

INTRODUCTION

Chloroplast development and chlorophyll biosynthesis have been under intensive investigation for a number of years but, due to their complex control by both nuclear and cytoplasmic genomes (Akoyunoglou and Argyroudi-Akoyounoglou 1978), the genetic and biochemical mechanisms have not been fully elucidated. Many attempts and approaches have been used to study the chloroplast complex system including those on greening of ethiolated plants (Bengston, et. al., 1977, Shlyk, et. al., 1970), biochemical, physiological and genetic studies of mutants defecttive in chlorophyll biosynthesis (Gough 1972, Mascia 1978, Kahn, et. al., 1977, Nielso and Kahn 1973). Most of the mutants studied have been nuclear-controlled chlorophy-l dificiencies, especially in higher plants, since sytoplasmic-controlled mutant cells generally appear less frequently and mostly as small sectors of white tissue among green cells (Kirk and Tilney-Bassett 1978). Thus, they are not readily available as experimental materials. Cytoplasmic mutants of lower organisms, single cell algae Chlamydomonas, have been studied extensively (Sager 1972, Sager 1970, Sager and Ramanis 1971, Chiang 1971, Gilham 1969) but their chlorophyll biosynthetic mechanism differs significantly from higher plants. Therefore, we used tissue culture techniques to provide plantlets of both nuclear and cytoplasmic inherited albinos of P. inflata for analysis of chlorophyll precursors which accumulated under controlled environmental conditions following feeding with δ-aminoleuvulinic acid

(Granick 1959, Mascia 1978). Also, the effect of selected chemicals on the stability of albinism was investigated since chlorophyll formation has previously been found to be regulated to some degree by the chemica ingredients in the culture medium in lower organisms (Horrum and Schwartzbach 1980, Ellis, et. al., 1975, Aoki, et. al., 1965) and iron has also been found to correct albinism in maize (Bell, et. al., 1958, Bell, et. al., 1962).

MATERIALS AND METHODS

Plant Materials

Three genetic strains of P. inflata, the wild-type plants, a cytoplasmic and a nuclear inherited albino were used in these studies. All are diploid 2n = 2x = 14. The cytoplasmic albino originated as a mutant callus culture derived from wild-type protoplasts (J. B. Power - personal communication) and the nuclear albino by gamma irradiation of seeds (K. C. Sink - personal communication). These three stocks were propagated in vitro monthly by terminal and internode cuttings plated in modified Murashige and Skoog (1962) medium (MS-C). The modified MS medium contained MS salts and iron with 0.8 mg/l thiamine-HCl, 100 mg/l myoinositol, 3.0 mg/l indoleacetic acid (IAA), 0.3 mg/l kinetin (K) and 3% sucrose. It was solidified with 9.0 mg/l agar and the pH was adjusted with 0.1 N HCl or 0.2 N NaOH to 5.8 prior to autoclaving. All leaves of the cuttings were removed to prevent any carry-over chlorophyll contamination to the etioplasts. Subcultures one month old were used for all studies. The three genotypes were grown under 1) light $Em^{-2}s^{-1}$ 16-hour photoperiod 25± 2°C for 30 days; 2) 15 days in the same light condition and then transferred to the dark for another 15 days; 3) in the dark by wrapping the culture vessels with aluminum foil and kept in the dark for 30 days.

Chlorophyll and Precursor Analysis

Plant tissue, leaves and stems above the agar line of the light, half-light, half-dark (light/dark) and the dark-grown plants were removed and some were incubated with 0.01 N δ-aminoleuvulinic acid (δ-ALA)(Mascia 1978). There were three classes of δ-ALA feeding: 1) no feeding; 2) feeding for 6 hours; 3) feeding for 24 hours. All steps were performed in the dark under a dim green safe light. The green safe light was constructed using a fluorescent tube wrapped with several layers of green cellophane. The spectrum of the light emitted was measured with a spectroradiometer.

The leaves were homogenized with ammoniacal aqueous acetone (1 ml of 25% NH_4OH:9 ml H_2O:90 ml acetone) at 4°C under the green safe light, centrifuged at 10,000 rpm and the supernatant was collected. The detailed procedures of Kahn, et. al., 1976, were followed. The absorbance of the extracts was measured on a Beckman spectophotometer Model 25 and the pigment concentrations were calculated from the millimolar extinction coefficient by Kahn, et. al., 1976. The equations of Arnon (1949) were used to determine the amount of chlorophyll a and b:

Millimolar extinction coefficient

$$\text{Pchlide} = 0.0426\ OD_{628} + 0.0072\ OD_{590} - 0.0343\ OD_{575}$$

$$\text{Proto IX} = 0.1962\ OD_{575} + 0.0466\ OD_{590} - 0.0585\ OD_{628}$$

$$\text{Mg-Proto} = 0.0618\ OD_{590} + 0.0238\ OD_{575} - 0.0035\ OD_{628}$$

Millimolar extinction coefficient (continued)

(μ-moles/ml extract)

$C_a = 0.0127\ OD_{663} - 0.00269\ OD_{645}$

$C_b = 0.0229\ OD_{645} - 0.00468\ OD_{663}$

$C_{total} = C_a + C_b$ or $\frac{OD_{652} \times 1000}{34.5}$

All mg/l extract experiments were conducted with five replicates for all plants grown in an experimental environmental condition and feeding with δ-ALA. The mean values were used to calculate the precursor quantity, chlorophyll accumulation and likewise to establish absorbance curves.

Effect of Chemicals on Chlorophyll Formation

In the previous section of this thesis, some of the tested chemicals, casein hydrolysate (CH) and plant extract (PE), showed an inhibitory effect on chlorophyll formation by visual evaluation in the cytoplasmic and nuclear albino plants. In addition, certain forms of iron have previously been found to correct albinism in maize (Bell, et. al., 1958 and Bell, et. al., 1962) and were included in our studies to elucidate the influence of these chemicals on chlorophyll formation.

Nuclear and cytoplasmic albino plants were grown in modified MS medium as previously described. The light was 19 $Em^{-2}s^{-1}$ 16-hour photoperiod at 25$\pm$ 2°C for one month after which the leaves were cut

into small pieces (0.2 - 0.5 cm^2) and incubated in the chemical solutions. The aqueous test solutions had 3% sucrose with 0, 0.5, 1, 2, 5, 10, 100 and 500 ppm iron from $FeSO_4$, $7H_2O$, $FeCl_3$, or $Na_2FeEDTA$. The incubation proceeded for 2 - 3 days in plastic petri dishes placed in an environmental chamber under 54 - 60 $Em^{-2}s^{-1}$ light (GE F40CW, RS, WM), at a temperature of $25^{\pm}$ 2°C. Changes in pigmentation were evaluated visually.

Table 1. Chlorophyll Accumulation in the Wild-type Petunia inflata, Cultured in the Light, Light/Dark or Dark and Fed with 0.1 N δ-ALA for 0, 6 and 24 hours.

Environment	Time of δ-ALA Feeding	Chlorophyll Accumulation (mg/l)			
		Chl a	Chl b	Chl (total)	Chl a/b ratio
Light	0 hr.	31.1980	29.0130	60.2100	1.0753
	6 hr.	30.7200	18.1500	48.8670	1.6926
	24 hr.	30.8940	20.3090	51.2030	1.5212
Light/Dark	0 hr.	22.1500	6.6748	22.8170	3.3185
	6 hr.	23.1200	6.3914	29.5110	3.6174
	24 hr.	19.9820	5.9211	25.9040	3.3741
Dark	0 hr.	0.1864	0.0891	0.2755	2.0920
	6 hr.	0.5734	0.0891	0.2755	2.3047
	24 hr.	0.3363	0.2397	0.5760	1.4030

Table 2. Chlorophyll Accumulation in the Cytoplasmic albino *Petunia inflata*, Cultured in the Light, Light/Dark or Dark and Fed with 0.1 N δ-ALA for 0, 6 and 24 Hours.

Environment	Time of δ-ALA Feeding	Chlorophyll Accumulation (mg/l)			
		Chl a	Chl b	Chl (total)	Chl a/b ratio
Light	0 hr.	1.3562	0.4533	1.8095	2.9918
	6 hr.	1.1433	0.6522	1.7955	1.7530
	24 hr.	1.5320	1.3152	2.8472	1.1648
Light/Dark	0 hr.	0.5215	0.2460	0.7675	2.1199
	6 hr.	0.3343	0.1647	0.4990	2.0298
	24 hr.	0.3597	0.3823	0.7420	0.9409
Dark	0 hr.	0.0087	0.0297	0.0384	0.2929
	6 hr.	0.0863	0.1654	0.2517	0.5218
	24 hr.	0.0374	0.0958	0.1332	0.3904

Table 3. Chlorophyll Accumulation in the Nuclear Albino *Petunia inflata* Cultured in the Light, Light/Dark or Dark and Fed with 0.1 N δ-ALA for 0, 6 and 24 Hours.

Environment	Time of δ-ALA Feeding	Chlorophyll Accumulation (mg/l)			
		Chl a	Chl b	Chl (total)	Chl a/b ratio
Light	0 hr.	7.5495	3.0676	10.6170	2.4610
	6 hr.	6.2590	3.0480	9.3070	2.0535
	24 hr.	6.3070	3.3194	9.6264	1.9000
Light/Dark	0 hr.	1.4417	0.6562	2.0979	2.1970
	6 hr.	0.9931	0.5235	1.5166	1.8970
	24 hr.	0.8492	0.6839	1.5331	1.2417
Dark	0 hr.	0.1169	0.1015	0.2184	1.1517
	6 hr.	0.1440	0.0675	0.2115	2.1333
	24 hr.	0.1556	0.1442	0.2998	1.0791

Table 4. Chlorophyll Precursor Accumulation in the Wild-type Petunia inflata, Cultured in the Light, Light/Dark or Dark and Fed with 0.1 N δ-ALA for 0, 6 and 24 Hours.

Environment	Time of δ-ALA Feeding	Precursor Accumulation (n moles/ml)		
		Proto-IX	Mg-Proto	Pchlide
Light	0 hr.	107.2900	60.0130	28.3340
	6 hr.	76.8690	43.4030	21.0000
	24 hr.	85.6300	47.3900	23.4200
Light/Dark	0 hr.	43.5800	23.4330	10.4640
	6 hr.	41.6400	22.9500	10.9170
	24 hr.	40.0100	21.4200	9.5008
Dark	0 hr.	0.9678	0.4206	0.1080
	6 hr.	2.8800	1.2670	0.3052
	24 hr.	3.2940	1.3430	0.2461

Table 5. Chlorophyll Precursor Accumulation in the Cytoplasmic Albino *Petunia inflata*, Cultured in the Light, Light/Dark or Dark and Fed with 0.1 N δ-ALA for 0, 6 and 24 Hours.

Environment	Time of δ-ALA Feeding	Precursors Accumulation (n moles/ml)		
		Proto-IX	Mg-Proto	Pchlide
Light	0 hr.	3.5529	1.7705	0.6478
	6 hr.	7.8882	3.7804	1.3802
	24 hr.	16.3600	7.3261	2.4088
Light/Dark	0 hr.	2.2097	1.0421	0.3049
	6 hr.	2.3327	0.9980	0.3394
	24 hr.	4.8383	2.0773	0.6525
Dark	0 hr.	0.4209	0.1969	0.0133
	6 hr.	2.1100	0.8705	0.1126
	24 hr.	1.9118	0.7662	0.0626

Table 6. Chlorophyll Precursor Accumulation in the Nuclear Albino *Petunia inflata*, Cultured in the Light, Light/Dark or Dark and Fed with 0.1 N δ-ALA for 0, 6 and 24 Hours.

Environment	Time of δ-ALA Feeding	Precursors Accumulation (n moles/ml)		
		Proto-IX	Mg-Proto	Pchlide
Light	0 hr.	14.7500	8.1225	3.7160
	6 hr.	17.9400	9.8018	5.4683
	24 hr.	39.5370	12.2225	7.5250
Light/Dark	0 hr.	3.6914	1.8740	0.7309
	6 hr.	4.4682	2.0166	0.6251
	24 hr.	10.5030	3.6156	0.9086
Dark	0 hr.	0.9261	0.4275	0.1249
	6 hr.	1.2321	0.5063	0.0546
	24 hr.	1.5778	0.6706	0.2125

Table 7. Visual Observation for Chlorophyll Formation 2 Days Following Feeding of Albino Petunia inflata mutants with $FeSO_4$

Mutants	$FeSO_4$ (ppm) + 3% Sucrose							
	0	0.5	1	2	5	10	100	500
Nuclear	W	Y+ANT	Y+ANT	Y+ANT	Y	Y	Toxic	Toxic
Cytoplasmic	W	Y	Y	Y	Y+ANT	Y+ANT	Toxic	Toxic

W - White (loses the traces of chlorophyll)

A - Greenish-yellow color (with traces of chlorophyll)

ANT - Anthocyanin Formation

Table 8. Visual Observation for Chlorophyll Formation 2 Days Following Feeding of Albino Petunia inflata mutants with $FeCl_3$

Mutants	$FeCl_3$ (ppm) + 3% Sucrose							
	0	0.5	1	2	5	10	100	500
Nuclear	W	Y	Y+ANT	Y+ANT	Y+ANT	Y+ANT	Toxic	Toxic
Cytoplasmic	W	Y	Y	Y	Y	Y	Toxic	Toxic

Table 9. Visual Observation for Chlorophyll Formation 2 Days Following Feeding Albino *Petunia inflata* Mutants with Na_2EDTA

Mutants	Na_2EDTA (ppm) + 3% Sucrose							
	0	0.5	1	2	5	10	100	500
Nuclear	W	Y+ANT	Y+ANT	Y+ANT	Y+ANT	Y+ANT	Toxic	Toxic
Cytoplasmic	W	Y	Y	Y	Y	Y	Toxic	Toxic

RESULTS

Chlorophyll and Chlorophyll-Precursor Accumulation in Normal Green, Nuclear and Cytoplasmic Albinos of P. inflata

Normal green plants, with or without feeding with δ-ALA, accumulated considerably greater amounts of chlorophyll a, b and total chlorophyll in comparison to the albinos (Tables 1, 2 and 3). The nuclear albino accumulated higher quantities of chlorophyll than the cytoplasmic albino. The total chlorophyll content of the nuclear albino was about six-fold less than that of the normal green plants when grown in the light, irrespective of the duration of δ-ALA feeding periods. The chlorophyll content of the cytoplasmic albino was 20 to over 30 times lower than that of normal green plants under similar conditions. In the light/dark regime, the chlorophyll content of the normal green plants was only 2 - 3 times lower than those under light grown conditions. But, when compared to the albinos, the chlorophyll content of the nuclear albino was five to six times lower than the light grown condition and 10 - 20 times lower than the normal green plants grown under the same condition. For the cytoplasmic albino, under the light/dark growing condition, the chlorophyll content decreased two to 4.5 times in comparison to the light grown plants. This response is similar to the decrease in the normal green plants and when compared to the normal green plants in the same growing condition, is about ten- to twenty-fold lower. In the

dark, the chlorophyll content of the green plants was reduced sixty to 200-fold, significantly lower than that for light grown plants. In contrast to the normal green plants, the chlorophyll content of the dark grown nuclear albino decreased less than fifty-fold, whereas it was much lower for the cytoplasmic albino. The comparison was made for all durations of feeding with δ-ALA.

Even though there were contrasting differences in the chlorophyll content between the normal green and albinos, the chlorophyll a/b ratio was similar. However, the chlorophyll a/b ratio tended to increase when the normal green plants were exposed to dark conditions. A reverse response occurred in the albinos, for plants grown in light/dark or total darkness.

The chlorophyll precursor analyses are summarized in Tables 4, 5 and 6. The level of precursors accumulated in normal green P. inflata were higher than those of the nuclear albino. In turn, the nuclear albino accumulated higher levels of precursors than the cytoplasmic albino. The nuclear albino had obvious traces of chlorophyll on the leaves, especially at the tips and the mid-rib regions while the cytoplasmic albino had a uniform pale yellow color. The amount of precursors accumulation is known to be controlled by the feedback inhibition of the precursors themselves; therefore, plants were externally fed with δ-ALA in order to more precisely determine accumulation. The resulting data were compared by computing the ratio between Proto IX to Mg-Proto and Pchlide, by setting Pchlide at 1.0 because accumulation is a relative value.

The normal green plants did not accumulate a significantly higher amount of any precursor upon feeding with δ-ALA. Only dark grown plants

had a small increase in Proto IX. However, the Pchlide of the dark grown normal green P. inflata did not increase.

Unlike the normal green plants, the precursors present in albinos accumulated significantly higher quantities following feeding with δ-ALA. This increased precursor accumulation occurred not only in plants under the dark grown condition but also for those under light/dark and light grown conditions. When Proto IX and Mg-Proto were compared to the Pchlide, the quantity of Mg-Proto did not increase but Proto IX increased three to 4.5-fold under dark grown conditions and 1.2 to 2-fold in the light/dark grown condition. The increases in the precursors were more obvious in the cytoplasmic as compared to the nuclear albino.

Effect of Iron Concentration and Sucrose on Chlorophyll Formation in Nuclear and Cytoplasmic albinos

Iron, in all forms tested, maintained traces of chlorophyll content in both the nuclear and cytoplasmic albinos. Leaf pieces floated in the sugar solution without iron turned white. Leaf pieces incubated in the sucrose solution with iron ions maintained traces of chlorophyll. The nuclear albino leaf pieces did not exhibit traces of chlorophyll in the sucrose solution alone. However, chlorophyll content was present on pieces incubated with sucrose solution plus 0.5 - 1.0 ppm iron from $FeCl_3$, $FeSO_4$ and Na_2EDTA. The iron sources were found to be toxic at 100 and 500 ppm. No obvious differences were found in the degree of greening of top leaves incubated in sucrose solution containing

0.5 - 10 ppm iron from Na_2EDTA or $FeSO_4$. However, with $FeCl_3$ the quantity of the chlorophyll for the nuclear albino was more intense in solutions with one to five ppm. Less greening occurred in the 0.5 and 10 ppm $FeCl_3$ solutions.

Anthocyanin pigment accumulated when the leaf pieces of the nuclear albino were incubated in the solution containing certain forms and levels of iron. $FeCl_3$ between one to ten ppm, Na_2EDTA at 0.5 to ten ppm and $FeSO_4$ at 0.5 to two ppm all induced anthocyanin accumulation in nuclear albino leaves. The anthocyanin pigmentation occurred as purple sectors on the otherwise pale, greenish-yellow leaves.

The cytoplasmic albino responded similarly to the nuclear albino in that traces of chlorophyll were not retained in the sucrose solution without iron. The greenish-yellow color was only retained in sucrose solutions with iron in the form of $FeCl_3$ at 0.5 to ten ppm or at 0.5 to ten and at 0.5 to 1.0 ppm of $FeSO_4$ or Na_2EDTA, respectively. Iron from all forms was toxic at one hundred and five hundred ppm. For $FeCl_3$, increases in the degree of greening was correlated with changes in concentrations. Greening was not pronounced in solutions with 0.5 ppm $FeCl_3$ and as the concentration increased from one to ten ppm, the greening decreased. The occurrence of anthocyanin pigmentation in the cytoplasmic albino was rare. A few sectors that contained faint anthocyanin were observed only at five to ten ppm $FeSO_4$.

DISCUSSION

This research indicated that the nuclear controlled Petunia inflata albino is unstable with respect to the small amount of chlorophyll accumulation. Conversely, the cytoplasmic albino mutant was quite stable. These observations were not entirely unexpected since visual observations on the degree of greening of stock cultures of these two mutant lines revealed the same pattern. The phenotypic variability of a mutant defective in chlorophyll biosynthesis was previously studied in Nicotiana tabacum var. "Samson" and was believed to be controlled genetically (Deshayes 1979). Regreening as a result of a back mutation may be observed more frequently in the nuclear albino which is inherited as a recessive trait, because one back mutation in the nuclear genome could directly correct the albinism in all the progeny cells. Since there are many chloroplasts per cell, the cytoplasmic albino would have to undergo the sorting-out process following a back mutation prior to the green pigmentation being readily observed.

That chlorophyll content decreased more in etiolated wild-type plants in comparison to the albinos was also expected, since it contains a much higher amount of chlorophyll. However, the observation that the chlorophyll a/b ratio increased in normal green plants but decreased in the albinos when grown in the dark needs more study to reach a definitive interpretation. Some chlorophyll inhibitors have been

found to change the a/b ratio. The antibiotic terramycin, digitoxin and plant growth regulators such as BAP and K altered the chlorophyll a/b ratio (Wolf 1977). BAP and K, which decrease the chlorophyll a/b ratio and change chlorophyll content (Parthier 1979) may stimulate back mutation in suspension cultures of albino P. hybrida Comanche (Ad Kool - personal communication). Thus, they might be responsible for this phenomenon as observed herein by green sector formation on leaves of the cytoplasmic albino P. inflata. The albinism might cause removal or accumulations of the inhibition; therefore, the chlorophyll a/b ratio decreased, whereas it increased in normal green plants. However, added cytokinins were not able to overcome a genetically-caused defect in chloroplast formation nor were they necessary for the regreening process in N-deficient leaves of plants (Parthier 1979). Changes in chlorophyll a/b ratio have also been observed in mutants of pearl millet where the ratio increased, decreased or remained constant (Kodurn and Kao 1980).

The discovery by Granick (1959) that plants accumulate more chlorophyll precursors upon exogenous feeding with δ-ALA has enabled studies in chlorophyll defective mutants to be investigated extensively. Both maize and barley have been studied relative to where blockage occurred in the chlorophyll biosynthetic pathway by determining chlorophyll precursor accumulation (Mascia 1978, Gough 1972). However, there are few reports on how to bypass the blocked steps (Bell, et. al., 1958, Bell, et. al., 1962).

The cytoplasmic and nuclear albinos were both found to be blocked

at the Proto IX synthesis step since it was accumulated when externally fed with δ -ALA. These Petunia mutants are thus similar to the mutants 1* Blandy-4, OY-1040 and I-13 in maize (Mascia 1978) and several other mutants as reviewed by Gough (1972). There are several possible reasons for the block in chlorophyll biosynthesis pathway. First, a change in a structural gene causing inhibition of enzyme production or alteration of the enzyme conformation so that the pathway cannot proceed. A second reason could be changes in the chloroplast membrane. Since the biosynthetic reactions following Proto IX are membrane-bound (Smith and Rebeiz 1979), mutations affecting conformation of the chloroplast membrane may result in defective chlorophyll biosynthesis. Bell, et. al., (1958) and Bell, et. al., (1962) also proposed that albinism can be caused by the inability of plants to take up iron for chlorophyll synthesis and can be corrected by external feeding with the propor iron form. Iron is known to be required for chlorophyll biosynthesis and chloroplast development (Machold and Stephan 1969 and Stocking 1978). The last possibility may be operative in the Petunia albino mutants. Traces of chlorophyll in albino leaves could be maintained by feeding with sucrose solutions containing iron, but the regreening response was only partial since the leaves did not turn completely green. Further efforts could be made by culturing the leaves in vitro on medium containing $FeCl_3$ instead of $Na_2FeEDTA$. However, the involvement of growth regulators, especially cytokinins, which are known to alter chlorophyll content and the optimal requirement of iron for growth, which does not relate to its optimal concentration for growing, have to be taken into account.

The cytoplasmic albino was observed to form green sectors when

grown *in vitro* with high levels of cytokinins (K or BAP). This phenomenon was not observed with the nuclear albino. The green sectors are believed to be formed by back mutation of the chloroplasts to wild-type. The progenitor heteroplastic cells divide and are accompanied by the sorting-out process which eventually leads to certain cells containing only normal green chloroplasts (Kirk and Tilney-Bassett 1978). Since the high cytokinin level in the culture medium induces multiple shoots, the sorting-out process may occur more frequently in the newly-formed shoots. Or, if the shoots are derived from callus at the base of terminal shoot cuttings, the green sectors are more liekly to be caused by back mutation and not by albinism correction since the other leaves remain phenotypically albino.

The previous experiments also showed that casein hydrolysate and plant extract supplied in the culture medium inhibited chlorophyll biosynthesis in the cytoplasmic and nuclear albino, respectively, since the usual traces of chlorophyll disappeared. The nutritional regulation of chlorophyll formation has previously been studied in algae (Horwen and Schwartzbach 1980, Ellis, et. al., 1975). The ratio of nitrogen to carbon is believed to control the quantity of chlorophyll in *Euglena* and elevated concentrations of acetate, especially combined with a low level of nitrogen, inhibit chlorophyll synthesis in *Goclenkinia*. More recently, McCarty and Rebeiz (1980) found that an extract from cucumber cotyledons inhibited chlorophyll biosynthesis and accumulation in the cotyledon itself. This response is similar to our experiment wherein the wild-type plant extract inhibited chlorophyll biosynthesis of the nuclear albino. As stated previously,

the mutants accumulated Proto IX; however, the block is incomplete. Plant extract may act as a secondary block for the bypass chlorophyll at Pchlide. Plant extract was found effective only for the nuclear albino. On the other hand, casein hydrolysate inhibited traces of chlorophyll formation only in the cytoplasmic albino. The mode of action of casein hydrolysate on chlorophyll biosynthesis has not been reported but can be considered to be different from plant extract since it differentially affected the nuclear and cytoplasmic controlled albinos.

In experiments on external feeding of iron in three different forms to albino leaf pieces, it was observed that anthocyanin pigment was formed when the cytoplasmic albino was fed with $FeSO_4$, but only faintly in a few sectors. In contrast, anthocyanin pigmentation occurred in abundance in the nuclear albino when supplied with any form of iron tested. Anthocyanin has been reported to accumulate in cell cultures of Happlopappus gracilis when grown in low auxin (Constabel, et. al., 1971) and Onslow (1925) reviewed the effect of sugar, physical and chemical factors, reaction with iron and mechanical injuries influence anthocyanin pigmentation. The formation of anthocyanin pigment in the albino leaves might occur through a combination of the above effects since sugar feeding alone was not stimulatory.

It is obvious that the nuclear albino is physiologically quite different from the cytoplasmic albino and these differences are quantitative. Nuclear genes controlling albinism have been studied extensively. Conversely, cytoplasmic albinos have been studied much less extensively, especially in higher plants, because of the unavailability

of plant materials. However, since both types of mutants result in the same phenotype expression, the inability to produce a normal quantity of chlorophyll, the background physiological processes can be assumed to be alike.

Kirk and Tilney-Bassett (1978) have suggested that tissue culture would be a useful tool to study the genetics and physiology of albinism in plants. This is obviously true, especially for the cytoplasmic determined albinos which need more intensive investigation in order to understand their role in chlorophyll biosynthesis and their interaction with nuclear genes.

SUMMARY

1. The nuclear albino Petunia inflata was more unstable than the cytoplasmic albino and accumulated total chlorophyll six times lower than the normal green plants. The nuclear albino accumulated twenty to thirty times less than the normal green wild-type plants.

2. Chlorophyll content of normal green plants decreased more than in either albino mutant when grown in the dark as compared to plants grown in the light.

3. The chlorophyll a/b ratio increased when normal green plants received the dark grown condition but hte situation was reversed for both albino mutants.

4. Etiolated normal green plants accumulated a small amount of Proto IX and the accumulation was not increased significantly when the plants were fed with δ-ALA. Pchlide was not observed to show any increase.

5. Both the cytoplasmic and the nuclear albinos accumulated Proto IX at a significantly higher amount when grown in the dark. The cytoplasmic albino accumulated relatively higher levels of Proto IX when compared to the nuclear albino.

6. The albino mutants retained traces of chlorophyll upon external feeding with $FeSO_4$, $FeCl_3$ or Na_2EDTA. $FeCl_3$ showed a shift effect on the greening level of the albino leaf pieces incubated in the sucrose solution with $FeCl_3$ at certain concentrations.

7. Anthocyanin pigmentation accompanied chlorophyll retention in the albino mutants. The nuclear albino, upon feeding with certain concentrations of $FeCl_3$, $FeSO_4$ or $Na_2FeEDTA$, consistently showed anthocyanin formation, but it was very rare with the cytoplasmic albino.

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