To the Graduate Council:

I am submitting herewith a thesis written by Myong Sheila Jeon entitled "Cyclamen Seed Germination and Tuber Regeneration in vitro." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Ornamental Horticulture and Landscape Design.

Effin T. Graham, Major Professor

We have read this thesis
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Vice Chancellor
Graduate Studies and Research

CYCLAMEN SEED GERMINATION AND TUBER REGENERATION IN VITRO

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Myong Sheila Jeon

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ABSTRACT

Cyclamen seeds which were sterilized for 2 minutes with a solution containing 10% Clorox and 90% Alconox and placed on Knudson's medium (- sucrose) were compared with seeds sown in a darkened bed in the greenhouse. Germination was 5 weeks earlier *in vitro* than in the greenhouse. Germination was 90% *in vitro* and 60% in the greenhouse. *In vitro* germination was similar at 20 and 22°C. Similar rates of germination were achieved *in vitro* with unsterilized seeds but contamination ensued. Even sterilized seeds were contaminated when the nutrient agar medium contained the recommended quantity of sucrose.

Cyclamen endosperm was shown to consist of small condensed protoplasts widely separated by massive cell walls in both dry and imbibed but nongerminating seeds. The histological results revealed that germination was accompanied by decomposition of endosperm cell walls.

Tubers were separated from seedlings germinated *in vitro*, sterilized in Clorox-Alconox solution for 30 minutes, divided in pieces, and placed on either Knudson's medium or Murashige shoot-tip rooting medium. Explanted tuber pieces regenerated roots and shoots when auxin and kinetin were included in the media. No regeneration occurred without the hormones. Tubers retained high regenerative capacity when divided in halves or thirds but not in fourths.

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CHAPTER I

INTRODUCTION

The genus Cyclamen belongs in the family Primulaceae. It is endemic to southeastern Europe, eastern Mediterranean islands, Asia Minor and the Middle East. Speciation is complex and there is controversy on classification of the fifteen or twenty species. However, there seems to be general agreement that only Cyclamen persicum Mill has been involved in derivation of the florist pot plant (Lyons and Widmer, 1980). This is the species which is under discussion in this thesis, and hereinafter is referred to as cyclamen.

Cyclamens are very beautiful herbaceous pot plants with gracefully nodding flowers and silvery green leaves arising from tubers. Flower colors are bright pearly white, pink, rose, scarlet or purple. A long blooming period is combined with outstanding flowers and foliage. Thus cyclamen should be a popular florist product. Prices have always been too high, however, due to the length of time required to produce salable plants, and this remains true today.

Cyclamen is propagated by division of tubers, but there is no rapid method for vegetative cloning of cultivars. Commercial propagation is entirely by seed which must be produced by hand pollination in the greenhouse and is very expensive. Furthermore, germination is slow and erratic and percentage yield of seedlings is low. Seedlings may continue to emerge up to five months, thus lowering efficiency of transplanting and potting and increasing

production costs.

In the older culture method for cyclamen, 18 months passed from sowing the seed until the plants bloomed. A recently developed cultural procedure has reduced production time to seven month but at the expense of substantial reduction in size of blooming plants. In both older and newer methods, however, the time taken from sowing seed to transplanting seedlings is similar, about five months. Any significant reduction of the excessively long time required for seedling production should increase profitability of cyclamen as a florist crop.

Since cyclamen seems to be very sensitive and responsive to its environment (Valaskova, 1975), it is desirable to test propagation in vitro where factors affecting growth can be isolated and controlled. The objectives selected for this thesis investigation were threefold. First, the major effort was given to establishing a technique for seed sterilization, a nutrient medium favorable for germination, and the time required for germination. Second, the seed was examined histologically to identify structural factors which may contribute to erratic and slow seed germination. Third, the possible regeneration of juvenile tubers was tested with tubers removed from seedlings produced in sterile nutrient culture.

CHAPTER II

LITERATURE REVIEW

A. Seed Germination

Although there has been no experiment of *in vitro* seed germination of cyclamen, many attempts have been made to improve seed germination in greenhouse conditions. Hill (1920) investigated the differentiation of cotyledon during the development of cyclamen seeds. Sumitomo and Kosugi (1963) reported the effects of temperature, pH, and seasonal changes on germination of cyclamen seed. Optimum temperature for germination was 16 - 20°C. All the seeds died at 30°C, whereas the seeds simply did not germinate at 25°C. Seeds which did not germinate at 25°C germinated when transferred to 16°C after 49 days. The best pH was between 5.5 and 6.5. Seeds were harvested on June 19 and sown every 10 days, until September 7. Germination was greater than 90%. However, germination duration was shortened as the number of days after the harvest increased.

Stephens and Widmer (1974) recommended that seed should be as fresh as possible. Storing seeds for long periods before use would significantly lower the germination percentage. They also recommended sowing 1/8 to 1/4 inch deep in flats filled with moist, nutrient-enriched peat moss. Seeds sown too deep, emerged late and the tuber's growing point was often below the soil surface. If seeds were sown too shallow, the seedling's root system was not properly anchored. Cyclamen seed germinated best in complete darkness at 18 -

20°C, a high humidity (70%) and with good air circulation. Widmer and et al. (1979) reported that because cyclamen seeds were susceptible to attack by Botrytis and other fungal pathogens and the germination chamber should be disinfected if possible. Seeds sown in nutrient-enriched peat germinated well. No fertilization was needed until 2 months after sowing.

Anderson and Widmer (1975) reported that cyclamen seed germination was improved with surface disinfestation and gibberellin treatments. For this experiment, the seeds were germinated on commercially available paper germination towels. Seeds were treated in various ways, such as hot water, alternating temperature, moist storage, fungicides and gibberellin. The numerous treatments utilized resulted in better germination than the controls. Dormancy was not a factor affecting germination of cyclamen seeds which were at least 6 months old as contrasted to freshly harvested seed. They concluded that "inadequate expression of seed vigor during germination under incompletely defined germination conditions appear to be the primary cause for slow and poor germination of cyclamen seed."

B. Morphlogy of Endosperm of Germinating Seeds

Anderson and Widmer (1975) reported that embryos with a minimal amount of endosperm surrounding them began to grow and 70% germinated and were growing normally after 19 days, whereas the embryos completely removed from the seed did not show any sign of growth. Gibberellin treatment accelerated seed germination. Mayer and Shain (1974) reported that gibberellins in some manner control the de novo

synthesis of α-amylase in the endosperm.

Jacobson et al. (1979) reported that although only a few cases have been examined, living endosperm cells in different plant families have a number of structural features in common. Furthermore, similar structural changes occur during germination. McNeil et al. (1975) found that in barley aleurone, its wall consisting largely of pentosans, is probably broken down by pentosanases originating in the aleurone cells. Taiz and Honigmen (1976) reported that the cell walls of barley aleurone layers undergo extensive degradation during the tissue's response to gibberellic acid. According to their findings, barley aleurone cell walls were similar to the wheat endosperm cell walls. The intracellular changes are usually associated with degradation of the cell walls. The cell wall breakdown commonly occurs during nutritional modification of endosperm. Cell wall breakdown apparently is important in embryo nutrition and penetration of endosperm by the embryo. It has been possible to establish embryonic control of endosperm breakdown in some seeds but only partial control or none at all in others. Information on the structure of mature endosperm is difficult to obtain, since the subject has only occasionally attracted attention of morphologists and anatomists.

Nothing has been reported on histological studies on endosperm of germinating cyclamen seeds. There have been few studies of living endosperm cells using modern light and electron microscopic techniques, but the available data confirm that there is a degree of uniformity in their structure and apparent composition.

C. Regeneration of Seedling Tuber

In 1929, Hill reported that adventitious leaves arose from the tuber just below the cut surface from a seedling of cyclamen from which the top of the tuberous hypocotyl with the cotyledon had been removed. In both the decapitated seedling and adult tubers one or more plumules developed in the course of time and normal cyclamen plants resulted.

Nakayama (1980) attempted vegetative propagation of cyclamen by notching the tuber. Regeneration occurred best if the tuber was scooped at one third of the upper part and the cut surface notched into one centimeter squares. However, propagation of cyclamen by tuber is laborious and not practical, except to maintain specific clones for breeding purposes.

Lowenberg (1969) performed cyclamen callus culture succesfully with adult tuber tissue. Mayer (1956) and Stichel (1959) observed organ formation and in some instances even whole plant regeneration in cultured tuber tissue explants. Nevertheless, Gier (1977) indicated that whole plant regeneration from tuber tissues was hard because of the difficulty of simultaneous induction of roots and buds and the generally high degree of internal microbial contamination of the tuber tissues. Gier (1977) experimented with antibiotics to suppress microbial contamination. Contamination of cyclamen tuber tissue explants could be substantially reduced without impairing growth and morphogenesis by precultivation in the presence of the antibiotic Achromycin and subsequent transfer of the explants to a medium without Achromycin.

Gier (1979) performed comparative studies on the morphogenetic potential of explants taken from different organs and also of callus cultures derived from somatic tissues of anthers. Whereas tuber tissues were easily stimulated to produce adventitious buds, other explants and callus show much less shoot forming capacity and often develop only isolated leaf organs. Moreover, simultaneous induction of bud and root formation could be achieved only in cultures of tuber tissues. Plants were raised in significant numbers from tuber tissue explants bearing both roots and buds. However, regenerating ability as well as the rate of microbial contamination showed considerable seasonal variation and was dependent on the age of the mother plant. It was concluded that although tissue culture technique at present does not permit mass propagation, it could be used to clonally multiply selected motherplants with good combining ability, thus greatly facilitating maintenance of breeding lines.

In 1982, Fersing et al. reported that a modified Murashige and Skoog medium was used to regenerate cyclamen plants from cultured organ fragments. The juvenile or adult tissues of cyclamen were capable of producing several types of organogenesis in vitro. It was concluded that young seedling tuber tissues have a high capacity for organogenesis.

CHAPTER III

MATERIALS AND METHODS

A. Seed Sources

Seeds of C. persicum 'Bambino Piano' were purchased in March, 1982, from Geo. W. Park Seed Co., Greenwood, SC. Altogether 300 seeds were used.

B. Sterilization Procedures

The seeds were soaked in a sterilizing solution for 2 min and rinsed three times with distilled water. A small tea strainer was used to strain seeds. The sterilizing solution was a mixture of one part Clorox and 9 parts Alconox solution. The Alconox solution contained 30 gm of Alconox powder in one gallon of warm water. For 100 ml solution, 0.75 gm of Alconox powder was dissolved in warm water. Clorox was measured directly from a commercial bottle obtained from a grocery market.

The seeds were placed on the culture medium using a pair of sterilized tweezers under a dead-air space transfer hood in which the working surface was sterilized with ultraviolet light followed by wiping with 75% ethanol. An alcohol lamp was used to sterilize the ends of tweezers and ends of culture tubes.

C. Media

The media used in this study are listed below.

a. Knudson's medium (- sucrose). The formula is as follows:

Medium-1 liter	
Knudson's stock solution	250 ml
Deionized water	750 ml
K_2HPO_4	0.125 gm
Ferric citrate stock solution	0.4 ml
B5 minor elements solution	0.5 ml
Bacto-Agar	0.8 gm

Knudson's Stock	
$Ca(NO_3)_2.4H_2O$	2.0 gm
$(NH_4)_2SO_4$	1.0 gm
$MgSO_4.7H_2O$	0.5 gm
Deionized water	1000 ml

Ferric citrate stock solution	
$FeC_6H_5O_7.5H_2O$	2.5 gm
Deionized water	100 ml

B5 minor elements solution	
H_2SO_4	0.5 ml
$MnCl_2.4H_2O$	2.5 gm
H_3BO_3	2 gm
$ZnSO_4.7H_2O$	0.05 gm

$CoCl_2.2H_2O$	0.03 gm
$CuCl_2.2H_2O$	0.015 gm
$Na_2MoO_4.2H_2O$	0.025 gm
Deionized water	1000 ml

b. Knudson's medium (+ sucrose): Same ingredients as above, but with 8 gm of sucrose added.

c. Knudson's medium with plant hormones: Same ingredients as above. (b) but 0.5 mg of auxin and 0.5 mg of kinetin were added.

d. Murashige shoot-tip rooting medium: The formula is listed below.

Medium-1 Liter	
Murashige and Skoog Salts	4.628 gm
1-Inositol	0.1 gm
Sucrose	30 gm
Thiamine HCl	0.40 mg
IAA	0.30 mg
Kinetin	1 mg
Buffering agent: phytion	0.159 mg
Deionized water	800 ml

D. Preparation of Media

a. Knudson's medium

The Knudson's stock solution was mixed first and then dibasic potassium phosphate, ferric citrate, B5 minor elements, and sucrose were added in the order given. After sucrose was dissolved, the pH

was adjusted to pH 5.5 with HCl and NaOH. This solution was heated to almost boiling and agar was slowly added with constant stirring until it dissolved completely. For Knudson's with plant hormones, auxin and kinetin were added before pH was measured.

This solution was dispensed into clean culture tubes, about 20 ml each. The vessels were plugged tightly with cotton, capped with plastic caps and autoclaved for 20 min at 121°C (15 psi). After autoclaving, the prepared media were cooled at room temperature, and stored in a refrigerator until use. The stored culture media were used for seed culture within a week. Some of the solution was autoclaved in flasks and poured into disposable Petri dishes (100 x 15 mm). Approximately 30 ml of the medium were added per plate.

b. Murashige shoot-tip rooting medium

Commercially available mixture in the powder form was measured out first, 20% less distilled water than the desired total volume of medium, and the powder was added while stirring constantly. Since the content of a package had been adjusted to pH 5.7, no further adjustment was made. The solution was heated to boiling and 6.4 gm of agar were added. After the solution became clear, 20 ml aliquots of the medium were dispensed into culture tubes. These were autoclaved for 15 min at 121°C. They were then cooled at room temperature and stored at temperatures below 25°C.

E. Germination Studies

Seeds were divided into nine groups and cultured as follows:

1. 50 sterilized seeds, Knudson's medium (- sucrose)
2. 50 unsterilized seeds, Knudson's medium (- sucrose)
3. 25 sterilized seeds, Knudson's medium (+ sucrose)
4. 25 unsterilized seeds, Knudson's medium (+ sucrose)
5. 25 sterilized seeds, Murashige's shoot-tip-rooting medium
6. 25 unsterilized seeds, Murashige's shoot-tip-rooting medium
7. 25 sterilized seeds, Knudson's medium (- sucrose)
8. 25 unsterilized seeds, Knudson's medium (- sucrose)
9. 50 seeds sown directly on the surface of the soil.

Groups 1 - 6 were kept in a dark incubator maintained at 20°C. Groups 7 and 8 were kept in a dark incubator at 22°C.

Seeds in group 9 were unsterilized, and the soil mixes used were Jiffy-7 peat pellets. Pellets were soaked in warm water for 10 - 15 min before use. Seeds planted on pellets were placed in a row on plastic trays and covered with a thick black plastic sheet to keep them in the dark. Trays were watered daily and kept in a moisture chamber made with clear plastic in the greenhouse. The greenhouse temperature fluctuated, especially during summer. As seedlings emerged from the soil they were transferred to light and kept in a moisture chamber until two leaves unfolded. Afterwards they were moved out of the moisture chamber and placed under a bench to avoid direct sunlight.

F. Histological Studies

Four groups of seeds were selected as follows:

1. Dry seeds soaked in distilled water for 24 hr

2. Germinating seeds which had formed tubers or roots, about 2 months after initial culture
3. Slow-germinating seeds which had been cultured for 2 months
4. Seeds that failed to germinate after 4 months of culture

Portions of endosperm about 1 mm^3 were fixed in paraformaldehyde-glutaraldehyde solution (Karnovsky, 1965) at 4°C for 24 hr. Tissues were then rinsed in cold 0.1 M sodium cacodylate buffer(pH 7.4) and postfixed in 1% buffered osmium tetroxide for 1 hr at 4°C. After fixation, the tissues were rinsed in 0.1 M cacodylate buffer three times at 5-min intervals. Fixed tissues were dehydrated in a graded series of ethanol. Toluene was used as a transitional solvent between the alcohol series and the embedding mixture. Finally the tissues were embedded in Epon 812 (Luft, 1961). Thin sections were cut with a diamond knife on a Porter-Blum MT-2 ultramicrotome.

For light microscopic observations, thick section (about 0.3 um thick) were cut, stained with 0.25% Azure II (Jeon, 1965) on a glass slide and examined. Desired sections were photographed in color using the Kodak Ektachrome film. For electron microscopic examination, thin sections with gold color were mounted on copper grids and double stained with saturated aqueous uranyl acetate and lead citrate for 15 and 5 min, respectively (Venable and Coggeshall, 1965). The sections were examined and photographed in a Hitachi H-600 microscope.

G. Regeneration Studies with Tubers Removed from Seedlings

Tubers from seedlings grown aseptically *in vitro* were divided into four groups and treated as follows.

1. Fifteen tubers were cultured without sterilization. Under the transfer hood, these tubers were cut in halves, in thirds, or in fourths. The tissue explants were placed on the culture media [Knudson's medium (- sucrose), Knudson's medium (+ sucrose), Murashige shoot-tip rooting medium, Knudson's medium (+ sucrose, + auxin, + kinetin)] and placed in an incubator in the dark at 20°C.

2. Fifteen tubers were sterilized for 5 min with Alconox-Clorox solution and rinsed with distilled water 3 times, and cultured on media as described above.

3. Fifteen tubers were sterilized as above for 15 min and cultured on media as above.

4. Fifty tubers were sterilized as above for 30 min and cultured on media as described above.

CHAPTER IV

RESULTS

A. Seed Germination

Media

Seeds cultured on Knudson's medium (- sucrose) germinated faster than seeds sown on flats, not being affected by different temperatures or sterilization, but germination was erratic (Table I and Figures 1 - 4.) However, unsterilized seeds showed a slight contamination within a week (Table II). This contamination remained at the same level until the experiments were terminated after 16 weeks (Table III).

Both sterilized or non-sterilized seeds cultured on Knudson's medium (+ sucrose) and Murashige shoot-tip and rooting medium were heavily contaminated (Table II and Table III, Groups 3, 4, 5 and 6; Figures 6 and 7).

Effect of sterilization

Germination of sterilized or unsterilized seeds was similar. Sterilization of seed coats for two min was sufficient to suppress contamination on Knudson's medium (- sucrose), whereas such sterilization did not prevent heavy contamination of media which contained sucrose such as Knudson's (+ sucrose) and Murashige shoot-tip-rooting media (Table III and Figures 5, 6 and 7). Germination rates for both sterilized and non-sterilized were similar (Table I, 1 versus 2, and 7 versus 8).

TABLE I

IN VITRO GERMINATION UNDER DIFFERENT ENVIRONMENAL CONDITIONS:
CUMULATIVE COUNTS OF GERMINATED SEEDS (WEEKLY)

Groups (# seeds)	Weeks	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1 (50)		.	.	2	5	7	25	39	40	40	40	45	46	46	46	48	49
	(%)			(4)	(10)	(14)	(50)	(78)	(80)	(80)	(80)	(90)	(92)	(92)	(92)	(96)	(98)
2 (50)		.	.	2	5	6	24	38	39	39	39	45	47	47	47	48	49
	(%)			(4)	(10)	(12)	(48)	(76)	(78)	(78)	(78)	(90)	(94)	(94)	(94)	(96)	(98)
7 (25)		.	.	1	2	4	13	19	20	20	20	23	24	24	24	24	24
	(%)			(4)	(8)	(16)	(52)	(76)	(80)	(80)	(80)	(92)	(96)	(96)	(96)	(96)	(96)
8 (25)		.	.	1	2	3	12	18	19	19	19	21	23	23	23	24	24
	(%)			(4)	(10)	(12)	(48)	(72)	(76)	(76)	(76)	(84)	(92)	(92)	(92)	(96)	(96)
9 (50)		.	.	.	.	.	.	.	2	5	10	25	30	.	.	.	.	.
	(%)								(4)	(10)	(20)	(50)	(60)				

Notes

Group 1 ; Sterilized and grown at 20°C
Group 2 ; Not sterilized and grown at 20°C
Group 7 ; Sterilized and grown at 22°C
Group 8 ; Not sterilized and grown at 22°C
Group 9 ; Grown in the greenhouse

All seeds except Group 9 were grown on Knudson's medium (- sucrose).

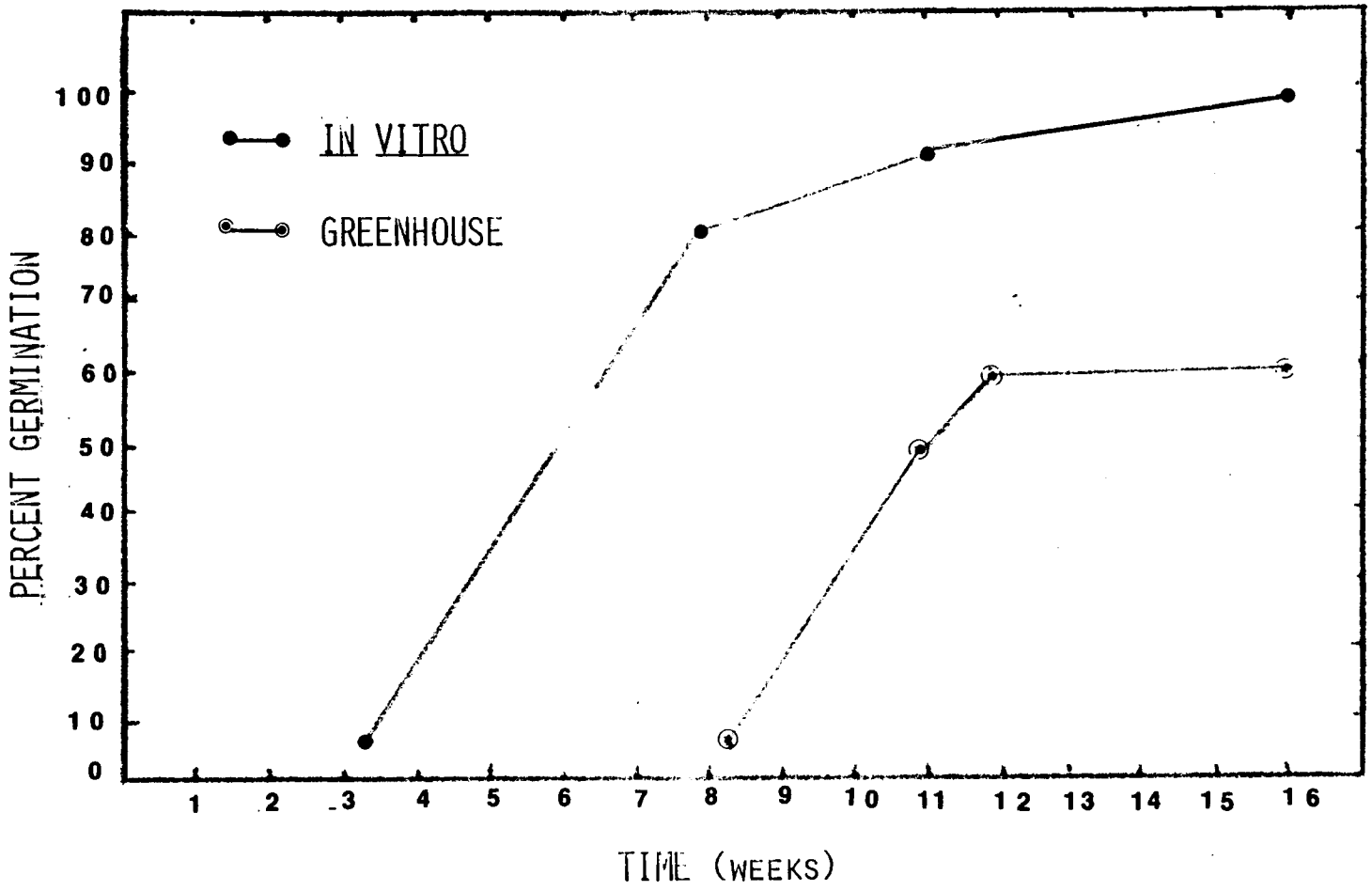

FIGURE 1. IN VITRO GERMINATION VERSUS GREENHOUSE GERMINATION.

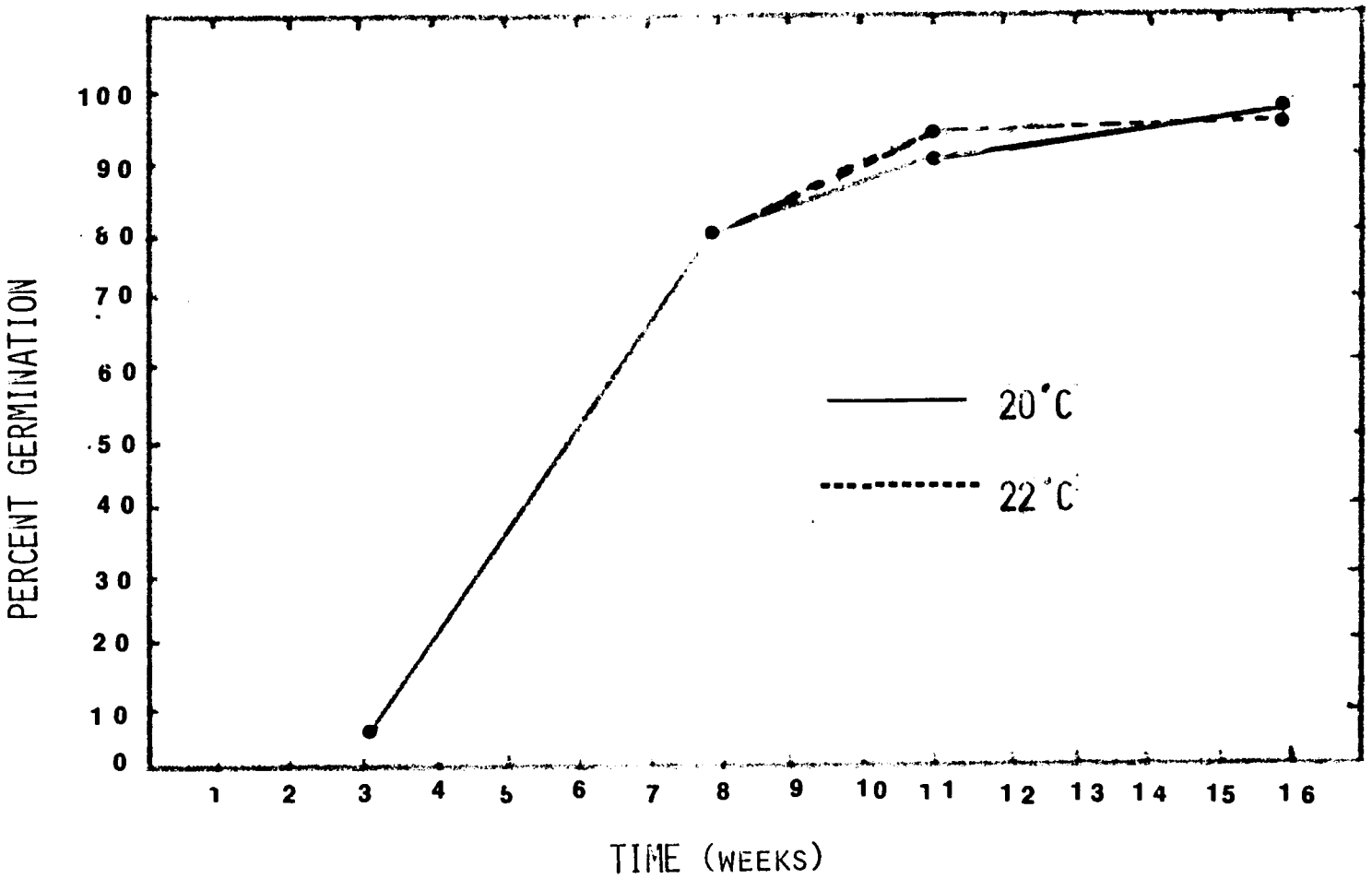

FIGURE 2. EFFECT OF TEMPERATURE ON IN VITRO GERMINATION.

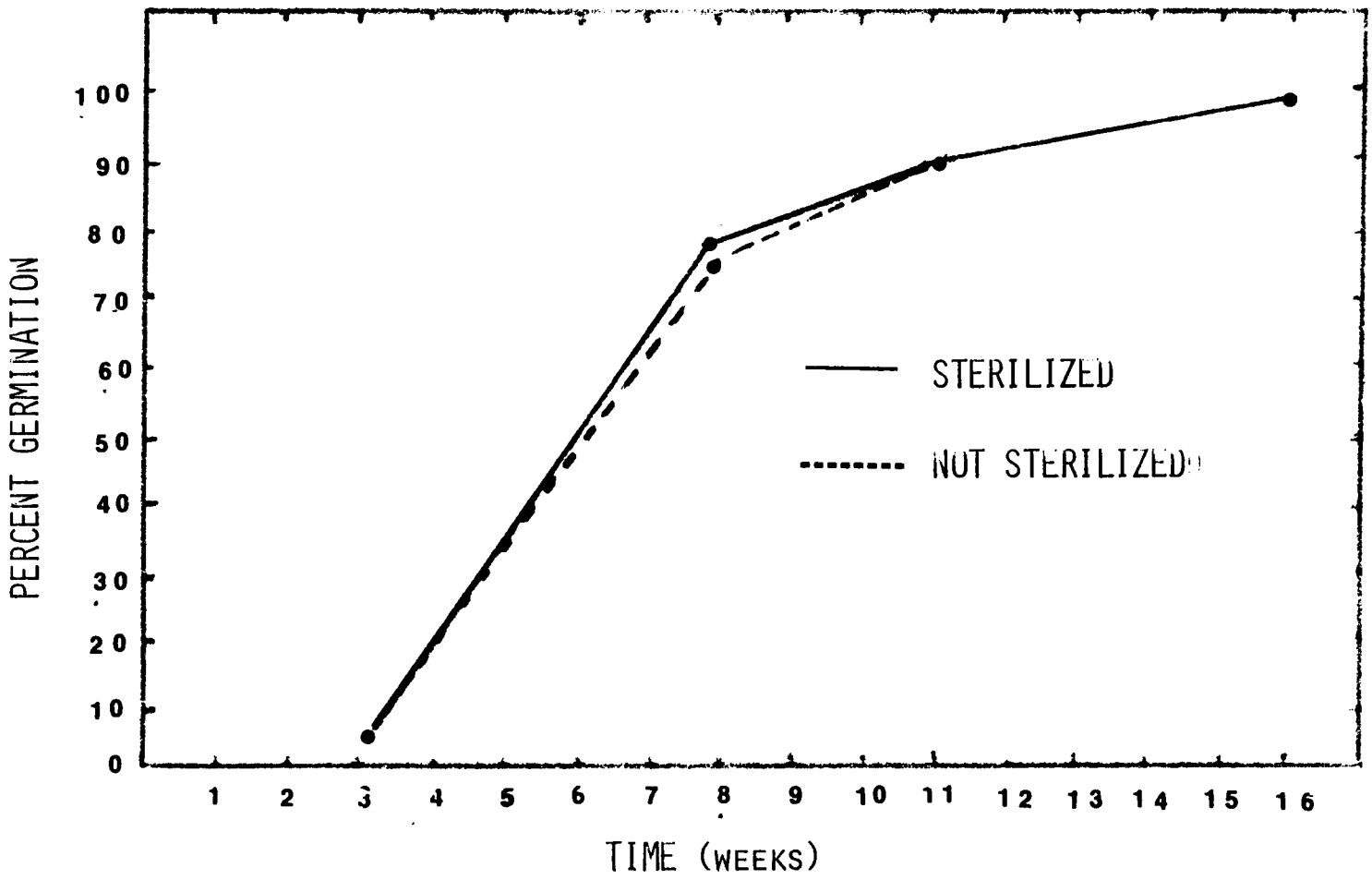

FIGURE 3. EFFECT OF STERILIZATION ON IN VITRO GERMINATION.

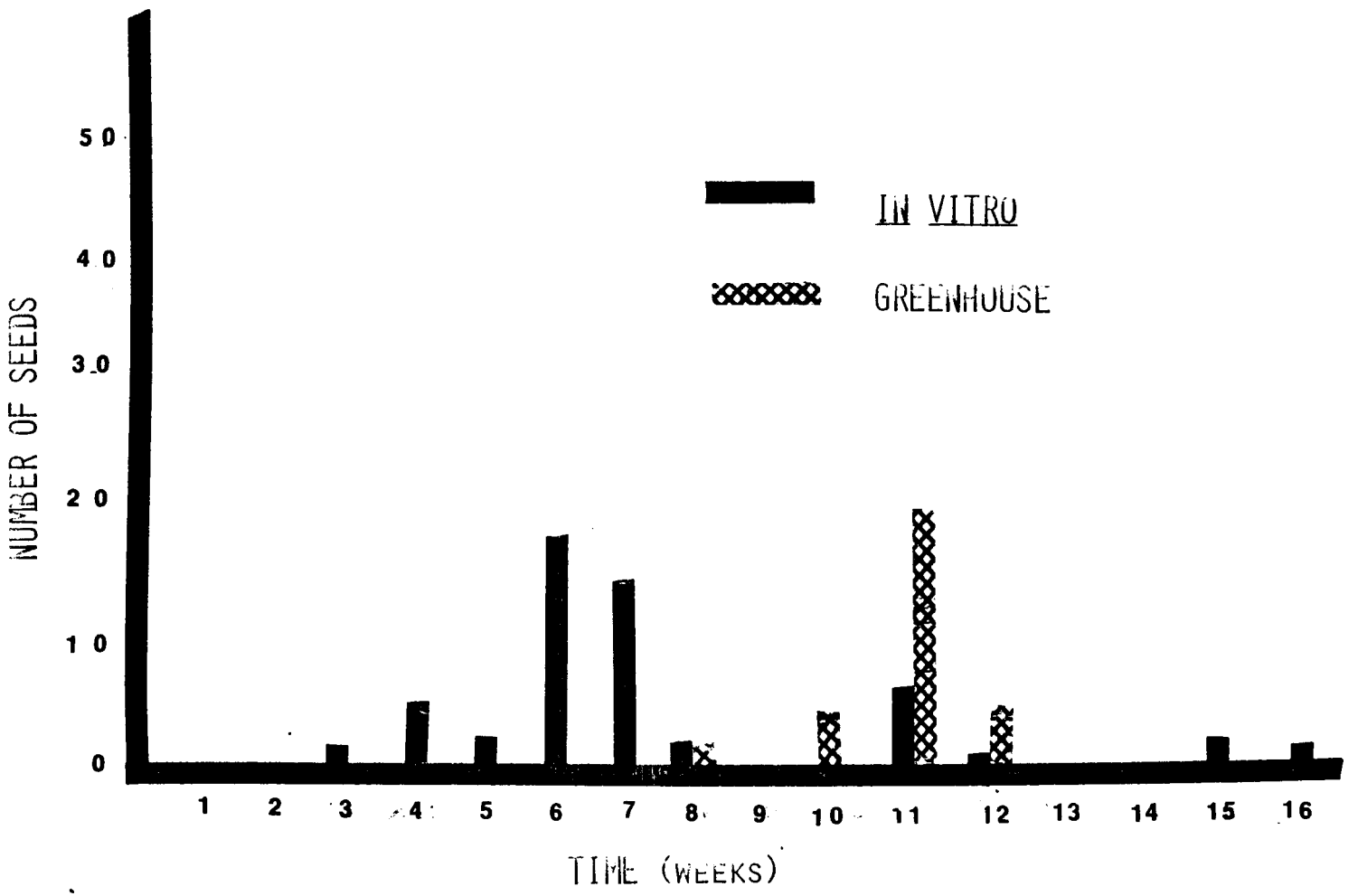

FIGURE 4. THE NUMBER OF GERMINATING SEEDS IN EACH WEEK.

TABLE II

MEDIA AND CONTAMINATION (EFFECT OF STERILIZATION ON CONTAMINATION)

Seed sterilization	Media: Knudson's (- sucrose)	Knudson's (+ sugar)	Murashige's
Sterilized (2 minutes)	-	+	+
Not sterilized	+	+	+

Notes
- ; Not contaminated
+ ; Contaminated

TABLE III

MEDIA AND CONTAMINATION DURING SEED GERMINATION (WEEKLY OBSERVATION)

Seed groups	Weeks 1	2	3	4	5	16
1	-	-	-	-	-	-
2	+	+	+	+	+	+
3	+	+++	+++	/	.	.
4	+++	+++	/	.	.	.
5	+	+++	+++	/	.	.
6	+++	+++	/	.	.	.
7	-	-	-	-	-	-
8	+	+	+	+	+	+

Notes

- ; No contamination
+ ; A slight contamination
+++ ; A heavy contamination
/ ; Experiment was terminated

Figure 5. Seed cultures on Knudson's medium (- sucrose). A slight contamination occurred on the left culture in which the seed had not been sterilized, whereas the right culture which contained a sterilized seed was not contaminated.

Figure 6. Seed cultures on the Knudson's medium (+ sucrose) (left) and Murashige's shoot-tip and rooting medium (right). Both were started with unsterilized seeds and were contaminated.

Figure 7. Seed cultures on the Knudson's medium (+ sucrose) (left) and Murashige's shoot-tip and rooting medium (right). Both contained sterilized seeds and were contaminated.

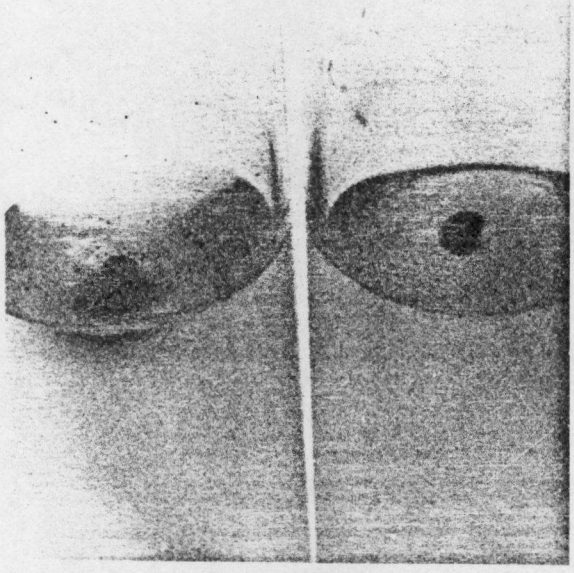

FIGURE 5

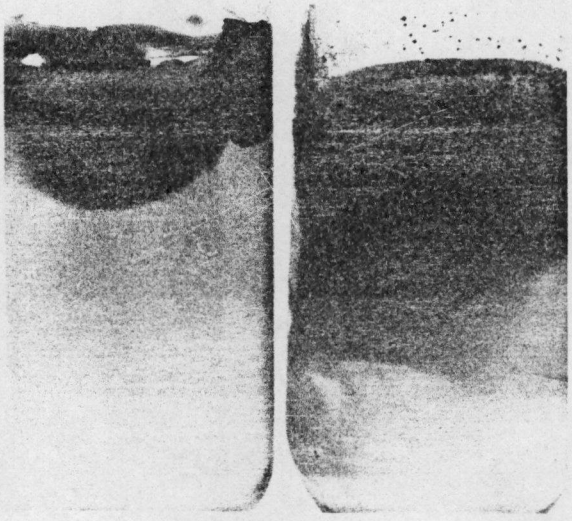

FIGURE 6

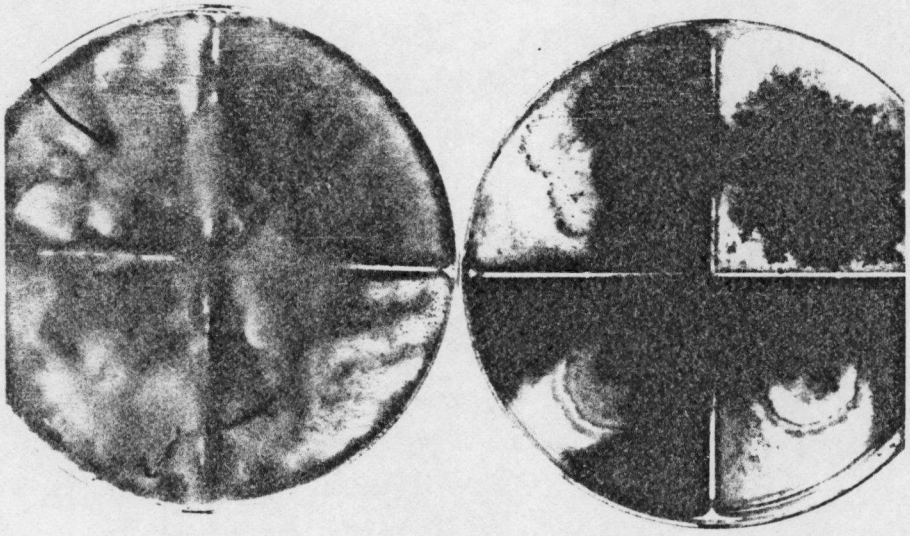

FIGURE 7

Effect of temperature

Both groups (1, 2 and 7, 8) germinated at the same rate (Table I, page 16 and Figure 2, page 18). This implies that there was no difference in germination at 20°C and 22°C.

Peculiarity of cyclamen seed germination

Seed germination was erratic and slow (Table IV and Figure 4). Germination peaked at six weeks *in vitro* whereas the greenhouse control took 11 weeks to reach that point (Table IV and Figure 4).

Total cumulative counts of germinating seeds

In vitro germination reached 98%, while only 60% of controls germinated (Figure 1, page 17).

In vitro germination versus greenhouse germination

Germination commenced at 3 weeks *in vitro* but at 8 weeks in the greenhouse. In both cases, a burst of germination occurred 3 weeks after the commencement of germination (Table IV). As for the total cumulative counts of germinating seeds, in vitro germination reached 90% in 11 weeks while the control reached to 60% in 12 weeks. The *in vitro* group had germinated 80% by the 8th week when the control began to germinate (Table I, page 16 and Figure 1, page 17).

Growth and development of seedlings

Hypocotyl emergence occurred after 3 weeks. The tuber had been formed by the time of hypocotyl emergence. Subsequently, the shoot started to grow (Figures 8, 9 and 10), and shoot elongation and root growth followed. One leaf blade emerged and two leaves were formed.

TABLE IV

NUMBER OF GERMINATING SEEDS (WEEKLY OBSERVATIONS)

Groups	Weeks 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1 (50 seeds)	.	.	2	3	2	18	14	1	0	0	5	1	0	0	2	1
2 (50 seeds)	.	.	2	3	1	18	14	1	0	0	6	2	0	0	1	1
7 (25 seeds)	.	.	1	1	2	9	6	1	.	.	3	1	.	.	0	0
8 (25 seeds)	.	.	1	1	1	9	6	1	.	.	2	2	.	.	1	0
9 (50 seeds)	.	.	.	.	.	.	.	2	3	5	20	5	.	.	.	.

Notes

Group 1 ; Sterilized and grown at 20°C
Group 2 ; Not sterilized and grown at 20°C
Group 7 ; Sterilized and grown at 22°C
Group 8 ; Not sterilized and grown at 22°C
Group 9 ; Grown in the greenhouse

All seeds except Group 9 were grown on Knudson's medium (- sucrose).

Figure 8. A tuber formed after 3 - 4 weeks.

Figure 9. A hypocotyl emerging after 3 - 4 weeks.

Figure 10. A hypocotyl elongating after 4 - 5 weeks.

FIGURE 8

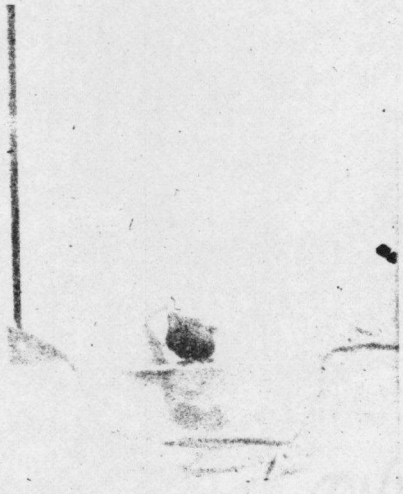

FIGURE 9

FIGURE 10

Generally, seedlings appeared to be longer than the ones grown in the greenhouse (Figures 11, 12, 13 and 14).

B. Histology of Germinating Seeds

Light microscopy

Both dry seeds and nongerminating imbibed seeds were characterized by very thick cell walls and were anatomically similar. Endosperm modification in germinating seeds was characterized by conspicuously reduced thickness of cell walls. Both slow germinating and fast germinating seeds showed similar patterns of modification in cell walls of endosperm, but vacuoles were fewer and larger in fast germinating seeds than in dry ones (Figures 15, 16, 17 and 18).

Electron microscopy

The dry seeds had dense thick cell walls, while the germinating seeds had loose, broken-down, thin cell walls (Figures 19 and 20). This electron micrograph supports the findings of light microscope observation, and shows the texture of cell walls. The germinating seeds contain less cytoplasmic material, and were occupied with large vacuoles, whereas the dry seeds were filled with cytoplasmic material. The germinating seeds contained fewer starch grains. The dry seed displayed a prominent nucleus.

C. Regeneration of Seedling Tuber

Media

Regeneration did not occur on Knudson's medium either with or without sucrose, while the Murashige and Knudson's media (+ sucrose),

Figure 11. Elongation of a shoot and growth of tap roots after 5 - 6 weeks.

Figure 12. One unfolding leaf after 7 - 8 weeks.

Figure 13. Two unfolding leaves after 8 - 9 weeks.

Figure 14. The formation of a spindly seedling.

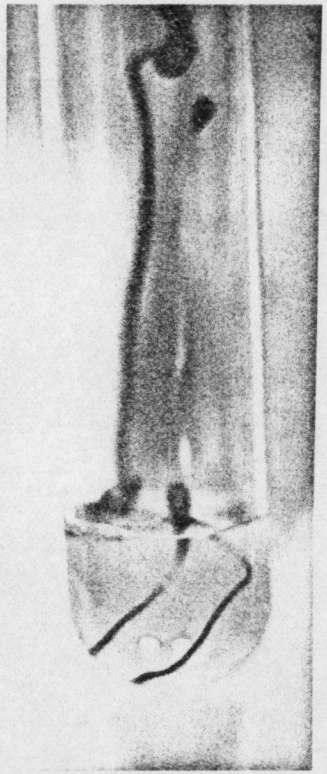

FIGURE 11

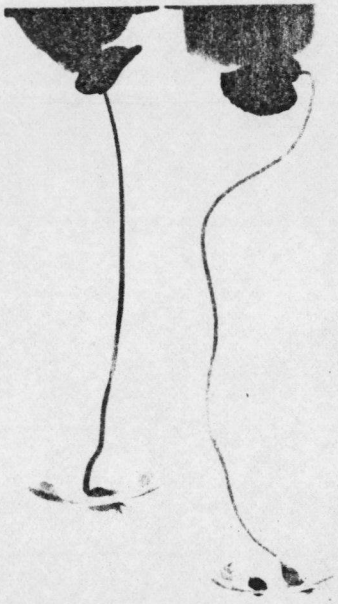

FIGURE 12

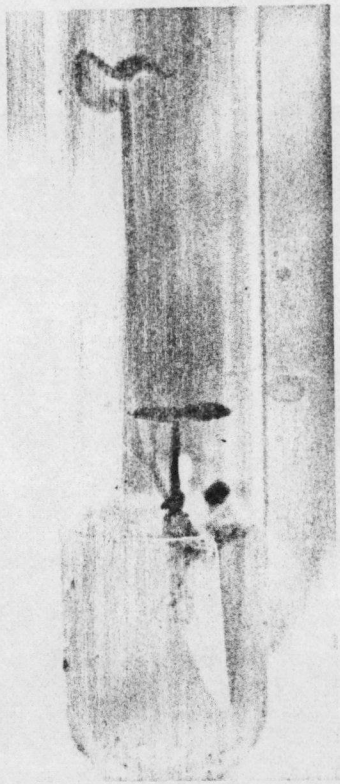

FIGURE 13

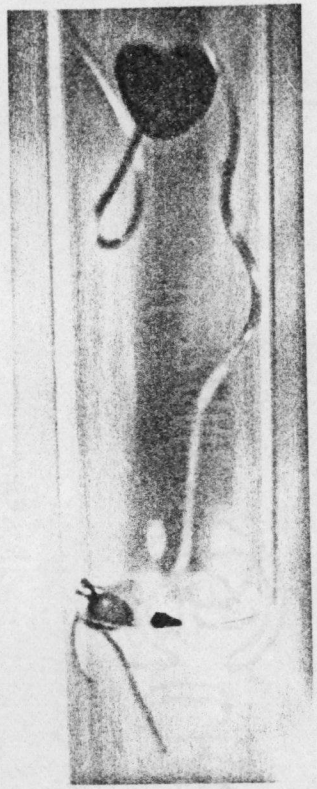

FIGURE 14

Figs. 15 - 18. Light micrographs of 0.3 um-thick sections of seeds embedded in resin and stained with Azure II.

Figure 15. The endosperm of a dry seed. Magnification, x 250.

Figure 16. The endosperm of a nongerminating seed after 4 months. Magnification, x 250.

Figure 17. The endosperm of a fast-germinating seed which formed tuber after 2 months. Magnification, x 125.

Figure 18. The endosperm of a slow-germinating seed which did not show any sign of germination outwardly after 2 months. Magnification, x 125.

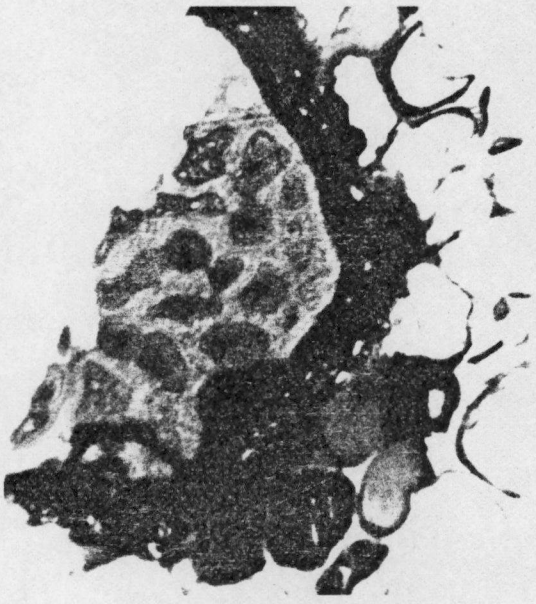

FIGURE 15

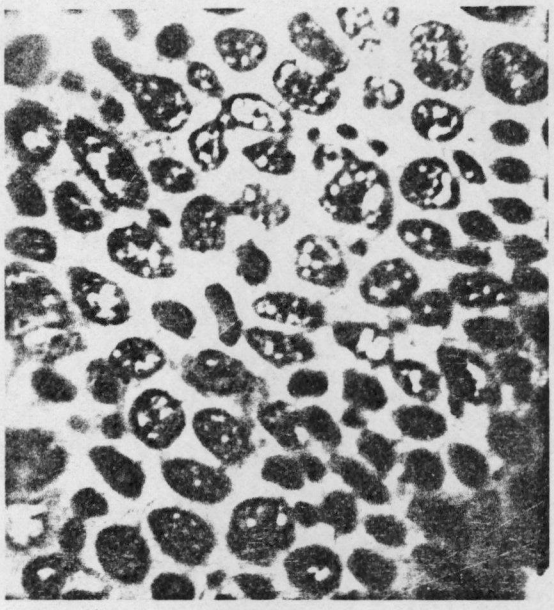

FIGURE 16

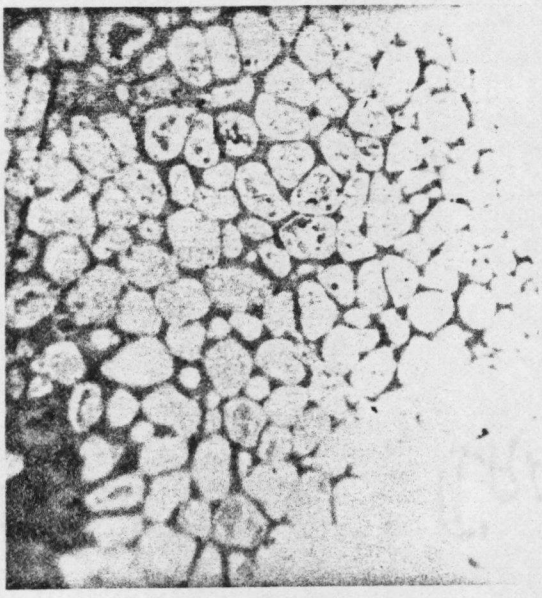

FIGURE 17

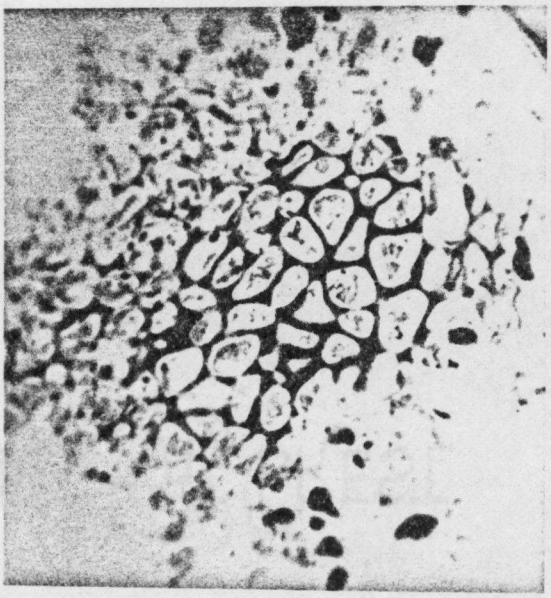

FIGURE 18

Figure 19. Electron micrograph of an endosperm of a dry seed, showing a thick cell wall (arrow). Magnification, x 4660.

Figure 20. Electron micrograph of the endosperm of a germinating seed, showing a broken-down cell wall (arrow). Magnification, x 6430.

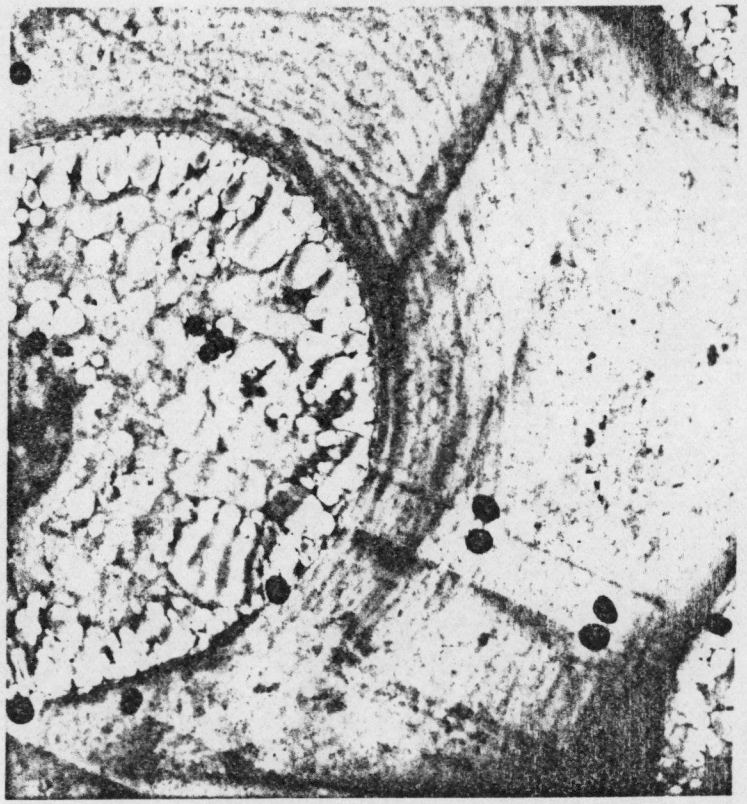

FIGURE 19

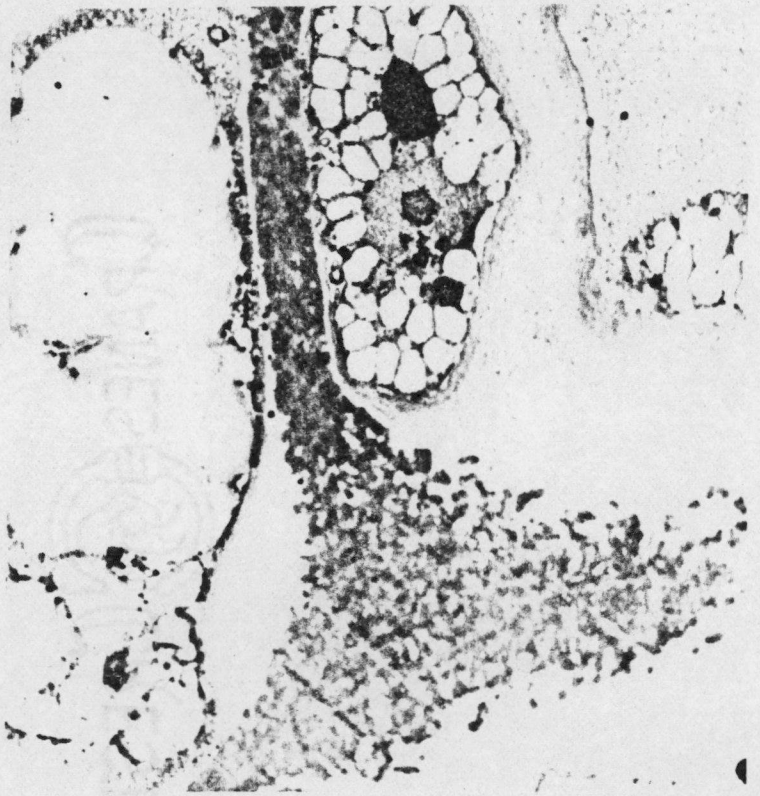

FIGURE 20

auxin, and kinetin proved to be suitable to induce callus and organ formation (Table V).

Contamination and sterilization

Knudson's medium (- sucrose) was not contaminated even if unsterilized tissues from seedlings grown aseptically were cu ltured. However, both Knudson's and Murashige media containing sucrose were heavily contaminated (Table VI). Sterilization for 30 min was required to eliminate contamination in all media containing sucrose.

The size of tuber and regeneration

All the tissue explants established callus within 4 weeks despite the difference in size of pieces. Both 1/2 and 1/3 size of tuber formed buds, and roots or shoots in another 3 weeks. However, the 1/4 sized tuber never formed any organs (Table VII).

Differentiation and growth of culutred tuber tissue explants

There were no changes within 3 weeks. Callus formation occurred in 3 to 4 weeks. The tissue explants placed on the Knudson's medium (- sucrose) never changed. When callus had formed after the third week, bud formation required another three weeks. Each bud produced three leaves (Figures 21, 22, 23 and 24). Some tissue explants formed more than one bud, whereas others formed only one bud per tissue piece. Some formed roots first, while others formed roots and shoots simultaneously (Figures 25, 26, 27 and 28).

TABLE V

MEDIA USED AND OCCURRENCE OF REGENERATION

	Media			
Results	Knudson's (- sucrose)	Knudson's (+ sucrose)	Murashige's	Knudson's (sugar/hormones)
Regeneration	-	-	+	+

Notes

- ; No regeneration occurred.
+ ; Regeneration occurred.

TABLE VI

STERILIZATION TIME AND CONTAMINATION OF MEDIA

Sterilization time	Media			
	Knudsons's (- sucrose)	Knudson's (- sucrose)	Murashige's	Knudson's (hormones)
0	-	+	+	+
5	-	+	+	+
15	-	+	+	+
30	-	-	-	-

Notes

+ ; °Contaminated
- ; Not contaminated

TABLE VII

SIZE OF TUBERS AND REGENERATION

Type of regeneration	Size of tubers 1/2	1/3	1/4
Callus	+	+	+
Organs	+	+	-

Figure 21. Callus formation after 3 weeks of culture.

Figure 22. Regenerating tubers in the presence and absence of plant hormones. Left, A medium containing no plant hormones; right, a medium supplemented with auxin and kinetin. Regeneration occurred only on a medium containing plant hormones.

Figure 23. A shoot emerging from a tissue explant.

Figure 24. Leaves forming on a tissue explant.

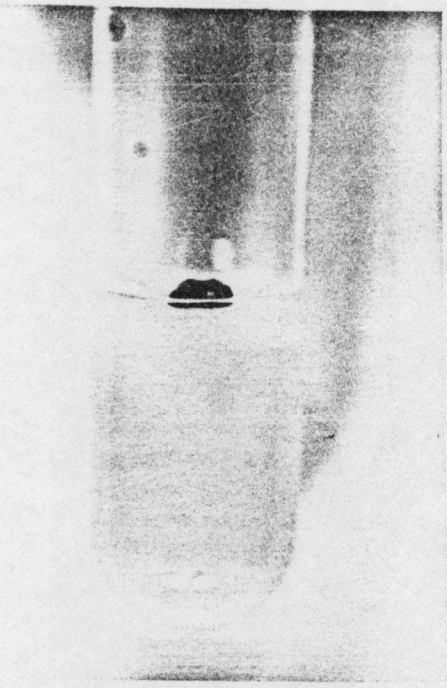

FIGURE 21

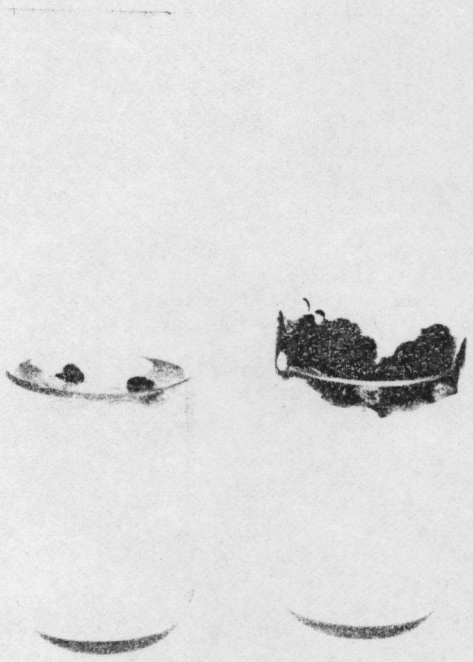

FIGURE 22

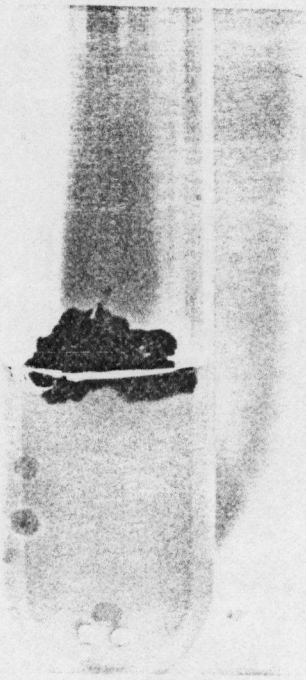

FIGURE 23

FIGURE 24

Figure 25. A tissue culture in which several buds formed at the same time.

Figure 26. A tissue cuture in which a shoot formed first.

Figure 27. A tissue culture in which roots formed first.

Figure 28. A tissue culture in which buds and roots were formed at the same time.

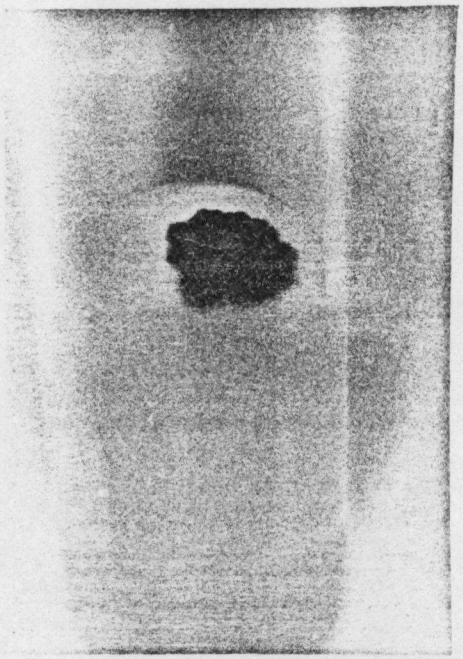

FIGURE 25

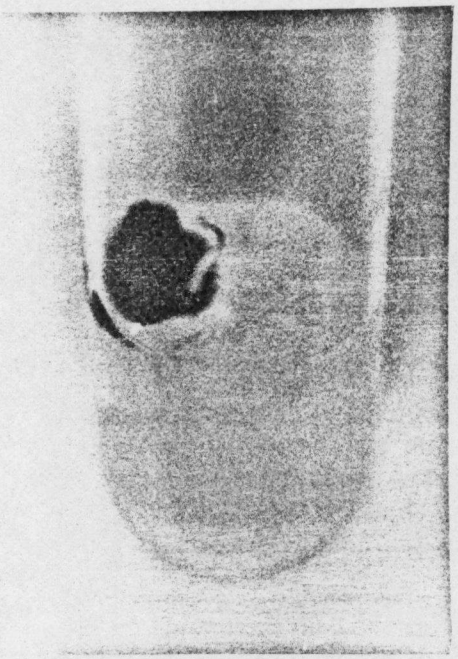

FIGURE 26

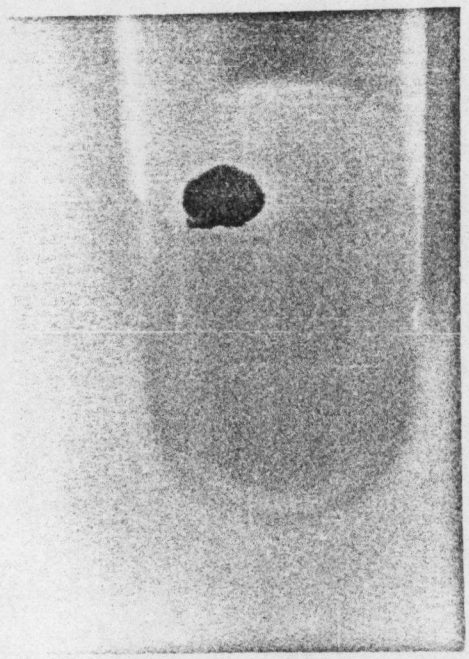

FIGURE 27

FIGURE 28

CHAPTER V

DISCUSSION

A. Seed Germination

Seeds placed on nutrient agar media and kept in an incubator germinated five weeks earlier than seeds sown in flats and kept in the greenhouse. This result may be related to temperature which fluctuated between 20 and 30°C in the greenhouse from April through the summer months when the experiment was conducted. Therefore, the greenhouse temperature was higher than the optimum 20°C, and even higher than the inhibitory 21°C, reported for cyclamen seeds (Stephens and Widmer, 1974). Both groups *in vitro* germinated at the same rate, and gave seedling yields over 90%. This is as high as germination can be expected with cyclamen seed (Sumitomo and Kosugi, 1963; Widmer *et al.*, 1979).

The results of experiments reported here suggest that poor germination of cyclamen seeds at temperatures above 20°C may not be due to direct physiological effects of temperature. It seems possible that growth of fungi is stimulated as temperature rises (Stephens and Widmer, 1974), and it is the presence of fungi in the seed beds under greenhouse conditions that inhibits germination. Both sterilized and unsterilized seeds germinated at the same rate on Knudson's nutrient agar medium (- sucrose). Thus, microorganisms carried into *in vitro* cultures on seed coats do not inhibit germination, but those that invade seed beds in the greenhouse may be inhibitory.

Knudson's medium (- sucrose) is suitable for the *in vitro* germination of cyclamen seed.

B. Histology of Germinating Seeds

Extraordinarily thick walls are characteristic of the endosperm cells of cyclamen seeds and may be an important factor in slow and erratic germination. If modification of cell walls is a prerequisite for penetration and emergence of embryonic shoot and root tips, then cyclamen probably would require a longer period of time for alteration of its thick endosperm cell walls compared with many other seeds which have much thinner endosperm cell walls. Treatment of seeds with substances which would cause or assist the degradation of cell walls may accelerate germination. For example, treatment of lettuce seeds with gibberellin resulted in the same morphological changes as in germinating seed (Jacobson *et al.*, 1979). Therefore, gibberellin is a suitable substance to test for its effect on germination of cyclamen seeds. In fact, Anderson and Widmer (1975) reported that gibberellin accelerated germination of cyclamen seeds, but this effect was accompanied by undesirable expulsion of embryos. Further experiments with gibberellin, especially *in vitro*, might lead to an improved method of germinating cyclamen seeds.

C. Regeneration of Seedling Tuber

All seedling tuber explants formed callus within four weeks on the Murashige shoot-tip-rooting medium containing auxin and kinetin. Knudson's medium without hormones failed to induce either organs or

callus from seedling tubers. These results prove that exogenous plant hormones are necessary for growth of seedling tubers. Seedling tubers of cyclamen have high regeneration potential when provided with favorable nutrition and environment, and appear to be excellent subjects for *in vitro* experiments.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Anderson, R. G. and R. E. Widmer, 1975. Improving vigor expression of cyclamen seed germination with surface disinfestation and gibberellin treatments. J. Amer. Soc. Hort. Sci. 100 (6): 597-601.

2. Fersing, J., A. Mouras and A. Lutz. 1982. First steps towards the industrial vegetative propagation of cyclamens by in vitro culture. Revue Horticole 27-30.

3. Gier, T. 1977. Morphogenesis and plant regeneration from cultured organ fragments of Cyclamen persicum. Acta Hort. 78: 167-174.

4.Gier, T. H., W. Kohlenbach and G. Reuther. 1979. Klonale vermehrung von Cyclamen persicum durch gewebekultur. Gartenbauwissenschaft 44: 226-237.

5. Hill, A. W. 1920. Studies in seed germination. Experiment with cyclamen. Ann. Bot. 31: 417-430.

6. Jacobsen, J. V. T., J. V Higgins and J. A. Zwar. 1979. Hormonal control of endosperm function during germination In "The Plant Seed: Development, Preservation, and Germination" (I. Rubenstein, R. L. Phillips, C. e. Green, and B. g. Gengenbach, Eds.), pp. 241-262. Academic press, New York.

7. Jeon, K. W. 1965. Simple method for staining and preserving Epoxy resin embedded animal tissue sections for light microscopy. Life Science. 4: 1839-1841.

8. Karnovsky, M. J. 1965. A Formaldehyde glutaraldehyde fixative of high osmolarity for use in electron microscopy. J. Cell Biol. 27: 137A.

9. Lowenber, J. R. 1969. Cyclamen callus culture. Can. J. Bot. 47: 2065-2067.

10. Luft, J. H. 1961. Improvement in epoxy resin embedding methods. J. Biochem. Cytol. 9: 409-414.

11. Lyons, R. E. and R. E. Widmer. 1980. Origin and historical aspects of Cyclamen persicum Mill. Hortsci. 15: 132-135.

12. Mayer, A. M. and Y. Shain. 1974. Control of seed germination. Annu. Rev. Plant Physiol. 25: 167-193.

13. Mayer, L. 1956. Wachstum und organbildung an in vitro kultivierten segmenten von Pelargonium zonale und Cyclamen persicum. Planta. 47: 401-446.

14.McNeil, M. P., Albersheim, L. Taiz and R. L. Jones. 1975. The structure of plant cell walls. VII. Barley Aleurone cells. Plant Physiol. 55: 64-68.

15. Nakayama, M. 1980. Vegetative propagation of cyclamen by notching of tuber. 11. Effect of scooping site and notching size on the regeneration of cyclamen tuber. J. Japan. Soc. Hort. Sci. 49: 228-234.

16. Sumitomo, A. and K. Kosugi. 1963. Studies of cyclamen. 1. On the germination of seed. Tech. Bull. Fac. Agr. Kagawa Univ. 137-140.

17. Stephens, L. C. and R. E. Widmer. 1974. Cyclamen seed germination. Minnesota State Flor. Bull. 6-8.

18. Stichel, E. 1959. Gleichzeitige induktion von sprossen und wurzeln an in vitro kultivierten Gewebestucken von Cyclamen persicum. Planta. 53: 293-317.

19. Taiz, L. and W. A. Honigman (1976). Production of cell wall hydrolyzing enzymes by barley aleurone layers in response to gibberellic acid. Plant Physiol. 58: 380-386.

20. Valaskova, E. 1975. Vliv ruznych zpusobu pudni dexinefekce na klicheni semen okrasnych rostlin. Sbornik UTVL. Zahradnictvi. 1974. 1(4):71-81. Vyrkummy Ustav Okraneho Zahradnictvi, Pruhonice, Czechoslovakia. (from Horticulture Abstract. 1975. n. 5959 p 525.)

21. Venable, J. H. and R. Coggeshall. 1965. A Simplified lead citrate stain for use in electron microscopy. J. Cell Biol. 25: 407-408.

22. Widmer, R. E., R. E. Lyons and M. C. Stuart. 1979. Fast-crop cyclamen. Flor. Rev. 164 (4240): 34-35. 77-78.

23. Widmer, R. E., R. J. Platteter and J. Gembis. 1976. Fast crop cyclamen. Minnesota State Florists° April 1, 3-9.

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