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dissertation entitled

STUDIES IN ALLIUM CEPA

I. DEVELOPMENT OF AN ANNUAL GENERATION CYCLE

II. PROTOCOL FOR PROTOPLAST ISOLATION AND CULTURE

presented by

Eric Ayeh

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Horticulture and Genetics

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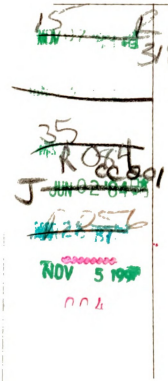
Major professor

Date August 9, 1982



STUDIES IN *ALLIUM CEPA*

I. DEVELOPMENT OF AN ANNUAL GENERATION CYCLE

II. PROTOCOL FOR PROTOPLAST ISOLATION AND CULTURE

By

Eric Ayeh

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Horticulture and Genetics

1982

ABSTRACT

STUDIES IN ALLIUM CEPA
I. DEVELOPMENT OF AN ANNUAL GENERATION CYCLE
II. PROTOCOL FOR PROTOPLAST ISOLATION AND CULTURE

By

Eric Ayeh

In an effort to decrease the time required to produce long-day hybrid onion cultivars, an annual seed-to-seed generation cycle was developed. A five x two x five factorial experiment was used in a randomized block design with 5 genotypes, 2 plant ages, and 5 vernalization periods. Significant differences were found among genotypes, vernalization periods, and the interaction genotype x plant age. Long-day genotypes bloomed earliest when young plants (86 days) were vernalized and short-day genotypes bloomed earliest when older plants (154 days) were subjected to cold treatment. The fastest seed to seed generation cycle observed was 10 months.

A second study was conducted to determine a protocol for onion protoplast isolation and culture. Two weeks and 3-month-old seedlings representing physiologically active cells and storage bulbs (dormant state of growth) were exposed to vernalizing and nonvernalizing temperatures. Protoplasts were isolated from leaf, flower scape, bulb and stem disc tissues and cultured in variable media. Protoplast yields and viability were significantly greater when derived from

physiologically active tissues exposed to vernalization temperature regardless of enzyme or asmoticum types. Similarly, streaming and division were only observed in protoplasts derived from vernalized tissues. Zeatin was required for cell wall regeneration in mesophyll and flower scape protoplasts, but bulb protoplasts regenerated cell walls in the absence of zeatin. The results suggest that protoplasts derived from cold stimulated plants mimic cold temperature effect on cells *in vivo*.

ACKNOWLEDGMENTS

My appreciation and thanks to Dr. Jon Forbes, my major advisor, for his guidance and encouragement during this study.

Thanks also to the other guidance committee members: especially Dr. Hugh Price for his advice and interest in my work. Drs. David Reicosky, Clifford Pollard, and James Hancock for their review of this manuscript.

Sincere appreciation is also expressed to Dr. Kenneth Sink for the use of his research facilities and to his advice and review of this manuscript. Others must also be thanked: The field study of the Department of Horticulture and fellow students for their support during my study.

Warmest appreciation to my parents for their patience and support throughout my education.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Male sterility has been described in many economic plant species. In both natural and cultivated plant populations, male sterility serves as a dependable outbreeding mechanism. Cytoplasmic male sterility (CMS) has been found and utilized in the production of hybrid seeds in: sugar beet (Lichter, 1978), sorghum (Shertz et al., 1978), corn (Duvick, 1965), carrot (Thompson, 1978), onion (Jones and Mann, 1965), radish (Ogura, 1968), and rice (Pearson, 1981). CMS has the advantage of being maternally inherited and large amounts of sterile progenies, can be produced depending on the presence or absence of nuclear gene restorer(s). Male sterility can also be used to eliminate detassling (i.e., in corn) and emasculation (i.e., in onion, carrots, etc.) in hybrid seed production.

The cultivated onion is self-compatible, but is predominately an outbreeder that is subject to inbreeding depression. Its outbreeding mechanisms protoandry flowering (pollen matures before stigma becomes receptive) and insect pollination (Pearson, 1981) make the use of CMS in hybrid seed production feasible, but hybrid seed yields are decreased as a consequence of backcrossing. Additionally, the biennial generation cycle of the crop makes time consuming the conventional breeding methods of simultaneously transforming CMS and developing inbred parent lines.

The characteristic pollen sterility of CMS in the onion is the result of microspore degeneration after meiosis, phenotypically expressed as brown anthers (Monsmith, 1928). It is under the control of a recessive nuclear gene and a cytoplasmic factor (Jones, 1943). Since bulbs are the most economically valued part of the onion plant, sterility of the F_1 plants is of little importance. With conventional breeding, a minimum of six backcross generations (12 yrs.) to the recurrent, CMS recipient parent is needed to insure successful incorporation. This is a relatively long period of time and shortening the generation cycle for a more rapid backcrossing scheme is desirable.

The somatic transfer of CMS via protoplast fusion has been achieved in two plants. Zelcer (1978) and co-workers transferred CMS from Nicotiana tabacum into the nuclear genome background of N. sylvestris by making the N. tabacum nucleus nonfunctional through x-irradiation. Izhar and Power (1979) used a selective media for the recipient Petunia axillaris to transfer CMS from P. hybrida. They used a media which selected against the genome of hybrida and nuclear hybrids between the two species. Successes via cell fusion transfer of plant cell genome or genes in other crop plants awaits refinement in cultural techniques to the point where functional plants can be regenerated from protoplasts and appropriate makers are available for selection in culture.

The examples with Nicotiana and Petunia demonstrate two different techniques which can transfer CMS in diploid organisms

while keeping intact the recipient nuclear genome. Use of enucleated protoplasts in creating cybrids provides a third alternative to combine specific cytoplasmic and nuclear genomes in plant cells. The successful use of high speed centrifugation and density gradients to isolate subprotoplast from cultured cells in Hordeum and Zea mays (Lorz et al., 1980) and the production of enucleated protoplast from the epidermis of onion bulbs (Bradley, 1978) suggest that CMS can be transferred via protoplast fusion in onion. However, the use of these techniques in onion has been limited since regeneration to plants from protoplasts has not yet been achieved.

The objectives of this research were to (1) develop an annual seed-to-seed cycle for greenhouse seed production, and (2) develop a protocol for onion protoplast isolation and culture as a prerequisite for the eventual transfer of CMS using protoplast fusion.

LITERATURE REVIEW

The ontogeny of organ formation and development in Allium varies among different species, but in A. cepa ontogeny also depends on the environmental conditions under which the plant is grown. The most important traits of the crop involve the bulbing response which is controlled by daylength and high temperatures, and the bolting response which is regulated by low temperatures (Jones and Mann, 1963; Health and Holdsworth, 1948; Heath, 1944, 1945; Hoffman, 1933). Seeds from the same packet, planted under different environmental conditions of temperature and daylength, may provide scallions, sets, large bulbs or seeds. To understand these differences and their possible effects on the annual breeding cycle and onion protoplast culture, a review on onion plant development is provided.

Morphology and Development

After the seedling is established, the young onion plant continues to produce new foliage and adventitious roots, and the stem slowly elongates and broadens. Early in development the entire leaf is solid and meristematic; at a later date only the sheath and base of the blade is meristematic; and still later only the base of the sheath has the capacity to grow since the rest of the leaf becomes hollow (Hoffman, 1933; Jones and Mann, 1963). Before this cavity is formed, all the inner cells of the leaf contain functional nuclei,

although the volume of the intracellular space is probably greater than that occupied by the cells. The mature leaf consists of this central cavity, surrounded by eight to ten layers of parenchyma with large intracellular spaces, the vascular bundles, the palisade layers and the epidermis (Hoffman, 1933). The shoot apex, found at the center and upper side of the broadstem (stem-disc) is protected at the bottom of the youngest tubular leaf. Each leaf surrounds the next younger leaf. The inflorescence axis or flower scape always arises at the apex of the stem. It follows the same growth pattern of a leaf with the scape becoming hollow at a later date. This pattern of growth, described for the common onion, is essentially the same in all cultivated species of Allium.

Effects of Temperature on Bulbing and Flowering

With inductive daylength, the onset of bulbing is characterized by the swelling of one or more of the innermost enlarged leaves and three or more of the outermost, nonemerged leaf initials. The swelling results from an increase in cell size and the development of intracellular spaces, without cell division (Heath, 1945). For a given daylength, bulbing is accelerated by high temperature and is greatly delayed or prevented by low temperature. Several workers have shown that the emergence of leaves normally ceases immediately or soon after bulb induction depending on temperature (Heath, 1943a, 1943; Heath and Holdworth, 1943a, Aoba, 1954). Thus, the entire process of bulbing and maturation does not include cell division as long as temperatures are kept at levels noninductive for flowering.

Cold temperatures revert the process of bulbing by inducing or maintaining cell-division at the shoot apex and at the young leaf initials (the same cells required for enlargement in bulbing). Photoperiod and noninductive temperatures for flowering may be required in concert to induce bulbing.

The existence of a critical bulb or plant size and a minimum requirement of cold temperature exposure for flower induction has been documented by several workers (Jones and Emsweller, 1939; Heath, 1944, 1945; Mather and Heath, 1944; Woodbury, 1950; Shishido and Saito, 1976; and Hesse et al., 1979). An effective vernalization temperature range of 5° to 12°C for flower induction (Demille and Vest, 1975) and a lack of effect of photoperiod on flower initiation or emergence has also been reported (Heath, 1945; Shishido and Saito, 1975). The plant or bulb size plays a major role in triggering cold temperature responses for flowering. A critical mother bulb size (Jones and Emsweller, 1939) and plant (Shishido and Saito, 1976) have both been recognized as factors which affect flower induction in the cultivated onion. Gregory (1936) refers to this critical size as "the ripeness to flower."

Somatic Cell Culture in Monocotyledons

The techniques of culturing *in vitro* single somatic cells, protoplasts or microspores under defined conditions, and proceeding via somatic embryogenesis or organ regeneration to a genetically stable plant has been demonstrated with a few species, mostly herbaceous dicots (Potrykus, 1979). Several workers have found that

monocotyledons often do not respond well to the wide range of conditions that successfully induce division of somatic cells in herbaceous dicotyledons (Carter et al., 1967; Krikoran et al., 1969; Hussey, 1975; Dudits, 1976; Koblitz, 1976; Thomas et al., 1978; Potrykus, 1979, 1980). However, within the last decade and a half, an increasing number of monocots have been regenerated via tissue or cell culture. Plant regeneration from mesocotyl derived callus has been obtained in all cereals (Potrykus, 1980). Propagation by tissue culture of members of the Liliaceae, Irideacea, and Amaryllidaceae has proven highly successful (Hussey, 1975, 1979). Plant regeneration from basal disc and root derived callus of A. cepa and from the leaf, stem and apical meristem of A. sativum has also been successful (Table 1). Callus has been derived from protoplasts in such cereals as Oryza sativa (Deka et al., 1976; Tsai et al., 1978). Triticum sp. (Dudits et al., 1976), Zea mays (Potrykus, 1977, 1979), Hordeum vulgare (Koblitz, 1976). Sorghum bicolor (Brar et al., 1980) and saccharum sp. (Maretzki et al., 1972), but attempts to regenerate plants from protoplasts in any of the aforementioned species has not been successful. So far only four monocots have not been recalcitrant in culture; successes have only been obtained in Bromus inermis (Kao et al., 1973) Asparagus officinalis (Ha and Mackenzie, 1973), Pennisetum americanum (Vasil et al., 1980) and Manihot esculenta (Shanin et al., 1980). Protoplast studies in the genus Allium have been limited to morphological and physiological studies of the cell, involving protoplasts isolated from the root and bulb epidermal layer of A. cepa (Table 1).

TABLE 1. Tissue Culture in the Genus Allium: State of the Art.

	References
Allium porrum (Leek)	
Basal disc → Callus	Dunstan and Short, 1979
Allium cepa (Onion)	
Basal disc → Callus	Dunstan and Short, 1977
Callus → Root	Fridborg, 1971
Callus → Shoot	Dunstan and Short, 1978
Root → Callus	Klein and Edsall, 1968 Selby and Collin, 1975
Callus → Shoot	Dunstan and Short, 1978
Callus → Protoplast	Bawa and Torrey, 1971
Bulb → Protoplast	Roland/Prat/Pilet, 1971 Vreugdenhill, 1957
Epidermal layer → Protoplast	Hepler/Zeigler, 1976 Schnaubl/Borman/Zeigler, 1978 Bradley, 1978
Flower head → Shoot	Dunstan/Short, 1978
Embryo → Callus	Guha/Johri, 1966*
Allium sativum (garlic)	
Leaf → Callus	Havranek/Novak, 1972, 1974
Callus → Shoot	Mostafa/El-Nil, 1976
Callus → Plant	Novak, 1979
Apical meristem → Callus → plant	Kehr/Schaeffer, 1976
Stem → Callus	Mostafa/El-Nil, 1976
Callus → Shoot	Mostafa/El-Nil, 1976
Callus → Embroids	Mostafa/El-Nil, 1976
Shoot buds → plant	Bhojwani, 1980
A. fistolosum	
Shoot tips → plant	Fujieda/Ando/Fujita, 1977

*Including *in vitro* development of ovary and ovules.

CHAPTER I

DEVELOPMENT OF AN ANNUAL GENERATION CYCLE IN *A. CEPA*

Introduction

External environmental factors (i.e., temperature and daylength) play an important role in the development of the onion. The literature on *A. cepa* indicates that there is a critical plant or bulb size and vernalization regime which affects flower induction. A vernalization range of 5 to 12°C is optimum for flowering in all cultivars, while the critical bulb size varies between genotypes--a minimum plant diameter of 3-6 mm across cultivars was observed by Shishido et al. (1976). Daylength does not appear to control flower initiation directly (Heath, 1945), but it seems to modify the effect of temperature on flowering (Shishido et al., 1975).

The objective of this study was to determine how plant age, genotype and vernalization period affect the generation cycle of onion under Michigan greenhouse conditions.

Materials and Methods

The experimental design was a 5 x 2 x 5 factorial in a randomized complete block. The variables were 5 genotypes, 2 plant ages, and 5 vernalization periods. Five plants per experimental unit

were used in each of two blocks, yielding a total of 250 plants per block.

Onion inbreds MSU 6693A, MSU 2399B, and their hybrid progeny MSU (6693 x 2399) were utilized as examples of longday (LD) onion. Dessert Seed Company inbred 986 and the hybrid cultivar 'Dessex' were included as short day (SD) onions.

Greenhouse Treatment

Seeds of all 5 genotypes were sown in January in separate flats filled with Houghton Muck soil and kept moist. Soon after germination (35 days), seedlings were transplanted into 10 x 10 cm clay pots and were placed in 2 greenhouses under conditions of 26 ± 2°C day/20 ± 2°C night. They were irrigated 4 times per week and fertilized twice a week with a 20-20-20 mix of 200 ppm of nitrogen per application. Greenhouse lighting was supplemented with fluorescent lamps to maintain an 18 hr. photoperiod. Sixty-eight days after the first sowing date, an additional set of seeds from all 5 genotypes were sown into similar flats and treated as above. The 50 treatment combinations were randomized in each greenhouse.

Cold Temperature Treatment

When the individuals sown on different dates were 86 and 154 days old, they were transferred into a cold room maintained at 7°C with an 18 hr. photoperiod. Watering was reduced to one application per week and fertilization to one application of 20-20-20 fertilizer every other week. Groups of 100 plants were returned to the greenhouse at 8, 10, 12, 14, and 16 week intervals. Upon return to the

greenhouse, plants were tagged on the first day of umbel (flower head) appearance.

Results and Discussion

Overall, LD genotypes yielded the highest number of umbels, with the young (86 day) plants being the most responsive. Although all vernalization regimes were effective in inducing flowering, the longer vernalization periods yielded greater amounts of flower heads (Tables 2 and 5); the 16 week treatment produced significantly more flowers than 14 weeks, although there was no significant difference between 8 and 10 weeks. The significant effects of longer vernalization periods on flower head formation (Table 5) and the cumulative response of increased vernalization period (Table 2) suggest that longer cold temperature exposure periods are a significant factor in flower initiation.

There was also a significant genotype x age interaction (Table 3). Both of the older (154 day) SD genotypes yielded more flower heads than the younger ones, but in the LD genotypes, 86-day-old plants produced twice as many flower heads as older ones (Table 4, Figure 1a). LD genotypes MSU 2399B and MSU (6693 x 2399) showed significant differences, but not MSU 6693A.

Considerable variability was found in the age necessary for flowering in long- and short-day onion genotypes. Long-day plants flowered after 86 days of vegetative growth, whereas short-day plants required longer periods of time. Umbels appeared within 30 days upon return of the plants to the greenhouse following vernalization. Thus,

TABLE 2. Interaction of Vernalization Period, Genotype, and Seedling Age on the Induction of Flowering in *Allium cepa* L.

Genotype	Photoperiod[1]	Age of Seedling (days)	Vernalization Period (weeks)					Total
			8	10	12	14	16	
			Number of Flowering Plants					
MSU 6693A	LD	86	1	1	5	7	10	24
		154	6	4	7	5	9	31
MSU 2399B	LD	86	3	4	4	8	9	28
		154	0	0	0	0	1	1
MSU (6693 x 2399)	LD	86	4	5	10	9	8	36
		154	3	1	4	6	6	20
Dessex	SD	86	0	0	0	0	0	0
		154	2	5	6	5	6	24
986	SD	86	0	0	0	0	1	1
		154	1	6	7	3	9	26
TOTAL			20	26	43	43	59	191

[1]Photoperiod necessary for bulbing; LD--long day, SD--short day.

TABLE 3. Analysis of Variance of the Effect of Vernalization Period, Genotype and Seedling Age on Flowering in *Allium cepa* L.

Sources of Variation	df	Sum of Squares	Mean Square	F
Genotype	4	50.55	12.66	8.3**
Age	1	1.70	1.70	ns
Vernalization Period	4	48.25	12.06	10.41**
Genotype x Age	4	110.05	27.50	23.75**
Genotype x Vernalization	16	24.15	1.50	ns
Vernalization x Age	4	3.55	0.88	ns
Genotype x Vernalization x Age	16	53.15	3.32	2.87*
Replication	1	4.04	4.04	
Error	49	56.76	1.16	
TOTAL	99	352.20		

n.s. = Not significant

* = Significantly different at the level of 5%.

** = Significantly different at the level of 1%.

TABLE 4. Genotype and Age Interaction with Long Day (LD) and Short Day (SD) Onion Cultivars.

Genotype	Photoperiod[1]	Age		F
		86 Days	154 Days	
		Number of Flowering Plants		
6693A	LD	24	31	2.45 n.s.
2399B	LD	28	1	36.45**
(6693 x 2399)	LD	36	20	12.80**
Dessex	SD	0	24	28.80**
986B	SD	1	26	31.25**

[1]Photoperiod necessary for bulbing; LD--long day, SD--short day.

**Significantly different at the level of 1%.

n.s. = not significant.

TABLE 5. Independent Comparisons for Vernalization Periods in *Allium cepa L.*

Vernalization Period	Totals	F
8 week vs. 10 week	20 vs. 26	0.9 n.s.
14 week vs. 16 week	43 vs. 59	6.4*
8 and 10 wks vs. 14 and 16 wks	20 and 26 vs. 43 and 59	39.2**
12 weeks vs. avg. of 8, 10, 14, 16 weeks	43 vs. avg. of rest	1.6 n.s.

n.s. = not significant

* = Significantly different at the level of 1%.

** = Significantly different at the level of 1%.

Figure 1. The mean and cumulative values of flowering plants against vernalization period (7°C).

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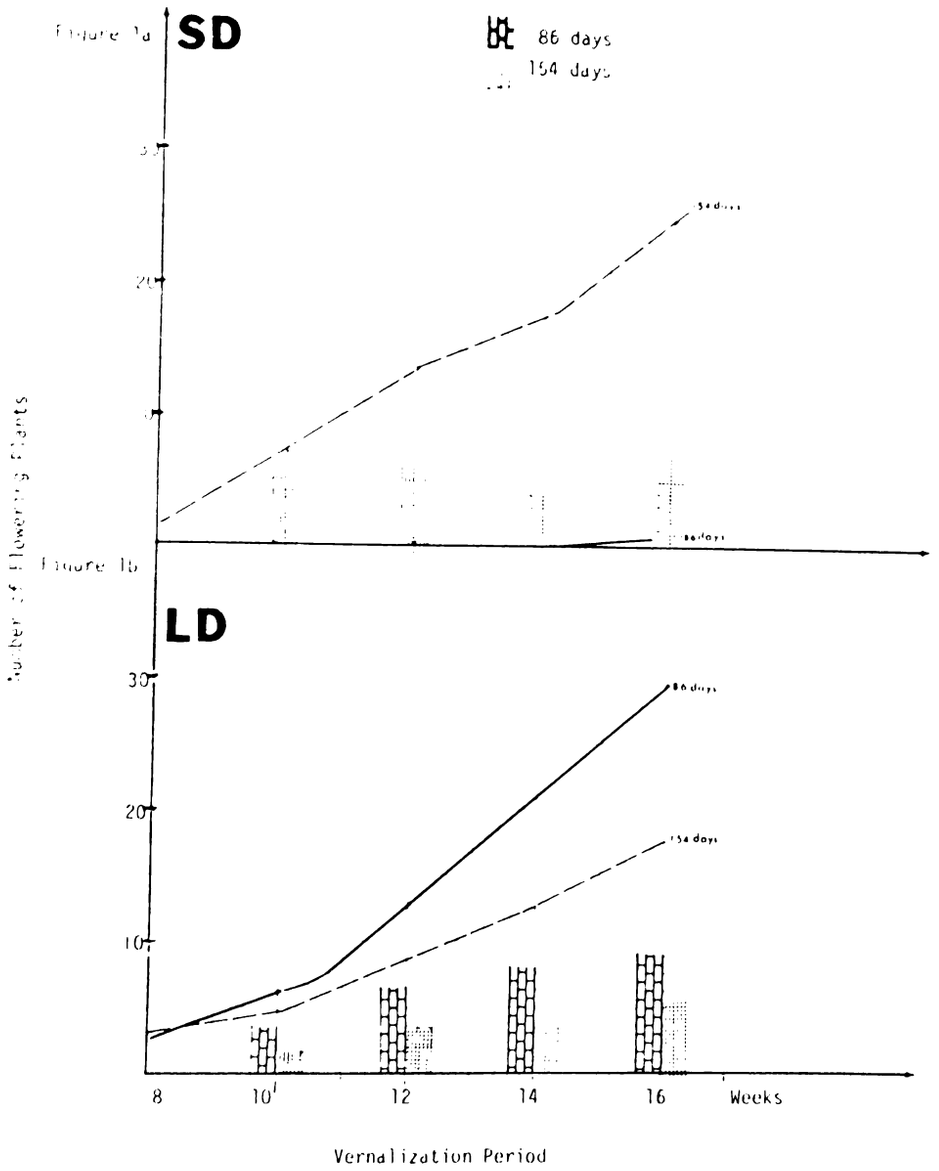

SD
86 days
154 days
LD
30
20
10
8
10
12
14
16
Weeks
Vernalization Period

the young 86 day plants all had umbels eight months after their seeding date, while all the 154-day plants were flowering after ten months. The leaves (tops) of all 154-day SD plants had fallen and their bulbs had ripened by the time of cold temperature exposure. While both age groups of LD genotypes achieved the minimum size required for flowering, only the 154-day treated SD genotypes attained this size. The younger SD plants were induced to bulb earlier under 18 hr. photoperiod, but could not respond to flowering stimulus.

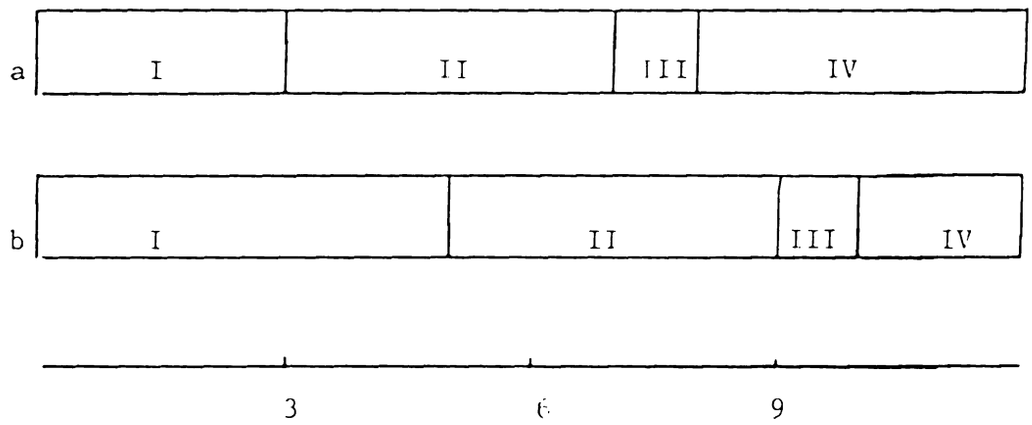
Eighty-six day old SD plants responded poorly to the flowering stimulus, perhaps because bulb formation was already induced by the 18 hr. photoperiod and bulbing cannot be reverted when temperatures are 7°C. The 154-day old SD genotypes may have responded to the flowering stimulus because their tops had fallen and their bulbs had ripened prior to cold temperature exposures.

Figure 2 represents the 4 phases in a generation cycle of the onion crop, reduced to an annual scale. Phase I and II represents the minimum plant size and duration of the vernalization regime respectively required for flowering (these appear to be the most critical in the cycle). Under both greenhouse and field conditions, the IV phase can be completed in a maximum of 2 months. Phase II can be extended as long as necessary for the final phases III and IV to be completed on an annual cycle. Under the conditions of this experiment, the minimum generation cycle was 10 months for LD and 12 for SD plants.

Figure 2. Relative length of phases for an annual generation cycle in onion: 18 hr photoperiod.

a. LD

b. SD



Phase I = Vegetative growth to achieve minimum size to flower.

Phase II = Vernalization phase.

Phase III = Umbel development to anthesis.

Phase IV = Fertilization to seed maturity.

CHAPTER II

ONION PROTOPLAST ISOLATION AND CULTURE

Introduction

Enzymatic isolation of protoplasts from onion roots and epidermal tissue has been accomplished (Table 1), but there has been little attempt to identify the conditions which most effectively promote cell division. Different tissues including flower petals, fruits, meristems, stems, tubers, bulbs, roots, and petioles have produced variant number of protoplasts in other species; but only leaf tissue has provided protoplasts which directly regenerate whole plants (Takebe et al., 1981; Ha and Mackenzie, 1973; Kartha et al., 1974; Shepard et al., 1977; Zapata et al., 1977; Sharin et al., 1980). The culture success of protoplasts has been affected by developmental stage (Green, 1975, 1981; Portrykus, 1977), pretreatment (Erickson, 1977; Shephard, 1981), genotype (Green, 1977) and the ratio of K^{+} and Ca^{++} ion concentrations (Vreugdenhill, 1957). Cell suspension cultures generally provide a more reliable and consistent source of protoplasts, but the incidence of cytological changes observed in cell culture (Novak, 1974) and the phenotypic changes observed in plants regenerated from cell cultures (Oono,1978; Krishnamurthi, 1977) does not render this protoplast source conductive to *in vitro* genetic manipulation.

The objective of this study was to develop a protocol for the isolation and culture of onion mesophyll, flower scape, and bulb protoplasts.

Materials and Methods

Seeds of MSU (6693 x 2399) were sown at 2-month intervals in flats filled with Houghton Muck Soil in the greenhouse (26°C day/ 20°C night, 18 hr photoperiod). Thirty-day-old seedlings were transplanted into 10 x 10 cm pots, watered 4 times per week and fertilized twice a week with 20-20-20 fertilizer mix at 200 ppm. Leaf explants were taken from three groups of plants which varied in age and environmental pretreatment. The groups were:

I. Nonvernalized vegetative tissue--Fifty, three-month-old seedlings were given environmental treatments of 26°C day/20°C night and 18 hr photoperiod in the greenhouse for 14 weeks. These plants did not flower and provided leaf and bulb tissues.

II. Reproductive tissue--Fifty, three-month-old seedlings were given environmental treatments of 10°C and 12 hr photoperiod in a growth chamber for 14 weeks before being returned to the greenhouse for 4 weeks. These plants flowered and provided leaf and flower scape tissues for protoplast isolation.

III. Vernalized vegetative tissue--Twenty, two-week-old seedlings were exposed to 10°C and 12 hr photoperiod in a growth chamber for 16 weeks and returned to the greenhouse. These plants did not flower and provided leaf tissue for protoplast isolation.

Harvesting of Explants

Bulbs--Explants were harvested from onion bulbs after the tops had fallen and the bulbs had matured.

Leaves--Leaf explants were taken from 10°C and 26°C treated plants at 4, 8, 10, and 12 week intervals. The second to the fourth youngest leaves were used.

Flower scape--Explants from flower scapes were harvested within the first seven days of umbel (flower head) development, prior to opening.

Stem discs--Bulbs from storage provided stem discs for protoplast isolation.

Surface Sterilization of Explants

Bulb--The outermost layers of 2-3.5 cm bulbs were removed and remnant roots were trimmed. The remaining portion of the bulb was then soaked in 1.57% sodium hypochlorite for 15 minutes with periodic agitation, followed by 4 rinses with sterile distilled water.

Leaf and flower scape. Explants were sterilized in 10% Clorox (0.25% active ingredient of sodium hypochlorite) for 6 minutes with constant agitation. They were then rinsed 3 times in sterile, distilled water.

Stem disc--Stem discs were obtained by trimming and discarding all the bulb scales, root and root initials of the bulb. The remaining broad stem including leaf and root meristematic regions, was surface sterilized using the same procedure as bulbs.

Protoplast Isolation

Bulbs were further trimmed under aseptic conditions to the two innermost scales and cut horizontally into 6-8 slices, each of which was cut horizontally and vertically into 2-5 mm square pieces. This procedure increased the surface area of the tissue for more efficient enzyme penetration. Stem discs were trimmed to their meristematic regions and likewise cut into 2-5 mm square pieces.

Leaves and flower scapes were also cut into 2-5 mm pieces and approximately 4 gms of tissue were incubated in 15 mls of the test enzyme solution and salt mixtures (Table 6). Leaf and flower scape tissues were incubated at 25°C for 5 and 4 hours respectively; bulb and stem disc at 20°C for 4 and 5 hours respectively.

Preparation of Protoplasts

At the end of the incubation period, protoplast release was facilitated by gently repipetting several times into a sterile petri-dish. Protoplasts from all sources were collected by filtering through a silk screen (0.5 mm^2 mesh) followed by centrifugation at 35 x g for 4 minutes. The precipitates, which appeared to be mostly protoplasts and debris, were gently mixed with 10 ml of 0.5 M sucrose and were centrifuged at 35 x g for 10 minutes. The floating protoplasts were then washed twice by centrifugation (35 x g for 15 minutes) in culture solution without hormones. The protoplasts were plated in liquid culture media (Table 7) and dispensed at variable densities in 4.0 ml proportions into 60 x 50.0 mm petri dishes.

TABL

Compon

Drise

Cellu

Pecti

Pect

Rhoz

CaCl

MgSO

KH_2

KNO

KI

Cus

Mar

KC

De

pH

P
F

TABLE 6. Enzyme Solutions Used in Onion Protoplast Isolation.

Component	Enzyme Solution[a]		
	E_1	E_2	E_3
Driselase	1%	1%	--
Cellulysin	1%	1.5%	2%
Pectinase	0.8%	4%	--
Pectinol R-10	--	--	3%
Rhozyme HP 150	--	--	1%
$CaCl_2 \cdot 2H_2O$	10 mM	10 mM	40 mM
$MgSO_4 \cdot 7H_2O$	10 mM	10 mM	25 mM
KH_2PO_4	2 mM	2 mM	2 mM
KNO_3	10 mM	10 mM	10 mM
KI	9.6 mM	9.6 mM	9.6 mM
$CuSO_4 \cdot 5H_2O$	1 mM	1 mM	1 mM
Mannitol	0.7 M	0.25 M	--
KCL	--	0.25 M	0.5 M
Dextran Sulfate	--	0.5%	0.5%
pH	5.0	5.0	5.0

[a]Enzyme solution 1 was most efficient in generating bulb protoplasts, solution 2 was most effective on stem discs and solution 3 proved most reliable for both leaf and flower scape tissues.

TABLE 7. Onion Protoplast Culture Media.

Compound	Protoplast Culture Medium[a] (mg/L)		
	Medium 1	Medium 2	Medium 3
$CaCl_2 \cdot 2H_2O$	440	150	150
KNO_3	253	253	253
NH_4NO_3	320.16	320.16	320.16
$NH_4H_2PO_4$	230.06	230.06	230.06
$(NH_4)_2SO_4$	134	134	134
$MgSO_4 \cdot 7H_2O$	370	247	247
$MnSO_4 \cdot 4H_2O$	13.2	13.2	13.2
$ZnSO_4 \cdot 7H_2O$	2.0	2.0	2.0
$CuSO_4 \cdot 5H_2O$	0.039	0.039	0.039
KI	0.75	0.75	0.75
$CoCl_2 \cdot 6H_2O$	0.025	0.025	0.025
H_3BO_3	3.0	3.0	3.0
$NaMoO_4 \cdot 2H_2O$	0.25	0.25	0.25
$NaH_2PO_4 \cdot 2H_2O$	172	172	172
$FeSO_4 \cdot 7H_2O$	27.85	27.85	27.85
Na EbTA	37.25	37.25	37.25
Nicotinic acid	1.0	1.0	1.0
Thiamine	5	10	10
Pyriodoxine	1.0	1.0	1.0
Folic acid	0.5	--	--
Biotin	0.05	--	--
M-Inositol	100	100	100

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TABLE 7. Continued.

Compound	Protoplast Culture Medium[a] (mg/L)		
	Medium 1	Medium 2	Medium 3
6BAP	0.3	0.3	0.3
2,4-D	0.3	0.3	0.3
Zeatin	0.5	--	--
Sucrose	15,000	15,000	15,000
Glucose	15,000	15,000	15,000
Mannitol	0.5 M	0.5 M	0.7 M
pH	5.6	5.6	5.6
Ampicillin	--	--	--
Tetracycline	--	--	1.0
Gentamicin 1	--	--	1.0

[a]Media 1, 2 and 3 were modified from BDS (Dunstan/Short, 1977; Gamborg, 1971), MS (Murashige/Skoog, 1962) and S2M (Short/Torrey, 1972).

Protoplast Count and Staining

Protoplast yield was determined by counting 0.2 ml aliquots of each 15 ml suspension with a modified Fusch-rose-nthal haemocytometer. Protoplast viability was determined with a fluorescein diacetate stain at pH 5.6 (Stadelman et al., 1972; Widholm, 1972). Cell wall regeneration was followed by Calcofluor White staining (Gamborg et al., 1975) where an equal volume of cell suspension was mixed with 0.1% solution of Calcofluor White stain in 0.5 M mannitol for 5 minutes, centrifuged at 35 x g for 4 minutes and then resuspended in 0.5 M mannitol. Fluorescence was observed in 364 nm U-V light.

Estimation of Percent Viability and Protoplast Survival

For each treatment, protoplast viability was estimated in 5 randomly chosen fields (200 x magnification) as the number of fluorescing protoplast divided by the total number of protoplasts. Protoplast survival was measured in 5 randomly chosen fields (100 x magnification) after a 48 hr period using the formula:

$$\frac{\text{Number of intact protoplasts}}{\text{Number of intact protoplasts after 48 hrs}} \times 100$$

Culture Media

Four mesophyll protoplast culture media were derived using the salts and vitamins of B5 (Fridborg, 1971), BDS (Dunstan and Short, 1977), MS (Murashige and Skoog, 1962) and S2M (Short and Torrey, 1977). These were each supplemented with 15 gm/l of glucose

and sucrose, 0.5M mannitol, 0.3 mg/l each of 2,4 dichlorophenoxy acetic acid (2,4-D) and 6-benzylaminopurine (6BAP), and 0.5 mg/l of zeatin. To determine the best source of protoplasts and physiological activity in culture (Table 14), a modified BDS medium labeled medium 1 (Table 7) was used. This was modified by increasing levels of calcium chloride and magnesium sulfate to that of MS, and the addition of thiamine at 0.5 mg/l, folic acid at 0.5 mg/l and biotin at 0.05 mg/l.

Bulb protoplasts were plated in Medium 3 (Table 7) derived from the salts and vitamins of BDS supplemented with glucose and sucrose at 15 gm/l each, mannitol at 0.7M and 2,4-D and 6 BAP at 0.3 mg/l, ampicillin 40 µg/l, tetracycline 1 mg/l and gentamicin 1 mg/l to control contamination. The same medium minus the antibiotics, but with the addition of 0.5 M mannitol was used for stem-disc protoplast culture (Medium 2). All media were adjusted to pH 5.6 and filter sterilized (0.20 micron filter, Nalgene Co., Rochester, N.Y.).

Enzyme Sources

Cellulase	Meiji Seika Kaisha Ltd., Japan
Cellulysin	Calbiochem, USA
Driselase	Kyowa Hakko, USA
Meicelase-P	Meiji Seika Kaisha Ltd., Japan
Macerase	Calbiochem, USA
Pectinase	Sigma Chemical Co., USA
Pectinol R10	Rohm and Haas, USA
Rhozyme HP-150	Rohm and Haas, USA

Results and Discussion

Isolation of Mesophyll and Flower Scape Protoplasts

Effects of enzyme mixtures.--Preliminary examination of several test enzyme mixtures revealed that Driselase and Pectinase were detrimental to mesophyll protoplast release. These enzymes caused browning of the tissue edges after two hours incubation and the released protoplasts showed browning at the end of a 5 hour enzyme incubation period. All plant materials including 2-week-old to 3-month-old plants, and vernalized and nonvernalized tissues showed browning in the presence of these enzymes.

Pectinol R-10, Rhozyme HP 150,and Cellulysin at lower concentrations in a mixture (E_3, Table 6), effectively released leaf and flower scape protoplasts without browning. The enzymes Cellulase, Macerase, and Meicelase-P in mixtures with other enzymes were not as effective in releasing either mesophyll or flower scape protoplasts.

Effects of an osmotic stabilizer.--Of the sugar compounds used for the osmotic stabilization of isolated protoplasts (mannitol, sorbitol, sucrose, and glucose) only mannitol appeared to be effective in isolating high numbers of intact mesophyll protoplasts. Potassium chloride (KCl) produced significantly more protoplasts than mannitol (Table 10) and high protoplast yields were observed from leaf explants exposed to temperature regimes of 10°C and 26°C (Figure 3) with no apparent effect on protoplast viability (Table 8).

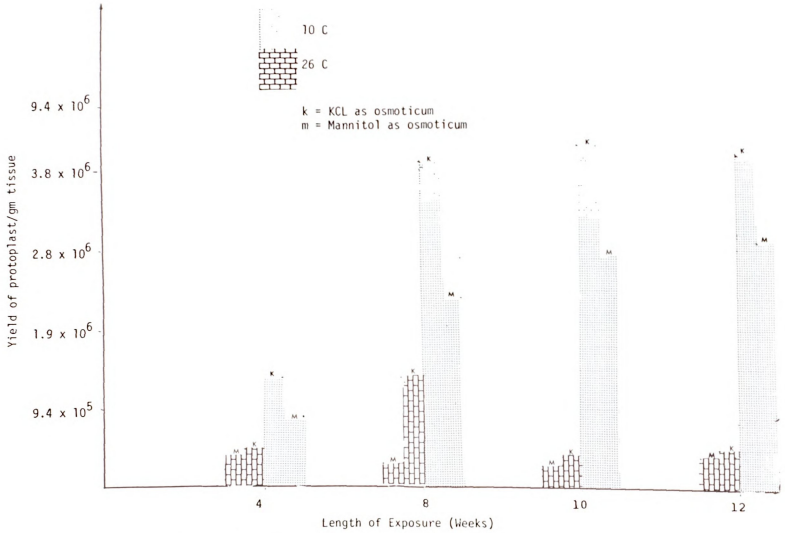

Figure 3. Histogram depicting yields of mesophyll protoplasts extracted in enzyme soultion E_3 (Table 6).

TABLE 8. Viability and Yield Estimates of Enzyme Extracted Protoplasts from Leaf Tissues using KCL as the Osmoticum.

Vernalization Time (Wks)	10°C		26°C	
	% Viability[a]	Yield[b]	% Viability[a]	Yield[b]
3 Month Old Seedlings				
4	79.3	3.5×10^5	39.5	1.4×10^5
8	61.7	1.1×10^5	45.0	3.5×10^5
10	70.5	1.6×10^6	31.6	0.9×10^5
12	62.3	1.3×10^6	27.5	1.2×10^5
2 Week-Old Seedlings				
16	67.0	2.8×10^5	*	*
Flower Scape	*	*	76.8	1.4×10^6

[a]Average count of 5 fields.

[b]Number of protoplast per ml of solution.

TABLE 9. Viability and Yield Estimates of Enzyme Extracted Protoplast from Leaf Tissues Using Mannitol as the Osmoticum.

Vernalization Time (Wks)	10°C		10°C	
	% Viability[a]	Yield[b]	% Viability[a]	Yield[b]
3-Month Old Seedlings				
4	69.0	2.1×10^5	50.5	1.2×10^5
8	63.5	6.0×10^5	31.0	0.7×10^5
10	72.0	7.2×10^5	33.0	0.8×10^5
12	76.0	7.6×10^5	27.3	1.0×10^5
2-Week-Old Seedlings				
16	76.0	1.7×10^6	*	*
Flower Scape	*	*	70.6	8.1×10^5

[a]Average count of 5 fields.

[b]Number of protoplast per ml of solution.

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TABLE 10. Yields of Mesophyll Protoplasts Extracted from Temperature Pre-Treated Plants.

Temperature	Osmoticum	Exposure Time in Weeks				Total
		4	8	10	12	
10°C	KCl	3.5[a]	11.0	16.0	13.0	43.5
	Mannitol	2.1	6.0	7.2	7.6	22.9
26°C	Kcl	1.4	3.5	9.0	1.2	7.0
	Mannitol	1.2	0.7	0.8	1.0	3.7
TOTAL		8.2	21.2	24.9	22.8	77.1

[a]All values are 1 x 10^5.

TABLE 11. Analysis of Variance of Temperature and Osmoticum Influences on Mesophyll Protoplast Yields.

Sources of Variation	df	Sum of Squares	Mean Square	F
Temperature	1	193.90	193.90	26.56***
Osmoticum	1	35.70	35.70	4.89*
Temp. x Osmoticum	1	18.75	18.75	n.s.
Replication	3	42.60	14.20	n.s.
Error	9	65.81	7.3	

*Significantly different at the $p \leq 10\%$ level.

***Significantly different at the $p \leq 0.05\%$ level.

The higher yeild counts which resulted from high salt extraction with KCl suggest that the univalent K^+ ion affects protoplast integrity (Vreugdenhill, 1957) and quality (Simmonds et al., 1979). Calcium chloride was not equally effective in enhancing protoplast yields.

Effects of temperature.--Figure 3 shows higher mesophyll protoplast counts from 10°C treated plants than those held at 26°C, regardless of the enzyme mixture. The difference observed between the two temperature conditions was highly significant (Table 11). Similarly, the flower scape yielded large amounts of protoplasts when extracted with either mannitol (Table 9) or KCl (Table 8).

Isolation of Bulb and Stem Disc Protoplasts

Among the enzyme mixtures tested on bulb and stem disc tissues, enzyme solutions E_1 and E_2 (Table 6) were the most effective in releasing protoplasts. Driselase and Pectinase enzymes did not brown these tissues. Bulb explants yielded more protoplasts than stem discs at the three incubation temperatures (Table 12). Unlike leaf explants, 10°C and 26°C exposure regimes had little effect on these protoplast yields.

Culture of Protoplast

Leaf and Flower Scape

Four protoplast media derived from the salts and vitamins of B5, BDS, MS, and S2M, all supplemented with sucrose, glucose, mannitol, and hormones were tested for their culturability of leaf and

TABLE 12. Protoplast Yields from Bulb and Stem-discs Maintained Under Differing Conditions.

Osmoticum Type	Storage Temp. °C	Tissue	Incubation Temperature °C		
			20	25	30
Potassium Chloride	10°	Bulb	2.3×10^{6}[a]	1.9×10^{6}	4.7×10^{5}
		Stem-disc	5.6×10^{4}	3.9×10^{4}	2.1×10^{4}
	26°	Bulb	2.0×10^{6}	2.5×10^{6}	1.4×10^{6}
		Stem-disc	7.0×10^{4}	9.1×10^{3}	3.1×10^{4}
Mannitol	10°	Bulb	6.1×10^{5}	6.2×10^{5}	1.7×10^{5}
		Stem-disc	1.1×10^{3}	1.0×10^{3}	0.7×10^{3}
	26°	Bulb	5.8×10^{5}	5.0×10^{5}	4.0×10^{5}
		Stem-disc	1.9×10^{3}	8.0×10^{2}	0.9×10^{3}

[a]Yield of protoplasts expressed as number of protoplast per ml of soln.

TABLE 13. Survival and Cytoplasmic Streaming in Protoplasts Derived from Different Sources and Conditions.

Protoplast Source	Culture Temp. °C	Character: Survival after 48 hrs. in culture[a]				Cytoplasmic Streaming[b]			
		Media[c] B5	BDS	MS	S2M	B5	BDS	MS	S2M
Mesophyll protoplast Nonvernalized vegetative tissue	10	51	62	45	50	V. poor	V. poor	V. poor	V. poor
	20	46	60	32	50	poor	poor	poor	poor
	25	40	55	30	30	poor	poor	V. poor	V. poor
Mesophyll Protoplast Vernalized Vegetative Tissue	10	86	97	86	70	poor	fair	poor	poor
	20	77	90	78	69	rich	rich	rich	rich
	25	73	85	72	63	rich	rich	fair	fair
Flower Scape Reproductive Tissue	10	90	96	84	73	poor	fair	poor	poor
	20	75	92	76	65	rich	rich	rich	rich
	25	72	78	72	60	rich	rich	rich	fair
2 wks-old Vernalized Vegetative Tissue	10	72	72	68	65	poor	poor	poor	poor
	20	66	78	62	46	poor	poor	poor	poor
	25	61	71	50	48	poor	poor	poor	poor

[a]Average of three plates

[b]Very poor = almost all protoplast dead by time of measurement; poor = protoplast turgid but no activity; fair = protoplast turgid with 50% of them streaming; rich = protoplast turgid with 50% of them streaming.

[c]Media compose of the salts and vitamins of B5, BDS, SM and S2M supplemented with glucose and sucrose at 15 gm-- eac, 0.5M mannitol, 2,4-D at 0.3mg-l, 6BAP 0.3 mg-l and Zeatin at 0.5 mg-l. All plants were incubated in the dark.

flower scape protoplasts. Protoplasts were incubated in the dark at three temperature levels and protoplast survival was estimated after 48 hrs. Leaf and flower scape protoplasts cultured in BDS supplemented with only 2,4-D and 6BAP remained alive for 7 days with no apparent cell wall formation. Cell wall formation could be detected by Calcufluor staining after 4 days in medium with zeatin added (Figure 5). Approximately 60% of the protoplasts regenerated cell walls during a 21-day culture period.

Leaf and flower scape protoplasts cultured in Medium I showed elongation and enlargement of the protoplast by the 5th day in culture (Figure 6). The resultant oblong-shaped protoplasts also showed cytoplasmic streaming characterized by an enlargement of the nucleus, and a more pronounced cytoplasm with diffused and dispersed cytoplasmic inclusions (Figure 7). The cytoplasms which were streaming were heavily channeled and chloroplasts could be seen in motion under the light microscope. Anywhere from 10 to approximately 90 percent of protoplasts were recorded streaming after 7 days in culture.

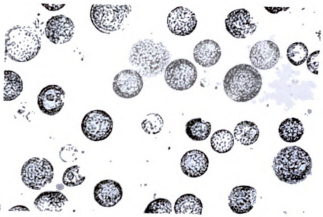
When treated in conjunction with 6BAP at 0.3 mg/l, both 2,4-D and zeatin at concentrations of 0.3 or 0.5 mg/l increased the number of protoplasts undergoing streaming. First division depicted by cytokinesis was also noticed in a few instances after ten days in culture (Figure 8).

Figure 4. Mesophyll protoplast of onion.

Figure 5. Fluorescence of regenerated cell walls of onion mesophyll protoplasts treated with Calcufluor White.

Figure 6. Onion mesophyll protoplasts showing enlargement and elongation prior to streaming.

4



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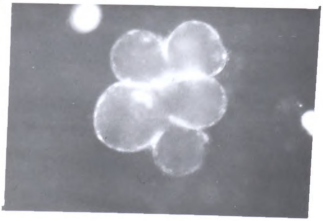

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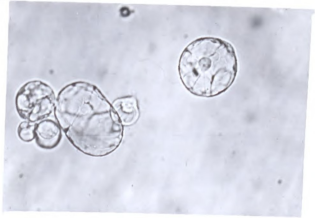

Figure 7. Flower scape protoplast showing cytoplasmic channeling and streaming.

Figure 8. Onion mesophyll protoplast undergoing apparent first and second division.

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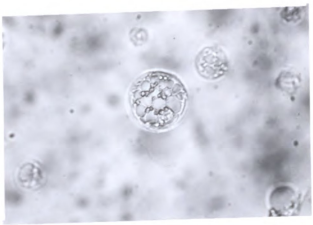

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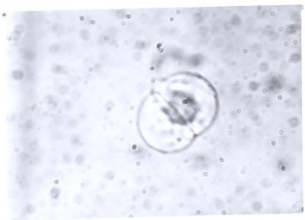

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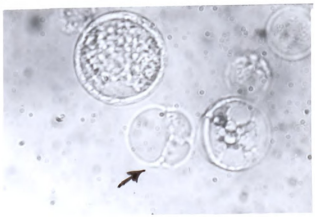

Bulb and Stem Disc

While the hormone zeatin was necessary for cell wall formation in leaf and flower scape protoplasts, bulb protoplasts regenerated a cell wall after a week in culture in the absence of zeatin. Bulb protoplasts survived up to 12 weeks in culture with no obvious signs of protoplast structural changes. Stem disc protoplasts survived up to 25 days in culture without apparent regeneration of cell wall.

Biennial Periodicity and Protoplast Culture

The yield and culturability of mesophyll protoplasts derived from 10°C treated plants, was superior to those pretreated at 26°C. Besides bolting, the response of the plant to cold temperature was also manifested in leaf explants from plants "ripe to flower" and from flower scape tissue during their development at 10°C. This response was characterized by nonhollow leaf and scape cavities (Figure 9). Apparently, the leaf meristematic region mentioned by Hoffman (1933) continued to divide once triggered when it is maintained under vernalization temperatures. In nutrient media solution, meristematic protoplasts float to the top of the tube after centrifugation for 15 minutes at 35 x g and can be separated from the precipitating mesophyll protoplasts or the denser parenchyma protoplast of the scape (Figure 10).

Mesophyll protoplast from "nonripe to flower" but vernalized seedlings (2 weeks-old seedlings) had the lowest yields at all 10°C

Figure 9. Onion flower scapes showing hollow and nonhollow cavities.

9-1 and 9-3. Onion flower scape showing cavities filled with cells. These scapes developed at 10°C.

9-2. Onion flower scape showing hollow cavity. This scape developed at 26°C

Figure 10. Onion flower scape protoplasts derived from plants maintained at 10°C. Note the large meristematic and small parenchyma protoplasts.

Figure 11. Mesophyll protoplast undergoing apparent budding.

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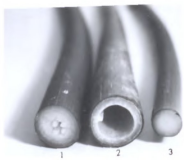

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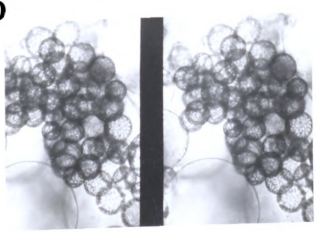

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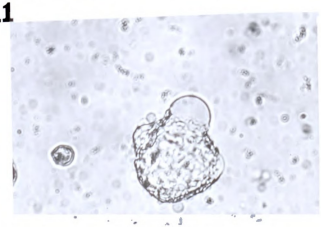

pretreated plants (Tables 8 and 9). None showed streaming or division in any of the four culture media tested.

The characteristic biennial periodicity known in the cultivated onion has been shown to be under the control of temperature. Inductive temperatures of 5°-12°C induce flowering and prevent bulbing (Jones and Emsweller, 1939; Heath, 1943, 1944, 1945; Aoba, 1954). This cold temperature effect, requiring a critical plant or bulb size, is responsible for a qualitative change at the cellular level and not the induction of ripeness to flower (Gregory, 1936; Shishido and Saito, 1976). The onset of or cessation of cell division in inducing flowering and bulbing respectively is a direct product of temperature in enhancing flower and bulb organs formation, the same organs defining biennial periodicity in the onion crop. The data generated in this study reveal that vernalization temperature also influences the onion cells' behavior *in vitro*. Only protoplasts from plants responsive to vernalization stimulus showed cytoplasmic streaming and division in culture. The high protoplast yields and viability of vernalized plants and the continuous and vigorous cell division within the cavities of vernalized leaf tissues and flower scapes are direct evidence of qualitative changes at the cellular level, induced by temperature, and expressed in culture.

LITERATURE CITED

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Abo El-Nil, M. M. 1977. Organogenesis and embryogenesis in cultures of garlic. Allium satirum. Plant Science Letters 9:259.

Aoba, B. 1954. On bulb formation and dormancy in onion II. On the process of bulb formation and development of scales. J. Hort. Ass. Japan 23:249.

Bawa, S. B. and Torrey, I. G. 1971. "Budding" and nuclear division in cultured protoplasts of corn, convolvulus and onion. Bot. Gaz. 132(2):240.

Bidney, S. L. and Shepard, J. F. 1980. Colony development from sweet potato petiole protoplast and mesophyll cells. Plant Science Letters 18:335.

Bhojwani, S. S. 1980. In vitro propagation of garlic by shoot proliferation. Scientia Horticulturae 13:47.

Bhojwani, S. S., Evans, P. K., Cocking, E. C. 1977. Protoplast technology in relation to crop plants: progress and problems. Euphytica 26:343.

Bradley, P. M. 1978. Production of enucleated plant protoplasts of Allium cepa. Plant Science Letters 13:287.

Carter, O., Yamada, Y., and Takashi, E. 1967. Tissue culture of Oats. Nature. London 214:1029.

Cocking, E. C., and Riley, R. 1981. Application of tissue culture and somatic hybridization to plant improvement. In Plant Breeding II. Frey, K. (ed.). Iowa State University Press, Ames, pp. 85.

Davey, M., Mackenzie, I. A., Freeman, G. G., Short, K. C. 1974. Studies of the growth, fine structure and flavor production of onion tissue growth in vitro. Plant Science Lett. 3:113.

Deka, P. C. and Sen, S. K. 1976. Differentiation in Calli originated from isolated protoplasts of rice, Oryza sativa through plating technique Molec. Gen. Genet. 145:239.

Dudits, D. 1976. The effect of selective conditions on the products of plant protoplast fusion. Cell genetics in Higher Plants. Dudits, Farkas, G. L., and Maiiga, P. (eds.). Akademia Kiado, Budapest. pp. 153.

Dudits, D. and Nemet, G. 1976. Methods of somatic plant genetics in cereal research. Proc. Int. Study Week Cereal Crops. Dudits & Nemet (eds.). Gembloux, pp. 127.

Dunstan, D. I., and Short, K. C. 1977. In vitro studies on organogenesis and growth in Allium cepa tissue cultures. Acta Horticulturae 78:139.

Dustan, D. I., and Short, K. C. 1977. Improved growth of tissue cultures of the onion, Allium cepa. Physiol. Plant 41:60.

Dustan, D. I., and Short, K. C. 1979. Shoot production from the flower head of Allium cepa. L. Scientia Horticulturae 19:345.

Dustan, D. I., and Short, K. C. 1979. Shoot production from cultured Allium porrum tissues. Scientia Horticulturae 11:37.

Duvick, D. M. 1965. Cytoplasmic pollen sterility in corn. Adv. Genetics 13:1.

Erickson, T. 1977. Technical advances in protoplast isolation and cultivation. Plant Tissue Culture and Its bio-Technological Application. Barz, W., Reinhard, E., and Zenk, M. H. (eds.). Springer-Verlag, N.Y., pp. 313.

Freeman, G. G., Whenman, R. J., MacKenzie, I. A., Davey, M. R. 1974. Flavor components in tissue cultures of onion, Allium cepa. Plant Science Lett. 3:121.

Fridborg, G. 1971. Growth and organogenesis in tissue cultures of Allium cepa var. Proliferum. Physiol. Plant 25:436.

Fujieda, K., Ando, Y., and Fuyita, Y. 1977. Propagation of Welsh-onion through shoot tip culture. Journal of the Faculty of Agriculture. Kyushu Univ., Japan, 22:89.

Gamborg, O. L. 1975. Plant Tissue Culture Methods. Gamborg and Wetter, L. R. (eds.), pp. 21.

Green, C. E. 1977. Prospects for crop improvement in the field of cell culture. Hort. Science 12:131.

Green, C. E. 1981. Tissue culture in grasses and cereals. Genetic Eng. for Crop Improvement. Rachie, K. O. and Lymann, J. M. (eds.), pp. 107.

Green, C. E., and Phillips, R. L. 1975. Plant regeneration from tissue cultures of maize. Crop Sci. 15:417.

Gregory, F. G. 1936. The effect of length of day on the flowering of plants. Sci. Hort. 4:143.

Guha, S., and Johri, B. M. 1966. In vitro development of ovary and ovule of Allium cepa. Phytomorphology, pp. 353.

Ha, B. D. D., and Mackenzie, I. A. 1973. Division of protoplasts from Asparagus officinalis L. and their growth and differentiation. Protoplasma 78:215.

Havranek, P., and Novak, F. J. 1973. The bud formation in the callus cultures of Allium sativum L. Z. Pflazenphysiol. 68:308.

Heath, O. V. S. 1943a. Studies in the physiology of the onion plant. An investigation of factors concerned in flowering of onions grown from sets and its prevention. Part I. Production and storage of onion sets and field results. Ann. Appl. Biol. 30(4):208.

Heath, I. V. S. 1943. Part II. Effect of daylength and temperature on onions grown from sets. General Discussion. Ann. Appl. Biol. 30(4):308.

Heath, O. V. S. 1945. Formative effects of environmental factors as exemplified in the development of the onion plant. Nature 155, No. 3943:623.

Heath, O. V. S., and Holdsworth, M. 1948. Studies in the physiology of onion plant. IV. The influence of daylength and temperature on the flowering of the onion plant. Journal of Expt. Botany 1, No. 3:353.

Heath, O. V. S., and Holdworth, M. 1948a. Morphogenetic factors as exemplified by the onion plant. In: Growth in relation to differentiation and morphogenesis. Symp. Soc. Exp. Biol. No. 11. Academic Press, N.Y., pp. 326.

Hesse, P. S., Vest, G., and Honma, S. 1979. The effect of 4 storage treatments on seed yield components of 3 onion inbreds. Scientia Horticulturae 11:207.

Hoffman, C. A. 1933. Developmental morphology of Allium cepa. Botanical Gazette 95:279.

Hussey, G. 1979. Propagation of some members of the Liliaceae, Iridaceae, and Amaryllidaceae by tissue culture. Acta Horticulturae, pp. 33.

Hussey, G. 1975. Totioptency in tissue explants and calli of some members of the LiLiaceae, Iridiaceae, and Amaryllidacea. J. Experimental Botany 26:253.

Izhar, S. and Power, J. B. 1979. Somatic hybridization in Petunia: A male sterile cytoplasmic hybrid. Plant Science Letters 14:49.

Jones, H. A. 1943. Inheritance of male sterility in the onion and the production of hybrid seed. Proc. Amer. Soc. Hort. Sci. 43:189.

Jones, H. A., and Boswell, V. R. 1922. Time of flower primordia formation in the onion Allium cepa. Proc. Amer. Soc. Hort. Sci. 19:144.

Jones, H. A., and Emsweller, S. 1939. Effect of storage, bulb size, spacing, and time of planting on production of onion seed. Agricultural Exp. Station Bulletin No. 628. University of California, Berkely.

Jones, H. A., and Emsweller, S. 1936. Development of the flower and macrogametophyte of Allium cepa Hilgardia 10 (11):415.

Jones, H. A., and Mann. L. K. 1963. Onions and Their Allies. In World Crop Books. Polunin, N. (ed.). Leonard Hill.

Kao, K. N., Gamborg, O. L., Michayluk, M. R., and Keller, W. A. 1973. Effects of sugars and inorganic salts on cell regeneration and sustained division in plant protoplasts. Collog. Int. Cent. Nat. Rech. Sci. 212:207.

Kao, K. N., Constabel, and Michayluk, M. R. 1974. Plant protoplast fusion and growth of intergeneric hybrid cells. Planta 121:215.

Kartha, K. K., Michayluck, M. R., Kao, K. N., and Gamborg, O. L. 1974. Callus formation and plant regeneration from mesophyll protoplast or rape plants. (Brassica napus L. cv. Zephyr). Plant Science Lett. 3:265.

Kavaljian, L. G., and Jensen, W. A. 1958. An analysis of cell morphology and the periodicity of division in the root-tip of Allium cepa. American Journal of Botany 45:365.

Kehr, A. E., and Schaeffer, G. W. 1976. Tissue culture and differentiation of garlic. Hort. Science 11(4):422.

King, P. J., Potrykus, I., and Thomas, E. 1978. In vitro genetics of cereals: Problems and perspectives. Physiol. Veg. 16:381.

Klein, R., and Edsall, P. C. 1968. Cultivation of callus from monocot roots. Phytomorphology 88:204.

Koblitz, H. 1976. Isolation and cultivation of barley protoplasts. Biochem. Physiol. Pflanzen. 170:287.

Koblitz, H. 1976. Isolation and cultivation of protoplasts from callus cultures of barley. Biochem. Physiol. Pflanzen 170:92.

Krishuarmurthi, M. 1977. Sugar-cane improvement through tissue culture. Proc. Congr. Int. Soc. Sugar Cane Technol. 16:23.

Krikoran, A. D., and Berguam, D. L. 1969. Plant cell and tissue cultures: The role of Herberlandt. Bot. Rev. 35:59.

Krikoran, A. D., and Koblitz, G. M. 1968. The aseptic culture of onion roots and root tissue. A preliminary report. Phyto-morphology 88:207.

Leaver, C. J. 1980. Mitochondrial genes and male sterility in plants. TIBS 5:248.

Lewis, A. C. 1901. Contributions to the knowledge of the physiology of Karyokinesis. Bot. Gazette 32:423.

Lichter, R. 1978. The restoration of male fertility in the CMS sugar beet by heat treatment and meristem culture. Z. Pflanzenzuchtung 81:159.

Lorz, M., and Potrykus, I. 1980. Isolation of subprotoplasts for genetic manipulation studies. Advances in Protoplast Research. Proceedings of the 5th Int. Protoplast Symposium. Ferency, L. and Farkas, G. L. (eds.), pp. 377.

Maretzki, A., and Nickell, L. G. 1972. Formation of protoplast from sugar cane cell suspension and regeneration of cell cultures from protoplasts. Proc. Int. Sym. Versailles, pp. 51.

Markinen, Yrjo. 1963. The mitotic cycle in allium cepa, with special reference to the diurnal periodicity and to the seed-ling aberration. Ann. Bot. Soc. Vananu Tom 34, No. 6.

Mather, P. B., and Heath, O. V. S. 1944. Studies in the physiology of the onion plant. II. Inflorescence initiation and develop-ment and other changes in the internal morphology of onion sets, as influenced by temperature and daylength. Ann. of Applied Bot. 31(3):173.

Meyer, Y. 1974. Isolation and culture of tobacco mesophyll proto-plasts using saline medium. Protoplasma 81:363.

Monsmith, H. R. 1928. Male sterility in Allium cepa L. Ph.D. Thesis, University of California, Davis.

Mostafa, M., and Abo-El-Nil. 1977. Organogenesis and embryogenesis in callus cultures of garlic, Allium sativum L. Plant Science Letters 9:259.

Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15:473.

Novak, F. J. 1980. Phenotype and cytological status of plants regenerated from callus cultures of Allium sativum L. Z. Pflanzenphysiol. 84:250.

Novak, F. J. 1974. The changes of Karytoype in callus cultures of Allium sativum L. Caryologia 27, No. 1:45.

Oono, K. 1978. Test-tube breeding of rice by tissue culture. Trop. Agric. Res. Ser. 11:109.

Ogura, H. 1968. Studies on the new male-sterility in Japanese radish with special reference to the utilization of this sterility toward the practical raising of hybrid seeds. Mem. Fac. Agr. Kagoshima Univ. 6:39.

Ohyama, K., Pelcher, L. E., and Horn, D. 1977. A rapid, simple method for nuclei isolation from plant protoplast. Plant Physiol. 60:179.

Pilet, P. E., Prat, R., and Roland, J. C. 1972. Morphology, RNASE and transaminase of root protoplasts. Plant and Cell Physiol. 13:297.

Potrykus, I. 1980. The old problem of protoplast culture: Cereals. Advances in Protoplast Research. Proceedings of the 5th Int. Protoplast Symposium. Ferency, L., and Farkas, G. L., (eds.), pp. 243.

Potrykus, I., Harms, C. T., and Lorz, H. 1979. Callus formation from cell culture protoplasts of corn. Zea mays L. Theoret. Appl. Genet. 54:209.

Schnabl, H., Bornman, C. H., and Zeigler, H. 1978. Studies on isolated starch containing Vicia faba and starch deficient Allium cepa guard cell protoplast. Planta 143:33.

Schertz, K. F., and Ritghey, J. M. 1978. Cytoplasmic-genic male sterile systems in sorghum. Crop. Sci. 18:890.

Selby, C., and Collin, H. A. 1976. Clonal variation in growth and flavor production in tissue cultures of Allium cepa L. Ann. Bot. 40:911.

Shanin, E. A., and Shepard, J. F. 1980. Cassava mesophyll protoplast isolation, proliferation and shoot formation. Plant Science Lett. 17:459.

Shepard, J. F., and Rotten, R. E. 1977. Mesophyll cell protoplasts of potato: Isolation, proliferation and plant regeneration. Plant Physiol. 60:313.

Shepard, J. F. 1981. Protoplast isolation and regeneration as aids in crop improvement. Genetic Eng. for Crop Improvement. Rachie, K. O., and Lymann, J. M. (eds.), pp. 107.

Shishido, Y., and Saito, T. 1976. Studies on the flower bud formation in onion plants. II. Effects of physiological conditions on the low temp. induction of flower bud on green plants. J. Japan Soc. Hort. Sci. 45:160.

Short, K. C., and Torrey, J. G. 1972. Cytokinin production in relation to growth of pea-root callus tissue. J. Exp. Bot. 23:1099.

Simmonds, J. A., Simmonds, D. H., and Cummings, B. G. 1979. Isolation and cultivation of protoplasts from morphogenetic callus cultures of Lilium. Canadian Journal of Botany 57(5):512.

Stadelman, E. J., and Kinzel, H. 1972. Vital staining of plant cell methods. Cell Physiol. 5:325.

Thomas, E., Potrykus, I., and King, P. J. 1978. In vitro genetics of cereals: Problems and perspectives. Physiol. Veg. 16(3):381.

Thompson, D. J. 1961. Studies on the inheritance of male sterility in the carrot. Daucas carota L. var. Sativa. Proc. Amer. Soc. Sci. 78:332.

Tsai, C., Chien, Y., Chou, Y. and Wu, S. 1978. A further study on the isolation and culture of rice, Oryza Sativa protoplasts. In Proc. Symp. Plant Tissue Culture, Beijin Science Press, Beijin, China, pp. 317.

Vasil, I. K., and Vasil, V. 1980. Embryogenesis and plantlet formation from protoplasts of Pearl Millet, Pennisetum americanum. Advances in Protoplast Research. Ferency, L., and Farkas, G. L. (eds.), pp. 255.

Vreugdenhill, D. 1957. On the influence of some environmental factors on the osmotic behavior of isolated protoplasts of Allium cepa. Acta Botannica Neerlanvica 6:472.

Widholm, J. 1972. The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Technol. 47:189.

Zapata, F. J., Evans, P. K., Power, J. B., and Cocking, E. C. 1977. The effect of temp. on division of leaf protoplasts of L. esculetum and L. peruvianum. Plant Science Lett. 8:119.

Zeiger, E., and Helpler, P. K. 1976. Production of guard cell--protoplasts from onion and tobacco. Plant Physiol. 58:492.

Zelcer, A., Avis, D., and Galen, E. 1978. Interspecific transfer of cytoplasmic male sterilty by fusion between protoplasts of Normal Nicotiana sylvestris and X-ray irradiated protoplast of male sterils N. tabaccum. Z. Pflanzenphysiol. 90:397.