HISTOCHEMICAL AND MORPHOLOGICAL ANALYSIS OF IN VITRO CULTURED EMBRYOS OF PELARGONIUM X HORTORUM BAILEY, IN COMPARISON TO NORMAL IN VIVO EMBRYOLOGY AND SEEDLING DIFFERENTIATION

FRANKLIN SCOTT ADAMS

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University of New Hampshire, Ph.D., 1967
Botany

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BY
FRANKLIN SCOTT ADAMS
B. S. JOHNSON STATE COLLEGE, 1956
M. S., UNIVERSITY OF PENNSYLVANIA, 1960

A DISSERTATION
Submitted to the University of New Hampshire
In Partial Fulfillment of
The Requirements for the Degree of
Doctor of Philosophy

Graduate School
Department of Botany
June, 1967
This dissertation has been examined and approved.

Charlotte J. Mast
R. W. Schreiber
A. R. Hedigan
Caren M. Rogers
A. E. Tori

May 25, 1967
Date
To My Devoted Wife
and
My Deserving Children
ACKNOWLEDGEMENTS

I wish to express my gratitude to Professor Charlotte G. Nast for her most generous assistance in planning and criticizing this study; to Drs. Albion R. Hodgdon, Richard W. Schreiber, Owen M. Rogers and Arthur E. Terri, who substituted for Dr. Douglas G. Routley who was absent on leave during the final year, for their helpful suggestions and corrections in preparing this dissertation; to the members of the Department of Chemistry for their advice and permission to use the facilities of their laboratories; to Dr. Willard E. Urban for his generous help with statistical matters; and to all the other members of the University faculty and staff for their aid, both direct and indirect. I am also indebted to Dr. Philip R. White, retired, formerly of the Jackson Memorial Laboratories, Bar Harbor, Maine, for the opportunity to visit in his laboratory, and for his expert advice and encouragement.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES.</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF ILLUSTRATIONS.</td>
<td>x</td>
</tr>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>II. LITERATURE REVIEW</td>
<td>4</td>
</tr>
<tr>
<td>III. MATERIALS AND METHODS</td>
<td>12</td>
</tr>
<tr>
<td><strong>A. Parental Stocks</strong></td>
<td>12</td>
</tr>
<tr>
<td>1. Selection of experimental material</td>
<td>12</td>
</tr>
<tr>
<td>2. Culture procedures</td>
<td>13</td>
</tr>
<tr>
<td><strong>B. Embryo Stocks</strong></td>
<td>18</td>
</tr>
<tr>
<td>1. Reproductive habit</td>
<td>18</td>
</tr>
<tr>
<td>2. Continuous supply of embryos</td>
<td>18</td>
</tr>
<tr>
<td>3. Handling of source materials at harvest</td>
<td>20</td>
</tr>
<tr>
<td><strong>C. Transfer Room and Growth Chamber Procedures</strong></td>
<td>22</td>
</tr>
<tr>
<td>1. Physical facilities</td>
<td>22</td>
</tr>
<tr>
<td>2. Procedures for sterilization and starting cultures</td>
<td>22</td>
</tr>
<tr>
<td>3. General culture conditions</td>
<td>23</td>
</tr>
<tr>
<td><strong>D. Nutrient Media</strong></td>
<td>25</td>
</tr>
<tr>
<td>1. Composition</td>
<td>25</td>
</tr>
<tr>
<td>2. Methods employed in preparing media</td>
<td>29</td>
</tr>
<tr>
<td>3. Heat sterilization</td>
<td>29</td>
</tr>
<tr>
<td>4. Chemical sterilization</td>
<td>30</td>
</tr>
<tr>
<td><strong>E. Histological Techniques</strong></td>
<td>31</td>
</tr>
<tr>
<td>1. In vivo studies</td>
<td>31</td>
</tr>
<tr>
<td>2. In vitro studies</td>
<td>31</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS (cont.)

IV. EXPERIMENTS AND RESULTS..............................................33
   A. The In Vivo Embryo.................................................33
      1. Sequence of development....................................33
   B. In Vivo Germination................................................36
      1. The germinating seed.........................................36
   C. In Vitro Precocious Germination.................................38
      1. The morphology and nucleic acid chemistry of normal germination vs. in vitro precocious germination.................40
   D. Statistical Analysis of Variance...................................47
      1. Design of the experiment....................................47
      2. Results.........................................................50
      3. Interpretation of data summarized in table 7...............53
      4. Interpretation of data summarized in table 8..............53

V. DISCUSSION.............................................................57

LITERATURE CITED........................................................65

APPENDIX A: In Vitro Precocious Germination:
photomicrographs of typical embryos in culture...............73

APPENDIX B: In Vitro Precocious Germination:
photomicrographs of sectioned embryos............................75

APPENDIX C: Differential Extraction of RNA and DNA:
confirmation photomicrographs....................................79

viii
LIST OF TABLES

Table 1. List of potentially suitable plants for use in experimental embryology.........13

Table 2. Recommended procedures for maintaining parental stocks as source materials..........14

Table 3. Composition of nutrient media used for maintaining parental stocks and advanced embryo cultures (Koths, 1966)................17

Table 4. Relative effectiveness of several trial media in supporting growth of P. x hortorum embryos of differing ages..............26

Table 5. A comparison between White's modified medium (1963) and Raghavan and Torrey's Capsella medium (1964)......................27

Table 6. A summary of the fixation and dehydration techniques used throughout this study..........32

Table 7. Summary results of the statistical analysis of variance between treatments..............51

Table 8. Summary results of the 2 x 2 factorial arrangement of significant treatments using mean squares..................52
LIST OF ILLUSTRATIONS

Figure 1. Principal reproductive features of *Pelargonium x. hortorum* Bailey......................19

Figure 2. The external morphology of *P. x. hortorum* floret development: Prefertilization to maturity........................................21

Figure 3. Dissection and evaluation area......................24

Figure 4. *In vivo* embryology of *P. x. hortorum*...........34

Figure 5. *In vivo* localization of nucleic acids in embryos corresponding to the starting stages used in culture .....................35

Figure 6. *In vivo* localization of nucleic acids in germinating seeds of *P. x. hortorum*........37

Figure 7. Summary comparison of growth rates between control, *in vivo* germinated embryos and *in vitro* precociously germinated embryos expressed as mean daily gain..................43

Figure 8. Precociously germinated embryos of *P. x. hortorum* growing independently of culture medium in sterile microgreenhouses............44
INTRODUCTION

Plant tissue culture as a useful adjunct to the more classical methods of Botanical investigation is a rapidly expanding field. Since the late nineteenth century, one phase of such investigations, namely that of embryo culture, has received particular attention.

Successful in vitro culture of plant embryos has stimulated the development of numerous and frequently divergent lines of investigation. Such programs range from the practical applications of the plant breeder seeking methods of overcoming certain seed inviabilities resulting from otherwise impossible hybrid crosses, to the theoretical considerations of the experimental embryologist concerned with the physico-chemical parameters of in vitro embryogenesis. As a consequence of these divergencies, the number of different species thus far utilized in embryo culture is phenomenal, and their attending reports are widely distributed throughout the literature, necessarily cutting across several investigational disciplines e. g., anatomy, morphology, cytology, biochemistry, genetics and pathology.

Since the earliest beginnings of in vitro experimental embryology, two major problems of a highly technical nature continue to persist. Of critical importance is the inability of immature embryos to grow in culture when excised at stages of development smaller than the familiar ball stage embryo. This problem continues despite the important successes of Steward et al (1963, 1964) using carrot tissues and Vasil, Hildebrandt and Riker (1964) and again Vasil and Hildebrandt (1965) using endive and tobacco respectively, in obtaining
pro-embryos and ultimately mature plants from cultured callus tissue derivatives. Secondly, many embryos, ball stage and older, germinate precociously under a variety of culture conditions and thus proceed directly to seedling stages and eventual maturity averting some or all of the usual stages associated with normal embryology.

In the case of the former, this investigator has experienced little additional success, the consequences of which will be discussed below. The latter has been examined experimentally with considerable success and, as a consequence, provides the basis for the present thesis.

Precocious germination of in vitro cultured embryos was first observed simultaneously with the earliest attempts to culture immature embryos. Subsequent confirmation of this phenomenon and continued observance with several species under a wide range of nutrient conditions provided little reason to suspect that such aberrant germinations possessed any useful significance for the embryologist.

Preliminary investigations in this laboratory designed primarily to test the effects of different media upon a wide range of immature embryos of Pelargonium x hortorum Bailey (early ball stage to mature), although completely inadequate for the purpose for which they were designed, produced observable differences in the development of certain of the embryos at specific stages. Further preliminary experiments utilizing completely defined media supplemented with auxin and other growth substances suggested that the observed differences were consistent and reproducible. Thus, as so often occurs in science, the consequence of apparent failure was the very real possibility of success.
This report attempts to define the morphological, anatomical and histochemical significance of in vitro precocious germination as it relates to normal in vivo germination in several stages of immature embryos of P. x. hortorum. Also included are the results of preliminary investigations designed to determine the optimum conditions required for routine maintenance of P. x. hortorum embryos in culture. Further evidence is presented which defines the role of growth substances in stimulating or inhibiting the differentiation and subsequent growth of the culture explants.

Because embryos of P. x. hortorum have not been previously utilized in culture and because the embryology of Pelargonium has not been reported in the literature, it has been necessary to present a classical study of normal embryogenesis which provides a standard for comparison with in vitro studies.

Extensive modifications to several recommended procedures for maintaining source plants under greenhouse conditions are also included.
LITERATURE REVIEW

The history of plant embryo culture has been variously reviewed. The first comprehensive review to include extensive discussion of the physical and chemical factors affecting embryo growth and development was published by Jacques Rappaport in 1954. More recently, S. Narayanaswami and Knut Norstog (1964) have included additional material and provided an extensive summary of the varied applications of embryo culture to experimental embryology. A discussion of the physical and morphological bases for embryo culture during the late nineteenth century and the first two decades of the twentieth century has been presented by Dieterich in 1924.

A chronological view of Embryo culture prior to 1941 has been compiled by Merry (1941, 1942).

Literature pertaining to cultivation of plant embryos excised from the ovule during embryogenesis is summarized by Rijven (1953).

Plant tissue and organ culture as viewed philosophically by an embryologist (Maheshwari and Ranga Swamy, 1963).

Nutrition, morphogenesis and differentiation, and adventive embryos as components of embryo culture studies are detailed by Raghavan (1965).

The number of different plant genera and individual species used in embryo culture has been extensively tabulated (Narayanaswami and Norstog, 1964). Up to date information of additional new species in culture may be obtained through the newsletter, "Explant", edited by Lipetz, Stonier and Tulecke, beginning in 1963 and continuing as a supplementary
publication to the 1963, Pennsylvania State University
International Plant Tissue Culture Meeting; P. R. White,
Chairman.

Although the number of different genera subjected to
in vitro embryo culture is exceedingly large, the number of
genera used routinely in experimental embryology is sub­
stantially less. In order of their frequency of use, based
upon a sampling of the literature since 1945, the following
genera are apparent: Datura, Capsella, Gossypium and Hordeum.

Prior to the present study, embryos of Pelargoniums
had not been used experimentally in culture. Conversely,
the use of Pelargonium stem tissues as a source of callus
derivatives has received limited attention (Hirth and Rybak,
1949; Mayer, 1956; Gressel, Moore and Narayana, 1958, 1959,
1963; Chen and Galston, 1964). Since the findings of these
workers possess little relevance to the in vitro culture of
Pelargonium x. hortorum embryos, further discussion will be
most effectively included in the discussion pertaining to
the development of suitable culture media.

Geraniums, especially those included in the genera
Pelargonium, are among the world's most popular plants as
evidenced by their economic worth (Clifford, 1958). The
flowers and stem tissues are used extensively in anatomy and
morphology classrooms. Despite this high popularity, their
use as experimental material in other than genetics investi­
gations has been sorely neglected (Adams, unpublished,
1965).

Only one detailed reference pertaining to geranium
embryology is available (Soueges, 1923). The development of
the embryo of Geranium molle L. from the two-celled zygote
through the initiation of cotyledons is described. Although
the sequence is similar to that displayed by *Pelargonium x. hortorum*, it has been necessary to work out the complete embryology in support of the present study.

The economic importance of Pelargoniums as popular garden plants and their additional value as a source of aromatic oils has provided the basis for their extensive world wide cultivation (Adams, unpublished, 1965). As a consequence, the logistical problems of large scale routine propagation and culture of disease free plants have been largely solved (Craig, 1959, 1960, 1963; Gasiorkiewicz, 1958; Munnecke, 1956). Conversely, the specific use of *Pelargonium x. hortorum* as a source of experimental material has required extensive modification of some procedures.

The literature abounds with references pertaining to the formulation of nutrient media suitable for embryo culture (Gautheret, 1959; Rappaport, 1954; White, 1963; Narayanaswami and Norstog, 1964; Raghavan, 1965). There are basically two types of nutrient media used extensively throughout. The first, appropriately described as undefined media, is characterized by the presence of one or more components of relatively unknown or partially determined composition. The unknowns may involve either qualitative or quantitative differences, however, in most instances, both factors are operational simultaneously. The use of such undefined media e. g., containing yeast extract, coconut milk, or embryo and ovule extracts, is justified by the realization that in certain situations e. g., overcoming seed inviabilities resulting from hybrid crosses, there is no other method available that will work.
Conversely, nutrient media, all components of which are known and their relative concentrations specifically stated, are described as defined media. Although there is some disagreement, it is the contention of most workers that experimental embryology requires the use of defined media, (White, 1963; Steward et al, 1954, 1958, 1961, 1963, 1964, 1965; Raghavan, 1965). In the present study the latter has been considered essential.

Another aspect of nutrient media formulation is the continuing search for plant growth regulators. Two major divisions are recognized and reviewed i. e., synthetic compounds and naturally occurring substances (Salisbury, 1957; Steward and Shantz, 1959; Cleland, 1961; Steward and Ram, 1961; Wain, 1965). As in the case of nutrient medium definition, there can be no doubt concerning the justification for using synthetic growth regulators when no other means is available. However, in view of this author's agreement with the position that meaningful research in experimental embryology requires precise control over all the varied parameters, the use of defined media and naturally occurring growth regulators is to be preferred where possible. Consequently, the literature concerning the use of artificially derived synthetic growth regulators is ignored.

At least three classes of compounds have been identified as naturally occurring growth regulators for which there has accumulated a substantial body of research. Specifically, these are the indole compounds, characterized by indole-3-acetic acid; the Kinins or n-6-substituted purines, characterized by Kinetin, 6-furfuryl-amino-purine; and the Gibberellins or Lactone derivatives, characterized by Gibberellic acid or G.A_3 (Audus, 1959; Salisbury, 1957;
Thimann, 1963; Steward and Shantz, 1959). Others have been identified and their universal occurrence suspected, however, since none of these were used in the present study, their inclusion in this review is omitted.

In general, the activity of these growth regulators both as natural constituents of biochemical growth phenomena (i.e., endogenous) and as adjunct constituents (i.e., exogenous) has been described in terms of their observable effects upon plant material (Salisbury, 1957). Thus IAA is characterized primarily as a regulator of growth by influencing the rate of cell division and elongation (Salisbury, 1957; Audus, 1959). Kinetin, when operating independently in a given system, chiefly stimulates cell division (Skoog et al. in Salisbury, 1957). In combination with other growth substances, particularly with IAA, Kinetin seems to affect the rate and order of differentiation (op. cit.). Gibberellins also elicit varied responses, however, their most common effect is to increase cell elongation (op. cit.).

A wide range of specific effects other than those mentioned has been described in the literature. However, an adequate discussion of these diverse effects would require individual treatment of several hundred papers which lies beyond the scope of this report. Therefore, further discussion will be limited to those papers that deal most directly with the responses observed in this investigation of precocious germination. Furthermore, because the problem of precocious germination has not been subjected to direct experimentation elsewhere, the following references have been selected on the basis of their overall applicability to this problem. No attempt has been made at this point to integrate these references as this will be more meaningfully accomplished in the final discussion.

That protein synthesis and nucleic acid synthesis are essential regulators of growth i.e., cell enlargement and cell elongation as well as cell division is well documented (Nooden and Thuman, 1963; Key, 1964 and Key et al, 1966). The relationship between the activity of auxins and other growth substances and the synthesis of proteins and nucleic acids has long been suspected (Biswas, 1959; Veen, 1963). Fox in 1964 demonstrated the incorporation of kinins into the RNA of plant tissues. In addition, Bendana et al, in 1965 recovered labeled RNA following the administration of labeled auxin to *Pisum* stem sections. Recently, in a well designed experiment, Cherry (1966) demonstrated a 1:1 correspondence between the addition of auxin and the synthesis of nucleic acids in peanut cotyledons. Most exciting, however, is the report from Liao (1966) demonstrating through the technique of autoradiography the precise intracellular localization of labeled growth hormones as occurring in the nucleus in direct association with the chromosomes. Similar
data is available from Roychoudhury et al (1965) and Datta and Sen (1965) wherein labeled growth substances have been correlated with increased DNA and RNA synthesis, RNA release into cytoplasm and amino acid incorporation into nuclear protein.

Under certain in vitro culture conditions, excised immature embryos of many plants will germinate prematurely, and in the presence of suitable nutrient conditions, will grow directly into seedling-like plants thereby averting one or more stages of development associated with their normal in vivo embryology. This phenomenon has been most often observed when embryos were cultured on the surface of the nutrient agar. Conversely, embryos cultured below the surface continue normal embryological growth until mature length is reached. The phenomenon of premature germination was first reported by Hannig in 1904. Twenty years later, Dieterich observed similar premature germination in culture with embryos of the Cruciferae and Gramineae. Dieterich termed this phenomenon, "Kunstliche Frühgeburten" (Dieterich, 1924). Rijven (1952) has interpreted this to mean "pre­cocious germination"...which appeared later also in several other investigations, and gained special attention as a deviation of normal development".

The terms "...deviation of normal development" are critical to the present study. On one hand, this could mean that the act of premature germination, resulting in the by­passing of certain stages of normal embryology, is abnormal. On the other hand, abnormal development could point specifically to the process of germination i. e., the morphology of germination; normal vs. abnormal.
In the case of the former, precocious germination would be equated with normal seed germination and seedling development. In the latter, the process of precocious germination would itself be considered abnormal. It is necessary at this point to assume that Rijven was concerned with the latter. This seems reasonable as evidenced by the following quotation.

"...Several investigators mentioned a premature germination of the embryos in culture; this abnormality is due to an insufficient knowledge of the factors controlling this phenomenon.

Germination is defined here to start at the moment that the embryonic tissue -- in a state of cell division and plasmatic growth -- becomes separated by an intercalated section of incipient cell-elongation."

Apparently, the phenomenon of precocious germination and its attending cause (s) have not been thoroughly investigated. Several authors have observed this phenomenon, and have commented variously about its significance, however, no study per se. is known to exist (Tukey, 1933; LaRue, 1936, 1933; Merry, 1941, 1942; Rijven, 1965). Consequently, a thorough investigation of the attending anatomical and morphological changes throughout is considered basic to an understanding of the processes involved. Hence the justification for the present study.
MATERIALS AND METHODS

Parental Stocks

Selection of experimental material: As a result of comparing the innumerable reasons why specific experimental materials had been selected for use in embryo culture, a list of basic requirements was developed. Accordingly, any embryo which best conformed to the following conditions was judged worthy of consideration.

1. angiospermous
2. highly developed when mature
3. large in size at maturity
4. readily available at all seasons (could be maintained in greenhouse)
5. not previously used in experimental embryology
6. ease of culture of parental stocks
7. disease free seeds (naturally sterile ovules)

In addition, the suggestions offered by Mrs. J. Sanborn of the University of New Hampshire Seed Laboratory, and Dr. P. R. White of the Jackson Laboratories, Bar Harbor, Maine, helped immeasurably in assembling a list of possible selections (see Table 1). Mature seeds, where obtainable, from each species were collected and the final choice was made commensurate with the ease by which the embryo could be excised.
Table 1. List of potentially suitable plants for use in experimental embryology.

<table>
<thead>
<tr>
<th>Family</th>
<th>Genus*</th>
<th>Species*</th>
</tr>
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<tbody>
<tr>
<td>Cruciferae</td>
<td>Barbarea</td>
<td>B. vulgaris R. Br.</td>
</tr>
<tr>
<td>Trapaeolaceae</td>
<td>Tropaeolum</td>
<td>T. peregrinum L.</td>
</tr>
<tr>
<td>Geraniaceae</td>
<td>Pelargonium</td>
<td>P. hortorum Bailey</td>
</tr>
<tr>
<td>Balsaminaceae</td>
<td>Impatiens</td>
<td>I. Sultani Hook</td>
</tr>
<tr>
<td>Apocynaceae</td>
<td>Vinca</td>
<td>V. rosea L.</td>
</tr>
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Culture procedures: The literature dealing with methods of geranium culture is voluminous. In the interest of purposeful writing, the several methods used are presented in tabular form (Table 2). The recommended options selected for use in support of the present study are marked by a marginal single asterisk. Changes or additions found advantageous for the purposes of this study are indicated by a marginal double asterisk.
Table 2. Recommended procedures for maintaining parental stocks as source materials.

<table>
<thead>
<tr>
<th>Ordered sequence of events</th>
<th>range of recommendations</th>
</tr>
</thead>
</table>

1. Propagation

   a. Rooting of cuttings

   ** (1) medium: 2:1 jiffy mix plus sandy loam, (add 200 ml. of nutrient solution once weekly when kept longer than four weeks).

   (2) procedure
   (a) do not use knife to prepare cuttings (often induces infection) alternative - snap off, include lower leaf and stipules in break then remove.
   (b) air dry (avoid direct sunlight) soft cuttings for 6-12 hours. (will reduce damping off losses)
   (c) avoid use of mist (enhances chance of spreading foliage disease)
   (d) cuttings should be ready in 6-8 weeks. (less time required in spring than in fall)
   (e) 4 weeks is sufficient in (1) above.

   ** b. Seed propagation (not used in cases of extreme heterogeneity)

   procedure
   (a) remove husk with forceps
   (b) scarify seed. (see footnote) remove section of seed coat with sharp sterile instrument at small end; don't damage the embryo.
   (c) place in germinating container (ideal - autoclaved petri dishes with filter paper inserts; rate - 25 seeds per 100 mm plate).
   (d) conditions - in light, temp. - 65-67°F. (transfer seedlings in 5-7 days).

2. Potting and maintenance

   ** a. Soil: 2:1 jiffy mix - sandy loam mixture.

   ** b. Procedure: place cuttings directly into 6" clay pots well embedded in sand bench following rooting.
Ordered sequence of events  range of recommendations

c. Fertilization
(1) every two weeks for liquid fertilizers when mixed at 3 pounds per 100 gallons of water.
(2) same nutrient requirements for seedlings and cuttings.
(3) seedling will show nutrient deficiencies sooner than cuttings.
** (4) Under the extreme demands of continuous embryo formation, severe iron, manganese and magnesium deficiencies were induced. These were effectively countered with the addition of Koths (1966) nutrient solution applied at the rate of 250 ml per 6" pot on alternate days, in addition to routine watering. (see Table 3)

d. Watering
(1) geraniums should not be watered too frequently after they are in 4" pots.
(2) the need for watering is primarily a function of the rate of drying.
(3) running the plants as dry as possible will make for shorter, well budded plants.
** (4) daily watering was necessary under the conditions of the present study. (exception - do not water or feed under 100% cloud cover, except if duration of cloud cover exceeds two days)

e. Light: increased light tends to produce a shorter, more differentiated plant (result of increasing the sugar concentration and decreasing the water so development is shifted toward differentiation) (Craig and Walker, 1959a)

f. Temperature
** (1) days - 67°F
** (2) nights - 60°F
Ordered sequence of events | range of recommendation
---|---
g. Pinching  
(1) to produce a well branched plant, pinching is necessary  
  (a) hard pinching - removal of 3-4 inches of growth  
*  
  (b) soft pinching - removal of only the shoot tip  
*  
(2) the more leaves present below the site of pinching, the faster additional branches will appear.  
**  
(3) a minimum of 3 branches and a maximum of 5 per plant were empirically determined as suitable. No more than one flower-head per branch was pollinated at a time (absolute maximum - 5 heads per plant).

Note: Of the many methods tried, acid treatment, presoaking, abrasive tumbling, etc., scarification has consistently produced the best results (Craig and Walker, 1959a).
Table 3. Composition of nutrient medium used for maintaining parental stocks and advanced embryo cultures (Ko'ths, 1966).

<table>
<thead>
<tr>
<th>Stocks</th>
<th>Amount Per. L.</th>
<th>Equivalents</th>
</tr>
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<tbody>
<tr>
<td>(1) Ca(NO₃)₄·H₂O Calcium nitrate</td>
<td>118g/L</td>
<td>(20,000 ppm N)</td>
</tr>
<tr>
<td>(2) KNO₃ Potassium nitrate</td>
<td>143g/L</td>
<td>(35,000 ppm CA)</td>
</tr>
<tr>
<td>(3) MgSO₄·7H₂O Magnesium sulfate</td>
<td>246g/L</td>
<td>(20,000 ppm N)</td>
</tr>
<tr>
<td>(4) KH₂PO₄ Monopotassium phosphate</td>
<td>39g/L</td>
<td>(62,000 ppm K₂O)</td>
</tr>
<tr>
<td>(5) Chelated iron, 3g (10% iron chelate)</td>
<td>100ml</td>
<td>(24,000 ppm Mg)</td>
</tr>
<tr>
<td>(6) Micronutrients all in 1 liter</td>
<td></td>
<td>(32,000 ppm S)</td>
</tr>
<tr>
<td>(a) H₃BO₃ (boric acid) 2.5 g.</td>
<td></td>
<td>(13,000 ppm K₂O)</td>
</tr>
<tr>
<td>(b) MnCl₂·4H₂O (manganese chloride) 1.75 g.</td>
<td></td>
<td>(20,000 ppm P₂O₅)</td>
</tr>
<tr>
<td>(c) ZnCl₂ (zinc chloride ) .1 g.</td>
<td></td>
<td>(3,000 ppm Fe)</td>
</tr>
<tr>
<td>(d) NaMnO₄·2H₂O (sodium molybdate) .025 g.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Embryo Stocks

Reproductive habit: Pelargoniums are unique in their reproductive characteristics (see Figure 1). As 5 carpeled, 10 ovulated florets, each possesses a potential of containing a maximum of 10 embryos. In over 100,000 pollinations, no more than 5 embryos per floret have been found; 4 are common. The actual number of embryos formed appears empirically to be a function of plant vigor and the time of day pollination occurs.

One hundred to three hundred florets develop per umbel, depending on the age of the plant and the number of umbels present. The greater the number of umbels, the fewer the number of florets. The florets mature sequentially at a rate of 4 to 10 daily over approximately a 15 day period.

Continuous supply of embryos: In order to have available a continuous supply of embryos, in sufficient numbers and at all stages of development likely to be needed, a routine procedure was established. Several series of 5/8", colored marking tags were prepared as follows. The tags were arranged into blocks of 150 tags of each color; the tags were numbered 1 thru 15, with ten tags of each number. Twenty individual florets were hand pollinated daily, and individually tagged. With each floret possessing a potential of 5 embryos, the two blocks of 10 tags each, presented a potential supply of 100 embryos daily. An average daily yield of 84 embryos was achieved in 20 observations. In a thirty day period, as many as 3,000 embryos would be produced on 24 plants.

By maintaining a daily calendar of tag numbers according to color, a group of embryos of known age could be harvested at any time. At the end of 30 days, all tagged
Figure 1. Principal reproductive features of *Pelargonium x. hortorum* Bailey.
florets, not harvested for use in culture, were removed and dried for seed. The tags were then reused in a subsequent sequence.

No more than 4 florets per umbel were pollinated and tagged per day. The reason for this was empirically determined in response to optimum embryo set; greater numbers of pollinations per umbel appeared to decrease the number of embryos produced per floret. The reason for this response is not at all clear and merits experimental treatment.

Under the culture conditions used in support of this study, pollinations were most effective if made between 9 and 12 A.M. daily. Routine pollinations at precisely the same time each day, combined with the observed rate of embryo development, allowed the routine harvesting of embryos at any desired stage of development, once the sequence of development had been determined (seasonal changes modify this sequence only slightly). Thus the logistical problems, common with other plants such as Capsella which require the manipulation of large numbers of ovules in order to assemble adequate numbers of identical embryos, have been avoided.

Unfortunately, it is not possible to ascertain precisely the stage of development of embryos inside the ovary on the basis of external morphology alone (see Figure 2). There is, however, a remarkable correspondence between the day of successful fertilization and the stage of embryonal development, by days, within the culture parameters used herein.

Handling of source materials at harvest: A heavy gage polystyrene open topped plastic box measuring 2 1/4 X 2 1/2 X 12 1/2 inches, and divided internally into four semi-chambers of equal size was filled to 1/2" of the top with 200 mls of nutrient solution (see Table 3) plus tap
Figure 2. The external morphology of *Pelargonium x. hortorum* floret development. (Pre-fertilization to maturity)
water. A parafilm cover was stretched tightly over the top and secured with large rubber bands. The compartments were marked with grease pencil corresponding to the age of the ovaries to be harvested. The entire ovary was excised in toto, including the supporting stem, with a sterile razor blade and placed immediately into the container; the stem end was inserted through a small hole punched in the parafilm cover with a dissecting needle. In this manner, the experimental material could be easily transported to the laboratory. Immature embryos treated in this manner could survive upwards to 5 days, although all embryos intended for culture purposes were placed in culture within 5 hours of harvest.

Transfer Room and Growth Chamber Procedures

Physical facilities: In general, the facilities provided were those recommended by White (1963). The transfer room and growth chamber were necessarily combined due to space limitations. An unused darkroom was cleared, cleaned thoroughly, painted and refitted. Provisions were made for constant temperature control and automatic lighting control in the culture area.

Procedures for sterilization and starting cultures: The contents of a single ovary were processed as one, producing from three to five ovules routinely. The sepals were stripped from the floret base, and the carpels removed individually with forceps and placed on sterile paper. The ovule was removed from the carpel and placed in a 10% chlorox solution bath for two minutes. Since the ovules of Pelargonium are naturally sterile internally, sterilization was thus assured (Munnecke, 1956). The ovules were then transferred
to a small covered dish of sterile liquid nutrient medium where they were held until excision of the embryo occurred. (Originally, an intermediate 95% ethyl alcohol rinse was used, however, this was later found to be unnecessary and in some cases, resulted in embryo necrosis due to excessive desiccation).

The ovule was aseptically removed from the nutrient dish with flamed forceps and placed on a sterile depression slide in a drop of sterile nutrient under the dissecting microscope (see Figure 3). Excision could be accomplished in from 5 to 30 seconds depending upon the stage of development and the skill of the operator. Two pairs of forceps were used; one relatively blunt pair for positioning and holding the ovule, and a second needle-pointed pair for the actual excision and subsequent transfer to the culture vessel. Even ball-stage embryos have been efficiently handled in this manner under 20X magnification. Sterile conditions were maintained throughout.

**General culture conditions:** During the preliminary studies of nutrient suitability, 1 ounce french square bottles containing 8 mls of agar nutrient were used for each embryo (White, 1963). When it became necessary to quantitate the data for statistical purposes, the french square bottles were abandoned in favor of 20 X 100 mm petri dishes containing 40 mls of nutrient which could hold four embryos simultaneously and more easily facilitated routine microscopic examination and measurement.

Cultures maintained in the dark were stored in a sterile 18" x 20" x 24" metal cabinet within the transfer room. Cultures maintained in the light were sealed with parafilm and scotch tape to insure sterility and facilitate
Figure 3. Dissection and evaluation area.
gas exchange. They were then stored on open shelves within the transfer room. The temperature was maintained at 23°C, ± 2°C (White, 1963). Lighting for each replicate was derived from 2-40" Sylvania "gro-lux" fluorescent lamps at 800 mv absolute energy (Thomas and Dunn, 1964). The culture vessels were rotated daily to equate intensity variations which are characteristic of long fluorescent lamps.

Cultures maintained in the french square bottles required routine transfer every 20 to 25 days. Conversely, the cultures grown in petri dishes and sealed with parafilm resisted desiccation throughout the 50 day culture period.

Nutrient Media

Composition: Originally, it was hoped to develop a medium capable of supporting the growth of very small embryos i.e., zygote stage. Reluctantly, it is conceded that the task has proven more difficult than presently available time or equipment permit. More will be said in the discussion.

Preliminary experiments were used to determine the effectiveness of several nutrient media found in common usage at the time the present study was initiated (White, 1963). The results of these trials are summarized in Table 4, and the effectiveness of each expressed simply as a relative function of the ability to support growth.

The final selection of a suitable nutrient medium was made between a modified White's 1963 medium, and the published Capsella medium of Raghavan and Torrey (1964). The basic formulations and modifications are presented in Table 5. White's medium was sufficiently superior both in total growth achieved and duration of effectiveness to warrant its use in the final quantitative study.
Table 4. Relative effectiveness of several trial media in supporting growth of *P. x. hortorum* embryos of differing ages.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Age of Embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 da</td>
</tr>
<tr>
<td>White 1943b</td>
<td>E</td>
</tr>
<tr>
<td>Gautheret</td>
<td>0</td>
</tr>
<tr>
<td>Knop</td>
<td>0</td>
</tr>
<tr>
<td>Torrey</td>
<td>0</td>
</tr>
</tbody>
</table>

0 -- no growth -- death resulting
00 -- no growth -- color and size maintained
E -- enlargement -- no differentiation
EE -- enlargement -- roots and shoots developed
Table 5. A comparison between White's modified medium (1963) and Raghavan and Torrey's Capsella medium (1964).

<table>
<thead>
<tr>
<th>Salts</th>
<th>White (1963)</th>
<th>Raghavan and Torrey (1964)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca(NO₃)₂·4H₂O</td>
<td>288.0</td>
<td>480.0</td>
</tr>
<tr>
<td>KNO₃</td>
<td>80.0</td>
<td>63.0</td>
</tr>
<tr>
<td>KCl</td>
<td>65.0</td>
<td>42.0</td>
</tr>
<tr>
<td>MnSO₄·4H₂O</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td></td>
<td>60.0</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>KI</td>
<td>0.75</td>
<td></td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>1.5</td>
<td>0.551</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>737.5</td>
<td>63.0</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>200.0</td>
<td></td>
</tr>
<tr>
<td>NaH₂PO₄·H₂O</td>
<td>19.0</td>
<td></td>
</tr>
<tr>
<td>MnCl·4H₂O</td>
<td></td>
<td>0.36</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td></td>
<td>0.0425</td>
</tr>
<tr>
<td>CuCl₂·2H₂O</td>
<td></td>
<td>0.027</td>
</tr>
<tr>
<td>(NH₄)₆Mo₇O₂₄·4H₂O</td>
<td></td>
<td>0.155</td>
</tr>
<tr>
<td>Fe₂(SO₄)₃</td>
<td>* 2.5</td>
<td></td>
</tr>
<tr>
<td>Salts</td>
<td>White (1963)</td>
<td>Raghavan and Torrey (1964)</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>--------------</td>
<td>----------------------------</td>
</tr>
<tr>
<td>ferric tartrate</td>
<td>* 0.31</td>
<td></td>
</tr>
<tr>
<td>Sequesterene - 10% iron chelate</td>
<td>6.0</td>
<td>6.0</td>
</tr>
</tbody>
</table>

Vitamins, sucrose, and agar concentrations used were the same as White's (1963) medium in both cases.

*Not used.
Methods employed in preparing the media: In general the procedures for nutrient preparation recommended by White (1963) were routinely employed. Those procedures recommended for the safe handling of heat labile growth factors were found to be time consuming, cumbersome and subject to contamination within the limited facilities of the laboratory. Two alternative methods were tried, and the results monitored through standard microbiological plating techniques and ultraviolet absorption spectra determined on the Perkins-Elmer Model no. 4000 recording spectrophotometer.

The range of compounds examined included \( \beta \)-indolyacetic acid: 6-furfurylamino purine; gibberellic acid, \( \text{GA}_3 \); \( \gamma \)-(indole-3)-butyric acid; \( \alpha \)-(indole-3)-propionic acid; \( \beta \)-naphthoxyacetic acid; \( \alpha \)-naphthylacetic acid; \( (2,4\text{-Dichlorophenoxy}) \text{ acetic acid and 2,4,5-(Trichlorophenoxy) acetic acid. Since only the first three compounds were used in subsequent culture experiments, further discussion will be limited correspondingly.

Heat sterilization: IAA, Kinetin and \( \text{GA}_3 \) at \( 1 \times 10^{-3} \) molar, \( 1 \times 10^{-4} \) molar and \( 1 \times 10^{-3} \) molar concentrations respectively were prepared in water, alcohol and the salt solutions of White and Raghavan (See Table 5). Presterilization spectra were recorded followed by 15 minutes of autoclaving at 15 psi, at which time the solutions were cooled and returned to the spectrophotometer, and a second profile determined. In each case there occurred a slight depression of the peaks, which is indicative of a corresponding change in concentration. This could be explained as a materials loss in autoclaving. However, in the case of IAA, there was a corresponding shift of the spectral maxima with the development of a shoulder in the area of 250 cm\(^{-2}\), which is
indicative of by-product formation. Further observations designed to determine the precise nature of the molecular alteration would have required the use of the mass-spectrophotometer. Since this lies beyond the scope of this report, such studies were deferred, although their potential value is recognized.

**Chemical Sterilization:** 1 X 10^{-2} Molar solutions of the identical factors employed above were prepared in C. P. absolute ethyl alcohol. Appropriate dilutions were made and the U. V. spectra determined. The results were consistent with the literature values and the previous study except that no materials loss nor by-product formation was observed. Subsequent use of these factors in the nutrient medium was accomplished through serial dilutions in single distilled, demineralized water, using aseptic-quantitative technique throughout. From the alcohol 1 X 10^{-2} M stock, a water 1 X 10^{-5} stock was derived. It is theorized, that in the process of serial dilution, any negative effects of the original solvent would be completely countered, particularly in view of a final dilution from 1 X 10^{-5} M stock to the nutrient concentration of 1 X 10^{-7} molar strength. All growth factors were added to the final medium following autoclaving just before the culture vessels were pipetted prior to solidification.

Routine plating tests on agar were entirely negative throughout. No tests were conducted to ascertain the presence of alcohol dependent bacteria as such would be incapable of survival in the complete medium.

Such tests are necessarily empirical in nature due to the frequent reliance on negative data. They are therefore justified only in the sense that they worked satisfactorily for the purposes required in this study.
Histological Techniques

**In vivo studies:** Standard histological techniques were employed after Johansen (1940) and Sass (1958). Because of difficulties encountered with differential hardness in ovular tissues at various stages of development, a modified FAA and tertiary-butyl alcohol procedure was devised (see Table 6) followed by embedding in paraplast. Despite the usual problems associated with embryological sectioning (Johansen, 1945) satisfactory median sections were obtained for staining.

Sharman's (1943) Tannic Acid and Orange G. stain was used for the standard in vivo series. This procedure was specifically designed for use in studies of the shoot apex, but its value as an embryo stain has been recognized (Gray, 1954).

**In vitro studies:** All prestaining procedures used with the cultured materials were identical with those above. Differential staining for nucleic acids in support of the statistical data was accomplished according to the method of Brachet, 1953, as summarized in Jenson (1962).
Table 6. A summary of the fixation and dehydration techniques used throughout this study.

<table>
<thead>
<tr>
<th>Fixative ml/L</th>
<th>Dehydration schedule</th>
<th>(time)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Special F.A.A.</td>
<td>Fixation</td>
<td></td>
</tr>
<tr>
<td>50% EtOH wash</td>
<td>12-24 hrs</td>
<td></td>
</tr>
<tr>
<td>50% EtOH - 880 ml</td>
<td>10% TBA</td>
<td>3 times/15 min. ea.</td>
</tr>
<tr>
<td>CH₃CO₃ - 70 ml</td>
<td>20% TBA</td>
<td>2 hrs</td>
</tr>
<tr>
<td>CH₂O - 50 ml</td>
<td>35% TBA</td>
<td>2 hrs</td>
</tr>
<tr>
<td>12 to 24 hours</td>
<td>55% TBA</td>
<td>2 hrs</td>
</tr>
<tr>
<td>depending upon age of embryos</td>
<td>Pure, reused TBA</td>
<td>2 hrs</td>
</tr>
<tr>
<td>Pure, 1st change</td>
<td>Pure, 2nd change</td>
<td>2 hrs</td>
</tr>
<tr>
<td>1/2 Pure and 1/2 Paraffin oil</td>
<td>Pure Paraffin oil</td>
<td>2 hrs</td>
</tr>
<tr>
<td>1/4 Pure and 3/4 Paraffin oil</td>
<td>10 drops low melting point paraffin</td>
<td>2 hrs</td>
</tr>
<tr>
<td>Pure Paraffin oil</td>
<td>add 10 drops low melting point paraffin</td>
<td>1/2 hr</td>
</tr>
<tr>
<td>10 drops low melting point paraffin</td>
<td>add 20 drops low melting point paraffin</td>
<td>15 min</td>
</tr>
<tr>
<td>Pure Paraffin oil</td>
<td>Pour off 1/2 add back l.m.p.</td>
<td>overnight</td>
</tr>
<tr>
<td>replace with h.m.p.</td>
<td>replace with paraplast embedd.</td>
<td>overnight</td>
</tr>
<tr>
<td>1 change</td>
<td>replace with paraplast embedd.</td>
<td>1 day</td>
</tr>
</tbody>
</table>
EXPERIMENTS AND RESULTS

The In Vivo Embryo

A classical study of normal in vivo embryology was undertaken in order to provide a comprehensive standard for subsequent interpretation of in vitro development and differentiation. The study was divided into two parts. The first consisted of a standard embryo series according to Sougees (1923) and expanded to include all mature stages of development not usually presented in studies of this nature, but essential to the interpretations required (see Figure 4). A second series was prepared using sections cut at 25 microns of those stages of development corresponding to the embryos used in culture and stained for morphological localization of nucleic acid concentrations (see Figure 5). The in vivo embryology of \textit{P. x hortorum} as revealed by the aforementioned studies is described after the manner of Rijven (1952) as follows.

**Sequence of development:** Although the embryology of \textit{Pelargonium} is widely known, no unified account of the embryogenesis of a specific species is available in the literature. One embryological study characterized as being typical of the family Geraniaceae was reported by Sougees in 1923. Embryogenesis of \textit{P. x hortorum} differs only slightly.

Ovules of the family Geraniaceae are uniform throughout and of the campylotropous type (Eames, 1961). The type of embryo sac development typical of the family is in question. Johansen (1950) with reservation has suggested the Onagrad type, or monosporic i.e., four nucleate; egg apparatus plus
Fig. 4. *In vivo* embryology of *P. x. hortorum*
Fig. 5. *In vivo* localization of nucleic acids in embryos corresponding to the starting embryos used in culture.
one polar nucleus. Johri in Maheshwari (1963) describes three cases found in the order Geraniales as mono, bi or tetrasporic, but does not detail their occurrence in the various families. In this study, no more than four nuclei; egg apparatus and one polar nucleus, have been observed in any single ovule. However, since precise embryo sac definition lies beyond the scope of the present study, further discussion is omitted.

In vivo embryo development has been equated with time as average stage of development since the day of fertilization based on a total of ten observations of ovules from 5 florets, each from a different plant. This procedure is much simplified from that of Rijven (1952) but is considered more than adequate for the species used within the uniform culture parameters employed. Major alteration of nutrient composition and physical parameters has altered but slightly the developmental sequence.

The stages of development for young embryos in the ovule and older excised embryos may be seen from the microphotographs of histological sections (Figure 4).

**In Vivo Germination**

A study of normal in vivo seedling germination was undertaken in order to provide an adequate standard for subsequent interpretation of in vitro precocious germination and differentiation. Selections of several stages of germination and seedling development were serially sectioned at 15 microns and stained for localization of nucleic acids (see Figure 6). The process of in vivo germination and differentiation was found to be normal, i.e., not unlike any other fully developed, dicotyledonous, angiospermous seed.
Fig. 6. *In vivo* localization of nucleic acids of germinating seeds of *P. x. hortorum*
Scarified seeds showed visible signs of germination e.g., splitting of the seed coat or the appearance of the primary root through the area of scarification, within as few as twelve hours after setting up the experiment. This event always resulted from cell elongation as described above. Within a few hours (seldom more than six hours) division figures could be found within the primary root meristem, which constitutes the second sequential event. Following the inception of growth in the primary root meristem, within as few as four hours and as many as six days, the epicotyl began to proliferate. The final event, lateral root initiation, which occurs sequentially in petri dishes, has been observed to occur much earlier in soil. Since lateral root initiation is greatly affected by the presence of artificial media, lateral root initiation in vivo shall also be considered sequential. These several events and their attending photomicrographs (Figure 6), constitute the standards upon which interpretation of precocious germination is based.

One additional aspect needs discussion. In retrospect, the overall process of in vivo germination is remarkably coordinated and once initiated, proceeds to its logical conclusion without aberration (genetic mutations and related phenomena excepted).

**In Vitro Precocious Germination**

A study of in vitro immature embryo development was undertaken for two purposes; (1) to determine the requirements for inducing precocious germination and (2) to precisely define the morphological, anatomical and histochemical
significance of precocious germination as compared and contrasted with normal in vivo germination and development.

Preliminary studies revealed that contrary to the findings of other workers that precocious germination is limited to a surface position of contact with nutrient agar, immature embryos of *P. x. hortorum* were observed to germinate precociously in a variety of circumstances e.g., on an agar surface; partially embedded in agar; fully embedded in agar; in liquid nutrient media either totally submersed or on a sterile filter paper slant partially immersed in the medium with either light or dark conditions throughout. It cannot be denied, however, that immature embryos germinated precociously with greater uniformity and highest reproducibility when in surface contact with an agar nutrient medium in the light.

Consequently, as a result of these observations and commensurate with those described in part III, B-2, immature embryos of five different beginning ages were placed in culture and their development followed. The beginning ages employed were 23, 15, 11, 9 and 7 days. Their equivalent stages of ovular development are illustrated in Figure 5.

After considerable growth had occurred, selections of the developing embryos were removed from culture, processed for sectioning at 15 microns and stained for morphological and anatomical localization of nucleic acid distribution. Representative photomicrographs selected from serial sections of useable embryos of all ages and all treatments are presented in appendix B. *In situ* photographs illustrating some of the characteristic external features of precocious germination are presented in appendix A.
The morphology, anatomy and nucleic acid localization of in vitro precocious germination: Precocious germination was observed to occur variously in immature embryos of at least four of the five starting ages. This response was uniformly observed in embryos of 23, 15 and 11 day origins. Precocious germination occurred less uniformly in 9 day old embryos and in many instances was impossible to interpret histologically due to gross distortion of the natural configuration of the embryo resulting from the development of an amorphous callus. Many roots and occasional shoots arose from this amorphous callus, but the exact origin of these structures whether from hypocotyl, epicotyl or cotyledons could not be precisely determined. (See appendix B, plate 4). The development of roots and shoots, etc., following extensive callus development is not without precedent (Steward et al, 1958a, 1958b; Vasil et al, 1964, 1965).

Precocious germination occurred rarely in 7 day old embryos, if in fact it occurred at all. That is to say, some of these embryos were observed to enlarge in a distorted manner; occasionally a callus-like-tissue was observed, and what little growth may have occurred ceased within fourteen days of initiation. Attempts to fix and section this material by conventional methods failed. No further observations were conducted.

The anatomy and morphology of precocious germination is unique i. e., differs significantly from normal in vivo germination. In embryos of all starting ages, the first detectable response in culture is enlargement (i. e., at right angles to the axis of the embryo) of the root end of the hypocotyl, followed immediately by elongation (i. e., in the plane of the axis of the embryo). These responses
occur most rapidly and most directly for 23 day old embryos and correspondingly less in each case of 15, 11 and 9 day old embryos. Such responses are in part similar to incipient cell elongation of the hypocotyl region of the in vivo embryo. However, where in vivo incipient elongation is followed closely by sequential division of the root meristem which results in the coordinated development of a primary root, in vitro elongation of the hypocotyl is followed by the development of callus-like-tissues of varying complexity in the area of the root meristem. (See appendix B, plates 1, 2, 3, and 4). In all cases, regardless of variations in anatomical complexity, such divisions and atypical enlargement result in the partial to total destruction of the normal radicle structure. Such destruction varies from the displacement of the radicle as a unit (see appendix A, plate 1), to its disintegration into several fragmentary pieces (see appendix B, plates 1 and 2), or to its complete alteration in response to the production of callus-like-tissues (see appendix B, plate 3). Such destruction is more apparent in the younger embryos.

In the older embryos, 23 and 15 day, a region of callus-like-tissue (called intercalary callus in this case) forms between the hypocotyl and root. Unfortunately, it has not been irrevocably determined whether this callus area formed prior to root formation, during root formation or after root formation in all cases. Although the possibility of intercalary callus formation preceeding the development of roots is strongly suspected, the presence of at least partially continuous vascular tissue through the area of disturbance requires cautious interpretation and suggests the need for further study.
It is also possible that these roots have arisen from the callus and not the primary root meristem, which is strongly suspected to be the case particularly in the younger embryos. (See appendix B, plates 3 and 4). If such can be precisely confirmed, these roots would have to be considered as substitute primary roots or secondarily derived roots.

The marginal tissues of cortex and epidermis in the area of callus-like-tissue formation are not continuous nor typical in appearance. Although the capacity of such tissues for serving the usual functions of storage and protection can be questioned, such was not subjected to experimentation. In addition, the absence of uniformity and the presence of intercalary callus-like-cells, individually arranged or grouped, and the regular occurrence of surface ruptures presents mute testimony to the abnormal processes of development which have occurred. Were it not for the maintenance of sterile conditions throughout until these wound-like-areas healed, the cultures most certainly would have been lost to contamination. Subsequent transfer of some of these embryos to "micro-greenhouses" (see Figure 8) and their careful nurture has resulted in the successful establishment of healthy seedling geraniums.

In the 11 day old embryos, the lack of continuity between hypocotyl and root is even greater. Here there is often no recognizable cortex or epidermis, and although the vascular tissue between these structures is connected, the cells are irregularly arranged and the endodermis displaced or several irregular layers substituting for normal arrangement are found (appendix B, plate 3). The main body of the embryo (i.e., hypocotyl, epicotyl and cotyledons) is usually discernable although its subversion by callus tissue
Figure 7. Summary comparison of growth rates between control, in vivo germinated embryos and in vitro precociously germinated embryos expressed as mean daily gain.

Measurements for 35 day old seedlings and 23 day old embryos were suspended at 24 days.

Upper limits = mean highs

Lower limits = mean lows

Note the relationships in time between ages relative to day of germination, rate of growth once initiated and the range of growth between theoretically identical individuals.
Figure 8. Precociously germinated embryos of *P. x. hortorum* growing independently of culture medium in sterile "micro-greenhouses".
invasion and disruption is most obvious.

In the 9 day old embryos, the absence of continuity between the embryo proper and the post-germinal structures is even more obvious (appendix B, plate 4). In fact, it is most often impossible to discern any area that would clearly constitute the body of the embryo; at best there are fragmentary areas of embryonic-like cells that are suspected to have been part of the original embryo but may actually be regenerated or secondarily derived embryonic cells (appendix B, plate 4). Never-the-less, despite the presence of copious amounts of derived callus-like-tissue, eventually roots or shoots were observed to develop in at least one or two embryos of each treatment. However, both roots and shoots developed in only four cases and did so irrespective of treatments.

The inception of epicotylonal growth and development was observed to occur following the prior formation of the substitute primary root. Epicotylonal development occurred independently of treatments as indicated in Table 7, for 23, 15, and 11 day old embryos, and apparently so for 9 day old embryos which were not subjected to statistical analysis of variance. However, at this stage, shoots were occasionally observed to develop in the absence of clearly differentiated roots. Quantitation of this data was not performed due to the impossibility of externally measuring internal differentiation which could be interpreted as the point in time of initiation of meristematic activity. Such could be accomplished through routine sacrificing of culture material for serial histological analysis which would ultimately involve many hundreds of individual embryos in order to obtain a reliable sample. These problems, although not
insurmountable, necessarily lie beyond the scope of the present study.

Lateral root formation or substitute primary root formation, whichever the case may be, occurred significantly with respect to treatments but often out of sequence with epicotylary development for 15 and 11 day old embryos and would have been reasonably expected to do so had 9 day old development been quantitated. Lateral roots of 23 day old embryos were initiated sequentially in time and independently of treatments.

The localization of nucleic acids within precociously germinated embryos, although of considerable interest, does not lend itself to precise interpretation. That nucleic acids were in fact being stained is confirmed by the differential perchloric acid extraction method for RNA and DNA recommended by Jensen, (1962). (See appendix C). Intense RNA staining of pregerminal mature embryonic tissue, which is totally removed by perchloric acid extraction, does not seem specific for RNA. An RNA staining response which occurs after the first extraction (RNA) step suggests that an unknown staining reaction is occurring. Similarly, the secondary cell walls of vascular tissues stain heavily, and extraction, although removing approximately 90% of this staining response does not adequately explain why secondary cell wall material should stain at all. Efforts to derive significance from these observations continue to be frustrated, primarily because the prepared sections can not be quantitated with presently available equipment. However, the necessity for exact quantitative analysis is recognized and such experiments are anticipated for the future.
Despite this unfortunate circumstance, it is suspected that treatment-induced and reproducible quantitative differences in nucleic acid distribution are in fact being observed, and it is emphatically suggested that such data, accurately derived, could give meaning to some of the presently unexplained statistical data reflecting treatment differences presented in the next section.

Statistical Analysis of Variance

Design of the experiment: As previously described (see section I, p. 3) preliminary experiments, designed to test the effectiveness of established nutrient media on P. x hortorum embryos, suggested that quantitative differences between embryos of different ages within different nutrient conditions were being observed. The first attempts to quantitate these observed differences were completely frustrated. This was probably due to the combined effects of three independent factors. One involved the use of french square culture bottles. The second resulted from attempts to measure growth and development on the basis of weight differences, which resulted in losses of culture material due to coincidental contamination. In addition, other negative effects derived from injury due to handling could not be predicted nor compensated for. The third involved complications resulting from suspected pre-conditioning differences derived from nutrient deficiencies in the parental stock material.

The following corrective measures were implemented. As a result of consultation with Professor Willard E. Urban, Statistician, the required experimental parameters for
statistical analysis were established, and the specific quantitative measurements to be performed were identified.

A factorial experiment (Snedecor, 1956) was designed as follows. Five regimes involving in vitro embryos of five ages (see, section IV-C) were established under identical conditions. Eight treatments: (1) control group; (2) IAA; (3) Kinetin; (4) GA$_3$; (5) IAA x Kinetin; (6) Kinetin x GA$_3$; (7) IAA x GA$_3$; and (8) IAA x Kinetin x GA$_3$, with two replications of each, all at $1 \times 10^{-7}$ Molar concentrations were used identically for each age group. A sixth regime, using mature seeds within the same treatment parameters, but without complete nutrients, was conducted simultaneously. Four embryos were used per culture dish in the 7, 9, 11 and 15 day regimes and three embryos per dish for the 23 day regime. Four culture dishes were used for each treatment replication. Thus the experimental design involved a total of 304 embryos. From this number, six subsamples from each replicate were used as the primary subjects for quantitative observations of the in vitro embryos. In the germination regime, 25 seeds per dish, in 2 replications with 4 dishes in each times 8 dishes per treatment required 1,600 seeds.

Random measurements were taken of 12 subsamples throughout for each of the traits listed below.

1. day of primary root initiation
2. day of epicotyl initiation
3. day of lateral root initiation
4. total number of lateral roots formed
5. total length of hypocotyl
6. primary root length expressed as
7. epicotyl length daily gain
Of the seven traits measured, all but number 5 were suitable for analysis. In culture, the hypocotyl elongated in a coiled and irregular manner; undoubtedly a phototropic response which effectively precluded accurate measurement. In the seedling experiment, the hypocotyls were straight.

Initially it had been decided to use Duncan's Multiple Range Test for analysis. Final experimental design and the frequency distributions of the derived means, which showed a skewed distribution resisting efforts toward normalization, combined to present justifiable objections to the use of Duncan's test (Duncan, 1955). Upon advisement, it was decided to use a standard analysis of variance employing least squares. The data were fitted into a standard program and computed by machine.

The problem of finding an appropriate culture vessel was solved by using 20 x 100 mm petri dishes, vapor sealed with parafilm and securely fastened with plastic tape. Thus a clear surface, top and bottom, facilitated observation and measurement. Since direct measurements would have resulted in contamination, indirect measurements were made with adjustable micro callipers and a millimeter rule. Accuracy of ± 0.2 mm was achieved through practice on materials of known dimensions.

The problem of nutritional pre-conditioning of embryo stocks has not been examined experimentally. A change in nutrient media satisfactorily improved the vigor and overall appearance of the parental stocks. It was empirically decided that the effect of nutrient preconditioning would be no greater than the influence of unknown genetic effects, i.e., heterozygous stocks. Both conditions are discussed at some length in section V.
Results: None of the seven day old embryos grew significantly in culture. Several, irrespective of treatment, were observed to enlarge slightly. However, in all cases, enlargement ceased within fourteen days of initiation, and the embryos appeared bleached or necrotic. No further observations were conducted.

The nine day old embryos grew irregularly and inconsistently. A number formed leaves, and others even formed roots, only four formed both. The only response observed consistently throughout was the formation of callus tissue. The presence of callus tissue was not observed uniformly in the remaining regimes and, as a consequence, could not be included in the quantitative analysis. The decision was made to subject this material to morphological and histochemical examination, the results of which are discussed in section IV-C.

Embryos in the remaining regimes i.e., 23 day, 15 day, 11 day and the germinated seeds, grew satisfactorily and provided useful data which is summarized in Table 7. The results are most interesting. The most consistent source of variation was an IAA x Kinetin (abbr. = Ixk) interaction. One additional source of variation was noted in three cases as an IAA response. However, in these cases, the I x k interaction approached significance and this absence of significance may have been due to chance. For the purposes of discussion, the presence of the I x k interaction shall be considered as highly probable.

In order to further elucidate the precise nature of the I x k interaction, the mean squares within treatments were compared in a 2 x 2 factorial arrangement (Snedecor, 1956). The results are summarized in Table 8.
Table 7. Summary results of the statistical analysis of variance between treatments.

<table>
<thead>
<tr>
<th>Traits</th>
<th>Group</th>
<th>Significant Sources of Variation - 5% level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary root initiation</td>
<td>23 da</td>
<td>IAA</td>
</tr>
<tr>
<td></td>
<td>15 da</td>
<td>IAA</td>
</tr>
<tr>
<td></td>
<td>11 da</td>
<td>IAA x Kinetin</td>
</tr>
<tr>
<td>Epicotyl initiation</td>
<td>23 da</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 da</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11 da</td>
<td></td>
</tr>
<tr>
<td></td>
<td>germination</td>
<td></td>
</tr>
<tr>
<td>Lateral root initiation</td>
<td>23 da</td>
<td>IAA x Kinetin *</td>
</tr>
<tr>
<td></td>
<td>15 da</td>
<td>IAA x Kinetin</td>
</tr>
<tr>
<td></td>
<td>11 da</td>
<td>IAA x Kinetin</td>
</tr>
<tr>
<td></td>
<td>germination</td>
<td></td>
</tr>
<tr>
<td>Total number of lateral roots</td>
<td>23 da</td>
<td>IAA x Kinetin</td>
</tr>
<tr>
<td></td>
<td>15 da</td>
<td>IAA x Kinetin</td>
</tr>
<tr>
<td></td>
<td>11 da</td>
<td>IAA x Kinetin</td>
</tr>
<tr>
<td></td>
<td>germination</td>
<td></td>
</tr>
<tr>
<td>Daily gain primary root</td>
<td>23 da</td>
<td>IAA</td>
</tr>
<tr>
<td></td>
<td>15 da</td>
<td>IAA</td>
</tr>
<tr>
<td></td>
<td>11 da</td>
<td>IAA</td>
</tr>
<tr>
<td>Daily gain epicotyl</td>
<td>23 da</td>
<td>IAA x Kinetin</td>
</tr>
<tr>
<td></td>
<td>15 da</td>
<td>IAA x Kinetin</td>
</tr>
<tr>
<td></td>
<td>11 da</td>
<td>IAA x Kinetin</td>
</tr>
<tr>
<td>Hypocotyl length</td>
<td>germination</td>
<td>IAA x Kinetin</td>
</tr>
</tbody>
</table>

*approaching significance
Table 8. Summary results of the 2 x 2 factorial arrangement of significant treatments using mean squares.

<table>
<thead>
<tr>
<th>Primary root initiation</th>
<th>Lateral root initiation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>1.50</td>
</tr>
<tr>
<td>I</td>
<td>6.89</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>58.14</td>
</tr>
<tr>
<td>I</td>
<td>63.23</td>
</tr>
</tbody>
</table>

Number of lateral roots

|                         | 0  | K   | 0  | K   |
| 0                       | 1.05 | 0.23 | 0  | 1.69  | 3.80  |
| I                       | 14.63 | 11.73 | I  | 5.76  | 0.49  |
|                         | 0  | K   | 0  | K   |
| 0                       | .01  | .00  | 0  | .09   | .02   |
| I                       | .95  | 2.50 | I  | 0.00  | 0.03  |

Daily gain - epicotyl

|                         | 0  | K   | 0  | K   |
| 0                       | 2.33 | 0.95 | 0  | .04   | 4.62  |
| I                       | 7.70 | 0.18 | I  | 0.01  | 9.61  |

I = IAA; K = Kinetin; O = no treatment
Interpretation of data summarized in Table 7: Because none of the mean squares for GA$_3$ variance approached significance, it may be reasonably assumed that within the present experiment, GA$_3$ is nonoperative.

The initiation of growth among 23 day old embryos occurred independently of treatments. Conversely, growth rates among older embryos responded to the presence of IAA in the case of roots and to the I x K interaction in the case of shoots. In younger embryos, the root response was generally lacking. However, the possibility of such a response developing had the embryos been retained in culture for a longer period of time can not be entirely ruled out.

The initiation of epicotyl development occurred independently of treatments at all age levels. Conversely, treatment effects were observed in epicotyl growth rates as an I x K interaction.

IAA and kinetin combine in a synergic interaction to produce significant acceleration of growth and development in young embryos (see Figure 8). An I x K independence has not been demonstrated within the experimental parameters employed herein. The possibility that such a dependence does exist prior to the onset of autotrophic synthesis of natural auxins is suspected; however, the necessity for its determination lies beyond the scope of the present study. Such experiments are planned.

Interpretation of data summarized in Table 8: There exists high uniformity relative to the precise nature of the I x K interaction between the several traits showing significance in respect to the growth phenomena being measured, i. e., initiation of growth and rate of growth. Since the
total number of lateral roots is a function of secondary meristem activity, the initiation of primary roots, lateral roots and total number of lateral roots as essential derivatives of meristematic activity when contrasted should reflect strong correspondence between treatments. In the case of 11 day old embryos there is perfect correspondence between treatments with respect to the direction of response of the 0; I; K; and I x K effects. In the case of 15 day old embryos a similar correspondence exists except for the effect of K in lateral root initiation. Although this apparent contradiction has not been subjected to experimental treatment, one possible explanation based on observations made during the evaluation periods is that there existed distinct differences between the sites of lateral root formation and the length of the primary root at the time the lateral roots were formed. Perhaps this, if quantitated, could account for the reversal in effect of K based on availability of K and the presence of autotropically derived gradients of growth factors along the axis of the primary root, or the presence of externally mediated chemotropic gradients. Unfortunately, the need for such an evaluation was not apparent until after the material had been processed for sectioning and the analysis of variance performed.

The directional relationships between treatments determined from growth rates are less uniform. This is to be expected because the traits measured involve plant parts that are not directly comparable, due to anatomical, morphological and location differences i.e., root, hypocotyl, shoot, all having different positions in relation to
nutrient availability, intercellular nutrient mediation and modification from root to shoot.

Daily gain in primary roots showed significance exclusively in the 23 day old embryos. These older embryos grew correspondingly faster and in greater amounts than younger embryos. The longer more numerous roots had a quantitative advantage over roots of younger embryos. This argument applies as well to the epicotyls of the 23 day old embryos and the differences in directional response to treatments can best be explained as a difference in the proximity of the tissues to the medium. In fact, any effect observed in the epicotyl must be viewed as an indirect effect due to mediation and modification of treatment components through autotrophic metabolic mechanisms.

Significant response to treatments in the hypocotyl of germinating seeds needs to be viewed with reservation. In all other aspects of quantitative analysis, seedlings grew independently of treatments, and until such time as the hypocotyl response is re-examined, the suspicion of chance effect must receive priority consideration.

One additional results needs explanation, and in the final analysis, may prove to be the most important. When corresponding directional responses between similar quantitative traits, as defined above, are examined in terms of relative percentage effectiveness, no uniform correspondence exists. For example, the percent effect of I to 0 treatment in 11 day old embryos evaluated for primary root initiation is 92%. The same effect of I in 11 day old embryos evaluated for total number of lateral roots is only 1%, despite the fact that the directional relationships throughout are identical. Such inconsistencies defy explanation and clearly
suggest the need for additional experimentation designed specifically to equate these differences. Some recommendations can be made based upon certain morphological differences observed between embryos of different ages and their response to the nutrient environment. This will be more appropriately presented in the discussion to follow. Suffice it to say here that these considerations invoke the reoccurring notion that embryos of differing ages possess distinct differences in nutrient dependence based on autotrophic synthetic capabilities and nutritional preconditioning.
DISCUSSION

Despite the obvious necessity for "changing horses in midstream", significant insights into the precise nature of precocious germination have been achieved. Of greatest importance is the realization that precocious germination is not a singular process i.e., occurring identically among embryos of different pregerminal ages and among different treatment parameters.

The most significant difference between precocious germinations observed in embryos of differing ages is the variation in the extent and kind of callus-like-tissues formed. Other differences observed between ages and treatments involve cell numbers, cell size, arrangement of callus-like-cells (i.e., from separate interstitial arrangement to the formation of massive homogeneous clones) and response to nucleic acid stains. In addition, detailed examination by phase microscopy and electron microscopy would undoubtedly reveal important cytological differences.

Further differences between treatments and ages have been identified by statistical analysis of variance which demonstrates an IAA x kinetin interaction which has significantly influenced the rates of initiation of meristematic-like activity in roots and rates of growth in roots and shoots. Although it has not been possible to state unequivocally the precise nature of this demonstrated interaction, continued research employing the additional methods of autoradiography combined with high resolution microscopy should provide additional insights into the nature of the molecular interrelationships involved.
Despite the obvious limitations of the present study, several cautious conclusions can be reasonably made. Variations in the precise manner of precocious germination can best be explained as a function of the degree of specialization already built into embryonic cells in accordance with type of tissue present and embryo age at the time precocious germination is stimulated. Not until final maturity (greater than 23 days) when the synthesis phase of embryo development has climaxed and the storage phase of metabolism is well established, does the embryo constitute a sufficiently integrated whole to successfully preclude the precocious germination response to a variety of culture conditions.

Commensurate with the degree of total development, which reflects a slowed down synthesis phase and increasingly active storage phase, precocious germination can be more easily and more rapidly triggered. Less mature embryos which are more deeply committed to the synthesis phase of development require greater time to begin precocious germination and reflect the extent to which well established synthetic metabolism resists the kind of subversion which leads to the formation of increasing numbers of less specialized callus-like-cells.

Continuing this line of reasoning, eventually a point is reached where the autotrophic synthetic mechanisms phase out to be replaced by heterotrophic, extra-embryonic mediated mechanisms of differentiation. It is at this level i.e., in the case of P. x. hortorum embryos between 7 and 9 days of age, where the extinction point for potential survival of in vitro embryos is reached. In other words, very immature embryos (ball stage or less in most cases) can not respond to the kinds of in vitro nutrient media thus far developed.
It has been suggested that the embryo at this stage of development is completely dependent upon parental nutrient mediation (Rijven, 1952) and the inability of the experimental embryologist to sustain the life process in such immature embryos strongly suggests that these heterotrophic requirements have not been successfully duplicated. If this line of reasoning makes any sense at all, the next logical conclusion is that very young embryos must be viewed as transient obligatory parasites.

Despite all that precocious germination may or may not be, its existence as a deviation from normal embryological development has previously been suggested (Hannig, 1904; Dieterich, 1924) and its significance speculated upon (Rijven, 1952). As a result of the present study, a more meaningful appraisal of the question of normalcy vs. abnormalcy can be presented.

Any answer to such a question must necessarily reflect the position from which the question is asked. Viewed in terms of normal embryology, the only acceptable response must be abnormal; viewed in terms of the experimental parameters employed in the present study, precocious germination is the most normal response that could be expected. Consequently, the question of normalcy vs. abnormalcy quickly becomes irrelevant.

Because the term precocious germination lacks general definition, it seems reasonable to attempt such a definition based upon the experimental evidence accumulated to date. Its accuracy and longevity is certainly subject to review and reappraisal.
"Precocious germination is a normal response of immature embryos which have reached a critical stage in development of at least partial autotrophic independence, which, upon excision from the ovule and in good condition, (when placed on a complementary nutrient medium) will grow directly into mature plants thereby averting some of the usual phases of complete embryogenesis".

Reduced to its simplest terms, precocious germination is a special case of germination.

Another question that has occurred to the author concerns the value of precocious germination for the experimental embryologist. As a novice in the enterprise of science, any honest answer must necessarily reflect too much enthusiasm and overly high ideals.

The concept of totipotency of plant cells is well known and needs no citation. Similarly, most of the work that has been done to date in attempting to precisely define totipotency has been performed with callus tissue by workers too numerous to mention. Callus tissue culture and differentiation occurring therein, unfortunately possesses several built in complications, the most severe of which have been recognized (Steward et al, 1958a, 1958b; Vasil et al, 1964, 1965; White, 1963). Two of these reported observations are; (1) callus tissue regularly requires the development of a minimal mass before differentiation can occur; (2) depending on the kind of tissue giving rise to the callus, differentiation when it does occur, always occurs at a minimal distance from the nutrient medium, at the center of the clone in some cases or at the surface of the clone oriented away from the medium in other cases. In this author's view, these reoccurring observations combine to generate serious objections to the use of callus tissue in investigations of differentiation
and totipotency. My reasoning proceeds as follows. The intervening undifferentiated callus cells between nutrient source and the site of differentiation must necessarily be suspected of a capacity for nutrient modification during translocation which is beyond the control of the experimenter. In other words, nutrient components reaching the site of differentiation may differ substantially from their original composition within the medium. That this is so is best evidenced by the continuing experimental reliance upon undefined nutrient components and the absence of evidence demonstrating experimental control over the kind of differentiation that has occurred.

Therefore, precocious germination presents an opportunity to quantitate the phenomena of totipotency in a manner unrealized in callus cultures. Such is contingent upon experimental designs well grounded in cytological and histochemical observations between cells of known origin and subsequent specialization, which naturally occur in embryogenesis. From properly designed experiments, profound insights into the biochemical events that enhance or limit totipotency could be realized. This is especially true of the embryo, as totipotency appears to become inhibited by structural and biochemical specialization. Despite the intervention of callus-like-tissues occurring in precocious germination, their unique morphological and anatomical deviations from the usual undifferentiated callus employed elsewhere, strongly emphasized their suitability for studies of totipotency.

Another aspect of the present study which seems worthy of cautious speculation involves the several preliminary attempts to grow smaller than ball stage embryos using completely defined media. That reliance on previous work
and trial and error methods were employed is not denied. Such methodology is not without precedent as over 60 years of tissue culture work has demonstrated (White, 1963).

Before acceptable alternatives can be evolved, a major change in technique must be realized. At the risk of pretentiousness, I would like to propose one possible avenue of research that seems to offer the promise of success. Such research would be directed toward the development of a comprehensive "metabolic map".

Specific qualitative and quantitative studies equating related biochemical events in time with observed differentiation during in vivo embryology and in vivo germination would be expected to characterize:

1. inorganic constituents
2. free organic metabolites
3. bound macro-molecular components, (protein and lipid associations) (nucleo-protein), etc.
4. naturally occurring growth substances which when combined in time and space, should provide the basis for routine manipulation of all phases of pregerminal or postgerminal growth and development. Such a project would be a monumental undertaking, and to be practically considered, would require the unprecedented cooperation of several laboratories and countless experimenters. Short of this, I see no significant gains in the area of reproducible culturing of zygote to ball stage embryos as being at all realistically possible.

A final indirect response to the present study needs discussion. Based on observations involving nutrient pre-conditioning as an aspect of maintaining parental stocks which may affect the quantitative response of in vitro embryos, I would like to propose the following.
Studies specifically designed to induce controlled nutrient deficiencies in embryo stocks are urgently needed. Many of the inconsistencies observed in the preliminary experiments supporting this study are suspected to have involved such complications. Their eventual negation, as discussed in section III, resulting from improved fertilization of the parental stocks needs confirmation. One possible result of such studies can be speculated upon.

Inorganic deficiencies are likely to prevent growth providing the deficiency can not be compensated for in the culture medium. Conversely, too high concentrations in the embryo stocks may be enhanced by abnormal concentrations in the nutrient medium. Summarily stated, the following interrelationships are proposed to exist between nutrient medium inorganic components and parentally conditioned embryo stocks.

<table>
<thead>
<tr>
<th>Range of concentrations</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Range of response</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>deficient</td>
<td>compounded</td>
</tr>
<tr>
<td>(no growth or aberrant growth)</td>
<td>range of useable (inhibitory or activity toxic)</td>
</tr>
</tbody>
</table>

The use of \textit{P. x. hortorum} tissues for such a study would be well advised.
Regretably, a meaningful discussion of the precise relationships between exogenously applied growth substances and nucleic acid localization as was anticipated in section II, must be deferred until such a time as a usable quantitative method can be devised.
LITERATURE CITED


Sharman, B. C. 1943. Tannic acid and iron alum with safranin and orange G in studies of the shoot apex. Stain Technology. 18: 105-111.


APPENDIX A

Plate 1.
20X. 9 day old embryo, 10 days in culture, illustrating:

a. lateral and axial expansion of hypocotyl
b. atrophied cotyledons
c. ruptured epidermis and cortex of root area
d. remenant of suspensor

Plate 2.
20X. 11 day old embryo, 10 days in culture, illustrating:

a. fragmented radicle
b. emerging substitute primary root
c. enlarged and elongated hypocotyl
d. slightly expanded cotyledons

Plate 3.
20X. 15 day old embryo, 20 days in culture, illustrating:

a. cotyledon
b. first leaf
c. junction of hypocotyl and first root
d. lateral roots
Plate 4.

20X. 9 day old embryo, 20 days in culture, illustrating:

a. gross aberration of root area
b. third order lateral root growing as primary root
c. reduced cotyledons
d. distorted epicotyl

Plate 5.

20X. 15 day old embryo root development, 20 days in culture, illustrating:

a. first root replacing expected primary root
b. lateral roots

Plate 6.

80X. 15 day old embryo root development, 20 days in culture, illustrating:

a. first root replacing expected primary root
b. lateral roots
APPENDIX B

(23 day old embryos)

**Plate 1.**

<table>
<thead>
<tr>
<th>Plate</th>
<th>Region</th>
<th>Magnification</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-1</td>
<td>Transition region</td>
<td>5X</td>
<td>overview</td>
</tr>
<tr>
<td>A-2</td>
<td>Transition region</td>
<td>10X</td>
<td>vascular tissue</td>
</tr>
<tr>
<td>A-3</td>
<td>Transition region</td>
<td>10X</td>
<td>remnant of primary root area</td>
</tr>
<tr>
<td>B-1</td>
<td>Transition region</td>
<td>5X</td>
<td>overview</td>
</tr>
<tr>
<td>B-2</td>
<td>Transition region</td>
<td>10X</td>
<td>margin; hypocotyl-root cortex</td>
</tr>
<tr>
<td>B-3</td>
<td>Transition region</td>
<td>10X</td>
<td>remnant of primary root area</td>
</tr>
<tr>
<td>C-1</td>
<td>Transition region</td>
<td>5X</td>
<td>overview</td>
</tr>
<tr>
<td>C-2</td>
<td>Transition region</td>
<td>10X</td>
<td>regenerated embryonic tissue?</td>
</tr>
<tr>
<td>C-3</td>
<td>Transition region</td>
<td>10X</td>
<td>isolated tissue?</td>
</tr>
<tr>
<td>D-1</td>
<td>Epicotyl</td>
<td>10X</td>
<td>overview</td>
</tr>
<tr>
<td>D-2</td>
<td>Epicotyl</td>
<td>43X</td>
<td>second leaf</td>
</tr>
<tr>
<td>D-3</td>
<td>Epicotyl</td>
<td>43X</td>
<td>apical meristem</td>
</tr>
</tbody>
</table>
APPENDIX B

(15 day old embryos)

Plate 2.

A-1. Transition region  5X. overview
A-2. Transition region  10X. vascular tissue
A-3. Transition region  43X. margin: cortex and callus

B-1. Transition region  5X. overview
B-2. Transition region  43X. remnants of embryonic vascular cells
B-3. Transition region  43X. vascular callus

C-1. Lateral roots  5X. overview
C-2. Root tip  10X. meristem absent?
C-3. Root tip  43X. heavily staining secondary walls

D-1. Epicotyl  10X. overview
D-2. Epicotyl  43X. third and fourth leaves
D-3. Epicotyl  43X. annular protoxylem (staining less intense)
# APPENDIX B

*(11 day old embryos)*

**Plate 3.**

<table>
<thead>
<tr>
<th>Plate</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-1.</td>
<td>Transition region 10X. overview</td>
</tr>
<tr>
<td>A-2.</td>
<td>Transition region 43X. origin, lateral root</td>
</tr>
<tr>
<td>A-3.</td>
<td>Transition region 43X. remnants of primary root area</td>
</tr>
<tr>
<td>B-1.</td>
<td>Transition region 5X. overview - note atrophied epicotyl</td>
</tr>
<tr>
<td>B-2.</td>
<td>Transition region 10X. vascular tissue</td>
</tr>
<tr>
<td>B-3.</td>
<td>Transition region 10X. margin: hypocotyl and root</td>
</tr>
<tr>
<td>C-1.</td>
<td>Aberrant root tip 10X. overview</td>
</tr>
<tr>
<td>C-2.</td>
<td>Aberrant root tip 43X. apparent loss of meristem; second precocious germination</td>
</tr>
<tr>
<td>C-3.</td>
<td>Aberrant epicotyl 10X. overview</td>
</tr>
<tr>
<td>D-1.</td>
<td>Epicotyl 10X. overview</td>
</tr>
<tr>
<td>D-2.</td>
<td>Epicotyl 10X. margin: hypocotyl and cotyledon</td>
</tr>
<tr>
<td>D-3.</td>
<td>Cotyledon 10X. transition region</td>
</tr>
</tbody>
</table>
APPENDIX B
(9 day old embryos)

Plate 4.

A-1. Transition region 5X. overview
A-2. Transition region 10X. vascular tissue
A-3. Transition region 10X. remnants of primary root area

B-1. Transition region 5X. overview
B-2. Transition region 10X. same: 105 μ below; median of vascular bundle remnants of primary root area
B-3. Transition region 10X. remnants of primary root area

C-1. Lateral root 10X. overview
C-2. Lateral root 43X. apparent loss of meristem
C-3. Transition region 10X. remnants of primary root area

D-1. Cotyledon (1) 5X. overview
D-2. Cotyledon (2) 10X. overview
D-3. Cotyledon (1) 43X. region of aberration
APPENDIX C

(Differential Extraction of RNA and DNA)

**Plate 1.**

<p>| | | | | |</p>
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>A-1</td>
<td>+ RNA + DNA</td>
<td>10X.</td>
<td>mature <em>in vivo</em> embryo</td>
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<tr>
<td>A-2</td>
<td>-</td>
<td>+ DNA</td>
<td>10X.</td>
<td><em>in vivo</em> embryo</td>
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<tr>
<td>A-3</td>
<td>-</td>
<td>-</td>
<td>10X.</td>
<td><em>in vivo</em> embryo</td>
</tr>
<tr>
<td>B-1</td>
<td>+ RNA + DNA</td>
<td>10X.</td>
<td>30 day <em>in vitro</em> embryo</td>
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<tr>
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<td>-</td>
<td>+ DNA</td>
<td>10X.</td>
<td><em>in vitro</em> embryo</td>
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<tr>
<td>B-3</td>
<td>-</td>
<td>-</td>
<td>10X.</td>
<td><em>in vitro</em> embryo</td>
</tr>
<tr>
<td>C-1</td>
<td>+ RNA + DNA</td>
<td>10X.</td>
<td>10 day <em>in vivo</em> embryo</td>
<td></td>
</tr>
<tr>
<td>C-2</td>
<td>-</td>
<td>+ DNA</td>
<td>10X.</td>
<td><em>in vivo</em> embryo</td>
</tr>
<tr>
<td>C-3</td>
<td>-</td>
<td>-</td>
<td>10X.</td>
<td><em>in vivo</em> embryo</td>
</tr>
</tbody>
</table>